Conservation biology of the critically endangered red-headed wood pigeon *Columba janthina nitens* in disturbed oceanic island habitats

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Contents

Chapter 1

General introduction

The high endemism of fauna and flora due to long periods of evolution under highly isolated conditions has led oceanic island ecosystems to be considered as hotspots of biodiversity with high conservation priority (Carlquist 1974; Whittaker 2007). In oceanic islands, unique but disharmonic biota that lack amphibians, large mammals, and plants with low dispersal capacity are characterized by limitations in species immigration and have evolved without interspecific competition and external perturbation (Carlquist 1974; Shimizu 2002). As a result, island endemics are vulnerable to the effects of habitat destruction, introduced predators, competition, and disease (Loope *et al*. 1988; Duncan & Blackburn 2004). Furthermore, island endemic species exhibit smaller population sizes and lower genetic diversity than those of mainland populations, and thus further decreases in population size may cause inbreeding depression and increased extinction risks (Frankham 1997, 1998). Indeed, 59% of the known extinctions since 1600 have occurred in islands (60% for mammals, 81% for birds, 95% for reptiles, 79% for mollusks, 84% for insects and 36% for plants; Whittaker 2007). Hence, the conservation of endemic species in oceanic islands is one of the most pressing issues to reduce the loss of biodiversity.

Recently, molecular biological techniques have become widely used in the fields of ecology and conservation (Isagi & Suyama 2013). The techniques enable us to understand our target species in terms of evolutionary and ecological processes that are difficult to estimate based only on field observations. Thus, molecular techniques provide valuable information for the long-term conservation of endangered species. For example, highly variable microsatellite markers have been used to estimate the genetic characteristics of several endangered species at the population level (Bradshaw *et al*. 2007; Kaneko *et al*. 2008; Yokogawa *et al*. 2013) and individual level (Kaneko *et al*. 2013) to determine meaningful management units and to maintain genetic diversity within and outside their habitat. In particular, high-throughput sequencing techniques using next-generation sequencers have made revolutionary advances in the usage of genetic information in ecology (Shokralla *et al*. 2012), as they allow us to recover DNA sequence data directly from environmental samples. Data from this technique have been used in a variety of applications, such as the estimation of community composition for root-associated fungi (Toju *et al*. 2013) and bacteria in the ocean (Kembel *et al*. 2011) and animal diet composition from feces or stomach contents (reviewed by Pompanon *et al*. 2012). Multifaceted approaches using molecular biological techniques may provide us with a better understanding of the genetic and ecological features of endangered island endemics and may lead to

effective conservation planning.

Many bird species have been the most seriously exposed to human disturbances such as habitat loss, overexploitation, and predation by invasive mammals (Gill 2007). It has been found that 37% of island endemic birds face the risk of extinction (BirdLife International 2008). A major threat to island endemic birds are predation by invasive mammals (Courchamp *et al.* 2003; Medina *et al.* 2011) because they have evolved without the risk of predation by terrestrial predators. Thus, eradications of invasive mammals (Howald *et al.* 2007; Horikoshi *et al.* 2009; O'Donnell *et al.* 2012) and reintroductions of captive bred populations to pest-free habitats (Ewen *et al.* 2012) have been the main conservation practices. Around the last decade, genetic issues have also been considered for the conservation of island endemic birds. The genetic diversity of island endemic birds, which was estimated by microsatellite markers, was lower than that of continental birds (Jamieson *et al.* 2006), and the extinction risks were higher in the species with lower genetic diversity (Evans *et al.* 2008). In New Zealand, Jamieson *et al.* (2006) also found inbreeding depression in small wild populations and captive populations that were established without considering their genetic diversity. In the case of the endangered Pink pigeon *Columba mayei*, the effects of inbreeding on egg fertility and squab, juvenile and adult survival were more serious in captive and free-living populations with low founder diversity of mitochondrial control region sequences (Swinnerton *et al.* 2004). These results indicate the importance of genetic management within and outside the habitats of focal species for the long-term conservation of threatened island endemic birds.

The estimation of population genetic structure is also important to detect an evolutionarily significant unit (ESU). Each ESU can be treated as a single management unit for habitat conservation by considering the evolutionary potential of wild populations; it is also important to avoid outbreeding depression by individual translocation among populations (Frankham *et al.* 2010). Island endemic birds are well known for evolution with reducing flying capacity and species differentiation by adaptive radiation (Weiner 1994; Whittaker 2007), and thus each island population tends to be regarded as a separate management unit. However, in the Galapagos Islands, the degree of genetic differentiation among island populations, estimated by microsatellite markers, differs among species. Island populations of Darwin's finches (Petren *et al.* 2005) and the Galapagos hawk *Buteo galapagoensis* (Bollmer *et al.* 2005) were genetically differentiated and treated as different management units. However, all the estimated island populations of the Galapagos dove *Zenaida galapagoensis* were regarded as a single management unit due to their weak genetic differentiation (Alarcon *et al.* 2006). This may be due to the strong flying capacity of Columbiformes (Gibbs *et al.* 2001), which cause gene flow among island populations by inter-island movement. The genetic

characteristics of endangered species appear to reflect their historical background and life history, and thus their genetic management should be conducted based on each situation. Considering these situations, conservation genetics studies of threatened island birds have not been fully explored and utilized in the face of the existing 431 endangered species.

The restoration of appropriate foraging habitat is an essential task for the habitat conservation of threatened birds in seriously disturbed island habitats. However, habitat restorations with massive eradications of introduced species may not cause a positive effect on their foraging condition. Some introduced species have exhibited interspecific interactions with native species due to long-term naturalization (Foster & Robinson 2007; Kawakami *et al*. 2009), which has led to complex food webs that include native and introduced species (Zavaleta *et al*. 2001; Kawakami 2008). In these situations, the consumption of introduced plants by endangered birds was reported in several studies, such as in New Zealand endemic birds (Williams *et al*. 1996) and endangered Azores bullfinches *Pyrrhula pyrrhula* (Ceia *et al*. 2011). If these threatened birds are strongly dependent on introduced species for food, the rapid eradication of introduced species may negatively impact their foraging conditions. To conduct appropriate restoration in the habitat of threatened island birds, detailed understanding of their feeding ecology, particularly the degree of dependence on introduced species and the significance of introduced species as food, is quite important.

Various methods have been employed to understand the diet of birds. Among these methods, direct observation and fecal analysis are common. The simplest approach is the direct observation of foraging behavior, which revealed the diet adaptation of Darwin's finches in the Galapagos Islands (Weiner 1994). However, in many circumstances, food identification by direct observation is difficult or impossible, particularly for forest birds that forage in complex environments with many types of food resources. Fecal analysis is widely used as a non-invasive approach to study the diet (e.g., Oliveira *et al*. 2002) and seed dispersal of birds (e.g., Kawakami *et al*. 2009). However, some bird species strongly crush food in their stomach, which makes it difficult to identify fragmented food items in fecal samples. In such cases, it takes a significant amount of time to identify food items using a microscope, and the resolution of food identification strongly depends on the skill of the observer (Holechek *et al*. 1982a, b).

Recently, fecal analysis using a DNA barcoding taxon identification system with a standardized DNA region (Hebert & Gregory 2005) has attracted attention as a new method of diet studies. In this method, DNA is extracted from fecal samples, and the fragments of the target region are amplified and sequenced. By referring to the sequence database, food items in the fecal samples are identified by their DNA sequences. This method seems to be less time consuming and less biased

than traditional microhistological analyses. Furthermore, high-throughput sequencing (HTS) by next-generation sequencing provides a large amount of sequences from each sample, which will increase the comprehensiveness of food identification (Valentini *et al*. 2009a, b). To date, the method has been used for diet analyses of various types of animals, including herbivorous ungulates (Valentini *et al*. 2009a; Raye′ *et al*. 2011; Kowalczyk *et al*. 2011; Willerslev *et al*. 2014), insectivorous bats (Burger *et al*. 2013), marine animals (Deagle *et al*. 2009) and carnivores (Shehzad *et al*. 2012). This method may also be useful for the diet analysis of threatened species, but it has rarely been used in conservation biology studies.

The Ogasawara Islands are a chain of small oceanic islands located 1000 km south of the main islands of Japan. The Ogasawara Islands consist of two island groups: the Bonin Islands (including Mukojima group, Chichijima group and Hahajima group) and the Volcano Islands (Kita-Iwojima, Iwojima and Minami-Iwojima), as well as several isolated islands (Nishinoshima, Minamitorishima and Okinotorishima). The climate is subtropical, with an average annual temperature on Chichijima of 23°C and an annual rainfall of approximately 1300 mm (Japan Meteorological Agency 2001). A high level of endemism is found on the islands due to the physical isolation of the flora and fauna. The rates of endemic species or subspecies on the Ogasawara Islands are as follows: 40% of the plants (Toyoda 2003), 80% of the land birds (Ornithological Society of Japan 2012) and more than 90% of the native land snail species (Kurozumi 1988). The Ogasawara Islands were registered as a World Natural Heritage Site in 2011.

Despite its high conservation value of biodiversity, the fauna and flora of the Ogasawara Islands have experienced serious ecological disturbances (Kachi 2010; Kawakami 2010). In the case of bird species, five endemic species (Bonin thrush *Cichlopasser terrestris*, Bonin pigeon *Columba versicolor*, Bonin grosbeak *Chaunoproctus ferreorostris*, rufous night heron *Nycticorax caledonicus crassirostris* and the Bonin Islands white-eye *Apalopteron familiare familiare*) became extinct by the 1930s due to habitat loss or overexploitation (Greenway 1967, Ministry of the Environment 2002). In recent years, the major threat for native bird species in the Ogasawara Islands has been predation disturbance by species. For example, feral rats (*Rattus norvegicus*, *Rattus rattus*) frequently cause fatal damage to small-sized seabird populations through direct predation (Kawakami & Higuchi 2010). Feral cats *Felis catus* prey upon both terrestrial birds and seabirds (Kawakami & Higuchi 2010). Grazing and trampling by feral goats *Capra aegagrus* have caused the destruction of forest vegetation, which represents important habitat for bird species (Hata *et al*. 2010). Invasive plants such as *Leucaena glauca* and *Bischofia javanica* have also changed the habitat conditions of native birds by disturbing succession and replacing the niches of native trees (Yamashita *et al*. 2010; Hata *et al*. 2010),

which may have caused negative impacts on the foraging and/or nesting of native birds in the Ogasawara Islands.

Today, the eradication of introduced predators and plants have been actively conducted, and the habitat condition for endemic bird species of the Ogasawara Islands is expected to gradually recover (Horikoshi *et al*. 2009). However, many bird species have experienced substantial population declines, and populations are likely to have undergone deleterious bottlenecks, thus increasing the risk of inbreeding and the loss of genetic diversity. At present, the genetic status of endangered endemic bird species in the Ogasawara Islands has been understudied, and the genetic information of these species has never been considered in their conservation planning (e.g., identifying management units, captive breeding and reintroduction programs).

Regarding the habitat conservation of endangered species in the Ogasawara Islands, an additional complication is that some introduced plants and animals have become the dominant components of the current ecosystem (Kawakami 2008). For example, 90% of the diet of the endangered Ogasawara buzzard *Buteo buteo* subsp. *toyoshimai* is thought to be composed of introduced animals: mainly the black rat and the green anole *Anolis carolinensis* (Kato & Suzuki 2005). Therefore, it is a concern that the rapid eradication of these introduced species may negatively affect the population of threatened birds. Thus, the foraging ecology of native bird species, particularly the degree of dependence on introduced species, should also be estimated for effective habitat conservation and nature restoration in the Ogasawara Islands.

I investigated the genetic characteristics and feeding ecology of the red-headed wood pigeon *Columba janthina* subsp. *nitens* (Columbidae), a critically endangered and endemic subspecies of the Ogasawara Islands. This subspecies is thought to have a reduced population size due to habitat loss and predation by feral cats (Horikoshi 2008). As of 2008, the population of this pigeon likely included approximately 100 individuals according to observation records (Horikoshi 2008), and this species is listed as critically endangered on the Japanese Red List (Ministry of the Environment 2012). The red-headed wood pigeon is widely distributed in the Ogasawara Islands, including the Bonin and the Volcano Islands. This subspecies is morphologically and genetically distinct from the two other subspecies: the nominate subspecies of the Japanese wood pigeon *Columba janthina janthina* (distributed in small islands near the Japanese mainland and the Korean Peninsula) and Stejneger's wood pigeon *C. janthina stejnegeri* (distributed in the Sakishima Islands) (Gibbs *et al*. 2001, Seki & Takano 2005, Seki *et al*. 2007), suggesting that this taxon has evolved in isolation and is an important lineage for biodiversity conservation.

To prevent the extinction of the red-headed wood pigeon, a captive breeding program was

started in 2001. However, the genetic characteristics of the captive population has never been estimated despite the existence of signs of inbreeding depression, such as chicks having a low survival rate, in addition to the appearance of malformed individuals (Tokyo Zoological Park Society pers. comm.). For appropriate conservation planning of the wild populations, it is important to reveal whether the Bonin and Volcano populations should be treated as a single management unit. Recent observations of banded individuals of the pigeon revealed frequent movements between the islands of the Bonin Island group, which are more than 50 km apart (e.g., Chichijima to Hahajima and Mukojima to Chichijima, Suzuki *et al.* 2006). It may also be possible for the pigeon to move between the Bonin and Volcano Islands and maintain gene flow between the populations. However, an estimation of the population genetic structure has never conducted.

The detailed diet of the red-headed wood pigeon is also unknown, except that it mainly eats fruits and seeds (Takano *et al.* 1995). Thus far, dietary analyses of the pigeon have mainly been carried out by direct observation (Kanto Regional Forestry Office 2005, 2006; Kanto Regional Environmental Office 2011) and microhistological analysis (Shibazaki & Hoshi 2006). The existing information on the diet of this species is fragmented due to the difficulty of continuous observation and species identification of digested food items in pigeon stomachs. The native forest habitat of the red-headed wood pigeon has been destroyed due to human settlements in the 19th century and World War II (Kachi 2010; Kawakami 2010). Instead, several introduced plants have expanded their populations and generated a large abundance of fruit (Ecological Society of Japan 2002; Toyoda 2003; Hata *et al.* 2010; Tanaka *et al.* 2010). The red-headed wood pigeon may frequently consume introduced species to supplement a lack of native food resources, and these introduced species will be eradicated during restoration projects in the near future. Hence, understanding the foraging ecology of the pigeon (diet composition, food preference and importance of introduced plants as food) is essential to conduct restoration in pigeon habitat with an appropriate timing and order.

The main objective of this study is to comprehensively examine the genetic characteristics and foraging ecology of the red-headed wood pigeon, which is difficult to estimate only using field data, using molecular techniques. The results of this study can be used for effective conservation planning of the pigeon within and outside their habitat and the restoration of the extremely disturbed Ogasawara Islands.

This thesis is composed of six chapters and three appendixes. In Chapter 2, I developed microsatellite markers for the red-headed wood pigeon and nominal subspecies of the Japanese wood pigeon *Columba janthina* subsp. *janthina* and compared their genetic diversity.

In Chapter 3, I conducted genetic analysis of wild and captive populations of the

red-headed wood pigeon using sequences of mitochondrial control region and microsatellite markers that were developed in Chapter 2. I compared the genetic characteristics between wild and captive populations for the appropriate management of genetic diversity within and outside the habitat. I also estimated the genetic structure of two wild populations (Bonin Islands and Volcano Islands) to answer the following question: should the two populations be treated as a single evolutionarily significant unit (ESU)?

In Chapter 4, I estimated the applicability of the high-throughput sequencing (HTS) approach to understand the diets of the red-headed wood pigeon by comparing the results with traditional microhistological analysis using the same sample set of pigeon feces. I also confirmed the consumption of introduced plants by the pigeons.

In Chapter 5, I revealed the foraging strategy of the red-headed wood pigeon in isolated and disturbed habitat in the Ogasawara Islands. I conducted HTS diet analysis for the fecal samples collected for two years and estimated the seasonal and inter-island differences in diet composition. I also estimated food selection patterns and the importance of introduced fruit as food resources for the pigeon using the data of diet composition, food resource availability and fruit nutrient composition.

In the general discussion of Chapter 6, I discussed the genetic characteristics and foraging strategy of the red-headed wood pigeon, as well as effective conservation planning within and outside their habitat. At the same time, I discussed the effectiveness and problems of molecular approaches in the conservation biology of island endemics.

Chapter 2

Development of microsatellite markers for the Japanese wood pigeon *Columba janthina*, endemic to islands of East Asia, and comparison of genetic diversity between two subspecies

Introduction

Island endemic species, which by nature have restricted habitat range and smaller population sizes than mainland species, are usually vulnerable to ecological disturbances and environment change, such as deforestation, introduced disease, and competition and predation pressures from invasive species. Island species may also have lower genetic diversity than mainland species, and thus genetic factors, such as inbreeding depression, may more strongly affect their chance of extinction (Frankham *et al*. 2010). In the case of bird species, 80% of all species that have become extinct since 1600 were island endemics (Primack 1998). It is important to develop conservation plans for the long-term conservation of endangered island bird species (e.g. identifying management units, establishing a captive breeding program for future reintroduction) based on genetic information, such as genetic diversity and genetic structure, in addition to conservation of habitat.

The Japanese wood pigeon, *Columba janthina*, is endemic to islands of East Asia, including some small islands near the Japanese mainland and the Korean Peninsula (Seki *et al*. 2007). This species lives in mature forests of evergreen broad-leaved trees in subtropical and warm temperate regions, and feeds mainly on seeds and fruits of trees in evergreen forests (Gibbs *et al*. 2001). The population size of the Japanese wood pigeon has declined due to habitat loss by deforestation (Ministry of the Environment 2002), and this species is listed as "Near Threatened" under the IUCN criteria (IUCN, 2014) and designated as a National Monument by Japanese government. One of the subspecies, the red-headed wood pigeon *Columba janthina nitens*, which is endemic to the Ogasawara Islands in Japan and whose population size is critically small (about 100; Horikoshi 2008), is listed as "Critically Endangered" (Ministry of the Environment 2012).

I developed seven nuclear microsatellite markers for the nominate Japanese wood pigeon and the rarer subspecies the red-headed wood pigeon. I also determined the transferability and level of polymorphism of genetic markers developed for the domestic rock pigeon *Columba livia* var. *domestica* by Traxler *et al*. (2000). To confirm the utility of the markers, I performed preliminary analysis of genetic diversity and genetic structure between populations of each subspecies.

Materials and methods

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Microsatellite markers were developed using the improved technique for isolating codominant compound microsatellite markers of Lian & Hogetsu (2002) and Lian *et al*. (2006). An adaptor-ligated, restricted DNA library for *Columba janthina nitens* was constructed according to the following procedure: genomic DNA was extracted from tissue sample from a captive bred individual using QIAamp DNA Micro Kit (Qiagen, Velno, Netherlands) and digested with the blunt-end restriction enzyme *Eco*RV. The restriction fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and an 8-mer with the 3′-end capped with an amino residue: 5′-ACCAGCCC-NH2-3′) using the Takara DNA ligation kit (Takara Bio, Shiga, Japan). Fragments were amplified by PCR from the *Eco*RV DNA library using compound SSR primer (AG)6(AC)7, (TC)6(AC)7 or (AC)6(AG)7 and an adaptor primer (5′-CTATAGGGCACGCGTGGT-3′). The amplified fragments, ranging from 400 to 800 bp, were then separated on a 1.5% LO3 agarose gel (Takara) and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified DNA fragments were cloned using the QIAGEN PCR Cloning plus Kit (Qiagen) following the manufacturer's instructions. The cloned fragments were amplified using the M13 forward and reverse primers from the plasmid DNA. Amplified fragments were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). For each fragment containing a compound SSR sequence at one end, a specific primer was designed from the sequence flanking the compound SSR using Primer3 (v. 0.4.0, Rozen & Skaletsky 2000) (National Human Genome Research Institute).

Polymerase chain reaction amplifications were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 10 μl, which contained 5 ng of extracted DNA, 5 μl of 2x Multiplex PCR Master Mix, and 0.2 μM of each multiplexed primer. Compound SSR primers [(AG)6(AC)5, (TC)6(AC)5 or (AC)6(AG)5] were labeled with fluorochromes NED, VIC or 6-FAM (Applied Biosystems), respectively. Polymerase chain reaction amplifications were performed with the GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using the following conditions: initial denaturation at 95 ºC for 15 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing for each of the designed specific primers at the temperatures shown in Table 2.1 for 90 s, extension at 72 ºC for 1 min, and final extension at 60 ºC for 30 min. The size of the PCR products was measured using the ABI PRISM 3100 Genetic Analyzer, GENESCAN analysis and Genotyper software (Applied Biosystems).

I also tested seven microsatellite markers developed for the domestic rock pigeon (CliμD17, CliμT17, CliμD16, CliμD19, CliμD32, CliμT13, CliμD01, Traxler *et al*. 2000) for cross-amplification

with the nominate Japanese wood pigeon and the red-headed wood pigeon DNA. Polymorphism of the markers was evaluated from feather or tissue samples from wild-origin individuals of the nominate Japanese wood pigeon ($n = 15$) and the red-headed wood pigeon ($n = 25$). Sampling of The nominate Japanese wood pigeon was carried out in Izu Islands ($n = 8$) and Okinawa Islands ($n = 7$), and The red-headed wood pigeon in the two islands groups of the Ogasawara Islands: Bonin Islands ($n = 23$) and Volcano Islands ($n = 2$), which are about 150 km apart from each other. Molting feather samples were collected from individuals captured for banding, and from carcasses resulting from raptor kills between 2004–2010. I calculated number of alleles (NA) and expected heterozygosity (HE) and observed heterozygosity (HO). Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested using FSTAT (version 2.9.3; Goudet 2001). Significance levels were adjusted using Bonferroni correction for multiple testing. Allele frequency in each locus was calculated using GENALEX (version 6.0; Peakall & Smouse 2006). I performed individual-based clustering STRUCTURE analysis using the STRUCTURE 2.2.3 software (Pritchard *et al*. 2000) as a preliminary test to discern between each subspecies, and between populations within each subspecies. In this method, individuals are used as the unit and assigned to the most likely genetic group (cluster: K). I used admixture with LOCPRIOR and allele frequency correlated models. Ten runs of $K = 1$–8 were carried out at 1,000,000 MCMC and 100,000 burn-in repetitions. The appropriate K was chosen based on log likelihood. Based on the correlated allele frequency model, the amount of divergence for each cluster from a common ancestral population (F values; Falush *et al*. 2003) was calculated. To estimate the degree of population differentiation, I calculated pairwise FST values among three populations: Izu Islands, Okinawa Islands and Ogasawara Islands, and tested their deviations from zero using GENALEX. The population of Volcano Islands was excluded from the analysis due to its small sample size.

Results

Seven of the newly developed loci showed a clear, strong single peak for each allele (Table 2.1). In the cross-species amplification test, one locus (CliμD01) out of seven loci was found to show a clear, strong single peak for each allele (Table 2.1). There was no evidence of significant linkage disequilibrium ($P > 0.05$) between these loci in either subspecies. In the nominate Japanese wood pigeon, seven loci were polymorphic and the number of alleles per locus (NA) of the eight loci ranged from one to seven with an average of 3.13 (Table 2.2). The observed and expected heterozygosities (HO and HE) ranged from 0.00 to 0.67 and from 0.00 to 0.64, with averages of 0.28 and 0.31, respectively. No loci showed significant deviation from HWE. In *Columba janthina nitens*, two loci

(Cjan 365 and Cjan 419) were polymorphic and the number of alleles per locus ranged from one to three, with an average of 1.38 (Table 2.2). The observed and expected heterozygosities (HO and HE) ranged from 0.00 to 0.08 and from 0.00 to 0.08, with averages of 0.01 and 0.01, respectively. Two individuals were heterozygous at locus Cjan365 and one individual at locus Cjan419. Neither locus showed significant deviation from HWE.

Allele frequency in each locus is shown in Fig. 2.1. Although the nominate Japanese wood pigeon and the red-headed wood pigeon had common alleles, except for the locus CliμD01, their frequency differed greatly between subspecies. Each population of the nominate Japanese wood pigeon had several private alleles. Frequencies of common alleles were also different between populations at locus Cjan365, Cjan450, Cjan541 and CliμD01. In the red-headed wood pigeon, most of the individuals exhibited the same genotype. The allele sizes of two individuals from the Volcano Islands did not differ from that of the Bonin Islands population. In the STRUCTURE analysis, the log likelihood was maximized at $K = 2$ (Fig. 2.2). From $K = 3$ to $K = 8$, standard deviations got larger. When $K = 2$, each subspecies were clearly assigned to different clusters (Fig. 2.3). The proportion of each cluster was different between Izu Islands and Okinawa Islands in the nominate Japanese wood pigeon when $K = 3$. In the red-headed wood pigeon, the proportion of each cluster was nearly the same between populations of the Bonin Islands and the Volcano Islands. Higher F values, which indicate strong genetic divergence from a common ancestral population, were exhibited in the cluster that dominated in the red-headed wood pigeon. Pairwise FST values showed strong genetic divergence between the two subspecies ($P < 0.01$, Table 2.3). The FST value between the two populations of the nominate Japanese wood pigeon was also significantly different from zero ($P < 0.05$).

Discussion

In the nominate Japanese wood pigeon, the microsatellite markers showed a moderate level of polymorphism and no evidence of deviation from HWE. Genetic divergence between two populations of Izu Islands and Okinawa Islands was indicated by the allele frequency, results of STRUCTURE analysis and FST values, despite the small sample sizes. Thus the markers should be useful for estimation of genetic diversity, genetic structure within subspecies, or migration rates among island populations. This genetic information will enable the identification of genetically meaningful management units and the development of an appropriate conservation plan, including captive breeding and reintroduction programs. On the other hand, polymorphism of the markers in the red-headed wood pigeon is much lower than that of the nominate Japanese wood pigeon, despite the

fact that the markers were isolated from the red-headed wood pigeon. This does not indicate the low resolution of the markers, but rather low genetic diversity of the red-headed wood pigeon. The values of the number of alleles (NA) and expected heterozygosities (HE) were very low compared with the other island endemic endangered bird species, such as Mariana Crow *Corvus kubaryi* (NA = 2.2, HE = 0.24) and Seychelles Kestrel *Falco araea* (NA = 2.5, HE = 0.38) (Jamieson *et al*. 2006).

The nominate Japanese wood pigeon and the red-headed wood pigeon shared several alleles, but their frequencies were very different, as supported by the results of STRUCTURE analysis and the large FST value. In addition, higher F values were exhibited in the clusters that dominated in the red-headed wood pigeon in STRUCTURE analysis. These indicate that the red-headed wood pigeon has experienced strong genetic divergence from a common ancestral population. Habitats of the nominate Japanese wood pigeon and the red-headed wood pigeon are more than 500 km apart, and both subspecies are strongly genetically differentiated, as indicated by the results of this study and a previous study using mitochondrial control region sequences (Seki *et al*. 2007). A founder bottleneck, genetic drift due to small population size, and spatial and temporal isolation with restricted immigration is reflected in the low genetic diversity of the red-headed wood pigeon. Furthermore, the population of Ogasawara Islands, in which most of the samples were collected, experienced strong ecological disturbance, such as deforestation and predation by feral cats (Horikoshi, 2008). These factors might also have reduced genetic diversity of the subspecies. The population of the Volcano Islands did not have any unique allele compared with the population of the Bonin Islands. In this study, the sample size in the Volcano Islands (n = 2) was too small to estimate genetic diversity and genetic structure between populations. The degree of disturbance in the Volcano Islands has been less substantial than that of Bonin Islands, thus, higher genetic diversity may remain in the population, but was undetected. Further genetic study comparing the two populations is essential for the conservation planning of the red-headed wood pigeon. If the sample size is increased and genetically unique individuals exist on the Volcano Islands, it may be possible to estimate the genetic diversity and genetic structure or migration rates between populations in this subspecies, which are important information for identification of a meaningful management unit. The genetic information will also contribute to the design of a mating program in the captive population and to select appropriate individuals for reintroduction to the wild, in order to maintain genetic diversity in the red-headed wood pigeon.

In this study, I characterized microsatellite markers for two subspecies of the Japanese wood pigeon and confirmed their usefulness for future genetic study of the species. I also showed that the genetic diversity of the subspecies red-headed wood pigeon was at a critically low level,

comparing with the nominate Japanese wood pigeon. The results of this study indicated the genetic management of the red-headed wood pigeon within and outside of the habitat is an immediate concern.

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Table 2.1. Characteristics of seven compound microsatellite loci for *Columba janthina* and cross-amplification from *Columba livia* var. *domestica*

Locus	Repeat motif	Primer Sequence (5'-3')	Ta ($^\circ$C)	Size range (bp)	Accession no.	Reference
Cjan012	$(AC)_6(AG)_7$	ACACACACACACAGAGAGAGAG	57	206-244	AB615356	This study
		ATCTGAGGTGGTCCTATGAAAC				
Cjan292	$(TC)_6(AC)_8$	TCTCTCTCTCACACACACAC	57	73-101	AB615357	This study
		ACACATGCTCACAGGACTTTT				
Cjan365	$(AG)_6(AC)_{10}$	AGAGAGAGAGAGACACACACAC	57	202-214	AB615358	This study
		GCGTTCTCTTAGTTTGGTGTTA				
Cjan419	$(AC)_6(AG)_7$	ACACACACACACAGAGAGAGAG	57	99-111	AB615361	This study
		GTCTGCAGAATGGAGTGATG				
Cjan450	$(AG)_6(AC)_{11}$	AGAGAGAGAGAGACACACACAC	57	112-124	AB615359	This study
		AGGTTTTCATCCTAAAGCTGTG				
Cjan541	$(AG)_6(AC)_7CA(AC)_3$	AGAGAGAGAGAGACACACACAC	57	140-146	AB615360	This study
		AATGTCAATGCTTTGCTACAAC				
Cjan675	$(TC)_6(AC)_8$	TCTCTCTCTCACACACACAC	57	100-102	AB615362	This study
		CTGTTTATCATCTTCATCAATGG				
CliμD01	$(GT)_{30}$	GATTTCTCAAGCTGTAGGACT	57	65-97	AF188627	Traxler et al. (2000)
		GTTTGATTTGGTTGGGCCATC				

*T*a: annealing temperature of primer pair.

Table 2.2. Variability of eight microsatellite loci in the nominal Japanese wood pigeon *Columba janthina janthina* and the red-headed wood pigeon *Columba janthina nitens*.

	Microsatellite loci								
	Cjan012	Cjan292	Cjan365	Cjan419	Cjan450	Cjan541	Cjan675	CliµD01	Average
Columba janthina janthina									
Izu Islands (*n*=8)									
N_A	2	1	5	2	2	2	1	5	2.50
H_O	0.13	0.00	0.63	0.00	0.63	0.25	0.00	0.38	0.25
H_E	0.12	0.00	0.69	0.22	0.49	0.22	0.00	0.50	0.28
Okinawa Islands (*n*=7)									
N_A	1	1	4	1	3	2	2	5	2.38
H_O	0.00	0.00	0.43	0.00	0.71	0.43	0.29	0.57	0.30
H_E	0.00	0.00	0.53	0.00	0.50	0.34	0.41	0.67	0.31
All (*n*=15)									
N_A	2	1	6	2	3	2	2	7	3.13
H_O	0.07	0.00	0.53	0.00	0.67	0.33	0.13	0.47	0.28
H_E	0.06	0.00	0.64	0.12	0.53	0.28	0.23	0.61	0.31
Columba janthina nitens									
Bonin Islands (*n*=23)									
N_A	1	1	3	2	1	1	1	1	1.38
H_O	0.00	0.00	0.09	0.04	0.00	0.00	0.00	0.00	0.02
H_E	0.00	0.00	0.08	0.04	0.00	0.00	0.00	0.00	0.02
Volcano Islands (*n*=2)									
N_A	1	1	1	1	1	1	1	1	1.00
H_O	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
H_E	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
All (*n*=25)									
N_A	1	1	3	2	1	1	1	1	1.38
H_O	0.00	0.00	0.08	0.04	0.00	0.00	0.00	0.00	0.01
H_E	0.00	0.00	0.08	0.04	0.00	0.00	0.00	0.00	0.01

N_A: number of alleles per locus, H_O: observed heterozygosity, H_E: expected heterozygosity.

Table 2.3. Matrix of pairwise F_{ST} values (below diaganal) and probability (above diagonal) for three populations of the Japanese wood pigeon *Columba janthina*: Izu Islands (IZU), Okinawa Islands (OKN) and Ogasawara Islands (OGS).

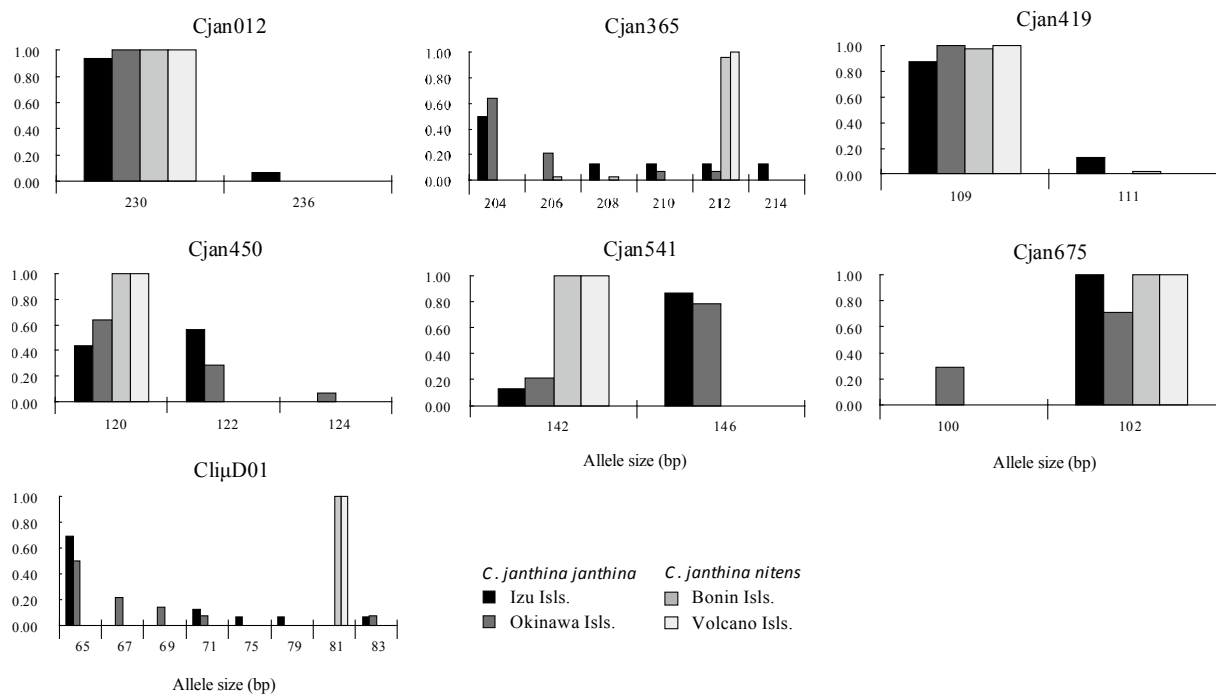|	IZU	OKN	OGS
IZU	—	0.032	0.001
OKN	0.063	—	0.001
OGS	0.806	0.715	—

Fig. 2.1. Allele frequency spectra at seven polymorphic microsatellite loci in two populations of the nominate Japanese wood pigeon *C. janthina janthina*; Izu Islands and Okinawa Islands, and two populations of the red-headed wood pigeon *C. janthina nitens*; Bonin Islands and Volcano Islands.
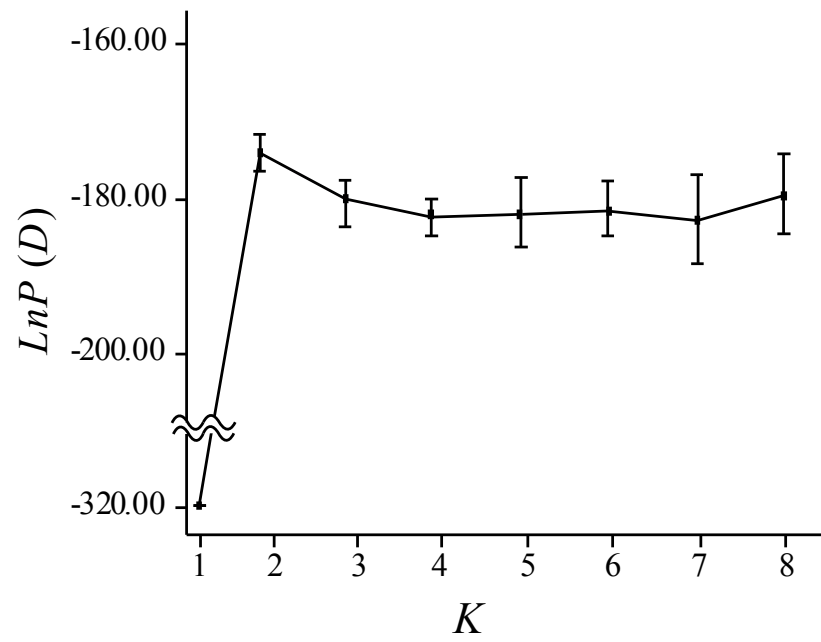
Fig. 2.2. Likelihood plot of STRUCTURE results. *Ln Pr* (*D*) is the log likelihood of each value of K, which is the number of simulated clusters. Where *ln Pr* (*D*) is maximized, K is the most likely. Black squares represent average values of *Ln Pr* (*D*), and vertical lines represent standard deviations.
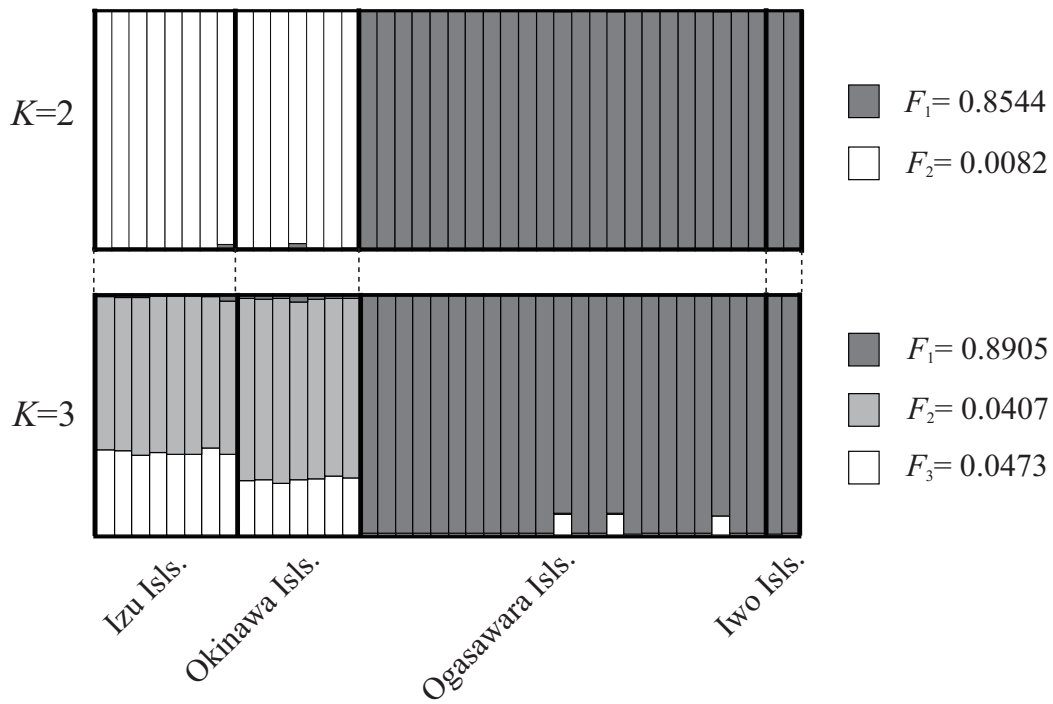
Fig. 2.3. Stacked bar chart from results of STRUCTURE analysis with maximum likelihood in $K =$ 2 and $K = 3$. Each individual is represented by a single bar, broken into K colored segments. The length of each segment represents proportional to the membership fraction in each cluster. Individuals are grouped by populations: Izu Islands and Okinawa Islands in the noinal Japanese wood pigeon *C. janthina janthina*; Bonin Islands and Volcano Islands in the red-headed wood pigeon *C. j. nitens*. The F values of each cluster are shown to the right.

Chapter 3

Genetic structure of wild and captive populations of the red-headed wood pigeon
Columba janthina nitens

Introduction

For the long-term conservation of endangered island species, the maintenance of genetic diversity within and outside the preferred habitat, and determining what are meaningful management units based on genetic structure in conjunction with habitat protection, are essential. In habitat conservation planning of endemic bird species on remote oceanic islands, each island population is often regarded as a separate evolutionarily significant unit. This may be because several species have evolved to avoid flying over water and exhibit strong genetic differentiation among island populations, for example Darwin's finches (Petren *et al*. 2005), the Galapagos Hawk *Buteo galapagoensis* (Bollmer *et al*. 2005) and the Bonin Islands White-eye *Apalopteron familiare* (Kawakami *et al*. 2008). However, some species such as Columbiformes are thought to be strong fliers and thus are able to move between islands (Gibbs *et al*. 2001). Thus, each discrete island Columbiform population should not necessarily be regarded as a separate evolutionarily significant unit due to the potential for weak genetic differentiation among island populations.

The habitat of the red-headed wood pigeon consists of two island groups about 150 km apart, the Bonin Islands (consisting of the islands of Mukojima, Chichijima and Hahajima) and the Volcano Islands (consisting of Kita-iwo-jima, Iwojima and Minami-Iwo-jima; Kanto Regional Forestry Office 2006, 2007, 2009; Fig. 3.1). Since the 19th century, the two largest Bonin Islands, Chichijima and Hahajima, have been inhabited by humans, and degradation of forest habitat and predation by feral cats are affecting the pigeon population on these islands (Horikoshi 2008, Kachi 2010, Kawakami 2010). Low genetic diversity in the population of red-headed wood pigeons on the Bonin Islands reported in previous studies (Seki *et al*. 2007; Chapter 2) may partly be due to this habitat loss and resulting population decrease. In contrast, since 1945, the degree of human disturbance has been lower in the Volcano Islands, with both Kita-Iwo-jima and Minami-Iwo-jima being uninhabited, and feral cats are not present on these islands. In the Volcano Islands, a more stable population with a higher genetic diversity than that observed on the Bonin Islands may remain. Recent observation of banded individuals of the Red-headed Wood Pigeon revealed frequent movement between the islands of the Bonin Island group, which are more than 50 km apart (e.g. Chichijima to Hahajima and Mukojima to Chichijima; Suzuki *et al*. 2006). It may also be possible for the red-headed wood pigeon to move between the Bonin and Volcano Islands, but this

is at present unconfirmed.

To prevent the extinction of the wild population, a captive-bred population of the red-headed wood pigeon was established in 2001. However, the present captive population (approximately 30 individuals) exhibits signs of inbreeding depression, as chicks have a low survival rate, in addition to the appearance of malformed individuals (Tokyo Zoological Park Society pers. comm.). This may be due to the small number of founder individuals, which consisted of only three individuals captured in Chichijima (Tokyo Zoological Park Society pers. comm.). Thus, analysis of the genetic characteristics of the captive breeding population is necessary in order to facilitate the selection and introduction of a suitable wild individual to the captive population.

To reveal genetic characteristics of the red-eheaded wood pigeon and design appropriate genetic management for it, several questions must be answered: 1) How much genetic diversity is present in each wild population, i.e. those present in the Bonin Islands and the Volcano Islands? 2) Has genetic differentiation between the Bonin Islands and the Volcano Islands occurred? 3) How should the genetic diversity of the captive population be managed? To answer these questions, I undertook genetic analyses using mitochondrial control region sequences and five polymorphic microsatellite loci.

Materials and methods

Sampling and DNA extraction

Sampling of wild populations was carried out at Chichijima and Hahajima in the Bonin Islands, and at Kita-Iwo-jima and Iwo-jima in the Volcano Islands between 2004 and 2010. In the captive population, blood or tissues stocked from 2001 at Ueno Zoo and Tama Zoo in Tokyo, Japan were used. The names of the populations are abbreviated to BON (Bonin Islands), VOL (Volcano Islands) and CAP (captive). In total 82 samples were collected. In the wild populations, 67 feather samples were collected from BON (n = 36) and VOL (n = 31), including 41 moulted feathers (Table 1). Feathers that had the following features were regarded as being from the red-headed wood pigeon (Gibbs *et al*. 2001): (1) dense plumulaceous portion, (2) sooty black colour, and (3) glossing body feathers with purple and green. In the Volcano Islands, most of the samples collected (29 of 31) were moulted feathers because of the difficulty in accessing and capturing birds during the limited opportunities available to undertake fieldwork. Several feathers that had fallen close to each other (< 2 m from each other) were regarded as being from the same individuals and used one of them for DNA extraction. However, even more than 2 m apart, some samples from the same individuals may have been collected unintentionally. In addition, 15 samples in the BON population

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were from a previous study by Seki *et al*. (2007). In the CAP population, I used samples from 15 individuals including the three founders captured in Chichijima. One of them was a chick born in Chichijima but the birthplaces of the two other individuals are unknown. The collected samples were stored at - 30 ° C before DNA extraction. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen) following the manufacturer' s protocol.

Mitochondrial analysis

A portion of the mtDNA control region was amplified using the primers KXH0 (5′ -TGTCCTATGTACTACAGTGCATCG-3′) and KXH5 (5′ -ATGGCCCTGACATAGGAACCAGAG-3′), designed by Seki (2006) and Seki *et al*. (2007). PCR amplification was carried out in a 30 L volume of the reaction mixture containing 5 ng of extracted DNA, 3 μL of 10×Ex Taq buffer, 2.4 μL of dNTP, 0.15 μL of Ex Taq (Takara) and 4 pmol of each primer pair. The PCR conditions were set according to the procedure of Seki *et al*. (2007), with an initial denaturation for 2 min at 94 ° C, 35 cycles of 94 ° C for 15 s, 52 ° C for 30 s and 72 ° C for 35 s, except in the first 10 cycles in which the annealing temperature was decreased by 1 ° C per cycle from 62 ° C to 53 ° C, with a final extension for 5 min at 72 ° C. PCR products were visualized on a 2.0% agarose gel to confirm unique amplification and product size. Prior to sequencing, the PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK). Cycle sequencing was performed using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the standard protocol. The cycle sequencing products were purified using Centri-Sep^{TM} spin columns and visualized using the ABI PRISM 310 Genetic Analyzer and Sequencing Analysis software (Applied Biosystems).

The sequences were aligned using CLUSTALW (Thompson *et al*. 1994). Some of the haplotype sequences from the BON population, which were previously reported by Seki *et al*. (2007), were included in the analysis. A haplotype network was constructed using TCS 1.2.1 (Clement *et al*. 2000) using the statistical parsimony method. In this analysis, I included four haplotypes found in the nominate subspecies of the Japanese wood pigeon and Stejneger' s wood pigeon *C. janthina stejnegerifrom* the data of Seki *et al*. (2007), in addition to the haplotypes recovered in the present study. The haplotypes Jn11 (Accession no. AB280807) and Jn19 (Accession no. AB280815) were the two most dominant haplotypes among the 25 haplotypes found in the nominate Japanese wood pigeon; Jn5 (Accession no. AB280801) and Jn8 (Accession no. AB280804) were the haplotypes found in the Stejneger' s wood pigeon.

The following analyses were performed using ARLEQUIN 3.0 (Excoffier *et al*. 2005). The numbers of haplotypes (N_H), haplotype diversity (h) and nucleotide diversity (p) in each population were calculated with their variances to quantify genetic diversity. The following analyses were performed only for the wild populations (BON and VOL). An analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) was performed to estimate the hierarchical genetic structure of each population. Genetic variances were partitioned between populations and within populations. The F_{ST} values of each pair of populations were calculated and their deviations from zero were tested to estimate the extent of genetic differentiation among populations. The software package LAMARC v2.1.3 (Kuhner 2006) was used to estimate the degree of population growth (G) based on the coalescent. The parameter G was estimated based on the current population mutation parameter $\theta_0 = 2N_0\mu$ (where N_0 is the current effective population size and μ is the mutation rate) in haploid individuals, assuming exponential population growth and expressed as $\theta_t = \theta_0 \exp(-Gt)$. The time t was measured backward in mutational units. Positive G values represent growing populations and negative values represent shrinking populations. I calculated the population growth rate using the mutation rate of the mitochondrial control region (0.2 substitutions per site per million years), according to Pérez-Tris *et al*. (2004). The migration rate (M) and number of immigrants per generation (N_m) were also estimated using LAMARC. The parameter M was calculated as $M = m/\mu$, where m is the immigration rate per generation and l is the mutation rate. I used the maximum-likelihood version with the default settings: 10 initial chains (10 000 steps), two final chains (200 000 steps) and the sampling of every 20th tree.

Microsatellite analysis

Genotypes of samples were determined at five polymorphic microsatellite loci (Cjan012, Cjan365, Cjan419, Cjan541 and Cjan675), which were originally developed by Chapter 2. PCR-amplification was carried out using a Qiagen Multiplex PCR kit (Qiagen). Each 5 μL volume of the reaction mixture contained 5 ng of extracted DNA, 3 μL of the 2× Multiplex PCR Master Mix, and 0.2 pmol of each primer pair. The PCR conditions were as follows: an initial denaturation for 15 min at 95 °C, 35 cycles of 94 °C for 30 s, 57 °C for 1.5 min, and 72 °C for 1 min, and a final step of 60 °C for 30 min. The sizes of the PCR products were measured using the ABI PRISM 3100 Genetic Analyzer, GeneScan, and Genotyper analysis software (Applied Biosystems).

The number of alleles per locus (N_A), allelic richness (R_S), expected (H_E) and observed heterozygosities (H_O), and the inbreeding coefficient (F_{IS}) were calculated to quantify genetic diversity. The number of alleles per locus and the expected and observed heterozygosities were

calculated using GENALEX 6 (Peakall & Smouse 2006), and the allelic richness and inbreeding coefficient were calculated using FSTAT 2.9.3 (Goudet 2001). The deviation of F_{IS} values from zero, which reflect deviation from Hardy–Weinberg equilibrium, and deviation from linkage equilibrium were tested using FSTAT. Individual-based clustering analysis was performed using the software STRUCTURE 2.2.3 (Pritchard *et al.* 2000) to estimate the genetic characteristics of each population on an individual level. In this method, individuals are used as the unit and assigned to the most likely genetic group (cluster). I used an admixture with LOCPRIOR implemented and allele frequency correlated models. Ten runs of K = 1–3 groups were carried out for 1 000 000 Markov chain Monte Carlo (MCMC) steps with a burn-in of 100 000 steps (10%). The appropriate value of K was chosen based on log likelihood. On the basis of the correlated allele frequency model, the amount of drift for each cluster from a common ancestral population (F values; Falush *et al.* 2003) was calculated. Genetic diversity (N_A, R_S, H_E, H_O, F_{IS} and deviation of F_{IS} from zero) was compared between moulted feather samples and individual identified samples to examine the effectiveness of moulted feather samples for the estimation of genetic diversity and the probability of repeat sampling. I could not estimate the deviation of F_{IS} from zero in individually identified samples in VOL because the sample size was small (n = 2).

The following analyses were performed only for the wild populations (BON and VOL). AMOVA was performed using GENALEX. The partition of genetic variance was the same as in the mitochondrial analysis. The R_{ST} values for each pair of populations were calculated and their deviations from zero were tested using GENALEX. The population growth and migration rate were estimated by LAMARC. To calculate the population growth rate, I used the typical microsatellite mutation rate of 10^{-3} to 10^{-4} per generation (Ellegren 2000, Schlötterer 2000). I used the Brownian model and other program settings were the same as in the mitochondrial analysis.

Results

Mitochondrial analysis

463 base pairs of sequence for all 82 samples of the red-headed wood pigeon were obtained. Five substitutions were observed and three haplotypes recovered (Nt1, Nt2 and Nt3; Table 3.2, Fig. 3.2): haplotypes Nt1 and Nt2 were also recovered by Seki *et al.* (2007); haplotype Nt3 is newly defined in this study. Nt2 was found only in moulted feather samples. The new sequence was deposited in the DDBJ nucleotide data bank (Accession no. AB698856). Most individuals were of the Nt1 haplotype and this haplotype is shared among all three sampled populations (Table 3.2, Fig. 3.2). In CAP, all the individuals were of the Nt1 haplotype. Thus, I only present data on the three

founders in Table 3.2 and Fig. 3.2. Nt2 was connected to Nt1 by four substitutions and was shared by BON and VOL. The Nt3 haplotype was observed in one sample from BON. Nt1 and Nt2 were connected to Jn11, the most dominant haplotype in the nominate Japanese wood pigeon (Seki *et al.* 2007), by 16 substitutions. Stejneger's wood pigeon shared all the haplotypes with the Japanese wood pigeon. This indicates genetic differentiation of the red-headed wood pigeon from the two other subspecies.

In the AMOVA results of sampled populations of the red-headed wood pigeon, based on F_{ST}, the entire genetic variance (100%) was observed among individuals within each population rather than among populations (Table 3.3). F_{ST} values among populations were not significantly larger than zero (Table 3.3). The results of the LAMARC analysis are shown in Table 3.4. The significantly positive *G*-value is indicative of historical population expansion of VOL. Because the 95% confidence interval included zero, the extent of population expansion of BON seems to have been limited. Migration rates were not significantly different between BON and VOL.

Microsatellite analysis

The estimators of genetic diversity in each population (N_A, R_S, H_O, H_E and F_{IS}) are shown in Table 3.5. In CAP, all loci were monomorphic and all the individuals exhibited the same genotype (Table 3.5, Fig. 3.3). In contrast, all loci were polymorphic in the wild populations sampled (BON and VOL). The loci Cjan012 and Cjan541 were polymorphic only in VOL, and Cjan675 was polymorphic only in BON. In BON and VOL, F_{IS} values were positive for all loci and significantly larger than zero. There was no evidence of linkage equilibrium among all pairs of loci. In the STRUCTURE analysis, the log likelihood was maximized at $K = 2$ groups. Individuals that have rare alleles were assigned to the cluster with the lower *F*-value in a higher proportion (Fig. 3.3). In total, 13 different genotypes were observed in VOL and seven genotypes in BON. In the AMOVA results, almost all the genetic variance was observed among individuals within each population (R_{ST}: 99%, Table 3.3). The R_{ST} value between populations was 0.014 and was not significantly different from zero (Table 3.3). The results of the LAMARC analysis (Table 3.4) revealed the *G*-value of BON to be significantly smaller than zero, indicating the apparent population decline of BON. In contrast, a population expansion for VOL was indicated by the significantly positive G-value. The migration rate from BON into VOL was significantly larger than that from VOL into BON.

To reduce the risk of repeat sampling of the moulted feather samples, I attempted to identify different individuals based on microsatellite genotypes. However, this proved unsuccessful

because of the critically low genetic diversity of the red-headed wood pigeon: 22 of 26 banded individuals exhibited the same genotype.

The comparison of genetic diversity between moulted feather samples and individually identified samples in each population is shown in Table 3.6. For both sample types recovered from BON, F_{IS} values were positive at all loci and significantly larger than zero. In VOL, the two individuals sampled exhibited the same genotype. Similar to BON, the moulted feather samples in VOL exhibited F_{IS} values significantly larger than zero.

Discussion

Taxonomic implication

The present study and several previous genetic studies indicated strong genetic divergence of the red-headed wood pigeon from the two other subspecies of Japanese wood pigeon (Seki & Takano 2005; Seki *et al*. 2007; Chapter 2). Sequence substitutions between the red-headed wood pigeon and the nominate Japanese wood pigeon in mitochondrial DNA were on average 1.4% divergent in the cytochrome-b region (Seki & Takano 2005) and 3.4% in the control region. These differences are slightly higher than that between the two sister species: the New Zealand pigeon *Hemiphaga novaeseelandiae* and the Chatham pigeon *Hemiphaga chathamensis* (1.3% in cytochrome-*b* region and 2.9% in control region; Goldberg *et al*. 2011). Considering the strong genetic differentiation and geographical isolation, the nominate Japanese wood pigeon and the red-headed wood pigeon may better to be regarded as separate species. However, further comparisons encompassing morphology and ecology are required to assess fully the species status of the red-headed wood pigeon.

Genetic diversity of the wild populations of the red-headed wood pigeon

The results of mitochondrial and microsatellite analysis indicate that the genetic diversity of the Red-headed wood pigeon is extremely low, consistent with the results of Seki *et al*. (2007) on the basis of mitochondrial data for the Bonin Islands population. In the mitochondrial data of the present study, the haplotype diversity (h = 0.12 in the wild population) is the lowest recorded with respect to other species of Columbidae, including the Puerto Rican plain pigeon *Patagioenas inornata wetmorei* (h = 0.48; Young & Allard 1997), the Dominican Republic plain pigeon *P. inornata inornata* (h = 0.59; Young & Allard 1997) and the pink pigeon *Nesoenas mayeri* of Mauritius (h = 0.45; Swinnerton *et al*. 2004). The genetic diversity at the microsatellite loci (N_A = 3.2, H_E = 0.12 in the wild populations) is similar to other island endemic threatened bird species

(Jamieson *et al*. 2006, Evans & Sheldon 2008), such as the Mariana crow *Corvus kubaryi* (N_A = 2.2, H_E = 0.24; Tarr & Fleischer 1999) and the takahe *Porphyrio hochstetteri* (N_A = 2.3, H_E = 0.40; Jamieson *et al*. 2006).

The low genetic diversity of the red-headed wood pigeon may reflect not only a recent bottleneck but also a founder event and/or the long term isolation of the island populations from the mainland. This is indicated by the results of the coalescence-based LAMARC analysis of mitochondrial data, which did not indicate population declines for either the BON or the VOL populations, and strong genetic divergence of the red-headed wood pigeon from the two other subspecies (Fig. 3.2). Indeed, the divergence time of the Bonin Island population from the nominate Japanese wood pigeon is 0.119–0.356 million years (Seki *et al*. 2007). Such isolation may be sufficient to cause genetic drift and reduce genetic diversity through the fixation of alleles. The extremely low level of heterozygosity in the microsatellite loci supports this scenario.

In contrast, the LAMARC analysis of the microsatellite data suggested a significant population decline in BON. This may be due to recent human disturbance and predation by feral cats in the Bonin Islands, because microsatellite data may reflect more recent population trends when compared with mitochondrial data due to the higher mutation rates seen in the microsatellite loci (Guichoux *et al*. 2011).

According to the results of molted feather samples, the VOL population exhibited a larger number of microsatellite genotypes (13) than did BON (7), possibly reflecting the presence of a larger population with higher genetic diversity. This seems to be due to the lack of predation pressure by feral cats in the Volcano Islands. Although the size of each island is small, favourable habitat conditions may be maintained in the Volcano Islands. In this study, moulted feathers played an important role as a source of material with which to estimate the genetic diversity of the VOL population. F_{IS} values significantly larger than zero were recovered in both the BON and the VOL populations. The same results were also recovered for both moulted feather samples and for individual identified samples in BON. Repeated sampling or genotyping errors are not likely to cause this result; it may instead be the result of occasional inbreeding within each island population, which is likely to occur given the small size of these populations. With careful strategies to avoid repeated sampling, the non-invasive collection of moulted feather samples can be a valuable source of genetic material for the study of endangered bird species, as well as from species or populations occupying habitats with difficult access.

Population genetic structure and migration between the Bonin Islands and the Volcano Islands

The results of AMOVA, F_{ST} and R_{ST} values suggest weak genetic differentiation between the BON and VOL populations. The LAMARC analysis indicates that the migration rate (estimated by the microsatellite data) from BON to VOL is higher than that from VOL to BON, despite the BON population exhibiting a trend consistent with population decline. Thus, I suggest that the weak genetic differentiation is due to immigration from BON into VOL, and is not due to population expansion. This result may reflect the recent habitat conditions of the Bonin Islands. Individuals in the BON population may move into VOL to avoid human disturbance and predation by feral cats, despite the relatively large distance between the two island groups (approximately 150 km).

More generally, the weak genetic differentiation between the two island groups reflects the movement capacity of Columbiformes. The results of the present study are similar to those of a study of the Galapagos dove *Zenaida galapagoensis*, a species with weak genetic differentiation among island populations separated by up to 100 km (Alarcon *et al*. 2006). These results together with the present study highlight an important aspect of the evolution of Columbiformes on islands; that is, that many species have maintained strong flying capacity even in remote islands habitat, whereas other island endemic terrestrial birds are known to avoid flying over water (Bollmer *et al*. 2005; Petren *et al*. 2005; Kawakami *et al*. 2008). Alarcon *et al*. (2006) suggested that the Galapagos dove might move among islands to track food resources and suitable environmental conditions. The higher genetic diversity of the less disturbed VOL population and higher migration rate from BON to VOL of the red-headed wood pigeon may support this hypothesis. The red-headed wood pigeon may thus have survived past catastrophic events, environmental changes and human disturbance by moving between the two island groups. Thus, consideration of inter-island movement is important for future habitat conservation planning.

Genetic characteristics of captive population and management issues

The CAP population, in which the most common mitochondrial haplotypes and microsatellite alleles among the five loci were completely fixed, is indicative of strong inbreeding. If the present inbred individuals of the CAP population are reintroduced to the wild, where environmental factors can increase mortality, they may not be able to survive. Further, even in captive conditions, long-term survival may be difficult due to inbreeding depression, which is found in the present captive population (Tokyo Zoological Park Society pers. comm.).

Reduced fitness of an inbred captive population can be overcome by introduction of a new genetic variant (Frankham *et al*. 2010). The captive breeding populations of the endangered pink pigeon, in which inbreeding depression has partly occurred, have maintained their genetic

diversity by exchanging genetically variable individuals among zoos and free-living populations, and have successfully increased their population size (Swinnerton *et al.* 2004). Genetic diversity of the CAP population of the red-headed wood pigeon should be restored via the introduction of wild individuals that have rare haplotypes or alleles. However, although introduction of individuals from BON or VOL to CAP may contribute to the restoration of genetic diversity of CAP, it may reduce genetic diversity and promote haplotype and/or allele fixation in the BON or VOL populations because the two populations also exhibit small poplation size and low genetic diversity. Indeed, in the present study, rare mitochondrial haplotypes were found in only five samples, and each rare microsatellite allele was found in only one to six samples, out of 67 samples from the wild population, in which the inferred number of individuals is 100 (Horikoshi 2008). The selection of individuals for introduction to the captive population and the timing of reintroduction into the wild population after the recovery of genetic diversity in CAP should be considered carefully.

This study revealed that the genetic diversity of the red-headed wood pigeon is in serious peril. Conservation of the remnant genetic diversity to the maximum extent possible within and outside the wild habitat is required. This study also suggests the occurrence of interisland movement between the Bonin and the Volcano Islands. The populations in the Bonin and the Volcano Islands should be considered to constitute a single evolutionarily significant unit.

Table 3.1. The number of individuals constituting the two sample types of the Red-headed Wood Pigeon: individual identified samples and moulted feather samples, as well as an overview of the four sites sampled. Numbers in parentheses are sample sizes from Seki *et al*. (2007).

	Bonin Islands		Volcano Islands	
	Chichijima I.	Hahajima I.	Kita-iwojima	Iwojima I.
Individual identified samples	17 (1)	7 (6)	2	0
Molted feather samples	5 (1)	7 (7)	28	1
Human	inhabited	inhabited	uninhabited	uninhabited
Cat	inhabited	inhabited	uninhabited	uninhabited
Latitide	27°04'	26°39'	25°26'	24°45'
Longitude	142°13'	142°9'	141°16'	141°17'
Size (km^2)	23.8	20.3	5.6	23.1

Table 3.2. Genetic characteristics of the sampled populations: Bonin Islands (BON), Volcano Islands (VOL), total wild caught individuals (All in Wild) and the Captive population (CAP), based on mitochondrial control region sequences. Variables: frequency (F_H) of three observed haplotypes, number of haplotypes (N_H), haplotype diversity (h) with variance, nucleotide diversity (p) with variance.

	F_H			N_H	$h \pm Vh$	$\pi \pm V\pi$
	Nt1	Nt2	Nt3			
BON ($n = 36$)	34	1	1	3	0.1126 ± 0.0718	0.001082 ± 0.001049
VOL ($n = 31$)	28	3	0	2	0.1806 ± 0.0860	0.001561 ± 0.001333
All in Wild ($n = 67$)	62	4	1	3	0.1153 ± 0.0523	0.001049 ± 0.001015
CAP ($n = 3$)	3	0	0	1	0.0000 ± 0,0000	0.000000 ± 0.000000

Table 3.3. The results of AMOVA for two wild populations, the Bonin Islands and the Volcano Islands, based on FST values for mitochondrial data and RST for microsatellite data.

	df	SS	Var.	%	
Mitochondrial analysis					
F_{ST}					
Between populations	1	0.13	-0.005	0%	$F_{ST} = -0.002$ ($P = 0.639$)
Within populations	65	19.37	0.298	100%	
Microsatellite analysis					
R_{ST}					
Between populations	1	6.58	0.049	1%	$R_{ST} = 0.014$ ($P = 0.156$)
Within populations	132	438.67	3.323	99%	

df, degrees of freedom; SS, sum of squares; Var, genetic variability; %, percentage of variability.

Table 3.4. The results of LAMARC analyses based on mitochondrial and microsatellite data for the Bonin Islands (BON) and the Volcano Islands (VOL).

	BON	VOL
Mitochondrial analysis		
θ	0.0027 (0.0019 - 0.0037)	0.0011 (0.0007 - 0.0018)
M	1606.452 (904.869 - 2600.909)	2070.771 (742.538 - 4450.683)
Nm	1.068	0.577
G	933.421 (-715.564 - 2208.908)	4269.767 (2868.473 - 5057.132)
G_R	0.0001	0.0005
Microsatellite analysis		
θ	0.0930 (0.0630 - 0.1300)	0.300 (0.226 - 0.419)
M	24.768 (20.502 - 29.340)	108.520 (91.428 - 126.575)
Nm	0.578	8.146
G	-236.6 (-332.121 - -170.001)	7.797 (6.098 - 9.790)
G_R	-0.2133	0.008 - 0.0008

The population mutation parameter (θ), migration rate into the presented population from the other (M), number of immigrants per generation (Nm), population growth parameter (G), population growth rate calculated using the intermediate mutation rates (Gr). The numbers in parenthesis shows the 95% confidence interval.

Table 3.5. Genetic diversity of the three populations sampled at five microsatellite loci: Bonin Islands (BON), Volcano Islands (VOL), all wild caught samples collectively (All in Wild) and Captive individuals (CAP) based on microsatellite data.

	Microsatellite Loci					
	Cjan12	Cjan365	Cjan419	Cjan541	Cjan675	Average
All in Wild (*n*=67)						
N_A	2	6	2	4	2	3.20
R_S	1.67	3.62	1.86	2.07	1.52	2.15
H_O	0.03	0.09	0.03	0.08	0.02	0.04*
H_E	0.07	0.29	0.11	0.09	0.05	0.12
F_{IS}	0.49	0.70*	0.74*	0.14	0.66	0.60*
OGA (*n*=36)						
N_A	1	5	2	1	2	2.20
R_S	1.00	3.17	1.92	1.00	1.78	1.77
H_O	0.00	0.09	0.03	0.00	0.03	0.02
H_E	0.00	0.20	0.13	0.00	0.08	0.08
F_{IS}	N.A.	0.55*	0.79	N.A.	0.66	0.65*
IWO (*n*=31)						
N_A	2	5	2	4	1	2.80
R_S	1.98	4.18	1.86	2.82	1.00	2.37
H_O	0.09	0.08	0.03	0.16	0.00	0.07
H_E	0.16	0.40	0.10	0.18	0.00	0.17
F_{IS}	0.47	0.80*	0.66	0.12	N.A.	0.58*
CAP (*n*=15)						
N_A	1	1	1	1	1	1.00
R_S	1.00	1.00	1.00	1.00	1.00	1.00
H_O	0.00	0.00	0.00	0.00	0.00	0.00
H_E	0.00	0.00	0.00	0.00	0.00	0.00
F_{IS}	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

The number of samples (*n*), the number of alleles per locus (N_A), allelic richness (R_S), the observed (H_O) and expected (H_E) heterozygosities and Inbreeding coefficient (F_{IS}) for five microsatellite loci in three populations. The asterisks show significant deviation of F_{IS} values from zero.

Table 3.6. Comparison of genetic diversity between sample types in the Bonin Islands (BON) and Volcano Islands (VOL) based on microsatellite data.

	Microsatellite loci					
	Cjan12	Cjan365	Cjan419	Cjan541	Cjan675	Average
Molted feather samples						
BON ($n = 12$)						
N_A	1	3	2	1	2	1.80
R_S	1.00	1.63	1.54	1.00	1.17	1.27
H_O	0.00	0.11	0.00	0.00	0.08	0.04
H_E	0.00	0.29	0.28	0.00	0.08	0.13
F_{IS}	N.A.	0.65	1.00	N.A.	0.00	0.72*
VOL ($n = 29$)						
N_A	2	5	2	4	1	2.80
R_S	1.34	1.92	1.20	1.39	1.00	1.37
H_O	0.10	0.09*	0.04	0.17	0.00	0.08
H_E	0.17	0.43	0.10	0.19	0.00	0.18
F_{IS}	0.47	0.80	0.66	0.11	N.A.	0.58*
Individual identified samples						
BON ($n = 24$)						
N_A	1	4	2	1	2	2.00
R_S	1.00	1.33	1.08	1.00	1.16	1.11
H_O	0.00	0.08	0.04	0.00	0.00	0.03
H_E	0.00	0.16	0.04	0.00	0.08	0.06
F_{IS}	N.A.	0.49	0.00	N.A.	1.00	0.57*
VOL ($n = 2$)						
N_A	1	1	1	1	1	1.00
R_S	1	1	1	1	1	1.00
H_O	0.00	0.00	0.00	0.00	0.00	0.00
H_E	0.00	0.00	0.00	0.00	0.00	0.00
F_{IS}	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

* $P < 0.05$

The number of samples (n), the number of alleles per locus (N_A), allelic richness (R_S), the observed (H_O) and expected (H_E) heterozygosities and Inbreeding coefficient (F_{IS}) for five loci. The asterisks show significant deviation of F_{IS} values from zero.
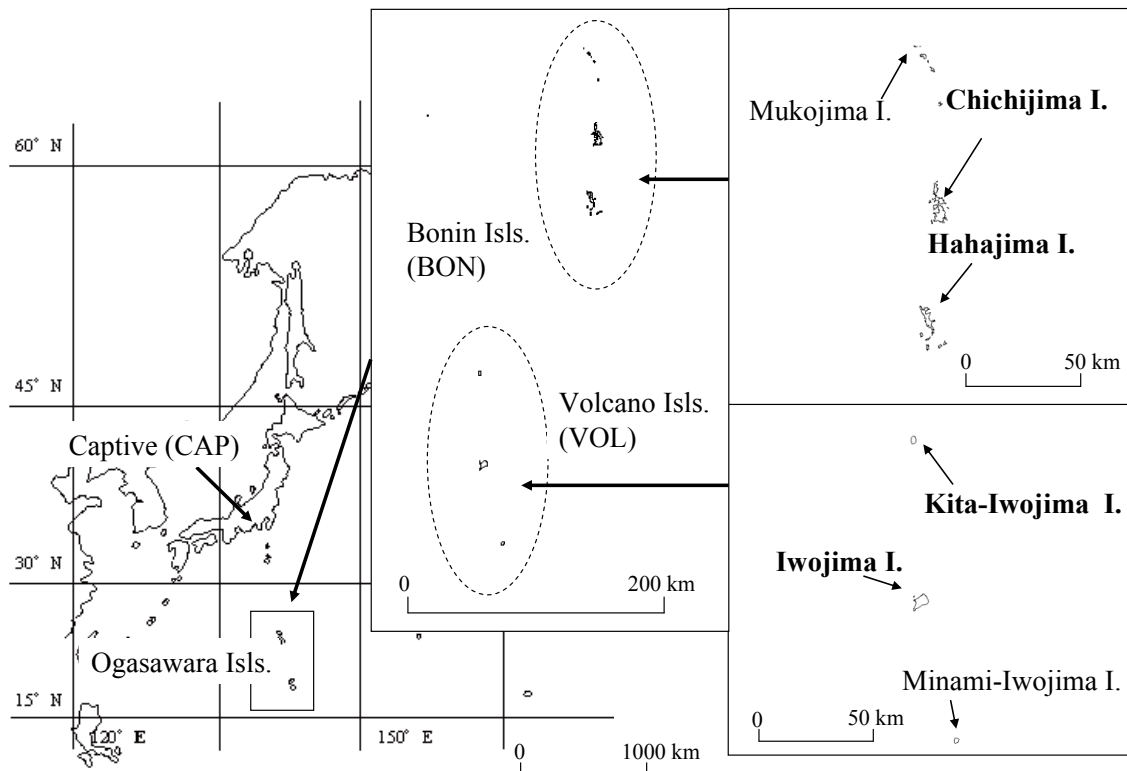
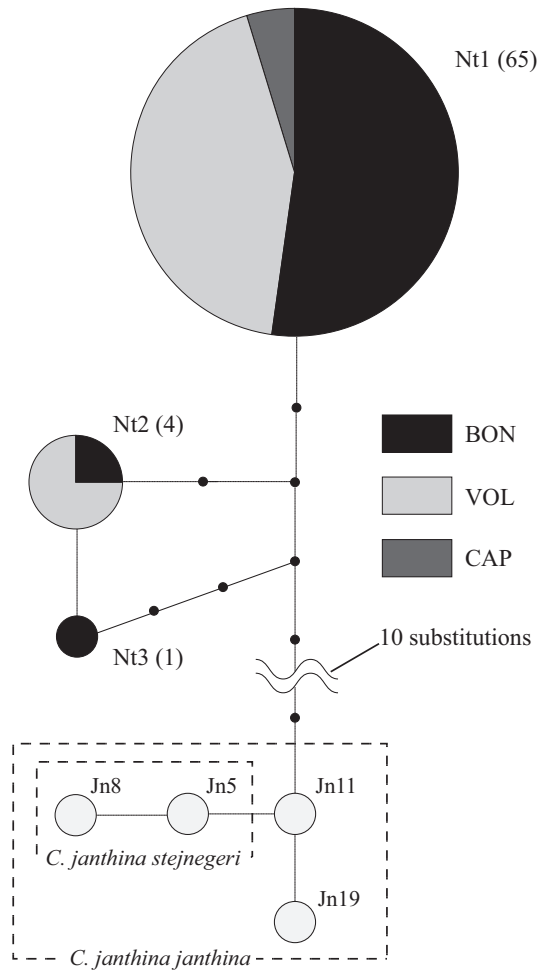**Fig. 3.1.** Distribution of red-headed wood pigeons. Sampling locations are in bold.

Fig. 3.2. Statistical parsimony network of mitochondrial control region haplotypes. Haplotypes Nt1, Nt2 and Nt3 were recovered in this study. Haplotypes Jn11 and Jn19 were the two most dominant haplotypes in the nominate Japanese wood pigeon recovered by Seki *et al*. (2007). Haplotypes Jn5 and Jn8 were the only two haplotypes recovered in Stejneger's wood pigeon. The size of each circle reflects the frequency of each haplotype. Black dots reflect missing or extinct haplotypes in the network that were not present in the samples collected. Lines connecting haplotypes represent a single mutational step.
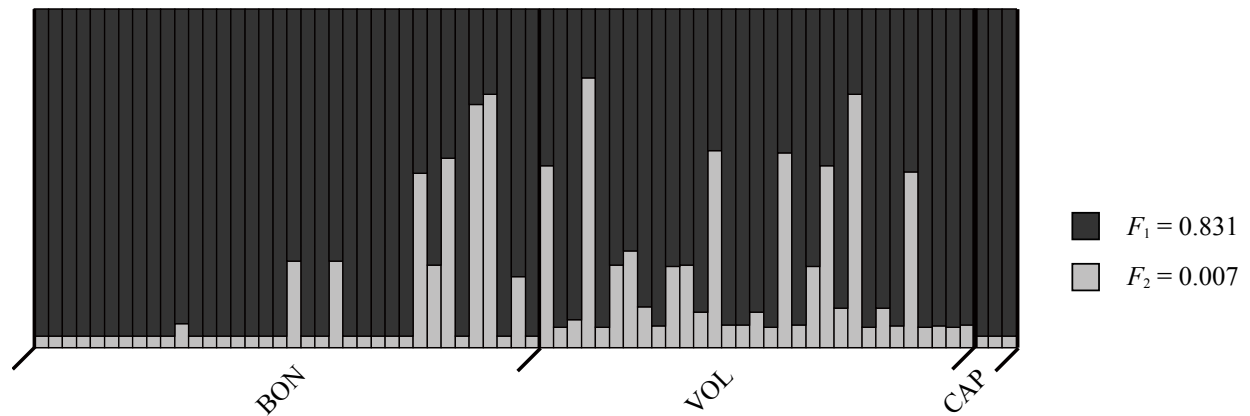
Fig. 3.3. Stacked bar chart depicting the results of STRUCTURE analysis with maximum-likelihood centred on $K = 2$ groups: each individual is represented by a single bar, broken into K coloured segments. The height of each segment (y-axis) is proportional to the membership fraction in each cluster. Individuals are grouped by population on the x-axis: BON, VOL and CAP.

Chapter 4

Application of High-throughput sequencing in detection of food plants for the
red-headed wood pigeon *Columba janthina nitens*

Introduction

  Understanding the diet of endangeres species is essential for appropriate nature restoration in their habitat. The red-headed wood pigeon is thought to be a seedeater (Takano *et al.* 1995) based on direct observations (feeding on *Elaeocarpus photiniifolius*, *Neolitsea boninensis*, *Melia azedarach* and *Ficus microcarpa*, etc. were recorded), similar to other *Columba* species (Gibbs *et al.* 2001). To maintain the foraging habitat of the pigeon, a forest must maintain its species diversity and supply seeds throughout the seasons (Kawakami 2008). However, the native forest of the Ogasawara Islands has been destroyed because of human settlements in the 19th century and World WarII (Kachi 2010; Kawakami 2010). Furthermore, several introduced plants, such as *Bischofia javanica*, *Ficus microcarpa*, *Leucaena glauca* and *Morus australis*, have expanded their populations and generate a large abundance of fruit (Ecological Society of Japan 2002; Toyoda 2003; Hata *et al.* 2010; Tanaka *et al.* 2010). The red-headed wood pigeon may consume introduced species, and these species will be eradicated during nature restoration projects in the near future.

  Fecal analysis via a DNA barcoding technique depends on a taxon identification system using a standardized DNA region (Hebert & Gregory 2005). This method may be an effective noninvasive approach for studying the diet of the red-headed wood pigeon. Thus far, dietary analyses of the pigeon have been carried out mainly by direct observation (Kanto Regional Forestry Office 2005, 2006; Kanto Regional Environmental Office 2011) and micro-histological analysis (Shibazaki & Hoshi 2006). However, the existing information on the diet of this species is fragmented because of the difficulty of continuous observation and species identification of digested food items in pigeon stomachs. Via the DNA barcoding method, high throughput sequencing (HTS) for fecal DNA using a next-generation sequencer (NGS) can provide large amounts of sequence data without a cloning step. Thus, the time and cost of analysis are reduced, and more detailed animal diet information can be collected (Valentini *et al.* 2009a,b; Pompanon *et al.* 2012). Although food DNA in fecal samples is often degraded, the universal short barcode primer *trn*L g-h (Taberlet *et al.* 2007) can effectively amplify plant DNA, as shown in previous herbivore diet analyses (Valentini *et al.* 2009a; Kowalczyk *et al.* 2011; Raye' *et al.* 2011).

The higher resolution resulting from the HTS approach in comparison with traditional micro-histological analysis (MHA) has been noted in several previous studies (Pompanon *et al*. 2012). However, there have been few attempts to compare the two methods using the same sample set. Soininen *et al*. (2009) suggested a comparison of the high-resolution HTS approach with a micro-histological analysis for the dietary analysis of stomach contents. The resolution difference between the two methods is unknown for fecal samples, in which food items are more degraded than stomach contents.

The aims of this Chapter were to estimate the usefulness of the HTS approach for fecal analysis of the red-headed wood pigeon, to compare the resulting resolution to that of the micro-histological analysis using identical sample sets, and to determine the current dietary composition (regarding whether this diet includes introduced species) of this pigeon.

Materials and methods

Figure 4.1 provides a general outline for the dietary analysis of the red-headed wood pigeons analyzed in this study. I conducted both HTS and micro-histological analysis using the same sample set and compared the results of the two methods. After determining the diet of the pigeons, I performed additional analyses using the HTS data.

Development of the trn*L reference database*

Samples from 222 seed plant species were collected from Chichijima and Hahajima in the Ogasawara Islands. 93% of the woody seed plants (118 species) described in Toyoda (2003) were covered. Other woody and herbaceous plants were collected by searching around the pigeon habitat. The samples were stored at -30°C before the DNA extraction, which was performed using a DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. The universal primer pair c–d (Taberlet *et al*. 1991) was used for PCR amplification of whole chloroplast *trn*L (UAA) introns (c: 5′-CGAAATCGGT AGACGCTACG-3′; d: 5′-GGGGA TAGAGGGACTTGAA C-3′). Each 10 μL of the total reaction mixture volume contained 5 ng of extracted DNA, 5 μL of 2 9 Multiplex PCR Master Mix, and 0.2 μmol/L of each primer pair. The PCR conditions were as follows: denaturation for 15 min at 95°C; 35 cycles of 30 s at 94°C, 1.5 min at 57°C, and 1 min at 72°C; and a final 10 min extension at 72°C. Cycle sequencing was performed with a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the standard protocol. The cycle sequencing products were visualized by the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). To build the reference database, P6 loop sequences (Taberlet *et al*. 2007) were

extracted from the entire *trn*L (UAA) intron sequence. The *Passiflora edulis* sequence was retrieved from GenBank and added to the database.

Fecal sampling and DNA extraction

48 fecal samples from the red-headed wood pigeons were collected in Chichijima and Hahajima from September 2009 to May 2011. Sampling on Chichijima was carried out continuously from September 2010 to May 2011. Of these samples, 35 were collected after directly observing pigeon elimination. The remaining 13 were collected from areas around nests and roosts, considering size, shape, and contents (mainly crushed seeds). The collected feces were stored at -30°C before DNA extraction. DNA was extracted from 20 mg of fecal dry weight using a DNeasy Plant Mini Kit (Qiagen). The remainder of each sample (more than half) was used for micro-histological analysis.

PCR amplification and sequencing from fecal DNA

To confirm that the 13 samples collected around the nests and roosts belonged to the red-headed wood pigeon, I amplified a portion of the mitochondrial COI region sequences (290 bp) with the primer pair PSF (5′-AAC CCGGCACCCTTCTAGGAGACGA-3′) and PSR (5′-ACCAGCTAGAGGTGGATAAACAGTT-3′). The primers were designed to avoid amplifying the mitochondrial DNA of other native bird species in the Ogasawara Islands (e.g., the brown-eared bulbul *Hypsipetes amaurotis* and scaly thrush *Zoothera dauma*; The Ornithological Society of Japan 2012) besides the red-headed wood pigeon. Each 10 lL of total reaction mixture volume contained 5 ng of extracted DNA, 0.05 lL of ExTaq (Takara), 1 lL of 10 9 ExTaq Buffer, 0.8 lL of dNTPs, and 0.12 lmol/L per primer pair. The PCR conditions were as follows: denaturation for 2 min at 94°C; 40 cycles of 15 s at 94°C, 30 s at 52°C, and 1 min at 72°C; and a final cycle of 5 min at 72°C. Ichecked for the presence of a PCR product of suitable length by electrophoresis on a 1.5% agarose gel.

The universal primer pair g (5′-GGGCAATCCTGAGCCAA-3′) and h (5′-CCATTGAGTCTCTGCACCTATC-3′; Taberlet *et al*. 2007) was used to amplify the *trn*L P6 loop. The forward primer was tagged with a multiplex identifier (MID, Roche Diagnostic) to identify the resulting sequences from each sample. PCR amplification was conducted using a Qiagen Multiplex PCR kit (Qiagen). Each 25 lL of total reaction mixture volume contained 20 ng of extracted DNA, 15 lL of 2 9 Multiplex PCR Master Mix, and 0.2 lmol/L of each primer pair. The PCR conditions were as follows: denaturation for 15 min at 95°C; 45 cycles of 30 s at 94°C, 1.5 min

at 57°C, and 1 min at 72°C; and a final cycle of 10 min at 72°C. The

PCR products were purified using exo/SAP (exonuclease I and shrimp alkaline phosphatase, Takara and Promega, Madison, WI) and a High Pure PCR Products Purification Kit (Roche Diagnostic, Basel, Switzerland) and then quantified using a NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The length of the PCR products was checked using a High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA) to confirm that the targeted region sequences were correctly amplified and that short fragments were completely excluded during the purification process. Next, the PCR products were mixed such that approximately the same number of molecules from each fecal sample was included in each mix (Pegard *et al.* 2009). Large-scale pyrosequencing was carried out on a 454 GS Junior Sequencer (Roche Diagnostic) following the manufacturer's instructions.

DNA barcoding

The resulting sequences were separated into each sample by MID tags. The sequences in each sample were assembled using the CAP Contig Assembly Program in BioEdit software (Hall 1999) for a 98% match. The short sequences (less than 40 bp) and contigs, which included fewer than four sequences in each sample, were excluded from DNA barcoding. The DNA barcoding for each contig was carried out using a local BLAST in BioEdit. Idetected a plant within the P6 loop database that exhibited the highest score with low e-values (<1.0e–25) for each contig. If two or more taxa are assigned the same score for a given contig, the contig was assigned to the lowest taxonomic level that included both taxa.

Micro-histological analysis

47 fecal samples remaining after DNA extractions were used for micro-histological analysis. All of one sample was used for DNA barcoding because of its small amount. I followed the modified method of Shibazaki & Hoshi (2006). The fecal samples were washed with water and filtered using a 0.5 mm screen. Any fragments remaining on the screen were examined under the microscope at 20 9 magnification. Fragment identification was performed using reference photos, and seed specimens collected in the Ogasawara Islands.

Data analysis

The discrimination rate for the P6 loop database at the three taxonomic levels (species, genus, and family) was calculated, as described by Raye′ *et al*. (2011). The discrimination rate for

the species level (Rs) was calculated by dividing the number of unique sequences by the number of species in the database. The discrimination rate at the genus level (Rg) was calculated by dividing the number of genera with one or more unique sequences by the total number of genera. The rate of discrimination for the species in each family (Rf) was calculated by dividing the number of unique sequences per family by the number of species in the family.

After DNA barcoding, I calculated the frequency of sequence reads (F_R) and the frequency of the presence (F_P) of each food item. To compare the resolution of HTS and, the numbers of detected food plants per sample and frequency of each food item (number of samples in which the specific food item was observed divided by the total number of samples) were calculated. Each food plant was classified as native or introduced. Using the data from HTS analysis, the following analyses were also performed. To compare the diets of pigeons on Chichijima and Hahajima, I performed island-based (samples were pooled for each island) and sample-based analyses. In the island-based analysis, the frequency of reads and the relative frequency of presence data for each plant taxa were calculated. The diet composition differences were tested by Pearson's chi-square tests. In the sample-based analysis, nonmetric multidimensional scaling (NMDS) on the Chao similarity index (Chao *et al*. 2004) and an analysis of similarities (ANOSIM, Clarke 1993) on a Chao matrix (Doi & Okamura 2011) were performed by the vegan package in R (Oksanen *et al*. 2010) using the number of reads and the presence/absence data of each plant taxa. I also compared the diet composition and diversity (number of reads vs. presence/absence of each plant taxa) measurements in these analyses.

Results

Plant discrimination using the trn*L P6 loop database*

The *trn*L P6 loop was sequenced for 222 plant species belonging to 175 genera and 76 families (accession numbers: AB817341-AB817701). Of the 222 sequences of the P6 loop database, 167 unique sequences were found. The sequence fragment lengths ranged from 66 to 148 bp. The discrimination rate at the species level, or Rs, was 75%, and the Rg, or genus level, was 89%. All families were identified using the P6 loop. Discrimination rates within families ranged from 14% to 100% (Table 4.1 and Table 4.2).

Resolution comparisons between HTS and micro-histological analysis

All 13 samples collected around the nests and roosts were confirmed as originating from the red-headed wood pigeon by amplification using the primers PSF and PSR. The sequencing of 48

fecal samples yielded 35,666 reads, corresponding to an average of 743 ± 338 (SD) bp and ranging from 157 to 1747. From the results of the DNA barcoding with the P6 loop database, 44 plant taxa were detected from 32,291 reads (approximately 90% of the total sequences). Most of the reads that were not assigned to a specific plant taxa in the P6 loop database (3225 reads) were short or had a low frequency. Of the 32,391 reads, more than 70% belonged to *Morus australis* (36.58%) and Gr. Lauraceae1 (34.94%; Table 4.3), indicating their high consumption by pigeons and/or the high PCR amplification efficiency of these plants. The number of detected food plants per sample in the HTS (6.73 ± 2.70) was significantly greater than that obtained from the micro-histological analysis (1.42 ± 0.62, *P* < 0.01, *t*-test). When comparing food items detected by HTS and micro-histological analysis, Lauraceae (identified as *Neolitsea aurata* in the micro-histological analysis), *Fagara boninsimae*, and *Planchonella* were frequently observed using both methods with similar frequencies of presence (Table 4.3). However, plants such as *Morus australis*, *Ficus*, and Poaceae were frequently observed using DNA barcoding only. Although they were identified only at low frequencies (observed in one sample each), *Distylium lepidotum* and *Wikstroemia pseudoretusa* were found only by using micro-histological analysis. In addition, the shells of snails (Pulmonata) and arthropods, which were not targeted by the DNA barcoding, were observed by micro-histological analysis.

Difference of diet composition between the islands, as estimated by HTS approach

There were significant differences between the dietary compositions of pigeons on Chichijima and those on Hahajima in both of the estimations (frequency of reads and relative frequency of presence data; Fig. 5). The results of the similarity analysis by NMDS and ANOSIM demonstrated the significant differences between the diet compositions on Chichijima and Hahajima based on the presence/ absence data for each plant taxa in each sample (*R* = 0.2236, *P* < 0.05, Fig. 4.2), and there was no significant difference according to the analysis based on the number of reads.

Discussion

Applicability of HTS approach for diet analysis of the red-headed wood pigeon

The HTS method via *trn*L P6 loops produced a much higher resolution than micro-histological analysis, as reported by some previous studies (Soininen *et al*. 2009; Valentini *et al*. 2009a,b; Raye′ *et al*. 2011). The comprehensiveness of the P6 loop database used in the present study appeared to be sufficient given the high rate of food plant identification from the pigeon feces. This finding may be explained by the high endemism and low species richness of the oceanic island

flora, which may improve the comprehensiveness of the local sequence database and can be a major limitation for food identification using the HTS approach (Valentini *et al*. 2009a). The results of the present study indicate the clear advantage of HTS over micro-histological analysis, the latter of which has a bias favoring easily identified food items (Soininen *et al*. 2009). Lauraceae, *Fagara boninsimae*, and *Planchonella*, which were frequently observed using both methods, have hard and large seeds. Thus, these plants may be easy to identify using micro-histological analysis because larger fragments may remain in feces, even after being crushed in a pigeon's stomach. In contrast, *Morus australis*, *Ficus*, and Poaceae, which were frequently observed only by HTS, have soft and small seeds and thus may be difficult to identify using micro-histological analysis; furthermore, most of these plants are introduced species. Although the P6 loop database could not identify the introduced plant *Ficus microcarpa* or other native *Ficus* species, the frequently observed sequence for Gr. Ficus1 may belong to the introduced *Ficus microcarpa*, considering the observation records of pigeon feeding and the great fruiting abundance of this plant in the Ogasawara Islands (Ecological Society of Japan 2002; Shibazaki & Hoshi 2006). These results indicate the underestimation of the pigeons' use of introduced plants in previous microhistological analyses (Shibazaki & Hoshi 2006).

However, some limitations of the HTS approach have also been presented. The first problem with this technique is its low discrimination rate within the P6 loop database for specific families (Lauraceae and Moraceae at 14% and 20%, respectively, Table 4.2). In the case of Lauraceae, *Neolitsea aurata* was detected using micro-histological analysis, although this species cannot be distinguished from the other Lauraceae species using P6 loop sequences. The performance of HTS approach will be improved by hierarchical barcoding (Moszczynska *et al*. 2009) using family-specific barcoding markers. The second problem is that some food items identified by micro-histological analysis were not observed using HTS (Table 4.3). In the case of the plants *Distylium lepidotum* and *Wikstroemia pseudoretusa*, there were no sequence mismatches in the primer-binding sites, which may have caused the strong amplification efficiency bias (Deagle *et al*. 2007). Thus, the inability to detect these plants may have been caused by a failure in the DNA extraction rather than a failure in the PCR amplification. These bias errors can occur when a DNA extraction sample is obtained from a single scat, particularly for dietary analysis of large mammals (Deagle *et al*. 2005). Although this bias may be small in the fecal analysis of small bird scat, the appropriate extraction strategies (e.g., subsampling and blending, Deagle *et al*. 2009; Kowalczyk *et al*. 2011) should be considered. The observation of snails and arthropods in the micro-histological analysis indicates that the pigeons ate small animals; thus, not only plants were targeted in the HTS

method. Diet analysis using animal-targeted markers (e.g. COI, Meusnier *et al*. 2008) may provide more detailed information regarding pigeon ecology.

Composition differences in pigeon diets between the islands

Comparing the pigeon food compositions based on the number of reads and presence data indicated significant differences between Chichijima and Hahajima (Figs. 4.3, 4.4A). Although *Morus australis* and *Ficus* dominated both of the islands, the results may indicate different patterns of food selection by the pigeons on each of the islands. The native Lauraceae species was observed only in Chichijima at a high frequency. This finding may be explained by its significant fruiting abundance in the forest of Chichijima (Toyoda 2003), indicating its importance as major native food resources on the island. In Hahajima, the read percentage of introduced *Morus australis* was much larger than that of Chichijima. These data may indicate the greater usage of this introduced species by the pigeons in Hahajima than those of Chichijima. *Leucaena glauca*, which exhibited the third highest frequency in terms of both read numbers and presence in Hahajima, is also an expanding introduced species in the Ogasawara Islands (Hata *et al*. 2010). This plant lives on the forest edge (Toyoda 2003), which is not the preferred forest habitat of the pigeon. Pigeons on Hahajima may eat Leucaena *glauca* to make up for a lack of food resources in the forest area. It is also interesting that the food composition varied among Hahajima samples (Fig. 4.4), despite the small sample size and restricted sampling site (five samples were collected in February around the same roost). The red-headed wood pigeons in Hahajima are more generalist than those of Chichijima because of a lack of food resources. Estimations of food resource availability and temporal possible diet variation using large number of samples on each island may reveal differences in pigeon feeding strategies between these locations.

I also compared data from the number of reads and the relative frequency of presence for each plant taxa. The reliability of quantitative diet analysis using the sequence count data has been discussed in several previous studies (e.g. Deagle *et al*. 2009, 2010; Soininen *et al*. 2009; Valentini *et al*. 2009a; Raye′ *et al*. 2011; Pompanon *et al*. 2012; Shehzad *et al*. 2012). The PCR efficiency and resulting number of sequences from each food item can be affected by several biological (e.g., number of DNA copies in a unit mass of tissues, digestion process) and technical factors (e.g. different sequence lengths among species, mismatches in primer-binding sites; Pompanon *et al*. 2012). Indeed, one of the shortest plant sequences dominates the dietary analysis of the alpine chamois *Rupicapra rupicapra* in Raye′ *et al*. (2011). Thus, Pompanon *et al*. (2012) recommended using the read count data for measuring spatial or temporal variations in the diet but not for absolute

diet quantification. In the case of the present study, comparing the proportion of each plant read number between the two islands can be reliable (e.g., sequence proportion of *Morus australis*). The sequence lengths of the Gr. Lauraceae1 (87 bp, only found in Chichijima), *Morus australis* (89 bp), and Gr. Ficus1 (89 bp), which exhibited high frequency read numbers, were not much shorter than those of the other detected food plants (ranging 72- 121 bp, average 89.9 bp), and their frequencies of presence were also high. Thus, pigeons may have actually eaten these plants in large amounts, as reported by Deagle *et al*. (2010) regarding the accuracy of quantitative diet data from the number of reads. However, the pigeon consumption of some plants with low PCR efficiency may be underestimated in the analysis based on the number of reads. For example, the low sequence proportion of Gr. Poaceae2 in Chichijima (4%) may be explained by its low PCR efficiency, despite its high frequency (75%) (Fig. 4.3A and C). No significant difference between Chichijima and Hahajima in the NMDS analysis was based on the number of reads, which may reflect the dominance of specific plant sequences (e.g., *Morus australis* and Gr. Ficus1) throughout the samples and the underestimation of some plants with low PCR efficiency. Another possibility is that the small number of reads for some plants may indicate secondary predation (Sheppard *et al*. 2005), such as plants eaten by snails or other invertebrate herbivores eaten by pigeons, or that the number of reads reflects the biomass to some extent (Yoccoz *et al*. 2012). This is an important issue to be considered in estimating pigeon food preferences. Given that appropriate methods to correct various biases in quantitative analysis have never been presented, it is advisable to use not only the number of reads but also the frequency of presence to estimate dietary composition and diversity, as in Soininen *et al*. (2009), Raye' *et al*. (2011), and Shehzad *et al*. (2012).

In this study, a diet analysis using DNA barcoding provided a high-resolution identification of food plants and clearly overcame the bias of traditional micro-histological analysis. The results of the DNA barcoding indicated frequent consumption of introduced species, rather than only native species, by the pigeons. The rapid eradication of some introduced species without restoration of the native seed plants may reduce available food resources for the red-headed wood pigeon. Although some existing technical problems must still be solved (e.g. the discrimination rate of the P6 loop database, sampling strategy), the HTS approach will contribute to a better understanding of foraging ecology of the red-headed wood pigeon, including its dependence on introduced species and appropriate nature restoration planning for the Ogasawara Islands.

Table 4.1. Species groups for the taxa whose sequences could not be assigned to spcies level in the P6-loop database and their associated families.

Group	Species	Family
Gr. Agavaceae1	*Dracaena spp.*	Agavaceae
	Sansevieria nilotica	Agavaceae
Gr. Asteraceae1	*Vernonia cinerea var. parviflora*	Asteraceae
	Youngia japonica	Asteraceae
	Youngia japonica	Asteraceae
Gr. Callicarpa1	*Callicarpa glabra*	Verbenaceae
	Callicarpa nishimurae	Verbenaceae
	Callicarpa subpubescens	Verbenaceae
Gr. Citrus1	*Citrus limon*	Rutaceae
	Citrus maxima	Rutaceae
	Citrus sinensis	Rutaceae
Gr. Cyperaceae1	*Cyperus kyllingia*	Cyperaceae
	Schoenus brevifolius	Cyperaceae
Gr. Cyperaceae2	*Carex hattoriana*	Cyperaceae
	Carex oahuensis	Cyperaceae
	Carex toyoshimae	Cyperaceae
Gr. Cyperaceae3	*Cyperus cyperoides*	Cyperaceae
	Cyperus microiria	Cyperaceae
Gr. Diospyros1	*Diospyros ferrea*	Ebenaceae
	Diospyros kaki	Ebenaceae
Gr. Ficus1	*Ficus benghalensis*	Moraceae
	Ficus boninsimae	Moraceae
	Ficus elastica	Moraceae
	Ficus iidaiana	Moraceae
	Ficus microcarpa	Moraceae
	Ficus nishimurae	Moraceae
	Ficus religiosa	Moraceae
	Ficus superba	Moraceae

Gr. Hedyotis1	*Hedyotis grayi*	Rubiaceae
	Hedyotis mexicana	Rubiaceae
Gr. Hibiscus1	*Hibiscus glanber*	Malvaceae
	Hibiscus tiliaceus	Malvaceae
Gr. Ilex1	*Ilex beecheyi*	Aquifoliaceae
	Ilex matanoana	Aquifoliaceae
Gr. Lauraceae1	*Cinnamomum pseudo-pedunculatum*	Lauraceae
	Machilus kobu	Lauraceae
	Machilus pseudokobu	Lauraceae
	Neolitsea aurata	Lauraceae
	Neolitsea boninensis	Lauraceae
Gr. Melastoma1	*Melastoma tetramerum*	Melastomataceae
	Melastoma tetramerum var. pentapetalum	Melastomataceae
Gr. Miscanthus1	*Miscanthus boninensis*	Poaceae
	Miscanthus condensatus	Poaceae
Gr. Morinda1	*Morinda boninensis*	Rubiaceae
	Morinda umbellata var. hahazimensis	Rubiaceae
Gr. Myrsine1	*Myrsine maximowiczii*	Myrsinaceae
	Myrsine okabeana	Myrsinaceae
Gr. Myrtaceae1	*Psidium cattleianum*	Myrtaceae
	Syzygium	Myrtaceae
	Syzygium buxifolium	Myrtaceae
	Syzygium cleyeraefolium	Myrtaceae
Gr. Palmae1	*Arenga engleri*	Palmae
	Caryota urens	Palmae
	Chrysalidocarpus lutescens	Palmae
	Mascarena verschaffeltii	Palmae
	Ptychosperma elegans	Palmae
	Veitchia merrillii	Palmae
Gr. Palmae2	*Clinostigma savoryana*	Palmae
	Neodypsis decaryi	Palmae
Gr. Pittosporum1	*Pittosporum chichijimense*	Pittosporaceae
	Pittosporum parvifolium	Pittosporaceae

	Pittosporum beecheyi	Pittosporaceae
	Pittosporum boninense	Pittosporaceae
Gr. Planchonella1	*Planchonella boninensis*	Sapotaceae
	Planchonella obovata	Sapotaceae
	Planchonella obovata var. dubia	Sapotaceae
Gr. Poaceae1	*Isachne* spp.	Poaceae
	Oplismenus compositus	Poaceae
	Paspalum urvillei	Poaceae
Gr. Poaceae2	*Arundo donax*	Poaceae
	Echinochloa crusgalli	Poaceae
	Paspalum notatum	Poaceae
	Paspalum scrobiculatum	Poaceae
Gr. Rosaceae1	*Osteomeles boninensis*	Rosaceae
	Osteomeles lanata	Rosaceae
	Photinia wrightiana	Rosaceae
Gr. Rutaceae1	*Boninia crassifolia*	Rutaceae
	Boninia glabra	Rutaceae
	Boninia grisea	Rutaceae
	Evodia nishimurae	Rutaceae
Gr. Stachyuraceae1	*Stachyrus macrocarpus*	Stachyuraceae
	Stachyrus macrocarpus var. *prunifolius*	Stachyuraceae
Gr. Symplocos1	*Symplocos kawakamii*	Symplocaceae
	Symplocos pergracilis	Symplocaceae

Table 4.2. Species discrimination rate (Rf) for 38 families that have more than one species in the Ogasawara Islands database.

Family	N species	N sequences	Rf (%)
Agavaceae	3	2	67
Anacardiaceae	2	2	100
Apocynaceae	2	2	100
Aquifoliaceae	3	1	33
Asteraceae	9	8	89
Caprifoliaceae	2	2	100
Cyperaceae	12	8	67
Ebenaceae	2	1	33
Euphorbiaceae	5	5	100
Fabaceae	11	11	100
Lauraceae	7	1	14
Liliaceae	2	2	100
Malvaceae	3	2	50
Melastomataceae	2	1	50
Moraceae	9	2	20
Myrsinaceae	3	2	67
Myrtaceae	6	3	50
Nyctaginaceae	3	3	100
Oleaceae	3	3	100
Palmae	9	3	33
Pandanaceae	2	2	100
Piperaceae	2	2	100
Pittosporaceae	4	1	25
Poaceae	24	17	71
Ranunculaceae	2	2	100
Rosaceae	4	2	50
Rubiaceae	13	12	92
Rutaceae	9	4	44

Sapindaceae	2	2	100
Sapotaceae	3	1	33
Solanaceae	3	3	100
Stachyuraceae	2	1	50
Symplocaceae	2	1	50
Theaceae	2	2	100
Ulmaceae	2	2	100
Urticaceae	2	2	100
Verbenaceae	7	5	75
Zingiberaceae	3	3	100

Table 4.3 List of the lowest taxonomic levels in the diet of the red-headed wood pigeon and its presence in high-throughput sequencing (HTS) and micro-histological analysis (MHA).

Food items	Native/Introduced	N reads	F_R (%)	F_P (%) HTS	F_P (%) MHA
Plants					
Morus australis	Introduced	11810	36.58	95.83	0.00
Gr. Lauraceae1	Native	11280	34.94	70.83	59.57
Gr. Ficus1	Native/Introduced	3902	12.08	93.75	4.00
Fagara boninsimae	Native	1328	4.11	33.33	31.91
Gr. Poaceae2	Introduced	922	2.86	66.67	0.00
Ligustrum micranthum	Native	514	1.59	6.25	0.00
Leucaena glauca	Introduced	335	1.04	12.50	0.00
Sambucus javanica	Introduced	297	0.92	20.83	0.00
Carex hattoriana	Native	231	0.72	2.08	0.00
Ardisia sieboldii	Native	226	0.70	18.75	0.00
Trema orientalis	Native	212	0.66	4.17	0.00
Gr. Planchonella1	Native	158	0.49	12.50	12.77
Poaceae	Introduced	115	0.36	31.25	0.00
Moraceae	Native/Introduced	114	0.35	14.58	0.00
Gr. Palmae2	Native/Introduced	90	0.28	31.25	0.00
Elaeocarpus photiniifolius	Native	87	0.27	10.42	2.00
Lantana camara	Introduced	77	0.24	18.75	6.38
Buxus liukiuensis	Introduced	72	0.22	6.25	0.00
Rhaphiolepis wrightiana	Native	67	0.21	8.33	0.00
Solanum nigrum	Introduced	63	0.20	2.08	0.00
Juniperus taxifolia	Native	62	0.19	4.17	0.00
Calaphyllum inophyllum	Native	51	0.16	10.42	0.00
Sonchus oleraceus	Introduced	43	0.13	4.17	0.00
Schima mertensiana	Native	41	0.13	10.42	0.00
Eurya boninensis	Native	23	0.07	6.25	0.00
Celtis boninensis	Native	21	0.07	10.42	0.00
Derris elliptica	Introduced	20	0.06	6.25	0.00

Hedyotis grayi	Native	18	0.06	2.08	0.00
Rutaceae	Native	17	0.05	4.17	0.00
Paederia scandens	Introduced	13	0.04	4.17	0.00
Livistona chinensis	Native	10	0.03	4.17	2.00
Gr. Myrtaceae1	Native/Introduced	10	0.03	2.08	0.00
Scaevola taccada	Native	9	0.03	4.17	0.00
Carica papaya	Native	9	0.03	2.08	0.00
Pinus luchuensis	Introduced	7	0.02	2.08	0.00
Terminalia catappa	Native	7	0.02	2.08	0.00
Osmanthus insularis	Native	6	0.02	4.17	0.00
Pennisetum purpureum	Introduced	5	0.02	2.08	0.00
Drypetes integerrima	Native	4	0.01	2.08	0.00
Elaeagnus rotundata	Native	4	0.01	2.08	0.00
Melastoma tetramerum	Native	4	0.01	2.08	0.00
Paspalum conjugatum	Introduced	4	0.01	2.08	0.00
Distylium lepidotum	Native	0	0.00	0.00	2.00
Wikstroemia pseudoretusa	Native	0	0.00	0.00	2.00
Animals					
Plumonata	Native/Introduced	-		-	0.15
Arthropoda	Native/Introduced	-		-	0.02

Frequency of sequence reads (F_R), frequency of the presence (F_P)
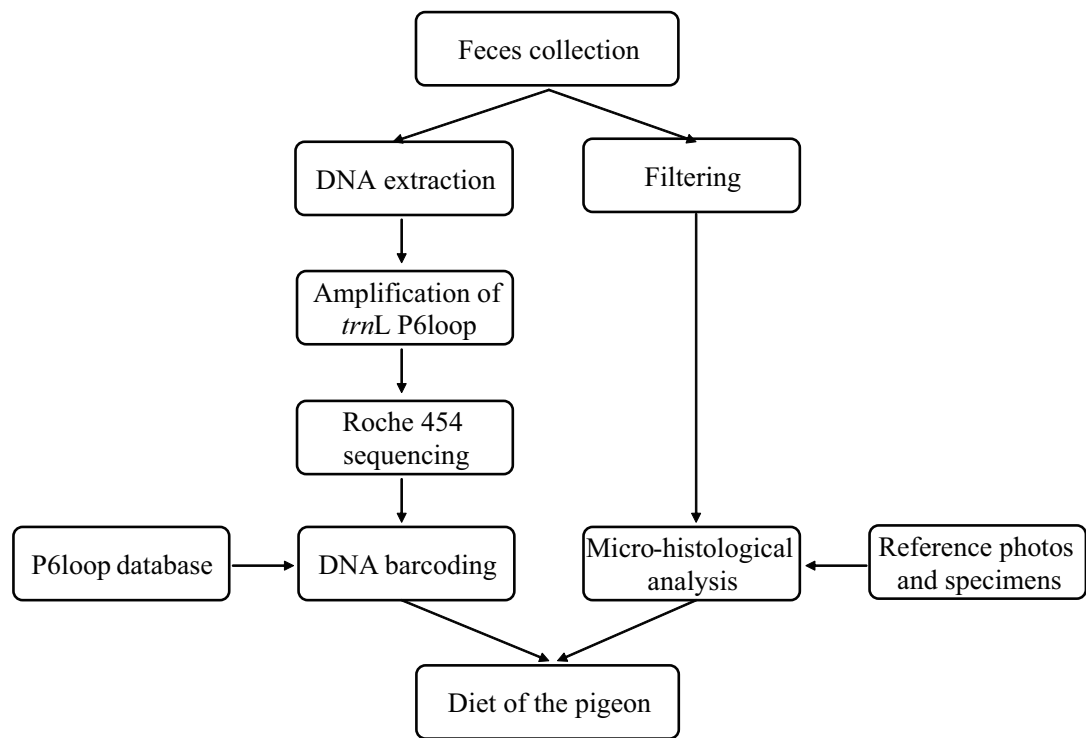
Fig. 4.1. General outline of the diet analysis for the red-headed wood pigeon.

Fig. 4.2. Comparison of diet composition between Chichijima Island and Hahajima Island based on the frequency of reads (A and B) and the relative frequency of presence data (C and D) for each food plant.
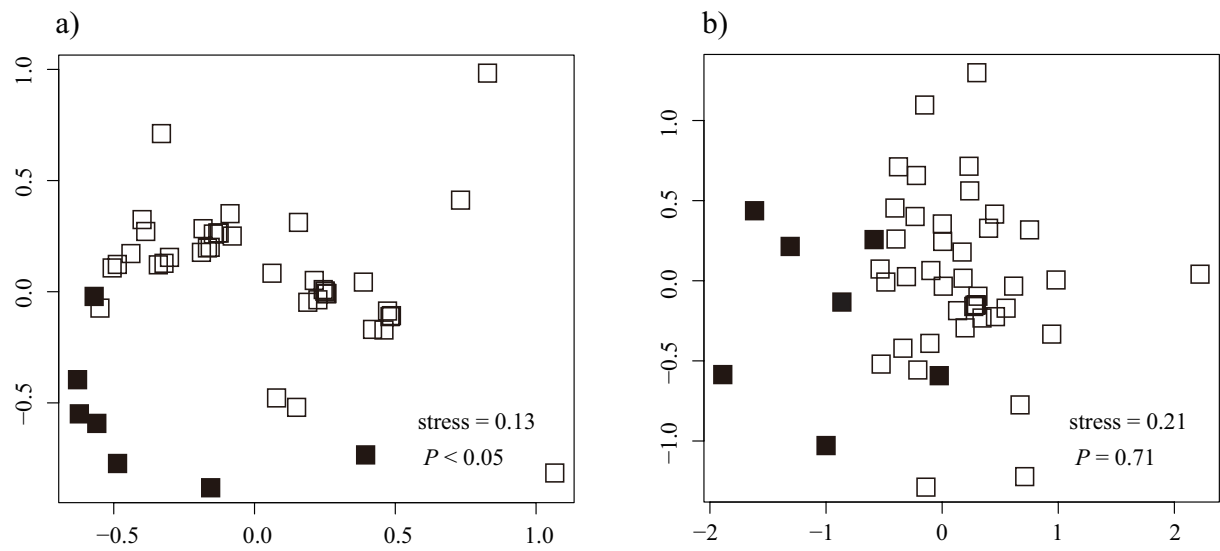
Fig. 4.3. The results of the similarity analysis with NMDS and ANOSIM, based on presence/ absence (A) and number of reads (B). White squares designate samples from Chichijima, and black squares designate samples from Hahajima.

Chapter 5

Foraging strategy of the red-headed wood pigeon *Columba janthina nitens* in highly remote and seriously disturbed oceanic island habitat

Introduction

Changes in the seasonality of available fruits due to deforestation and invasion by introduced plants may have an effect on the diet of forest frugivorous birds (e.g., Williams & Karl 1996). Particularly, the diet of island pigeons, which live in restricted island habitats and depend on fruits for most of their food resources (Gibbs *et al*. 2001), may be directly and continuously affected by the changing availability of fruits. Previous studies on the diet of island pigeons indicated pigeons' consumption of various fruits, which reflects food resource availability in forest habitats (Crome 1975; Frith *et al*. 1976; Powlesland *et al*. 1997; Oliveira *et al*. 2002; McConkey *et al*. 2005; Emeny *et al*. 2009). For island pigeons, plant diversity in forest habitats that can provide various fruits throughout the year seems to be essential. In disturbed forest habitats, pigeons may use introduced plants to complement native food resources. Alternatively, they may use introduced plants selectively when introduced plants are valuable in quantity and/or quality. Furthermore, their degree of dependence on introduced plants may differ among habitats and/or seasons. To conduct appropriate nature restoration in island pigeon habitats, it is important to understand seasonal food composition, food selection patterns, and the pigeons' dependence on introduced plants as their food resources.

Like to other *Columba* species (Gibbs et al. 2001), the red-headed wood pigeon mainly eat seeds (Shibazaki & Hoshi 2006; Chapter 4) and requires forests woth adequate species diversity and availability of edible seeds throughout the seasons. The forest habitat of the red-headed wood pigeon in the Ogasawara Islands has been seriously disturbed by human settlements in the 19th century and World War II (Kachi 2010; Kawakami 2010). Several introduced plants, including *Bischofia javanica*, *Ficus microcarpa*, *Leucaena glauca* and *Morus australis*, have expanded their populations and generate a large abundance of fruit (Ecological Society of Japan 2002; Toyoda 2003; Hata *et al*. 2010; Tanaka *et al*. 2010), causing changes in the seasonality of available fruits for pigeons. The results of the study in Chapter 4 indicated the effectiveness of HTS based analysis in detection of food plants of the pigeon comparing the result of micro-histological analysis, and also indicated pigeons' consumption of introduced plants (e.g., *Morus australis* and *Ficus*), which are planned to be eradicated. However, the importance of introduced plants on the pigeons' diet and the impact of introduced plant eradication on the pigeons' population are not clear. The results of

Chapter 4 remain preliminary due to a small sample size (N = 48), a limited sampling season (40 samples were collected in fall and winter), and a lack of data on food quantity and quality in the pigeons' habitat. To conduct an appropriate nature restoration considering the foraging condition of the red-headed wood pigeon, it is essential to understand the detailed food composition and food selectivity of the pigeon. In particular, whether the pigeons selectively use introduced fruit or just consume what is available should be clarified.

The aims of this study are 1) to estimate seasonal and inter-island differences in the food composition of the red-headed wood pigeon, 2) to detect the factors which influence the food selection of the pigeon, and 3) to reveal the value of introduced plants for the pigeon. I conducted HTS based diet analysis of the red-headed wood pigeon, which effectiveness was described in Chapter 4, for the fecal samples continuously collected over two years. Simultaneously, I conducted a survey of food resource availability in the pigeons' habitat, and nutrient analyses of major fruits, including those of native and introduced plants.

Materials and methods

Study sites

This study has been conducted in two subtropical oceanic islands, Chichijima (27˚ 04'N, 142˚13'E) and Hahajima (26˚ 39'N, 142˚09'E) in the Ogasawara Islands (Fig. 5.1). The vegetation of these islands can be classified into six types according to Shimizu (1992): *Elaeocarpus - Ardisia* mesic forest, *Pinus - Schima* mesic forest, *Dendrocacalia - Fatsia* mesic scrub, *Distylium - Schima* dry forest, *Distylium - Pouteria* dry scrub, and *Rhaphiolepis - Livistona* dry forest. *Dendrocacalia - Fatsia* mesic scrub occurs on the mesic ridges above 400 m on Hahajima, and *Distylium - Schima* dry forest is mainly distributed in Chichijima. The red-headed wood pigeon has been observed in all of the above vegetation types, but rarely in *Dendrocacalia - Fatsia* mesic scrub (Kanto Regional Environmental Office 2011). In addition, this pigeon also occurs in secondary forest and open areas near settlements, which exhibit a high density of introduced plants, such as *Bischofia javanica*, *Leucaena glauca* and *Ficus microcarpa* (Kanto Regional Environmental Office 2012).

Sampling and DNA extraction

In total, 683 fecal samples were collected in Chichijima and Hahajima in the Ogasawara Islands (Fig. 1). Sampling was carried out every month from June 2011 to May 2013 in Chichijima (average per month: 20.25 ± 13.38; range: 1 - 48) and from June 2012 to May 2013 in Hahajima (average per month: 12.91 ± 6.85; range: 4 - 24). Of these samples, 179 were collected after directly

observing pigeon elimination. The remaining 504 feces were collected from areas around nests and roosts and were identified by size, shape, and contents (mainly crushed seeds) per Chapter 4. The collected feces were stored at -30°C or in silica gel before DNA extraction. DNA was extracted from 20 mg of fecal dry weight using DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands).

General outline for diet analysis of the red-headed wood pigeon

Fig. 5.2 provides a general outline for the diet analysis of the red-headed wood pigeon. After feces sampling, the samples that were collected without observing pigeons' elimination were confirmed to be those of the pigeons using species-specific primers. For the samples that were confirmed to be those of the red-headed wood pigeon, a part of the chloroplast DNA: P6 loop region was amplified and sequenced by the HTS approach. DNA barcoding of obtained sequences was conducted using a local reference database of P6 loop including 230 seed plants that live in Chichijima and Hahajima, which was developed by (Chapter 4) and eight species were newly added in this study (accession numbers: AB985678 - AB985685). PCR using family/genus specific primers was performed on the samples in which Lauraceae and/or *Ficus* plant groups were found in P6 loop barcoding. This is because the discrimination rate of P6 loop sequences were low for these plant taxa (14% in Lauraceae and 20% in Moraceae (*Ficus*); Chapter 4), even though the red-headed wood pigeons may frequently consume them. Species or genus of Lauraceae and species (groups) of *Ficus* were identified by fragment analysis of each PCR product.

Amplification check of the fecal DNA using species-specific primers

A portion of the mitochondrial COI region sequences (190 bp) were amplified to confirm that the 504 samples collected around the nests and roosts belonged to the red-headed wood pigeon. The primer pair PSF and PSR2 (Table 5.1) was used for the amplification. PSF was designed by Chapter 4 and PSR2 was newly designed in this chapter. PCR amplification was conducted using a Qiagen Multiplex PCR kit (Qiagen). Each 5 μL of the total reaction mixture volume contained 20 ng of extracted DNA, 3 μL of 2 x Multiplex PCR Master Mix, and 0.2 μmol/L of each primer pair. The PCR conditions were as follows: denaturation for 15 min at 95°C; 40 cycles of 30 s at 94°C, 1.5 min at 57°C, and 1 min at 72°C; and a final cycle of 10 min at 72°C. The presence of a PCR product of suitable length was checked by electrophoresis on a 1.5% agarose gel.

High-throughput sequencing of the P6 loop

The universal primer pair *g* and *h* (Table 5.1; Taberlet *et al*. 2007) was used to amplify

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the chloroplast *trn*L P6 loop from the 628 feces DNA that were confirmed to be those of the red-headed wood pigeon. The forward primer was tagged with a multiplex identifier (MID; Hamady *et al*. 2008) to identify the resulting sequences from each sample. The concentration of the reaction mixture (10 μ L volume in this case) and PCR condition were the same as the COI amplifications. The PCR products were purified using exo/SAP (exonuclease I and shrimp alkaline phosphatase, Takara, Shiga, Japan and Promega, Madison, WI, USA) and a High Pure PCR Products Purification Kit (Roche Diagnostic, Basel, Switzerland). As a unit for a single sequence run, the 96 purified PCR products were mixed. The length of each unit of PCR products was checked using a High-Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA) to confirm that the targeted region sequences were correctly amplified and that short fragments were completely excluded during the purification process. A High Sensitivity DNA Kit (Agilent Technologies) was also used to calculate the concentration and number of molecules of PCR products. The sequencing was carried out on the Ion Torrent Personal Genome Machine (PGM) system with the Ion PGM^{TM} 200 Sequencing Kit and the Ion 314^{TM} Chip (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions.

Sequence filtering and DNA barcoding

The software Claident (Tanabe & Toju 2013) was used to separate sequences into each sample by MID tags and to filter sequences as follows. Sequences that met more than one of the following conditions were not used in DNA barcoding: 1) sequence length < 50 bp, 2) mean quality value < 20 and 3) minimum quality value of MID tags < 20. Trimming of low-quality 3$'$ tails was also conducted until three continuous sequences with a minimum quality value of 20 appeared. The DNA barcoding for each filtered sequence was carried out using a local BLAST in the software BioEdit (Hall 1999). I detected a food plant by referring to the partly modified P6 loop database created in Chapter 4. The match of each sequence to reference sequences was estimated based on having a BLASTN e-value less than 1e-25.

Fragment analysis using a family/genus specific primer

In this study, family/genus specific barcoding primers for Gr. Lauraceae1 and Gr. Ficus1, defined in Chapter 4, were newly designed using the chloroplast *trn*H – *psb*A region sequences (LAF-LAR and FIS-FIR; Table 5.1). The discriminated plant species or groups by *trn*H – *psb*A primers are shown in Table 5.2. Each plant (group) could be distinguished by its fragment size; thus, I conducted fragment analysis for the samples that included Gr. Lauraceae1 and/or Gr. Ficus1

sequences of P6 loop to identify the food plants at a lower taxonomic levels. PCR amplification was conducted using a Qiagen Multiplex PCR kit (Qiagen). Each 5 μL of the total reaction mixture volume contained 20 ng of extracted DNA, 2.5 μL of 2 x Multiplex PCR Master Mix, 0.01 μ mol/L of forward primer, 0.2 μ mol/L of reverse primer, and 0.1 μ mol/L of M13 (fluorescently labeled; Boutin-Ganache *et al*. 2001) primer. The PCR conditions were the same as those of the COI amplifications. The PCR product size was determined using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and GeneMapper software (Applied Biosystems). When there was no amplified *trn*H-*psb*A fragment, the number of read and presence of P6 loop sequences were used for data analyses (described as Gr. Lauraceae1 or Gr. Ficus1). When multiple peaks corresponding to *trn*H-*psb*A fragments were found (e.g., Gr. Machilus1 and Gr. Neolitseae1), presence data of each *trn*H-*psb*A group were separately included in the analyses, but the number of reads was included as single P6 loop groups (e.g., Gr. Lauraceae1).

Estimation of food resource availability

To estimate food resource availability in the pigeon's habitat, a fruiting census was conducted every month in Chichijima (from June 2011 to May 2013) and Hahajima (from June 2012 to May 2013). Census lines (6 km in Chichijima and 5.5 km in Hahajima, respectively; Fig. 5.1) were established in the pigeons' habitat to include typical native forests described in the *Study sites* section, secondary forests, and open areas. Fruiting abundances of all the fruiting trees within 5 m of the census lines were recorded using a three point score based on the count of fruit per tree: 1 pt. (< 100), 5 pt.(100 – 1000), and 10 pt. ($\geqq$ 1000). In the case of *Carica papaya*, *Passiflora edulis* and Fabaceae species, which have many seeds in a single fruit, scoring was weighted by the approximate number of seeds in a single fruit of each species because pigeons may eat the seeds of fallen fruits of these plants.

Nutritional analyses of fruits

In the pigeons' habitat in Chichijima and Hahajima, fleshy fruits of 37 fruiting plants that exhibit moderate to large fruiting abundance in a fruiting census and/or have records of pigeons' consumption (Shibazaki & Hoshi 2006; Chapter 4) were collected (40 g – 130 g per species). The collected fruits were weighted and stored at -30°C before the experiments were conducted. Fruit samples were thawed and dried at 40°C for 7 days. After weighting, fruits were ground and put into plastic tubes, and kept in a desiccator. Crude lipid was measured as diethylether extract by the Soxhlet method. Crude protein was estimated using the Kjeldahl procedure for total nitrogen and

multiplying by 6.25. Crude ash was measured by ashing at 550°C. These three estimations were carried out as described by Hasegawa (1993). Neutral detergent fiber (NDF) was estimated following the methods of van Soest *et al*. (1991). After quantification, proportions of each nutrient component in raw weight were calculated and used for statistical analyses.

Statistical analyses

The number of reads, frequency of sequence reads (F_R) and frequency of the presence (F_P) of each food plant were calculated for samples from Chichijima and Hahajima. Each food plant was classified as native or introduced. The diet composition of pigeons in Chichijima and Hahajima for each month was estimated by the relative frequency of detected plant taxa in each sample. The detected food plants that hold less than five reads in each sample were not included in the analyses to avoid the misidentification of plants by sequencing errors. The relative frequency of introduced plants for each month was also calculated. Estimated fruiting scores in fruiting censuses in Chichijima and Hahajima were summed for each month. Fruiting scores of several species that belong to the same barcoding plant group for where discrimination to the species level was not possible in the barcoding database were summed. The proportion of native and introduced plants for each month at each island was also calculated. The following analyses were carried out using the software R.3.0.2 (R Development Core Team 2013). To estimate seasonal change in the diet width, the number of plants detected from fecal samples was compared with the number of fruiting plants for each month. Based on difference in the sample sizes, five samples were randomly selected 1,000 times from the sample pool for each month, and a 95% confidence interval of diet width was calculated. To estimate the food selectivity of the pigeon, generalized linear mixed model (GLMM) analyses were carried out using R packages lme4 (Bates *et al*. 2014) and MuMIn (Bartoń 2013). The response variable of the model was the presence or absence of each plant detected in each sample, which was assumed to follow a binominal distribution. Candidate explanatory variables of the model were crude lipid, crude protein, crude ash, NDF, weight, and fruiting score. Sample ID was incorporated into the model as a random intercept. The variance inflation factors (VIFs) among explanatory variables was calculated and confirmed to be less than the cutoff value (5; Zuur *et al*. 2007) to avoid multicollinearity. All possible combinations of explanatory variables were examined, and appropriate models were selected based on the Akaike information criterion (AIC) (Burnham & Anderson 2002). I considered only the models that had ΔAIC (difference with the smallest AIC) of less than two. In addition, I calculated the relative importance of variables from the Akaike weights of all candidate models (Johnson & Omland 2004). Analyses were separately conducted in

Chichijima and Hahajima. To estimate the quality of introduced plants, the proportion of specific nutrients that strongly affect pigeons' food selection was compared between native and introduced plants that were detected from feces.

Results

Fruiting phenology in the two island habitats

Average fruiting scores per month per 1 km from the census line were 283.35 ± 140.55 in Chichijima and 130.09 ± 51.42 in Hahajima. Fruiting phenology was clearly different between Chichijima and Hahajima (Fig. 5.3c and d). The fruiting abundance of Schima mertensiana was the largest in Chichijima in both 2011 and 2012, and Leucaena glauca was the largest in Hahajima. The fruiting abundance of native plants (275 pt – 2,799 pt in Chichijima and 60 pt – 583 pt in Hahajima) fluctuated more drastically through the years than the fruiting abundance of introduced plants (235 pt – 820 pt in Chichijima and 162 pt – 830 pt in Hahajima). Thus, the proportion of introduced plants increased in the spring and early summer on both of the islands, when the fruiting abundance of native plants decreased. However, in Hahajima, fruiting abundances of introduced plants were larger than those of native plants in all months except for September 2012 and January 2013.

Diet composition of the red-headed wood pigeon in two island habitats

Six hundred and twenty-eight fecal samples of the 683 collected samples were confirmed with species-specific primers to be those of the red-headed wood pigeon (486 from Chichijima and 142 from Hahajima; Fig. 5.1). The list of detected plant groups and their frequencies is shown in Table 3. Of the 1,419,294 filtered sequences from seven independent runs of the Ion PGM sequencer, 122 plant groups were detected with a value < 1.0e-25. The relative frequencies of food plants clearly showed the differences in the diet composition between Chichijima and Hahajima (Fig. 5.3a, b). Gr. Planchonella1 and Gr. Neolitsea1, which were detected for long periods of time at high frequencies in the Chichijima samples, seem to be major food resources for the pigeons on the island. Gr. Planchonella1 was also detected in the Hahajima samples; however, Leucaena glauca and Morus australis were more frequently used in Hahajima. There were clear seasonal changes in the pigeons' diet on each island, and inter-annual changes on Chichijima (Fig. 5.3a, b). The proportion of introduced plants in the pigeons' diet increased in the summer of 2012 on both of the islands, but did not increase in the summer of 2011 on Chichijima. In Hahajima, the proportion of introduced plants was high (more than 50%) in all seasons except for the fall (September - November) of 2012.

Food selection patterns of the red-headed wood pigeon

Fig. 5.4 shows the comparison between the confidence interval of diet width (number of detected plants per one fecal samples) and the number of fruiting species (groups) estimated in Chichijima and Hahajima. Monthly diet width increased in specific seasons (August 2012 in Chichijima and April and May 2013 in Hahajima), and fluctuated more on Hahajima than on Chichijima.

All of the explanatory variables were included in the model selection because their VIF values were less than 5 (range 1.08 – 4.08). In the results of the model selection, crude lipid was included in all of the selected models in Chichijima and Hahajima (Table 5.4). NDF showed a positive effect in Chichijima and a negative effect in Hahajima. Fruiting score and weight showed negative effects in Chichijima and positive effects in Hahajima. Crude ash showed negative effects in Chichijima, and crude protein showed positive effects in Hahajima. Because lipid showed consistent positive effects in both islands, the proportion of lipids was compared between native and introduced plants that were used by pigeons. All of the values of lipid ratios in introduced plants were less than 10%, while several native plants exhibited lipid ratios of more than 10%, with a maximum of 30% (Fig. 5.5).

Discussion

The present study revealed the detailed and clear seasonal changes in food composition of the red-headed wood pigeon using HTS fecal analysis. Due to continuous sampling, the combination of universal and family specific markers, and the larger number of reads obtained from the Ion PGM sequencer, the number of detected food plants ($N = 122$) was much larger than that found by Chapter 4 ($N = 44$). This study indicates the usefulness of the HTS method on the wide range and the long-term monitoring of animal diets. Similar to biodiversity monitoring using environmental DNA (e.g., Baird *et al*. 2012), the HTS method may become a useful tool to estimate the impacts on food webs caused by environmental change, human disturbance and nature restoration, due to its objectivity and the increasing efficiency of sequencing technologies. In particular, clear results are expected in isolated and simple island ecosystems, in which potential food items are limited due to high endemism and low species richness.

Food selectivity of the red-headed wood pigeon

The HTS diet analysis, estimation of food resource availability and food quality revealed the foraging strategy of the red-headed wood pigeon in the seriously disturbed island habitat of the

Ogasawara Islands. Although selectivity for specific fruits was indicated, diet composition and diet width were flexibly shifted according to seasonal and inter-island variations in food resource availability. The results of the model selection indicated that pigeons prefer fruit that are rich in lipids. This feature is common between the two islands, and thus represents the food selectivity of the red-headed wood pigeon. This result concurs with the common finding that lipids are more preferred by birds than mammals (Levey & Carlos 2001; Bollen *et al*. 2004). Furthermore, lipids are easily emulsified and are a major component of pigeon milk (Levey & Carlos 2001; Gill 2007), indicating their importance in the reproduction of pigeons. Powlesland *et al*. (1997) suggested the preference of Parea *Hemiphaga novaeseelandiae chathamensis* for lipid-rich fruits and the importance of food quality on breeding success. Specific native plants such as *Fagara boninsimae*, *Planchonella obovata* (Gr. Planchonella1) and *Neolitsea aurata* (Gr. Neolitsea1) are especially rich in lipids (30%, 24% and 20%, respectively) and seem to be preferred by the red-headed wood pigeon (Table 5.3; Fig. 5.3), indicating their importance as high-quality food resources.

Food selectivity of the red-headed wood pigeon differed between the two islands

In Chichijima, pigeons seem to place food quality above food quantity, as indicated by the positive effect on lipid ratio and the negative effect on fruiting score and weight in model selection. The positive effect of the NDF ratio could be due to pigeons' consumption of *Pinus luchuensis,* which exhibited a large NDF ratio (74.4%) because the effect of NDF changed to be negative when the data were analyzed without this species. Fibers including NDF are known to be of low nutritional value (Thornburn & Willcox 1965), as the negative effect was detected in Hahajima. However, *Pinus luchuensis* may be easily used by pigeons in Chichijima because it lives near pigeons' nesting sites in Chichijima and includes lipids in moderate proportions (5.06%). It is unclear why crude ash negatively affected pigeons' food selection in Chichijima. Further analysis on the detailed ash contents in each fruit may be required to answer this question. Diet width in Chichijima increased when lipid-rich fruits (e.g., *Planchonella obovata* and *Neolitsea aurata*) were rarely consumed (especially summer in 2012; Fig. 5.3a, c, Fig. 5.4a), indicating lower selectivity in specific seasons. However, the diet composition and diet width differed between the years. In the summer (from June to August) of 2011, pigeons in Chichijima mainly consume Gr. Neolitsea1, but they consumed Gr. Ficus2 and various other plants in 2012. This difference may be due to the irregular bumper crop of *Neolitsea aurata* in 2010 and the remaining huge amount of seeds on the ground until the summer of 2011 (Kanto Regional Environmental Office 2012), which was not reflected in the results of the fruiting survey because fallen fruits on the ground were not counted

(Jun-Aug in 2011: 186pt, Jun-Aug in 2012: 221pt).

In Hahajima, two factors related to food quantity, fruiting abundance and weight also seem to be important for food selection. In terms of nutrition in Hahajima, both the protein and lipid ratios positively affected pigeons' food selection, indicating pigeons' lower food selectivity in Hahajima than in Chichijima. This may reflect differences in fruiting phenology and fruit density between the islands. Fruit density is much lower in Hahajima than in Chichijima (see Results), indicating the difficulty of obtaining food resources in Hahajima. Furthermore, the lipid-rich *Neolitsea aurata* does not live on Hahajima, and *Fagara boninsimae* is also rare on the island (Toyoda 2003). Pigeons in Hahajima may not be able to obtain sufficient lipid-rich food resources and therefore may be more likely to consume what is available. Fluctuations in diet width in Hahajima (Fig. 5.4) may also reflect this foraging condition in Hahajima, in which pigeons cannot continuously obtain preferable fruits.

Flexible shifts in diet composition and width seem to be essential strategy for island pigeons in order to survive in oceanic island habitats, which exhibit within-and among-year variations in food resource availability, as reported in previous studies (Oliveira *et al*. 2002; Emeny *et al*. 2009). Furthermore, this study indicated inter-island variations in the food selection patterns of the red-headed wood pigeon between the two islands, which are approximately 50 km apart. However, this does not indicate that pigeons in each island are specialized (adapted genetically) to each habitat because pigeons move between the islands irregularly, which were determined through observations of banded individuals (Suzuki *et al*. 2006; Kanto Regional Environmental Office 2010, 2011, 2012, 2013). The relationship between pigeons' foraging strategy and inter-island movement is unclear; however, pigeons may move among islands to search for food and to change their food selection patterns, following the food resource availability on each island. Such temporal and spatial shifts in foraging strategy may be an adaptation for isolated oceanic island in which habitat and food resources are restricted. In addition, such flexibility in food selection may be an advantage for the red-headed wood pigeon to survive serious ecological disturbance in the Ogasawara Islands, which caused six endemic birds in the Ogasawara Islands, including the Bonin wood pigeon *Columba versicolor*, to become extinct (Ministry of the Environment 2002).

Significance of introduced plants as food resources for the red-headed wood pigeon and management issues

The above-mentioned foraging strategy of the red-headed wood pigeon may also be a strategy to obtain food in seriously disturbed habitats invaded by introduced plants. Except for the

summer of 2011 in Chichijima, the frequency of introduced plants in pigeons' diets tends to increase when the fruiting abundance of native plants decreases (Fig. 5.3), indicating that the importance of introduced plants fluctuates temporally. In particular, Gr. Ficus2, which can be *Ficus microcarpa* considering its fruiting phenology, seems to be a major introduced food resource for the pigeon both on Chichijima and Hahajima. *Ficus* species are consumed by various animals due to their unique fruiting phenology, producing fruits when the fruits of many other plants are rare (Shanahan *et al.* 2001; Bollen *et al.* 2004). The importance of *Ficus* as food resources for forest pigeons is also indicated (Frith *et al.* 1976). *Ficus microcarpa* seems to have become a major food resource for the red-headed wood pigeon, instead of native *Ficus* species (e.g., *Ficus boninsimae*), which exhibit small fruiting abundance. Although its population size is not very large, fruits of *Ficus microcarpa* may be really available to the pigeons to use due to its large tree size and the fruiting abundance of individual trees (Ecological Society of Japan 2002; Toyoda 2003).

The quality of introduced fruit, which are consumed in high frequency by the red-headed wood pigeon was low as they contained less lipid than compared to the pigeons' prefered native fruit (Fig. 5.5). Thus, introduced fruit function as fallback food for the pigeons to complement the lack of preferable native fruits. Unlike the case of the Ogasawara buzzard *Buteo buteo toyoshimai*, which depends on introduced animals (e.g., the black rat *Rattus rattus* and the green anole *Anolis carolinensis*; Kato & Suzuki 2005) for 90% of its food, introduced fruit do not seem to be essential food resources for the red-headed wood pigeon, and may be less important for pigeons' reproduction. Thus, it may be appropriate to eradicate introduced plants that are used by pigeons and restore native food plants for the natural restoration of the Ogasawara Islands and the long-term conservation of the pigeons' habitat. However, considering the quantitative value of introduced fruit, their importance seems to differ between the two islands. Introduced plants may be more valuable in Hahajima, where introduced fruit are consumed frequently and food quantity is an important factor for food selection, than in Chichijima (Fig. 5.3, Table 5.4). The effect of introduced plant eradication on the foraging conditions for the red-headed wood pigeon may differ between Chichijima and Hahajima, considering the direct and indirect effects of introduced plant eradication (Kawakami 2008; Bergstrom *et al.* 2009; Simberloff *et al.* 2013). In Chichijima, positive impacts on the pigeons' foraging conditions may be expected following the recovery of various native food plants, which produce fruits throughout the year, after the eradication of introduced plants. In Hahajima, the rapid reduction of food resources following the eradication of introduced plants that are consumed frequently by the pigeons may cause negative impacts on pigeons' foraging condition. Range, speed and the order of introduced plant eradication and the restoration of native flora in each

island may be better decided by considering the different foraging strategies and degrees of dependence on introduced fruit by the red-headed wood pigeon on each island.

This study revealed temporal and spatial diet shifts of the endangered red-headed wood pigeon based on HTS fecal analysis. This study also discussed foraging strategies of the pigeon and the significance of pigeons' consumption of introduced plants in isolated and disturbed oceanic island habitats. The HTS diet analysis, combined with field data, may be a useful tool to monitor the effect of environmental change, ecological disturbance and nature restoration on the foraging conditions of endangered species. Furthermore, such studies are expected to reveal complicated food webs including native and introduced species, informing appropriate conservation planning for oceanic island biodiversity.

Table 5.1 Sequences of the primer pairs used in this study.

Name	Sequence	References
PSF	AACCCGGCACCCTTCTAGGAGACGA	Chapter 4
PSR2	GGTAGTAGTCAGAAGCTTATGTTGT	This study
*trn*L g	GGGCAATCCTGAGCCAAATC	Taberlet *et al*. (2007)
*trn*L h	CCATTGAGTCTCTGCACCTATC	Taberlet *et al*. (2007)
LAF	CCTTCCTGCTGAAATACAGA	This study
LAR	AATATCGTAGTTTCTTCTGT	This study
FIF	GACTAACAAATAATAATTTA	This study
FIR	CTACAAATGGATAAGACTTC	This study

Table 5.2 Plant groups for Lauraceae and *Ficus* defined by *trn*H-*psb*A sequences.

*trn*L P6 loop group	*trn*H-*psb*A group	Product size (bp)	Species	Accession No.
Gr. Lauraceae1	*Cinnamomum camphora*	169	*Cinnamomum camphora*	
	Cinnamomum pseudo-pedunculatum	163	*Cinnamomum pseudo-pedunculatum*	
	Gr. Machilus1	153	*Machilus kobu*	
			Machilus pseudokobu	
			Machilus boninensis	
	Gr. Neolitsea1	171	*Neolitsea aurata*	
			Neolitsea boninensis	
Gr. Ficus1	*Ficus iidaiana*	171	*Ficus iidaiana*	
	Gr. Ficus2	161	*Ficus benghalensis*	
			Ficus elastica	
			Ficus microcarpa	
			Ficus religiosa	
			Ficus superba	
	Gr. Ficus3	170	*Ficus boninsimae*	
			Ficus nishimurae	

Table 5.3 List of detected plant groups in the diet analysis of the red-headed wood pigeon.

Plant group	Native/Introduced	Chichijima			Hahajima			Total		
		N reads	F_R (%)	F_P (%)	N reads	F_R (%)	F_P (%)	N reads	F_R (%)	F_P (%)
Gr. Ficus2	Introduced	291310	28.64	25.10	85182	21.18	23.24	376492	26.53	24.68
Gr. Neolitsea1	Native	194576	19.13	43.42	277	0.07	1.41	194853	13.73	33.92
Gr. Planchonella1	Native	117731	11.57	42.39	36398	9.05	30.28	154129	10.86	39.65
Osmanthus insularis	Native	84254	8.28	10.70	0	0.00	0.00	84254	5.94	8.28
Elaeocarpus photiniifolius	Native	8315	0.82	9.05	59319	14.75	16.20	67634	4.77	10.67
Leucaena glauca	Introduced	9644	0.95	6.79	47279	11.76	46.48	56923	4.01	15.76
Morus australis	Introduced	29055	2.86	22.84	21776	5.42	56.34	50831	3.58	30.41
Gr. Lauraceae1	Native/Introduced	33022	3.25	11.52	625	0.16	10.56	33647	2.37	11.31
Rhaphiolepis wrightiana	Native	18006	1.77	10.70	10238	2.55	9.86	28244	1.99	10.51
Trema orientalis	Native	8157	0.80	7.61	19261	4.79	19.72	27418	1.93	10.35
Solanum nigrum	Introduced	3318	0.33	1.65	20481	5.09	14.79	23799	1.68	4.62
Cinnamomum pseudo-pedunculatum	Native	22332	2.20	4.12	0	0.00	0.00	22332	1.57	3.18
Fagara boninsimae	Native	21844	2.15	16.46	41	0.01	2.11	21885	1.54	13.22
Passiflora edulis	Introduced	8735	0.86	2.26	11011	2.74	9.86	19746	1.39	3.98
Gr. Ficus1	Native/Introduced	11083	1.09	6.38	6303	1.57	16.90	17386	1.22	8.76
Pinus luchuensis	Introduced	14943	1.47	18.93	519	0.13	5.63	15462	1.09	15.92
Gr. Machilus1	Native	3050	0.30	3.09	11288	2.81	4.23	14338	1.01	3.34
Dianella ensifolia	Introduced	13340	1.31	1.03	0	0.00	0.00	13340	0.94	0.80

Capsicum annuum	Introduced	230	0.02	0.62	12440	3.09	12.68	12670	0.89	3.34
Mucuna toyoshimae	Native	12371	1.22	1.65	0	0.00	0.00	12371	0.87	1.27
Erythina boninensis	Native	1686	0.17	1.03	9722	2.42	6.34	11408	0.80	2.23
Ardisia sieboldii	Native	11027	1.08	14.81	231	0.06	4.93	11258	0.79	12.58
Gr. Hibiscus1	Native	11128	1.09	5.56	99	0.02	0.70	11227	0.79	4.46
Musa spp.	Introduced	9906	0.97	2.06	14	0.00	1.41	9920	0.70	1.91
Psidium guajava	Introduced	6599	0.65	2.06	3099	0.77	4.23	9698	0.68	2.55
Gr. Callicarpa1	Native	8462	0.83	2.06	53	0.01	2.82	8515	0.60	2.23
Elaeagnus rotundata	Native	6690	0.66	7.20	32	0.01	0.70	6722	0.47	5.73
Gr. Palmae1	Introduced	898	0.09	2.47	5725	1.42	11.97	6623	0.47	4.62
Carica papaya	Introduced	19	0.00	0.21	6406	1.59	9.86	6425	0.45	2.39
Livistona chinensis	Native	760	0.07	1.85	5523	1.37	9.15	6283	0.44	3.50
Bischofia javanica	Introduced	4126	0.41	8.85	1955	0.49	19.72	6081	0.43	11.31
Hibiscus rosa-sinensis	Introduced	1709	0.17	0.82	3869	0.96	3.52	5578	0.39	1.43
Calaphyllum inophyllum	Native	3314	0.33	2.06	2053	0.51	17.61	5367	0.38	5.57
Celtis boninensis	Native	1128	0.11	2.67	3330	0.83	15.49	4458	0.31	5.57
Gr. Myrtaceae1	Native/Introduced	3559	0.35	4.53	159	0.04	4.23	3718	0.26	4.46
Lantana camara	Introduced	3582	0.35	13.79	0	0.00	0.00	3582	0.25	10.67
Bidens alba	Introduced	3119	0.31	2.67	302	0.08	5.63	3421	0.24	3.34
Drypetes integerrima	Native	2891	0.28	3.50	31	0.01	1.41	2922	0.21	3.03
Ochrosia nakaiana	Native	2703	0.27	2.47	17	0.00	0.70	2720	0.19	2.07
Acacia confusa	Introduced	0	0.00	0.00	2478	0.62	4.23	2478	0.17	0.96

Gr. Poaceae2	Introduced	2329	0.23	1.65	5	0.00	0.70	2334	0.16	1.43
Ageratum conyzoides	Introduced	1483	0.15	3.29	812	0.20	4.93	2295	0.16	3.66
Terminalia catappa	Native	851	0.08	2.06	1410	0.35	12.68	2261	0.16	4.46
Zanthoxylum arnottianum	Native	2154	0.21	8.44	0	0.00	0.00	2154	0.15	6.53
Gr. Rosaceae1	Native	597	0.06	6.58	1419	0.35	4.23	2016	0.14	6.05
Paederia scandens	Introduced	1107	0.11	1.03	805	0.20	9.15	1912	0.13	2.87
Freycinetia boninensis	Native	1821	0.18	2.47	0	0.00	0.00	1821	0.13	1.91
Vitex rotundifolia	Native	446	0.04	3.09	1202	0.30	3.52	1648	0.12	3.18
Juniperus taxifolia	Native	1353	0.13	3.29	227	0.06	2.82	1580	0.11	3.18
Phyllanthus tenellus	Introduced	29	0.00	0.41	1429	0.36	7.75	1458	0.10	2.07
Casuarina equisetifolia	Introduced	1365	0.13	3.09	29	0.01	1.41	1394	0.10	2.71
Schima mertensiana	Native	1227	0.12	6.38	137	0.03	2.11	1364	0.10	5.41
Distylium lepidotum	Native	1291	0.13	4.53	67	0.02	2.11	1358	0.10	3.98
Hernandia sonara	Native	1140	0.11	1.85	0	0.00	0.00	1140	0.08	1.43
Bothriochloa bladhii	Introduced	1097	0.11	1.23	40	0.01	1.41	1137	0.08	1.27
Rivina humilis	Introduced	736	0.07	1.44	372	0.09	9.15	1108	0.08	3.18
Caesalpinia crista	Native	0	0.00	0.00	1077	0.27	4.23	1077	0.08	0.96
Fatsia oligocarpella	Native	0	0.00	0.00	1033	0.26	3.52	1033	0.07	0.80
Messerschmidia argentea	Native	994	0.10	1.23	0	0.00	0.00	994	0.07	0.96
Oxalis corniculata	Introduced	0	0.00	0.00	929	0.23	6.34	929	0.07	1.43
Stachytarpheta jamaicensis	Introduced	844	0.08	1.23	0	0.00	0.00	844	0.06	0.96
Gr. Palmae2	Introduced	569	0.06	1.65	261	0.06	4.93	830	0.06	2.39

Paspalum conjugatum	Introduced	760	0.07	0.41	0	0.00	0.00	760	0.05	0.32
Gr. Citrus1	Introduced	99	0.01	0.82	621	0.15	2.82	720	0.05	1.27
Scaevola taccada	Native	198	0.02	1.44	506	0.13	7.04	704	0.05	2.71
Wikstroemia pseudoretusa	Native	606	0.06	2.88	0	0.00	0.00	606	0.04	2.23
Sonchus oleraceus	Introduced	554	0.05	2.47	35	0.01	2.82	589	0.04	2.55
Leptochloa panicea	Introduced	65	0.01	0.82	508	0.13	0.70	573	0.04	0.80
Euphorbia pilulifera	Introduced	538	0.05	1.03	0	0.00	0.00	538	0.04	0.80
Gr. Poaceae1	Introduced	516	0.05	2.47	0	0.00	0.00	516	0.04	1.91
Sapindus mukorossi	Introduced	497	0.05	1.03	0	0.00	0.00	497	0.04	0.80
Desmanthus virgatus	Introduced	32	0.00	0.62	443	0.11	14.08	475	0.03	3.66
Gr. Pittosporum1	Native	400	0.04	0.82	12	0.00	0.70	412	0.03	0.80
Castanopsis sieboldii	Introduced	258	0.03	2.67	83	0.02	3.52	341	0.02	2.87
Machaerina nipponensis	Native	331	0.03	0.82	0	0.00	0.00	331	0.02	0.64
Averrhoa carambola	Introduced	17	0.00	0.41	278	0.07	4.93	295	0.02	1.43
Pandanus boninensis	Native	215	0.02	2.26	10	0.00	0.70	225	0.02	1.91
Conyza sumatrensis	Introduced	219	0.02	1.23	0	0.00	0.00	219	0.02	0.96
Mimosa pudica	Introduced	9	0.00	0.21	209	0.05	3.52	218	0.02	0.96
Gr. Symplocos1	Native	159	0.02	3.91	27	0.01	2.82	186	0.01	3.66
Rhus succedanea	Introduced	171	0.02	1.23	0	0.00	0.00	171	0.01	0.96
Derris elliptica	Introduced	154	0.02	1.65	0	0.00	0.00	154	0.01	1.27
Rubus nishimuranus	Native	39	0.00	0.62	114	0.03	4.23	153	0.01	1.27
Psychotria homalosperma	Native	50	0.00	0.62	100	0.02	1.41	150	0.01	0.80

Trachelospermum asiaticum	Native	126	0.01	1.85	0	0.00	0.00	126	0.01	1.43
Sambucus javanica	Introduced	94	0.01	1.23	23	0.01	1.41	117	0.01	1.27
Geniostoma glabrum	Native	115	0.01	0.21	0	0.00	0.00	115	0.01	0.16
Ilex mertensii	Native	103	0.01	1.65	11	0.00	0.70	114	0.01	1.43
Melia azedarach	Native	101	0.01	0.41	0	0.00	0.00	101	0.01	0.32
Ligustrum micranthum	Native	99	0.01	2.06	0	0.00	0.00	99	0.01	1.59
Sporobolus diander	Introduced	92	0.01	0.82	0	0.00	0.00	92	0.01	0.64
Gr. Rutaceae1	Native	88	0.01	0.62	0	0.00	0.00	88	0.01	0.48
Gr. Diospyros1	Introduced	27	0.00	0.82	59	0.01	1.41	86	0.01	0.96
Psychotria boninensis	Native	77	0.01	0.41	6	0.00	0.70	83	0.01	0.48
Mangifera indica	Introduced	10	0.00	0.21	68	0.02	2.11	78	0.01	0.64
Gr. Morinda1	Native	0	0.00	0.00	74	0.02	0.70	74	0.01	0.16
Gr. Hedyotis1	Native	0	0.00	0.00	46	0.01	0.70	46	0.00	0.16
Lagerstroemia indica	Introduced	0	0.00	0.00	36	0.01	2.82	36	0.00	0.64
Agave americana	Introduced	31	0.00	0.21	0	0.00	0.00	31	0.00	0.16
Zoysia matrella	Introduced	31	0.00	0.41	0	0.00	0.00	31	0.00	0.32
Tridax procumbens	Introduced	23	0.00	0.41	7	0.00	0.70	30	0.00	0.48
Bacopa procumbens	Introduced	27	0.00	0.41	0	0.00	0.00	27	0.00	0.32
Sophora tomentosa	Native	23	0.00	0.21	0	0.00	0.00	23	0.00	0.16
Buxus liukiuensis	Introduced	21	0.00	0.41	0	0.00	0.00	21	0.00	0.32
Pennisetum purpureum	Introduced	12	0.00	0.21	9	0.00	0.70	21	0.00	0.32
Boehmeria boninensis	Native	19	0.00	0.41	0	0.00	0.00	19	0.00	0.32

Gr. Myrsine1	Native	16	0.00	0.41	0	0.00	0.00	16	0.00	0.32
Ipomoea pes-caprae	Native	16	0.00	0.21	0	0.00	0.00	16	0.00	0.16
Enteropogon dolichostachys	Introduced	15	0.00	0.21	0	0.00	0.00	15	0.00	0.16
Medicago lupulina	Introduced	15	0.00	0.21	0	0.00	0.00	15	0.00	0.16
Pahicum maximum	Introduced	15	0.00	0.62	0	0.00	0.00	15	0.00	0.48
Tarenna subsessilis	Native	11	0.00	0.21	0	0.00	0.00	11	0.00	0.16
Delonix regia	Introduced	0	0.00	0.00	10	0.00	0.70	10	0.00	0.16
Lepidium virginicum	Introduced	9	0.00	0.21	0	0.00	0.00	9	0.00	0.16
Eurya boninensis	Native	8	0.00	0.21	0	0.00	0.00	8	0.00	0.16
Metrosideros boninensis	Native	8	0.00	0.21	0	0.00	0.00	8	0.00	0.16
Clematis terniflora	Native	0	0.00	0.00	7	0.00	0.70	7	0.00	0.16
Cassia fistula	Introduced	0	0.00	0.00	6	0.00	0.70	6	0.00	0.16
Gr. Agavaceae1	Introduced	6	0.00	0.21	0	0.00	0.00	6	0.00	0.16
Morinda citrifolia	Native	6	0.00	0.21	0	0.00	0.00	6	0.00	0.16
Jasminum hemsleyi	Introduced	5	0.00	0.21	0	0.00	0.00	5	0.00	0.16
Lobelia boninensis	Native	5	0.00	0.21	0	0.00	0.00	5	0.00	0.16

Number of obtained sequence reads (N reads), frequency of sequence reads (F_R) and the frequency of the presence (F_P)

Table 5.4 Results of a generalized linear mixed model that predicts pigeons' food selection

Adopted factors						AIC	ΔAIC	Akaike weight (%)
Chichijima								
Ash-	Count-	Lipid+	NDF+		Weight-	8341.0	0.00	40.3
Ash-	Count-	Lipid+	NDF+	Protein+	Weight-	8341.5	0.44	32.3
Hahajima								
	Count+	Lipid+	NDF-	Protein+	Weight+	2522.5	0.00	62.7
Ash-	Count+	Lipid+	NDF-	Protein+	Weight+	2523.7	1.24	33.8

Akaike information criterion (AIC), difference with the smallest AIC (ΔAIC), relative importance of variables (Akaike weight), crude ash (Ash), fruiting score (Count), crude lipid (Lipid), neutral detergent fiber (NDF), crude protein (Protein), positive effect (+), negative effect (-)
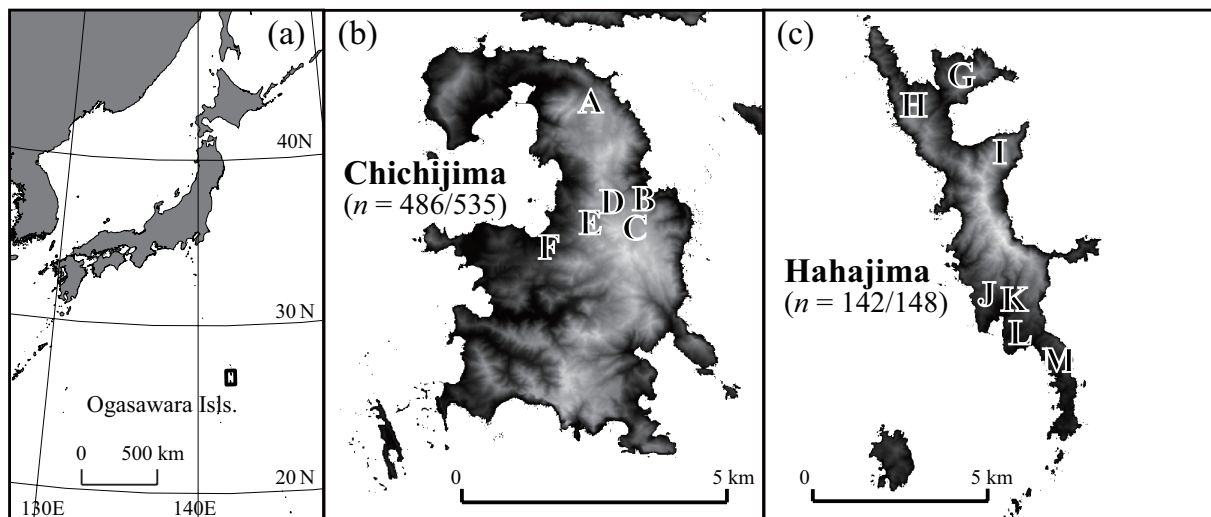
Fig. 5.1 (a) Locations of the Ogasawara Islands in Japan. Sampling locations of feces of the red-headed wood pigeon and fruiting census sites in (b) Chichijima and (c) Hahajima in the Ogasawara Islands. Each letter represents the locations of census lines for the fruiting census. Census lines and the name of each location are not shown to protect pigeons' habitat. The numbers in parentheses show the number of fecal samples that were confirmed to those of the red-headed wood pigeon/the number of collected fecal samples.

Feces collection

↓

DNA extraction

Elimination observed   Elimination not observed

PCR by pigeon
specific primer

Amplified   Not amplified

P6loop PCR   No use

↓

High throughput sequencing

↓

DNA barcoding with originally
developed P6loop database

Gr. Lauraceae1 and/or
Gr. Ficus1 detected

PCR by family/genus
specific primer

Neither Gr. Lauraceae1 nor
Gr. Ficus1 detected

↓

Fragment analysis

↓

Plant identification

Fig. 5.2 General outline for the diet analysis of the red-headed wood pigeon.
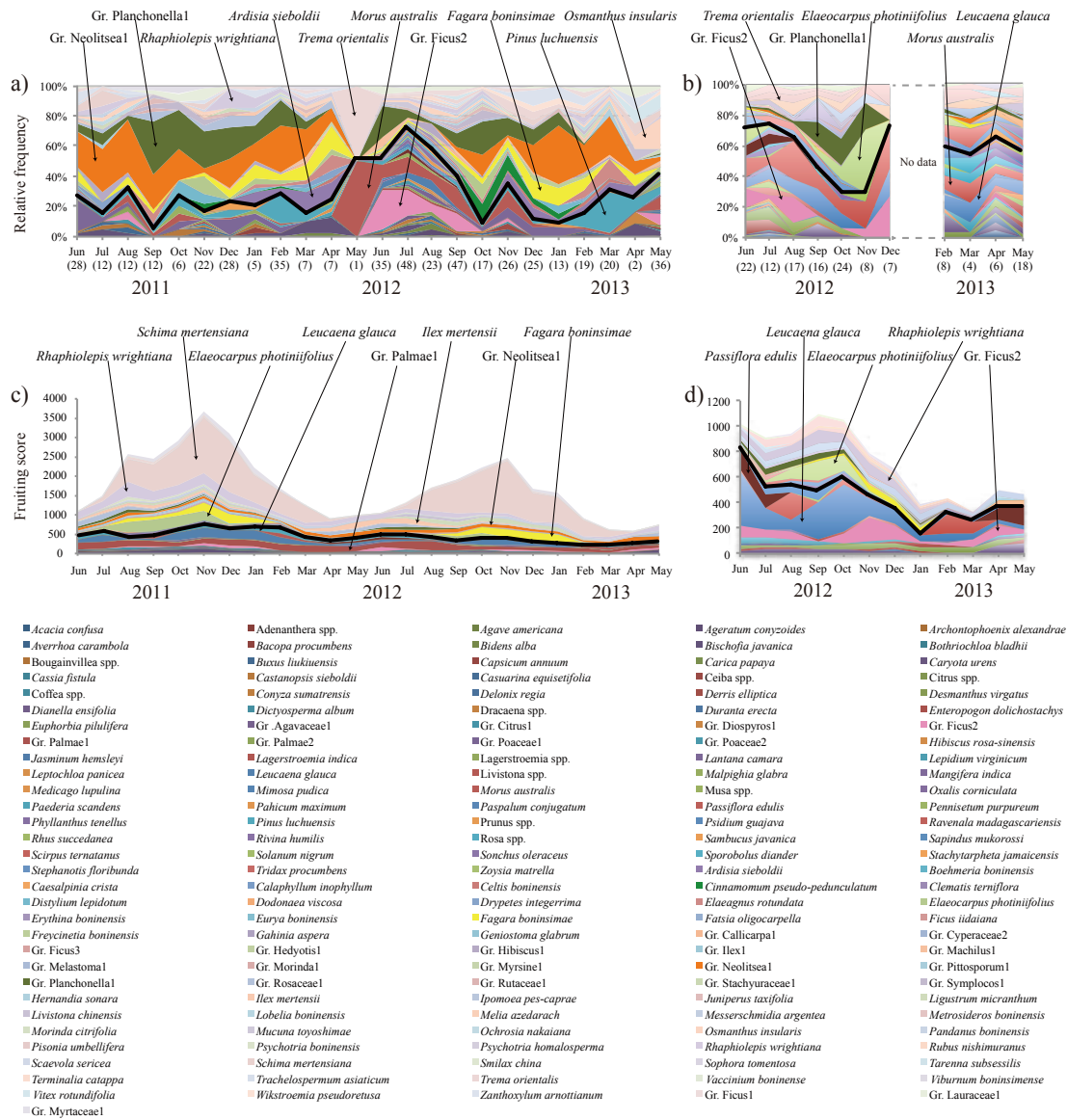
Fig. 5.3 Change in food composition of the red-headed wood pigeon based relative frequency of sequence data for (a) Chichijima, (b) Hahajima, and fruiting score for (c) Chichijima, and (d) Hahajima. Plants that are shown under black lines in each graph are introduced species. Numbers in parenthesis are sample sizes in each month.

a)

b)
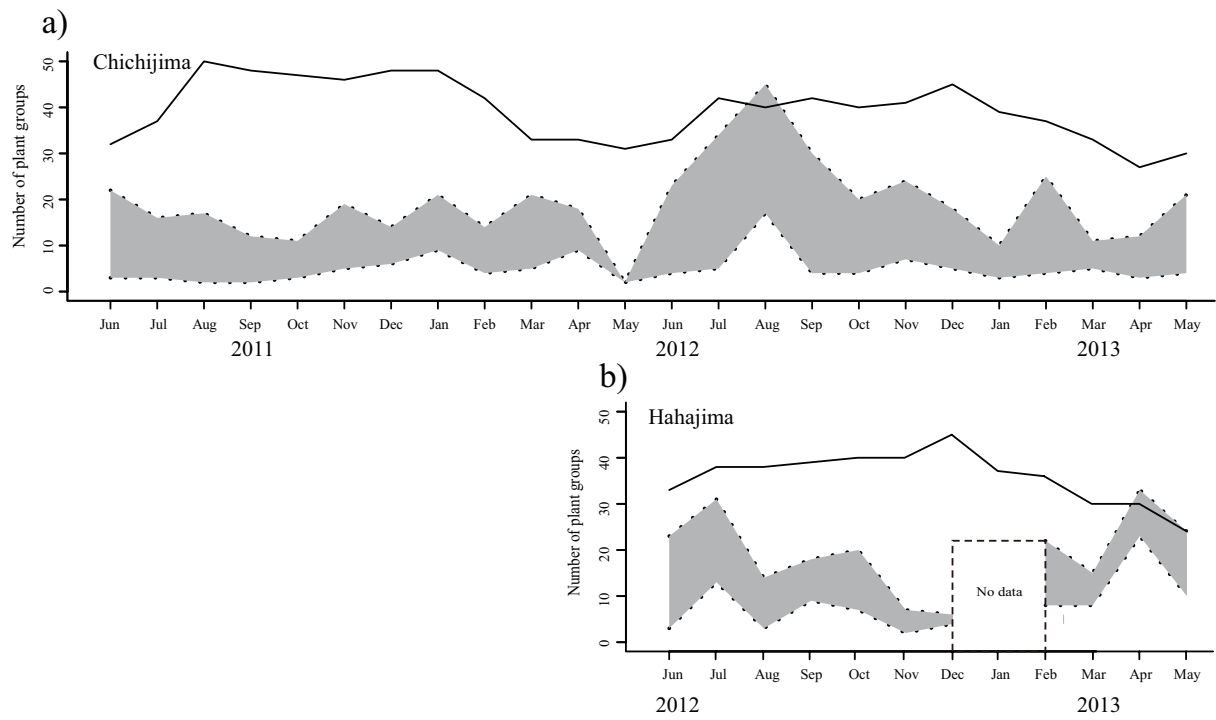
Fig. 5.4 Comparison between the estimated diet width of the red-headed wood pigeon and observed number of fruiting plant species (groups) in a) Chichijima and b) Hahajima. Grey areas show 95% confident interval of diet width (number of detected plants per one fecal sample). Solid lines show the number of observed plant species (groups) in fruiting census.
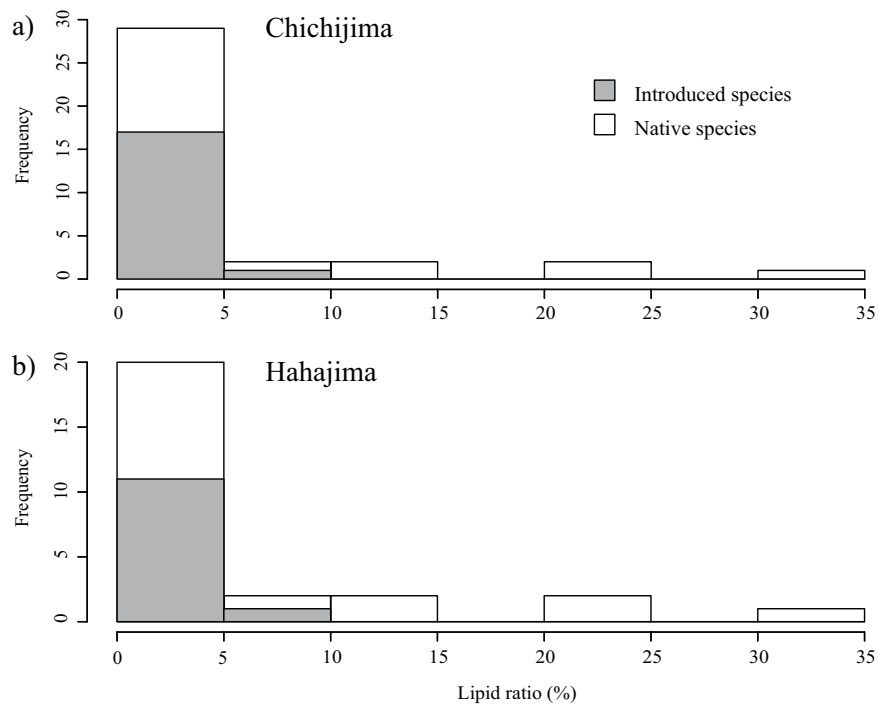
Fig. 5.5 Frequency distribution of native (white) and introduced (grey) food plants in a) Chichijima and b) Hahajima based on lipid ratio.

Chapter 6
General discussion

In this study, I revealed the genetic characteristics and foraging strategy of the critically endangered red-headed wood pigeon *Columba janthina nitens*. The multifaceted approach of molecular biological methods provided a better understanding of the ecology and the management issues for the subspecies, which are difficult to reveal using only field data. In this chapter, I discuss the management issues of the red-headed wood pigeon within and outside their habitat and restoration in the Ogasawara Islands. I will also discuss the effectiveness and future challenges of molecular biological methods on studies of conservation biology for endangered species in the Ogasawara Islands and other oceanic islands.

The results reported in Chapter 2 and 3 suggested that the genetic diversity of the red-headed wood pigeon is at the lowest level compared with other Columbidae species (Young & Allard 1997; Swinnerton *et al*. 2004) and island endemic threatened bird species (Jamieson *et al*. 2006; Evans & Sheldon 2008). In particular, the genetic diversity of the captive population is in serious peril. Conservation of the remaining genetic diversity to the maximum extent possible within and outside the wild habitat is required. Individuals who have rare haplotypes or alleles should be introduced into the captive population. To avoid further reduction in the genetic diversity of the wild population, any introduction of individuals into the captive population should only be conducted once the number of wild birds has increased as a consequence of habitat conservation or the eradication of cats. However, if a further decline in the wild population occurs, the wild population may become extinct. If the population becomes extinct, their genetic diversity is effectively a moot point. Therefore, individuals who have rare haplotypes or alleles should be introduced into the captive population as soon as possible. This is because such individuals seem to be very few, and rare haplotypes or alleles are likely to be lost from the wild population if the population continues to decline (Frankham *et al*. 2010).

In Chapter 3, the occurrence of inter-island movement between the Bonin and the Volcano Islands was also indicated, which was not detected in previous banding studies (Suzuki *et al*. 2006). The results of the study were consistent with that of the Galapagos dove (Alarcon *et al*. 2006), indicating an evolutionary aspect of island Columbiformes that maintained strong flying capacity even in remote island habitats, whereas other island endemic terrestrial birds avoided flying over water (Bollmer *et al*. 2005; Petren *et al*. 2005; Kawakami *et al*. 2008). The populations in the Bonin and Volcano Islands of the red-headed wood pigeon are considered to constitute a single

evolutionarily significant unit. Habitat conservation should consider the wide-range movements of the pigeon to be effective. Particularly, the population in the Volcano Islands, which exhibited higher genetic diversity and favorable habitat conditions, appears to be of special importance because the islands may be functioning as a sanctuary for the entire population in the Ogasawara Islands. Simultaneously, feral cat eradication in inhabited islands should be conducted to reduce further losses of genetic diversity and to conserve broader favorable habitat.

In Chapter 4 and 5, I revealed the detailed diet composition of the red-headed wood pigeon using an HTS approach, which had not been previously revealed by direct observation (Kanto Regional Forestry Office 2005, 2006; Kanto Regional Environmental Office 2011) and microhistological analysis (Shibazaki & Hoshi 2006). The results of HTS diet analysis and the estimation of food quantity and quality indicated the importance of introduced fruit for the red-headed wood pigeon to complement the lack of preferable native fruits. The rapid eradication of introduced plants without restoring native food resources may negatively impact the pigeons' foraging condition. Thus, a strategy that balances the eradication of introduced plants and the restoration of native food resources is important, which may be different between Chichijima and Hahajima. In Chichijima, in which the pigeons' consumption of introduced plants is concentrated in early summer, it may be appropriate to restore several native plants that fruit in the season (e.g., *Ficus boninsimae*, *Livistona chinensis*) after introduced plant eradications. In Hahajima, where pigeons frequently consume introduced plants in almost all seasons, the restoration of various native plants that provide fruits throughout of the year should be carried out in parallel with introduced plant eradications. In addition, the eradication of introduced rats should also be carried out in both islands to avoid food competition with pigeons, as noted by the Kanto Regional Forestry Office (2006).

This study has also revealed several important challenges that may be faced in the future. The results of mitochondrial and microsatellite analysis indicated wide-range movements of the red-headed wood pigeon including the Bonin and the Volcano Islands. However, it is only a generation-scale movement via gene flow. The pattern (range, direction and frequency) and significance of short-term inter-island movement is still unclear. Inter-island variation of food resource availability may be a reason for the pigeon to move among islands to obtain food throughout the year. Future research is required to reveal the relationship between the pattern of inter-island movement by the pigeon and food resource availability in each island habitat. This may provide a better understanding of the pigeons' foraging strategy in isolated oceanic island habitats and may enable us to suggest more concrete implications for creating sanctuaries and restoration

projects to improve pigeon habitat.

The restoration of foraging habitat for the red-headed wood pigeon, which has adapted to native forest in the Ogasawara Islands, will be valuable for long-term conservation of the forest ecosystem in the Ogasawara Islands. However, special consideration of the genetic structure of native plants is required in their restoration to avoid negative impacts such as outbreeding depression. This is because several native plant species in the Ogasawara Islands are strongly genetically differentiated among islands and even within each island due to geographical isolation by oceanic barriers and adaptive radiation within islands (e.g., Kaneko *et al*. 2008; Sugai *et al*. 2013; Tsuneki *et al*. 2014). For the appropriate restoration of forest ecosystems in the Ogasawara Islands, comprehensive ecological and genetic approaches including animals and plants are required in the future.

In addition, some technical challenges also exist. The microsatellite markers developed in this study could not identify each individual due to the low genetic diversity of the pigeon. Recently, the HTS approach has also been used for the development of microsatellite markers (e.g., Nakahama *et al*. 2012; Sakata *et al*. 2013; Kamioki *et al*. 2013) and genotyping, making it possible to isolate much larger numbers of polymorphic microsatellite regions that were not found using previous methods. Using the HTS microsatellite analysis, it may be possible to perform individual-based analysis, such as the estimation of foraging range using genotype data of feces or molted feathers. In the HTS diet analysis, various biological and technical factors that affect diet quantification using sequence reads (Pompanon *et al*. 2012) may be calibrated in the future, which will make it possible to conduct more accurate analyses of diet composition.

For the long-term conservation of threatened island endemic bird species within and outside favorable habitat, I first stress the importance of the careful selection of genetically suitable founders for the captive population to maintain the genetic diversity of the wild population, as well as its genetic management to minimize the loss of genetic diversity. Captive breeding populations of threatened species have often been established only after their wild population sizes have been critically reduced (Frankham *et al*. 2010), making it difficult to maintain enough genetically variable founders. The red-headed wood pigeon is a typical example of such cases, representing the critical importance of establishing a suitable captive population before the population has seriously declined, as recommended by the IUCN (1987). Individual-based genetic analysis using polymorphic genetic markers can help to select the suitable candidates for captive populations.

It is also important to note that the ideal habitat conservation plan that considers genetic structure, which may reflect the flying capacity, may differ among species. For some strong fliers

such as Columbiformes, each island habitat may not be treated as a single management unit, despite the general knowledge regarding the evolutionary traits of island endemic birds (Weiner 1994; Whittaker 2007). Thus, comprehensive genetic analyses, which cover the entire range of island habitat, are required to determine meaningful management units that reflect the evolutionary and ecological traits of the target species.

In terms of habitat conservation, nature restoration in disturbed oceanic islands should consider the interaction between native and introduced species, which may be temporally and spatially different. This is particularly important for the generalists with broader foraging ranges such as Columbiformes, which change their diet according to available food resources; wide-range and continuous surveys are required to reveal their dependence on introduced species and predict their response to the eradication of introduced species. The comprehensive and objective HTS diet analysis may help to understand such complicated interactions between native and introduced species. The method is less time consuming and may also enable us to conduct dietary studies on a more massive scale using a large number of samples. Furthermore, the development of local sequence databases will increase the resolution of taxon identification rather than simply using global databases (Pompanon *et al*. 2012; Nakahara et al. unpublished data). In particular, clear results are expected in isolated and simple island ecosystems, in which potential food items are limited due to high endemism and low species richness. A multifaceted approach of molecular biological techniques can provide valuable information to understand the ecology and conservation issues of island endemics. Furthermore, technical improvements in combination with field studies may contribute to the long-term conservation of oceanic island biodiversity.

Summary

Chapter 1

Oceanic island ecosystems are vulnerable to human disturbances and many of island endemics are at risk of extinction. Conservation of endemic species on oceanic islands is one of the most pressing issues in order to reduce the rate of biodiversity loss, but their long-term conservation planning is not conducted based on sufficient scientific data. Recent development of molecular biological techniques may enable us to understand ecology and genetic characteristics of island endemics, and to suggest more concrete conservation planning. I investigated the critically endangered red-headed wood pigeon *Columba jantnia nitens*, an endemic subspecies which are living highly isolated and disturbed oceanic island, the Ogasawara Islands. This chapter introduce the aims and overview of the study; genetic characteristics and foraging ecology of the red-headed wood pigeon, suggesting its appropriate conservation plan within and outside of the pigeons' habitat, as a case study of endangered island endemics.

Chapter 2

I developed seven new microsatellite markers for two subspecies of the Japanese wood pigeon *Columba janthina*, the nominate Japanese wood pigeon *C. janthina janthina* and the red-headed wood pigeon *C. janthina nitens*. I also confirmed the cross-use of one microsatellite marker developed for *Columba livia* var. *domestica*. Seven loci were polymorphic in the nominate Japanese wood pigeon, while two loci were polymorphic in the red-headed wood pigeon. Using the markers, I performed a preliminary analysis of genetic diversity and genetic structure within each subspecies. The expected heterozygosity ranged from 0.00 to 0.64 in the nominate Japanese wood pigeon and from 0.00 to 0.08 in the red-headed wood pigeon. Each subspecies and each population within the nominate Japanese wood pigeon had different allele frequencies. The red-headed wood pigeon exhibited far lower genetic diversity than the nominate Japanese wood pigeon. Furthermore, the red-headed wood pigeon appears to have experienced a strong genetic drift from a common ancestral population, inferred by STRUCTURE analysis. The markers described here may be useful for investigating genetic diversity and genetic structure of Japanese wood pigeon populations, and could be used to estimate appropriate evolutionarily significant unit and to guide development of a

captive breeding program based on the genetic information.

Chapter 3

I conducted genetic analyses of the red-headed wood pigeon *Columba janthina nitens* using the DNA sequences of a portion of the mitochondrial control region and five microsatellite markers to estimate the genetic characteristics of the two wild populations from the Bonin and Volcano Islands, as well as one captive breeding population. The genetic diversity of the wild individuals was exceptionally low in both the mitochondria (nucleotide diversity = 0.00105) and at the microsatellite (3.2 alleles per locus and H_E = 0.12) loci. Higher numbers of microsatellite genotypes were observed in the Volcano Islands population than in the Bonin Islands population, which maybe because of the relatively low impact of human disturbance in the Volcano Islands. The most common mitochondrial haplotypes and microsatellite alleles observed in the two wild populations were completely fixed in the captive population. These results suggest that the genetic diversity of the captive population needs to be increased. However, introduction of a wild individual into a captive population can lead to a decreased genetic diversity in the small wild population and therefore should be done with caution. The genetic differentiation between the Bonin and the Volcano island groups was low, and the populations of the two island groups should be regarded as a single evolutionarily significant unit. However, special consideration is required for habitat conservation in the Volcano Islands, which may be functioning as a sanctuary for the red-headed wood pigeon. For the long-term conservation of threatened bird species that live on remote oceanic islands, determination of management units considering gene flows dependent on their flying capacity and maintenance of genetically suitable wild and captive populations are essential.

Chapter 4

Knowing the diet of an endangered animal is important for adequate nature restoration on oceanic islands. This is because the eradication of introduced species may fatally affect the endangered species when the endangered species rely on the introduced species as diet. DNA barcoding techniques together with high-throughput sequencing (HTS) may provide more detailed information on animal diets than other traditional methods. I performed a diet analysis using 48 fecal samples from the critically endangered red-headed wood pigeon *Columba janthina nitens*

based on chloroplast *trn*L P6 loop sequences. The frequency of each detected plant taxon was compared with a micro-histological analysis of the same sample set. The HTS approach detected a much larger number of plants than the micro-histological analysis. Plants those were difficult to identify by micro-histological analysis after being digested in the pigeon stomachs were frequently identified only by HTS. The results of HTS analysis indicated the frequent consumption of introduced species, in addition to several native species, by the red-headed wood pigeon. The rapid eradication of specific introduced species may reduce the food resource available to this endangered bird, and thus special consideration of pigeons' diet may be required in nature restoration planning. Although some technical problems still exist, the HTS approach may contribute to a better understanding of ecology of island endemics and their conservation.

Chapter 5

I studied the feeding ecology of the critically endangered red-headed wood pigeon *Columba janthina nitens*, a subspecies endemic to a highly remote and disturbed oceanic island chain: the Ogasawara Islands. An analysis based on high throughput sequencing (HTS) was carried out for 628 fecal samples collected over two years on two island habitats. Food availability and the nutrient composition of common fruit found on the two islands were also estimated. The results of the HTS diet analysis detected 122 food plant taxa and showed clear seasonal and inter-island variations in the pigeons' diet. Although the pigeons' preference for lipid rich fruits indicated, the pigeons' diet were flexibly changed according to food resource availability. This flexibility of food selection may reflect the foraging strategy of the pigeon in isolated island habitats with poor food resources. Pigeons also temporally consumed introduced plants in high frequency, which may complement the lack of preferable native food resources. The degree of the pigeons' dependence on introduced plants seems to differ between the two island habitats; thus, different impacts of the eradication of introduced plants on the pigeons' foraging condition on each island should be considered. HTS diet analysis combined with field data may be a useful tool to monitor the foraging conditions of endangered species, which may vary according to environmental changes, ecological disturbance and nature restoration in their habitat. This study may also contribute to an appropriate conservation in oceanic island ecosystems with complicated food webs including native and introduced species.

Chapter 6

This conclusion chapter summarizes how my study employing a multifaceted molecular biological approach revealed genetic characteristics and foraging ecology of the red-headed wood pigeon, making it possible to inform conservation planning for the pigeon within and outside of the habitat. The results of this conservation biological study also suggested general management issues for island endemic birds: 1) careful selection of founder individuals for ex-situ conservation to maintain genetic diversity within and outside of the habitat 2) consideration of genetic structure, which may reflect flying capacity and may differ among species in detection of management units 3) nature restoration considering the interaction between native and introduced species, which may be temporary and/or spatially different. Further technical improvements of molecular biological methods and combination with field works may contribute appropriate conservation planning of the valuable but disturbed oceanic island ecosystems.

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Appendix 1
Lack of genetic differentiation among subpopulations of the black-footed albatross on the Bonin Islands

Summary

I performed genetic analyses, using 11 polymorphic microsatellite markers for six breeding colonies of the black-footed albatross *Phoebastria nigripes* on the Bonin Islands, an important breeding area in the western North Pacific, to determine appropriate management unit for long-tem conservation of genetic diversity. The results of AMOVA, STRUCTURE analysis, principal coordinates analysis, values of F_{ST}, R_{ST} and the migration rates suggested the lack of genetic differentiation aomong subpopulations. Although albatross species are strongly philopatric, the present results indicate a lack of population genetic differentiation among six subpopulations and the presence of sufficient gene flow to maintain the genetic homogeneity. In the principal coordinates analysis, a few individuals were genetically different from most of the other individuals, indicating a probability of immigration. The black-footed albatrosses on the Bonin Islands are in a good condition to maintain genetic diversity and can be treated as a single genetic management unit.

Introduction

Certain pelagic seabirds such as albatrosses and petrels exhibit strong nest fidelity and natal philopatry, despite wide-ranging movement (Tickell 2000). These biological features can strongly affect population dynamics and are considerable in conservation efforts. For example, in a recovery activity of the endangered short-tailed albatross *Phoebastria albatrus*, establishing a new breeding colony that was only 2 km away from an existing colony took 12 years, despite attracting individuals with decoys and a sound system (Hasegawa 2007). Nest fidelity and natal philopatry may also affect the population genetic structure. If there are few migrations among breeding colonies, gene flow among subpopulations would be restricted. In such a case, genetic diversity may be reduced because of genetic drift and inbreeding, especially in small subpopulations (Frankham *et al*. 2002). To understand the philopatric birds within their ecological context and draft an appropriate conservation plan, it is important to characterize the population genetic structure.

The black-footed albatross *Poebastria nigripes* is a seabird endemic to the North Pacific (BirdLife International 2005). This species also exhibits strong nest fidelity and natal philopatry; breeding birds have reportedly returned to the nest within > 5m of the previous year's nest, and

fledging chicks usually return to their natal colonies to breed (Cousins & Cooper, 2000). Black-footed albatross mainly breed in the Northwestern Hawaiian Islands in the eastern North Pacific (more than 59 000 pairs), the Izu Islands (~700 pairs), the Bonin Islands (~730 pairs) and the Senkaku Islands (>100 pairs) in the western North Pacific (Cousins & Cooper 2000; Tickell 2000; Chiba *et al*. 2007). A few pairs have been found to breed intermittently in US outlying islands, such as the Marshall Islands (Naughton *et al*. 2007). The breeding population of the Hawaiian Islands, which represents more than 95% of the total breeding pairs, is slightly declining due to incidental mortality in long-line fisheries (Cousins & Cooper 2000; IUCN 2008). In 2000, at least 2000 birds were reportedly killed in US-based fisheries and a further 6000 in Japanese and

Taiwanese fleets. If the present situation continues, > 60% of black-footed albatross populations are expected to decline over the next three generations (i.e. 56 years) (IUCN, 2008). The black-footed albatross is listed as 'endangered' under the IUCN criteria (IUCN 2008).

The Ogasawara Islands comprise one of the most important breeding areas of the black-footed albatross in the western North Pacific (Fig. 1). The islands consist of two groups: the Bonin Islands (including Mukojima Islands, Chichijima Islands, Hahajima Islands) and the Volcano (Iwo) Islands, and additional isolated islands, such as Minami-Torishima. Breeding of the black-footed albatross is recognized on seven islands in the Mukojima Islands (Nakanoshima, Muko-Torishima, Mukojima, Nakoudojima Nakoudo-Torishima, Meganeiwa and Yomejima) and two islands in the Hahajima Islands (Ane-Minamitorishima and Imouto-Torishima) (Chiba *et al*. 2007; Fig. 1). The Mukojima and Hahajima islands are about 120km apart. Breeding colonies on the Mukojima Islands have been known before 1930 (Muko-Torishima, Mukojima, Nakoudojima, Nakoudo-Torishima; Yamashina 1930; Chiba *et al*. 2007). The subpopulations on Muko-Torishima, Nakoudojima, and Nakoudo-Torishima were reported in 1930 (Yamashina 1930) and the subpopulation of Yomejima after 1945 (Chiba *et al*. 2007). The subpopulation on Mukojima became locally extinct around 1930 but was reestablished naturally in 1992. The number of breeding pairs on each breeding colony ranged from <10 to 200 (Suzuki *et al*. unpubl. data; Fig. A1). On the other hand, colonies on the Hahajima Islands have been established recently (in 2003–2004; Institute of Boninology 2004), and the number of breeding pairs at this location was much smaller than that of the Mukojima Islands (about 10; Fig. A1.1).

The population had a high mortality rate during the late 19th and the early 20th centuries because of overexploitation for feathers and meat (Cousins & Cooper 2000; Tickell 2000). Roughly 250 000 black-footed albatrosses are estimated to have been killed between 1898 and 1901 on Minami-Torishima (Eda *et al*. 2008). Furthermore, Pacific War battles on the Iwo Islands and a

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volcanic eruption on Nishinoshima Island in 1969 disturbed breeding colonies and caused a population decline (Kurata & Kaneko 1982; Cousins & Cooper 2000). In the 1930s, only a dozen black-footed albatrosses were seen on Muko-Torishima (Yamashina 1930, 1942; Tickell 2000).

Recently, the number of the black-footed albatrosses in the Bonin Islands has been recovering, but each breeding colony is scattered and the size of each island subpopulation remains small (10–200 pairs). In these small subpopulations, inbreeding depression and extinction could occur if there is no gene flow among subpopulations. Furthermore, the population of the Bonin Islands is genetically different from the Hawaiian population, as suggested by previous studies using the mitochondrial cytochrome b region (Eda *et al*. 2008). This population represents an important lineage for the conservation of genetic diversity within the species. It is important to assess the genetic traits of the Bonin Island population to assist in conservation efforts for this species.

This study aims to determine the present-day genetic status of the black-footed albatross on the Bonin Islands. I performed genetic analyses using microsatellite markers of samples from major breeding colonies in the Bonin   Islands in order to assess the genetic diversity and genetic structure of the population.

Materials and methods

Sampling and DNA extraction

Feather samples were collected from 77 black-footed albatross chicks at six breeding colonies on the following islands: Muko-Torishima (20), Mukojima (10), Nakoudojima (20) and Yomejima (20) in the Mukojima Islands, and Imouto- Torishima (four) and Ane-Minamitorishima (three) in the Hahajima Islands, in April and May 2008. The collected samples were stored at -30 °C before DNA extraction. DNA was extracted from the roots of feathers according to the SDS/Proteinase K protocol (Sambrook & Russell 2001).

Microsatellite analysis

Genotypes of the sampled individuals were determined at 11 polymorphic microsatellite loci (Dc5, Dc9, Dc20, De11, 10C5, 11F3, 11H1, 11H7, 12C8, 12E1 and 12H8; Burg 1999; Dubois et al. 2005) isolated from wandering albatross *Diomedea exulans*. PCR amplification was carried out using a Qiagen Multiplex PCR kit (Qiagen). Each 10 mL total volume of the reaction mixture contained 5 ng of extracted DNA, 5 mL of 2x Multiplex PCR Master Mix and 0.2 mM of each primer pair. The PCR conditions were as follows: first denaturation for 15 min at 95 °C, 25 cycles for 30 s at 94 °C, 1.5 min at 57 °C, 1 min at 72 °C and a final cycle for 30 min at 60 °C. The sizes of

the PCR products were measured using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and GENSCANTM analysis software (Applied Biosystems).

Data analysis

To quantify genetic diversity, the number of alleles per locus, allelic richness and expected and observed heterozygosities were calculated. The number of alleles per locus and expected and observed heterozygosities were calculated using the EXCEL MICROSATELLITE TOOLKIT (Park 2001); allelic richness was calculated using FSTAT 2.9.3 (Goudet 2001). Departures from the Hardy–Weinberg Equilibrium (HWE) and the linkage disequilibrium were tested using FSTAT. To estimate the hierarchical genetic structure of each population, an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was performed using GENALEX 6 (Peakall & Smouse 2006). Genetic variances were partitioned at three levels: between groups of subpopulations: Mukojima Islands and Hahajima Islands, among subpopulations within groups, and within subpopulations. The F_{ST} and R_{ST} values of each pair of the subpopulations and between groups of Mukojima Islands and Hahajima Islands were calculated, and I tested their deviations from zero using GENALEX. The performance of these estimators depends on the applicability of the underlying analytical models, and many other factors such as microsatellite mutation rates (Balloux & Lugon-Moulin 2002). I used both F_{ST} and R_{ST} because it is unclear which of these estimators reflects the genetic structure of our genotype data more exactly than the other. The migration rates among subpopulations were estimated using BayesAss 1.1, which applies the Bayesian method to multilocus genotypes and determines the migration rates between populations over the last several generations (Wilson & Rannala 2003). Because the BayesAss program does not assume migration-drift equilibrium, it is suitable for estimation among increasing and expanding subpopulations on the Bonin Islands. The program was carried out at 3,000,000 Markov chain Monte Carlo (MCMC), 1,000,000 burn-in repetitions and a sampling frequency of 2000. Delta was set to the default value of 0.15. Ten independent runs were carried out, and the mean values of migration rates were compared among subpopulations. In addition, 95% confidence intervals were reported for each estimate. To assess the genetic differences among individuals, two types of individual-based analysis were performed. First, STRUCTURE analysis was performed using the software STRUCTURE 2.2 (Pritchard *et al.* 2000), and the number of genetic groups (*K*) was estimated. The model with admixture and correlated allele frequencies was used. Twenty runs of *K* = 1–10 were carried out at 1,000,000 MCMC and 100,000 burn-in repetitions. The likelihood of each *K* was then calculated, and the appropriate K was chosen. Second, a principal coordinate

analysis (PCO) based on pairwise kinship and relatedness values among individuals were performed using GENALEX.I used kinship defined by Loiselle *et al.* (1995) and Ritland (1996) and relatedness by Hardy & Vekemans (1999), Queller & Goodnight, (1989), Lynch & Ritland (1999), Wang (2002) and Li *et al.* (1993). In the case of diploids in noninbreeding, the two coefficients are closely related and provide the same information (Hardy & Vekemans 1999). However, to reduce the error of analysis caused by the small sample sizes and the relatively low polymorphism of the markers, I used multiple coefficients including both kinship and relatedness. Coefficients were estimated using SPAGeDi 1.2 (version 1.2; Hardy & Vekemans 2002). I performed an assignment test, using GeneClass2 (Piry *et al.* 2004), for individuals that plotted separately from most other individuals and estimated how likely the genotypes of these individuals are in the Bonin Islands.

Results

The genotypes of all 77 individuals at 11 microsatellite loci were determined. The average values of the number of alleles on each subpopulation (N_A) were 2.4–3.9, with the overall average being 4.5. The allelic richness (R_S) values were 1.99–2.36, with an overall average of 2.08. The overall expected and observed heterozygosity (H_E and H_O) ranged from 0.33 to 0.44 (average: 0.36) and 0.29 to 0.48 (average: 0.32) respectively (Table A1.1). There were no departures from HWE in each microsatellite locus and no evidence of linkage disequilibrium among any pairwise microsatellite loci comparisons. However, the power of the test for HWE is limited in small sample sizes (~10) and virtually non-existent for the two samples for which n=3 (subpopulation in Ane-Minamitorishima). In the AMOVA results, almost the entire genetic variance (F_{ST}: 97%, R_{ST}: 100%) was attributed to among individuals of each subpopulation (Table A1.2). Most of the F_{ST} values among subpopulations were not significantly larger than zero, except for the two pairs of Mukojima-Nakoudojima (F_{ST} = 0.032, $P<0.05$) and Nakoudojima-Yomejima (F_{ST} = 0.030, $P<0.01$). All the RST values among subpopulations were 0.000 (Table A1.3). FST and RST values between groups of Mukojima Islands and Hahajima Islands are 0.020 and 0.000, respectively, and both of these were not significant (Table A1.2). The migration rates estimated by BayesAss 1.1 among the breeding colonies were relatively high (3.0–5.3%; Table A1.4). The migration rates were not found to be related to spatial distances among breeding colonies (Fig. A1.2). I checked the convergence of MCMC, but the log likelihood values did not peak at the initiation point of data collection. In the STRUCTURE analysis, the log likelihood was maximized at *K*=1, indicating that all individuals were clustered to the same genetic group (Fig. A1.3). However, the parameters (F_{ST} and a) did not converge even when MCMC and burn-in repetitions were increased to 10 000 000 for $K = 1$–2.

According to the results of the PCO analysis based on kinship (Loiselle *et al*. 1995) among individuals, most individuals were plotted concentrically as a single group, even though they were sampled at different breeding colonies (Fig. A1.4). On the other hand, two individuals (YM03 and NA09) were plotted separately from most of the other individuals. Several other PCO analyses using different kinships (Ritland 1996) and relatedness values (Queller & Goodnight 1989; Li et al. 1993; Hardy & Vekemans 1999; Lynch & Ritland 1999; Wang 2002) showed similar results (data not shown). The probabilities that the individuals YM03 and NA09 would be assigned to the Bonin Islands' population were low (0.7 and 6.8%, respectively). These two individuals may have genetic features that differ from those of the other individuals.

Discussion

Population dynamics and genetic structure of the black-footed albatross on the Bonin Islands

The results of RST, AMOVA, BayesAss, STRUCTURE, and PCO analyses and the small values of F_{ST} suggested lack of genetic differentiation among six subpopulations of the black-footed albatross on the Bonin Islands. These results indicated that the population of the black-footed albatross on the Bonin Islands is likely to be a metapopulation, which is genetically homogenized. This is despite the fact that strong nest fidelity and natal philopatry were reported for this species (Cousins & Cooper 2000) and that the breeding colonies are scattered in this area. However, the F_{ST} values were significantly greater than zero in two pairs of Mukojima-Nakoudojima and Nakoudojima-Yomejima. F_{ST} is based on the infinite allele model (IAM), which assumes that each mutation creates a novel allele. R_{ST} is based on the stepwise mutation model (SMM), which assumes that each mutation creates a novel allele either by adding or deleting a single repeated unit of microsatellite. F_{ST} is deflated by a high mutation rate of microsatellite, while R_{ST} is unaffected by the mutation rate under a strict SMM. On the other hand, F_{ST} reflects the population genetic structure more sensitively than R_{ST} in small and recently diverged populations wherein stepwise mutations are not accumulated and migration or genetic drift will be more influential than mutation events for population subdivision (Ruzzante 1998; Balloux & Lugon-Moulin 2002). The significance of F_{ST} in this study may be derived from small sample sizes, recent population expansion and migration among breeding colonies. The non-convergence of Markov chain in analyses based on the Bayesian method: BayesAss and STRUCTURE may have been affected by the low genetic differentiation among subpopulations. The performance of Bayesian clustering methods, which depends on the Hardy–Weinberg and linkage disequilibria, will decrease with decreasing genetic differentiation (Latch *et al*. 2006). In BayesAss analysis, Wilson & Rannala

(2003) pointed out that higher migration rates and smaller sample sizes increase the variance of posterior probability distribution. Although the non-convergence of Markov chains indicates higher migration rates among subpopulations, the confidence of the values may be low. In STRUCTURE analysis, Latch *et al*. (2006) suggested that the performance of the program decreases when the F_{ST} values decline below 0.03. In this study, the F_{ST} values of each pair of subpopulations ranged from 0.00 to 0.032, and this indicates that the STRUCTURE program might not perform well for clustering and detecting the number of clusters (*K*). Conversely, the non-convergence of MCMC indicates that $K = 1$.

Several previous studies also showed a lack of genetic differentiation among subpopulations of albatross species; these studies used microsatellite markers in a geographical scale similar to this study. In waved albatross *Phoebastria irrorata*, there was no genetic differentiation among three subpopulations whose breeding colonies were 5–15 km apart from each other (Huyvaert & Parker 2006). Three subpopulations of white-capped albatross *Thalassarche cauta* (30km apart; Abbott & Double 2003) and two subpopulations of Buller's albatross *Thalassarche bulleri bulleri* (126 km apart; Bekkum *et al*. 2006) showed similar results. On a larger scale, despite the fact that each breeding colony was about 1000 km apart, no genetic differentiation was found among the five subpopulations of the wandering albatross (*Diomedea exulans*; Burg & Croxall 2004) or two subpopulations of black-browed albatross *Thalassarche melanophris* (Burg & Croxall 2001). Furthermore, all subpopulations of the gray-headed albatross *Thalassarche chrysostoma*, whose colonies are dispersed around the Antarctic Ocean, were not genetically differentiated (Burg & Croxall 2001). In a study using amplified fragment length polymorphism markers, Milot *et al*. (2008) reported the probability of genetic homogeneity among 10 breeding colonies of the wandering albatross located in various geographic scales (5–7000 km).

In addition to the lack of genetic differentiation, there is a remarkable similarity in genetic diversity among black-footed albatross subpopulations on the Bonin Islands, despite a large difference in census/sample sizes. This pattern is not uncommon in albatrosses: it is seen, for example, in the waved albatross *Phoebastria irrorata* (Huyvaert & Parker 2006) and the wandering albatross *Diomedea exulans* (Burg & Croxall 2004; Milot *et al*. 2008). The pattern might be relevant to the population dynamics of philopatric but highly mobile albatrosses. In the wandering albatross, Milot *et al*. (2008) suggested two interpretations. First, current colonies might have been derived from one ancestral source. Second, recurrent colonization and extinction of metapopulation dynamics reduced the genetic diversity of subpopulations but occasional long-distance dispersal contributed to maintaining genetic panmixia.

The genetic homogeneity of black-footed albatross populations on the Bonin Islands can be explained by metapopulation dynamics, especially in re-colonization and current migration among subpopulations. Re-colonization is an important factor that has a huge effect on the genetic characteristics of newly established subpopulations. The recolonization of genetically variable founders could have contributed to transferring the genetic diversity from the original subpopulations to the newly established subpopulation and to inhibit genetic differentiation among these subpopulations. After a drastic decline in numbers due to a past bottleneck (Cousins & Cooper 2000; Tickell 2000; Chiba *et al*. 2007), the population on the Bonin Islands has slowly recovered and re-colonization has occurred in some islands, such as Mukojima, Imouto-Torishima and Aneminamitorishima (Tokyo Metropolitan Government, 2008). The similarity in the genetic diversity of the newly established populations and the other population suggested that these re-colonizations were attributable to the genetic variation in the founder individuals.

The current migration among subpopulations is another important factor for maintaining genetic homogeneity of scattered subpopulations on the Bonin Islands. Although the black-footed albatross exhibits strong nest fidelity and natal philopatry (Cousins & Cooper 2000), our results consistently suggested lack of genetic differentiation among six subpopulations of the black-footed albatross on the Bonin Islands. Thus, it is reasonable to assume that a certain amount of gene flow among the subpopulations occurred in the Bonin Island population due to migration, and this gene flow might contribute to the small genetic differentiation among subpopulations and similarity in genetic diversity among subpopulations in this area. Migrations of the black-footed albatross among breeding colonies on the Bonin Islands have been found occasionally by mark–recapture studies. However, the data for this species are fragmented, and its migration rate is unknown (Tokyo Metropolitan Government 2008) because all of the breeding colonies of the black-footed albatross on the Bonin Islands are located on uninhabited islands and frequent field examinations are challenging. Therefore, banding data in the Bonin Islands cannot precisely determine whether migrants are involved in reproduction. The present study results suggest that migrants join in breeding and strongly affect the genetic homogeneity among subpopulations on the Bonin Islands. The genetic structure of the population on the Bonin Islands is influenced by several factors acting together (including nest fidelity, philopatry and migration), but gene flow (including migration/colonization) appears to be the dominant factor that explains the genetic pattern over the various distances between the colonies on the Bonin Islands.

Genetic homogeneity of the population also means that there is no population fragmentation among subpopulations. This is an important condition for the population to maintain

genetic diversity because the population is insusceptible to genetic drift. In addition, the low to moderate genetic diversity, which was found in the seriously bottlenecked population of the black-footed albatross on the Bonin Islands, is not unusual for albatross species. Long lived species are considered to be less vulnerable to demographic stochasticity and are, to some extent, buffered against loss of genetic diversity when bottlenecks last for short periods of time (Sæther *et al.* 2004). Milot *et al.* (2007) suggested that the wandering albatross *Diomedea exulans* might have survived with low genetic diversity for long periods of time due to their life history or inbreeding avoidance behavior. The results of the present study indicate that the present genetic status of the black-footed albatross on the Bonin Islands is not in serious peril for its long-term conservation.

Migrations from outside of the Bonin Islands

Regarding the results of our PCO analyses, two individuals (YM03 and NA09) were plotted separate from the majority and were found to have genetically different features. These individuals have a rare allele, and they may be related to a minor strain in the population on the Bonin Islands or to immigrants. In particular, the individual YM03 can be excluded with a confidence level of 0.01. This individual may be related to an immigrant from outside of the Bonin Islands (e.g. the Hawaiian Islands). Walsh & Edwards (2005) performed a genetic analysis of populations of the black-footed albatross in Izu-Torishima and the Hawaiian Islands by using the mitochondrial cytochrome b region. They suggest that the migration rate from the Hawaiian to western North Pacific Islands is negligible. This conclusion was inferred via the maximum likelihood method. In the study of Eda *et al.* (2008), the migration rate was inferred via the Bayesian method, and genotypic data of the Bonin Islands population were added to the data of Walsh & Edwards (2005). Eda *et al.* (2008) suggest a low frequency of migration but also point to the apparent existence of migrants from the Hawaiian Islands into the western North Pacific Islands.

A mark–recapture study reported that a chick banded at Pearl Hermes Reef in the Midway Islands had been bred in the Bonin Islands in the 1970s (Tickell 2000). At sea, black-footed albatrosses disperse widely, with ranges of distribution overlapping between Hawaiian and western North Pacific populations in the non-breeding season (Cousins & Cooper 2000; Tickell 2000). Therefore, it is possible for the black-footed albatross to migrate between the Hawaiian and the Bonin Islands, despite the fact that the distance is 4000 km. Considering the results of genetic studies – including those of the current study and mark–recapture studies – migrants to the Bonin Islands might affect the recent distribution and help maintain the genetic diversity of the population on the Bonin Islands. Migrants from the Hawaiian populations might not strongly affect the

population genetic structure because haplotypes of mtDNA showed two clades that separate the Hawaiian and western North Pacific populations (Eda *et al*. 2008). However, even if they arrive in low numbers, migrants can increase the genetic diversity of the bottlenecked population on the Bonin Islands.

Conclusion

I performed the first microsatellite analysis of the black-footed albatross and obtained basic data pertaining to the long-term conservation of a population on the Bonin Islands. I suggested the effectiveness of genetic analysis by assessing the population dynamics of a seabird on the Bonin Islands, given that the undertaking of precise mark– recapture studies is difficult. Population fragmentation among subpopulations was absent. Neither nest fidelity nor natal philopatry seemed to affect the population genetic structure of the black-footed albatross on the Bonin Islands. In addition, there is the probability of migration from outside of the Bonin Islands. The population can be treated as a single management unit in terms of the conservation of genetic diversity within the species. At this moment, the risk of extinction due to inbreeding within each island subpopulation is low and artificial conservation activities such as reintroduction are not required. However, the population on the Bonin Islands is so small that it is vulnerable to fishery by-catch and change in the environment. If the population declines, the present genetic status might be disrupted due to genetic drift and inbreeding. For the long-term conservation of the black-footed albatross on the Bonin Islands, continuous monitoring of the population size, environment of breeding sites and genetic status is important.

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Table A1.1 Genetic diversity of the black-footed albatross *Phoebastria nirlipes* based on microsatellite data

	Microsatellite loci											
	10C5	11F3	11H1	11H7	12C8	12E1	12H8	Dc5	Dc9	Dc20	De11	Average
Mukojima Islands												
Muko-Torishima Island (MT, $n = 20$)												
N_A	3	1	7	9	2	3	2	4	3	2	3	3.6
R_S	1.80	1.00	3.76	3.91	1.49	1.43	1.15	1.95	1.79	1.28	2.36	1.99
H_E	0.30	0.00	0.79	0.81	0.18	0.14	0.05	0.34	0.27	0.10	0.58	0.33
H_O	0.35	0.00	0.85	0.85	0.20	0.15	0.05	0.40	0.30	0.10	0.35	0.33
Mukojima Island (MI, $n = 10$)												
N_A	2	2	8	6	2	3	1	2	3	1	3	3.0
R_S	1.98	1.30	3.97	3.85	1.87	1.60	1.00	1.30	2.17	1.00	2.26	2.03
H_E	0.51	0.10	0.79	0.81	0.39	0.19	0.00	0.10	0.47	0.00	0.54	0.36
H_O	0.60	0.10	0.70	0.90	0.30	0.20	0.00	0.10	0.40	0.00	0.60	0.35
Nakoudojima Island (NA, $n = 20$)												
N_A	2	1	7	8	2	2	2	5	3	1	4	3.4
R_S	1.28	1.00	4.04	3.95	1.88	1.71	1.15	1.98	1.54	1.00	2.40	1.99
H_E	0.10	0.00	0.83	0.82	0.41	0.30	0.05	0.32	0.19	0.00	0.58	0.33
H_O	0.10	0.00	0.80	0.65	0.45	0.15	0.05	0.30	0.20	0.00	0.50	0.29
Yomejima Island (YM, $n = 20$)												
N_A	2	1	9	10	2	4	2	4	3	1	5	3.9

R_S	1.92	1.00	3.67	4.25	1.58	1.92	1.15	2.01	2.52	1.00	2.65	2.15
H_E	0.45	0.00	0.75	0.86	0.22	0.31	0.05	0.35	0.57	0.00	0.62	0.38
H_O	0.45	0.00	0.80	0.95	0.25	0.35	0.05	0.30	0.60	0.00	0.55	0.39
Hahajima Islands												
Imouto-Torishima Island (IM, $n = 4$)												
N_A	2	1	4	7	2	3	1	1	3	1	3	2.6
R_S	1.75	1.00	3.46	5.46	1.75	2.93	1.00	1.00	2.75	1.00	2.75	2.26
H_E	0.25	0.00	0.75	0.96	0.25	0.71	0.00	0.00	0.68	0.00	0.68	0.39
H_O	0.25	0.00	0.75	1.00	0.25	0.00	0.00	0.00	0.50	0.00	0.50	0.30
Ane-Minamitorishima Island (AN, $n = 3$)												
N_A	2	1	4	4	1	3	1	3	3	1	3	2.4
R_S	2.00	1.00	4.00	4.00	1.00	3.00	1.00	3.00	3.00	1.00	3.00	2.36
H_E	0.33	0.00	0.87	0.87	0.00	0.73	0.00	0.60	0.73	0.00	0.73	0.44
H_O	0.33	0.00	1.00	1.00	0.00	1.00	0.00	0.67	0.33	0.00	1.00	0.48
All ($n = 77$)												
N_A	3	2	9	10	2	6	2	5	3	2	5	4.5
R_S	1.79	1.04	3.81	4.05	1.68	1.86	1.11	1.87	2.13	1.08	2.45	2.08
H_E	0.33	0.01	0.79	0.83	0.28	0.30	0.04	0.30	0.41	0.03	0.59	0.36
H_O	0.34	0.01	0.81	0.84	0.05	0.23	0.04	0.30	0.38	0.03	0.51	0.32

The number of individuals (n), the number of alleles per locus (N), the expected (H_E) and observed (H_O) heterozygosities, and allelic richness (R_S) for 11 microsatellite loci in six populations. The names of the populations are abbreviated as follows: Muko-Torishima Island (MT), Mukojima Island (MI), Nakoudojima Island (NA), Yomejima Island (YM), Imouto-Torishima Island (IM), and Ane-Minamitorishima Island (AN)

Table A1.2 The results of AMOVA based on F_{ST} and R_{ST} for subpopulations in the Bonin Islands

	df	SS	MS	Var.	%	
F_{ST}						
Between groups	1	2.951	2.951	0.031	2%	F_{ST}=0.027 (*P*=0.050)
Among subpopulations within groups	4	10.276	2.569	0.023	1%	
Within subpopulations	148	285.767	1.931	1.931	97%	
R_{ST}						
Between groups	1	18.265	18.265	0.000	0%	R_{ST}=0.000 (*P*=0.96)
Among subpopulations within groups	4	228.497	57.124	0.000	0%	
Within subpopulations	148	12957.018	87.547	87.547	100%	

d.f., degree of freedom; SS, sum of squares; MS, mean sum of squares; Var, genetic variability; %, percentage variability.

Table A1.3 Matrix of pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) values for six subpopulations in the Bonin Islands: Muko-Torishima Island (MT), Mukojima Island (MI), Nakoudojima Island (NA), Yomejima Island (YM), Imouto-Torishima Island (IM), and Ane-Minamitorishima Island

	MT	MH	NA	YM	IM	AN
MT	---	0.000	0.000	0.000	0.000	0.000
MH	0.013	---	0.000	0.000	0.000	0.000
NA	0.001	0.032*	---	0.000	0.000	0.000
YM	0.008	0.000	0.030**	---	0.000	0.000
IM	0.037	0.011	0.041	0.000	---	0.000
AN	0.039	0.044	0.039	0.000	0.000	---

Asterisks denote significant values as follows:

*$P < 0.05$, **$P < 0.01$

Table A1.4 Estimate of the migration rate (proportion of individuals) among six subpopulations in the Bonin Islands: Muko-Torishima Island (MT), Mukojima Island (MI), Nakoudojima Island (NA), Yomejima Island (YM), Imouto-Torishima (IM), and Ane-Minamitorishima (AN). The numbers in parentheses show 95% confidence interval

		Migration from					
		MT	MH	NA	YM	IM	AN
Migration	MT	0.781 (0.672-0.960)	0.038 (0.000-0.157)	0.039 (0.000-0.163)	0.031 (0.000-0.141)	0.039 (0.000-0.162)	0.034 (0.000-0.168)
into	MH	0.041 (0.000-0.170)	0.792 (0.671-0.971)	0.038 (0.000-0.149)	0.032 (0.000-0.144)	0.038 (0.000-0.162)	0.036 (0.000-0.169)
	NA	0.035 (0.000-0.141)	0.036 (0.000-0.156)	0.784 (0.671-0.951)	0.031 (0.000-0.148)	0.035 (0.000-0.146)	0.032 (0.000-0.150)
	YM	0.053 (0.000-0.180)	0.046 (0.000-0.179)	0.048 (0.000-0.183)	0.823 (0.673-0.990)	0.051 (0.000-0.211)	0.043 (0.000-0.189)
	IM	0.038 (0.000-0.153)	0.036 (0.000-0.150)	0.038 (0.000-0.147)	0.030 (0.000-0.141)	0.786 (0.671-0.963)	0.032 (0.000-0.156)
	AN	0.053 (0.000-0.187)	0.053 (0.000-0.199)	0.053 (0.000-0.188)	0.052 (0.000-0.204)	0.052 (0.000-0.193)	0.823 (0.673-0.991)
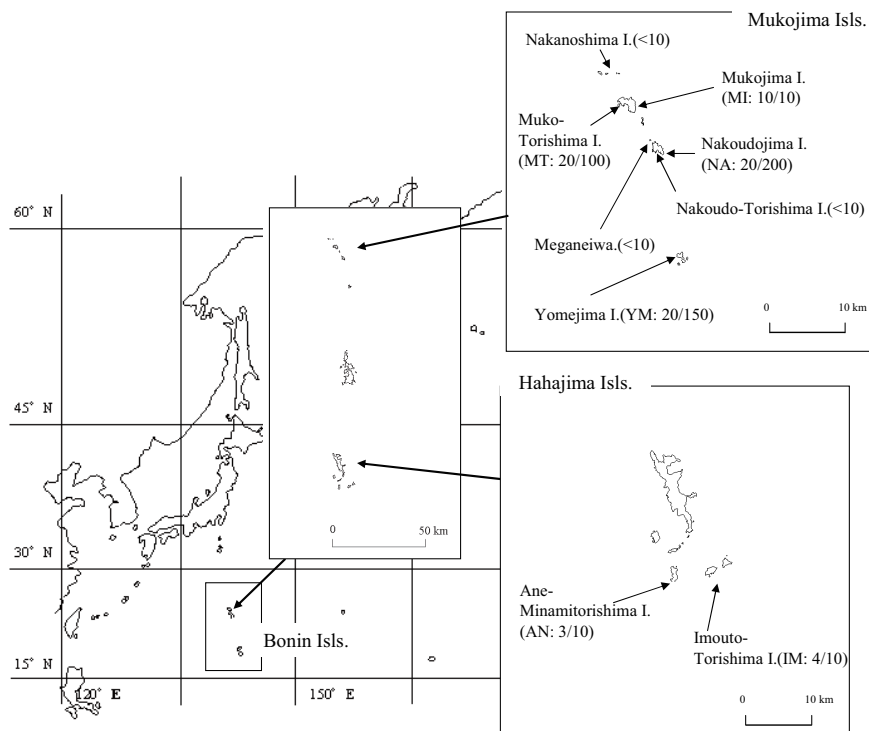
Fig. A1.1 Breeding range of black-footed albatross and sampling location in the Bonin Islands. The numbers in parentheses are the abbreviations of the study sites and the sample sizes/number of breeding pairs.
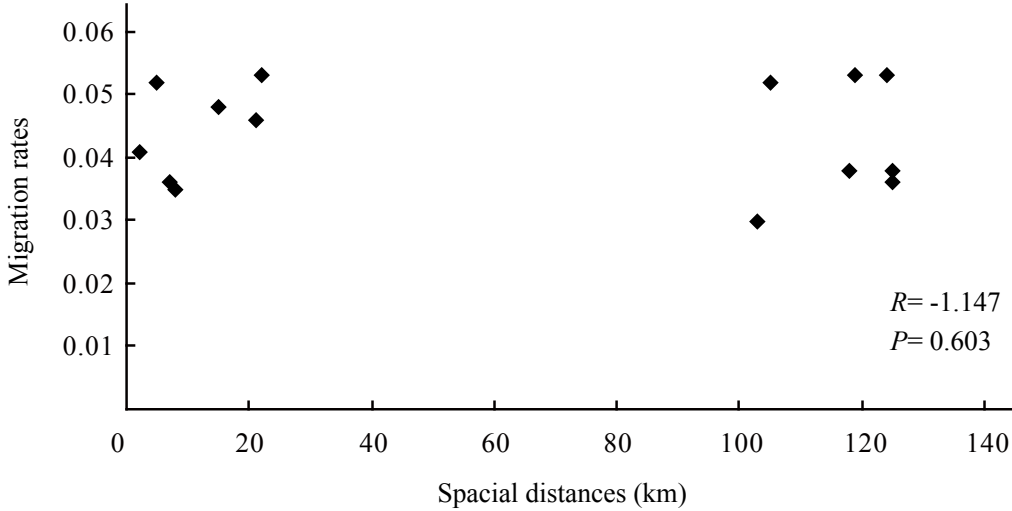
Fig. A1.2 Relationship between migration rates and spatial distance, among breeding colonies.
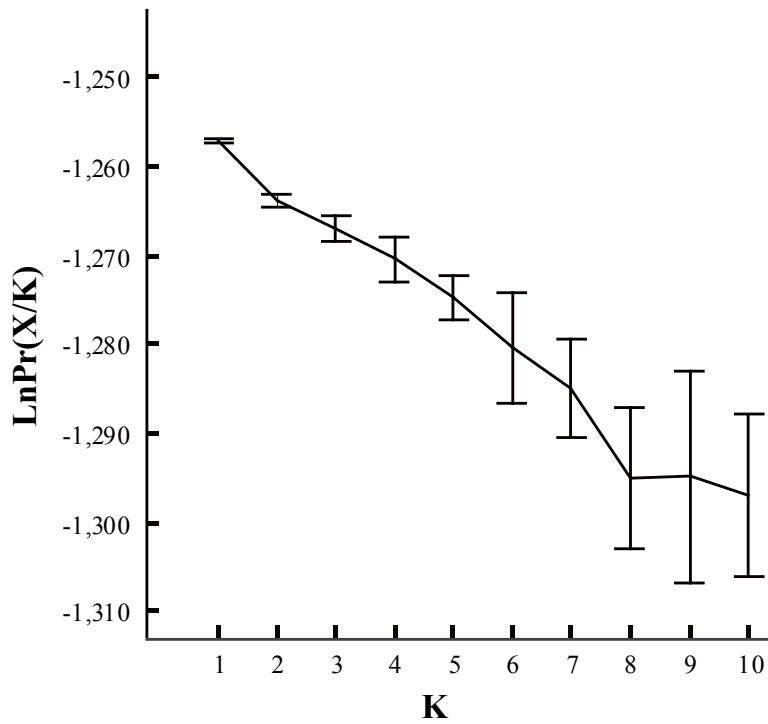
Fig. A2.3 Likelihood plot of STRUCTURE results. Ln Pr (X/K) is the log likelihood of each value of K, which is the number of simulated clusters. Where ln Pr (X/K) is maximized, *K* is the most likely. Black squares represent the average values of Ln Pr (X/K), and vertical lines represent standard deviations.
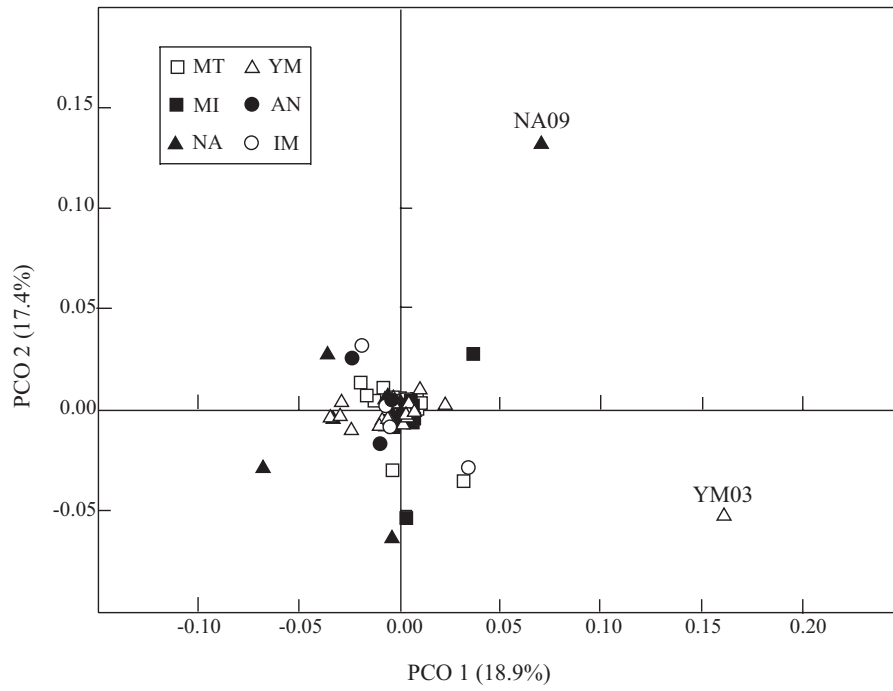
Fig. A2.4 Principal coordinate analysis plot of kinship (Loiselle *et al*. 1995) among individuals. The names of the populations are abbreviated as follows: Muko-Torishima Island (MT), Mukojima Island (MI), Nakoudojima Island (NA), Yomejima Island (YM), Ane-Minamitorishima Island (AN) and Imouto-Torishima Island (IM).

Appendix 2

Predominance of unbalanced gene flow from western to central north pacific colonies of the black-footed albatross (*Phoebastria nigripes*)

Summary

To estimate possible gene flow caused by dispersal between populations two remote breeding sites: western North Pacific and central North Pacific, I performed genetic analysis on six colonies of black-footed albatross *Phoebastria nigripes* using 10 microsatellite markers. The central and western North Pacific populations were genetically differentiated. However, an estimation of migrants per generation indicated directional dispersal from the western to the central North Pacific.Compared to other Central North Pacific populations, the population on Kure Atoll, the westernmost atoll in the Hawaiian Islands in the central North Pacific, exhibited weaker genetic differentiation from the western North Pacific populations, suggesting frequent immigration from the western North Pacific. The recent expansion of the western North Pacific population may be due to an increase in returning individuals, which may be caused by increased breeding success rates and /or survival rates. Range-wide and long-term monitoring of the black-footed albatross population using genetic markers may help to uncover dispersal dynamics of this highly mobile but philopatric albatross species and to make appropriate conservation decisions in light of environmental changes.

Introduction

For a highly mobile albatross species, the open ocean is not necessarily ageographical barrier that restricts dispersal (Weimerskirch & Wilson 2000; Croxall *et al*. 2005; Phillips *et al*. 2005). Long-range dispersal events may also influence the population dynamics of remote breeding colonies (Young 2010), which could be reflected by their population genetic structure, as estimated by highly variable markers (Rousset 2001). Several previous studies have reported gene flow among remote breeding colonies of albatross species (Burg & Croxall 2001, 2004; Abbott & Double 2003; Bekkum *et al*. 2006; Huyvaert & Parker 2006; Milot *et al*. 2008, Young 2010; Ando *et al*. 2011) despite their strong nest fidelity and natal philopatry (Tickell 2000). Information on the genetic structure of populations is important for determining dispersal dynamics and thus conservation planning for highly pelagic species.

The major breeding colonies of the black-footed albatross, *Phoebastria nigripes*, are restricted to two remote regions, approximately 4 000 km apart, in the North Pacific (BirdLife

International 2012). The colony in the Northwestern Hawaiian Islands in the central North Pacific is currently stable and holds more than 95% of the total population (approximately 59,000 pairs) distributed among eight breeding colonies. Future declines due to fishery bycatch and colony loss by sea level rise are a concern (Arata *et al*. 2009). In contrast, the populations in the western North Pacific, in the Izu Islands (approximately 1 000 pairs) and the Ogasawara Islands (approximately 1 000 pairs), are rapidly increasing and expanding (Tokyo Metropolitan Government 2008; Arata *et al*. 2009; H.S., K. Horikosi, T. Sasaki, and H. Chiba, unpubl. data), although the trend of another colony in the   Senkaku Islands (56 pairs [Arata et al. 2009]) is unknown. Indeed, the number of breeding pairs in the Ogasawara Islands has tripled in the last decade (H.S., K. Horikosi, T. Sasaki, and H. Chiba, unpubl. data).

Previous genetic studies using mitochondrial cytochrome *b* sequences indicated genetic differentiation between the central and western North Pacific populations (Walsh & Edwards 2005; Eda *et al*. 2008). Thus, the recent expansion of the western North Pacific population may be due to increasing natal recruitment of individuals from the western North Pacific population itself rather than to immigration from the central North Pacific population. However, the degree of gene flow between the two regions is still unresolved. Several sampled individuals in the western North Pacific colonies exhibited haplotypes common to the central North Pacific population (Walsh & Edwards 2005; Eda *et al*. 2008), and thus Eda *et al*. (2008) suggested that gene flow between the two regions should not be ignored. The results of a microsatellite analysis performed among breeding colonies in the Ogasawara Islands also indicated immigration from outside the Ogasawara Islands (Ando *et al*. 2011). All of these studies targeted a limited number of breeding colonies and had small sample sizes; thus these studies may have provided only fragmented information about the genetic structure of the black-footed albatross. In the case of the sympatrically distributed Laysan albatross, *P. immutabilis*, no clear genetic differentiation between the central and western North Pacific region was found, indicating that longrange dispersal may occur between the two regions in the North Pacific (Young 2010). Thus, similar results could be expected in the range-wide genetic analysis of the black-footed albatross using highly variable nuclear markers.

The study reported here sought to estimate recent possible gene flow between the central and western North Pacific populations of the black-footed albatross. All of the major breeding colonies of the species were targeted and genetic analysis was performed using polymorphic microsatellite markers.

Materials and methods

Sampling and DNA extraction

Feather or blood samples were collected from 278 Black-Footed Albatross chicks or breeding adults at six breeding colonies on the following islands: Izu-Torishima (50) and the Ogasawara Islands (77) in the western North Pacific and on Kure Atoll (11), Midway Atoll (48), Laysan Island (44), and Tern Island (48) in the central North Pacific from 2003 to 2008 (Fig. A2.1). Samples in the Ogasawara Islands were analyzed by Ando *et al.* (2011). The collected samples were stored at −30°C before DNA extraction. DNA of the Izu-Torishima samples was extracted according to the SDS/Proteinase K protocol (Sambrook & Russell 2001). For the samples from the central North Pacific, DNA was extracted from tissue and feathers using extraction kits (DNeasy, Qiagen) following the manufacturer's protocols, and DNA from blood was isolated using DNA purification kits (IDetect, ID Labs Biotechnology Inc., London, Ontario, Canada) for whole animal blood following the manufacturer's protocols.

Microsatellite analysis

Genotypes of the sampled individuals were determined at 10 polymorphic microsatellite loci (Dc5, Dc9, Dc20, De11, 10C5, 11F3, 11H1, 11H7, 12C8 and 12H8; Burg 1999; Dubois et al. 2005) isolated from the wandering albatross, *Diomedea exulans*. Polymerase chain reaction (PCR) amplification was carried out (using a Multiplex PCR kit, Qiagen). Each 10 ml total volume of the reaction mixture contained 5 ng of extracted DNA, 5 ml of 2xMultiplex PCR Master Mix, and 0.2 mM of each primer pair. PCR conditions were as follows: first denaturation for 15 min at 95°C, 25 cycles of 30 sec at 94°C, 1.5 min at 57°C, and 1 min at 72°C and a final cycle for 30 min at 60°C. The sizes of the PCR products were measured (using the ABI PRISM 3100 Genetic Analyzer, Applied Biosystems) and GeneScan and Genotyper analysis software (Applied Biosystems).

Data analysis

The number of alleles per locus (N_A), allelic richness (R_S), and expected (H_E) and observed heterozygosities (HO) were calculated to quantify genetic diversity. The number of alleles per locus and the expected and observed heterozygosities were calculated using GENALEX6 (Peakall & Smouse 2006), and allelic richness was calculated using FSTAT 2.9.3 (Goudet 2001). The difference in allelic richness between the central and western North Pacific groups, departures

from Hardy-Weinberg Equilibrium (HWE), and linkage disequilibrium were tested using FSTAT. I also listed private alleles in each region and in the population. To estimate the hierarchical genetic structure of each population, an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) based on F_{ST} and R_{ST} was performed using GENALEX 6. Genetic variances were partitioned at three levels: between the western and central North Pacific populations, among populations within regions, and within populations. The F_{ST} and R_{ST} values between each pair of populations were calculated, and their deviation from zero was tested using FSTAT. I performed individual-based clustering STRUCTURE analysis using the STRUCTURE 2.2.3 software (Prichard et al. 2000). In this method, the appropriate number of genetic groups (clusters: *K*) is estimated based on log likelihood, and individuals are assigned to the most likely cluster. Iused admixture with LOCPRIOR and allele frequency correlated models. Ten runs of $K = 1$–8 were carried out with 1 000 000 Markov Chain Monte Carlo (MCMC) and 100 000 burn-in repetitions. Based on the correlated allele frequency model, the amount of divergence for each cluster from a common ancestral population was calculated (*F* values; Falush *et al.* 2003). The number of migrants per generation among populations was estimated using a maximum-likelihood approach in the software Migrate 3.1.5 (Beerli & Felsenstein 1999, 2001). Iused a Brownian motion mutation model with constant mutation rates over loci, and each MCMC run consisted of 10 short and three long replicate chains. The burn-in period was set to 100 000 steps.

Results

The genotypes of 278 individuals were determined at 10 microsatellite loci. The estimators of genetic diversity in each population (N_A, R_S, H_E, and H_O) are shown in Table A2.1. Each population exhibited a similar level of genetic diversity, despite large differences in population size. Some populations have private alleles (Fig. A2.1, Table A2.1, Table A2.2). The allelic richness was not significantly different between the central and western North Pacific groups ($P = 0.134$). There were no departures from HWE at each microsatellite locus, and there was no evidence of linkage disequilibrium among any pairwise microsatellite loci comparisons.

In the AMOVA, 6% of the genetic variance was attributed to variance between regions, and both the F_{ST} and R_{ST} values were significantly different from zero (Table A2.3). Comparing pairwise F_{ST} values among populations, the central and western North Pacific populations were clearly genetically differentiated (Table A2.4). In contrast, the R_{ST} values between Kure in the central North Pacific and Ogasawara and Izu in the western North Pacific were not significantly

larger than zero (Table A2.4). In fact, the R_{ST} values between Kure and all other populations were not significantly different from zero. In the STRUCTURE analysis, the log likelihood was maximized at $K = 2$ (Fig. A2.2). From $K = 3$ to $K = 8$, the standard deviations increased. When $K = 2$, the central and western North Pacific populations were clearly assigned to different clusters (Fig. A2.3). However, the cluster that dominated in the western North Pacific population appeared in larger proportion in the Kure population than in the other populations in the central North Pacific. The F values of each cluster were nearly the same. The number of migrants per generation estimated using Migrate 3.1.5 is shown in Table 5. Significantly larger numbers of migrants were estimated from Ogasawara and Izu in the western North Pacific to each population in the central North Pacific compared with those in the opposite direction.

Discussion

Genetic characteristics and population history of the western North Pacific population

The results of the AMOVA, the F_{ST} values, and the STRUCTURE analysis suggested that the central and western North Pacific populations are clearly genetically differentiated. These results are consistent with previous studies comparing mitochondrial cytochrome *b* sequences between the populations (Walsh & Edwards 2005; Eda *et al*. 2008). In addition, both the western and central North Pacific populations had several private alleles and exhibited similar levels of genetic diversity. Black-Footed Albatross in the western North Pacific seem to have maintained a genetically unique population, despite the serious population decline due to human disturbance in the late nineteenth and early twentieth centuries (e.g. overexploitation for feathers and meat; Yamashina 1930, 1942; Cousins and Cooper 2000; Tickell 2000). This may be because many of the individuals, particularly nonbreeders, survived the over exploitation on the sea and maintained their genetic diversity. Rapid population recovery after the population bottleneck could prevent further loss of genetic diversity due to genetic drift. This kind of situation could be possible for the long-lived (40 or more years), latematuring (starts breeding at 7 yr old) Black-Footed Albatross (Cousins & Cooper 2000). A similar situation was reported in the short-tailed albatross, *P. albatrus*, whose breeding population had been reduced to 50 – 60 individuals in the 1940s in the Izu Islands (Tickell 2000; Hasegawa 2003) but has maintained high genetic diversity (29 haplotypes in mitochondrial control region; Kuro-o *et al*. 2010). The results of the study reported here suggest that recent population growth and expansion of the western North Pacific population is not due to immigration from the central North Pacific population but to natal recruitment from the western

North Pacific breeding colonies, as previous studies indicated (Walsh & Edwards 2005; Eda *et al.* 2008). This suggestion is supported by the estimated number of immigrants from the central North Pacific (0.0002–1.6727; Table A2.5), which is much smaller than the actual increase of the breeding population in the last two decades (H.S., K. Horikosi, T. Sasaki, and H. Chiba, unpubl. data). The increased natal recruitment in the western North Pacific population is likely a combination of high reproductive success rates (fledging success: 93%; Deguchi *et al.* 2012) and /or high postfledging survival. In the Ogasawara Islands, nesting conditions seem to have improved after the eradication of feral goats, carried out in 2004 (Hasegawa 1992; Tokyo Metropolitan Government 2008). This eradication may have increased the breeding success rate of the Black-Footed Albatross in the Ogasawara Islands. However, the population of the Laysan Albatross, which use the same nesting site as the Black-Footed Albatross in the Ogasawara Islands, has not increased from 10 –20 pairs (Oka 1995, Tokyo Prefecture 2008) after first recognition of breeding in 1976 (Kurata 1978). The reason for this discrepancy is unclear. To understand the basis of the population increase and expansion of the Black-Footed Albatross in the western North Pacific, an analysis of reproductive success, survival, and foraging areas in the breeding and nonbreeding season is required, including comparisons with the populations of the central North Pacific and other North Pacific albatross species.

Inferred long dispersal between the central and western North Pacific populations

The results of our Migrate analysis indicated that a large number of migrants exist from the western North Pacific to the central North Pacific. Our calculated values were much higher than those of previous studies using mitochondrial cyt b sequences (Walsh & Edwards 2005; Eda &. 2008). This result may indicate the long dispersal of the western North Pacific population. The genetic structure of the Kure population in the central North Pacific may reflect immigration from the western North Pacific population, indicated by the larger number of estimated immigrants per generation from both Ogasawara and Izu in the western North Pacific. Our R_{ST} values and STRUCTURE analysis also indicated weak genetic differentiation between the Kure and western North Pacific populations. In STRUCTURE analysis, all of the sampled individuals on Kure were assigned to the western North Pacific cluster with a higher probability (0.075–0.548) than the other individuals sampled in the central North Pacific, except for one individual from Midway (0.109). In contrast, none of the sampled individuals in the western North Pacific population, including the two genetically distinct individuals observed by Ando *et al.* (2011), were assigned to the central North

Pacific cluster with a high probability (< .033). The two genetically distinct individuals do not appear to be related to immigrants from the central North Pacific population but seem to belong to a minor strain in the Ogasawara Islands or to immigrants from the Senkaku Islands, where genetic study has never been conducted. Long dispersal may occur easily in the western North Pacific population, similar to results in a previous study of the wandering albatross (Gauthier *et al*. 2010). Gauthier *et al*. (2010) found that the smallest colony had the highest number of dispersing young because of the low availability of potential mates. Although the population size of the Black-Footed Albatross in the western North Pacific colonies is increasing, the colony is still small and of low density compared with the major breeding colonies in the central North Pacific (Tokyo Metropolitan Government 2008; Arata *et al*. 2009). Thus, dispersal of young individuals may be occurring, even over the long distances (approximately 4 000 km) between the western and central North Pacific colonies. This may be possible because the population distribution during the nonbreeding season on each side of the North Pacific overlaps (Cousins & Cooper 2000; Tickell 2000). Gauthier *et al*. (2010) also suggested that most dispersers colonize in a colony where the density is the lowest. Young dispersers from the western North Pacific colonies might easily colonize Kure Atoll, which exhibits a small population size and low density (Arata *et al*. 2009).

Migration between the central and western North Pacific populations has been discussed in two previous studies that assessed mitochondrial DNA sequences (Walsh & Edwards 2005; Eda *et al*. 2008). Eda *et al*. (2008) suggested the existence of low but apparent migration between the regions. However, the study reported here, using highly variable microsatellite markers and wide sampling, including Kure Atoll, indicated more frequent and directional dispersal between each side of the North Pacific.

Implications for conservation and future studies

The study reported here revealed that the recent increase and expansion of the western North Pacific population of the Black-Footed Albatross was not due to immigration from the central North Pacific population. The western North Pacific population, which exhibits unique genetic characteristics and a small population size, should be regarded as a high conservation priority. This study also suggested the existence of gene flow between each side of the North Pacific at an island population level, indicating a recent long dispersal. Thus, whether each population is to be regarded as a separate species, as Walsh & Edwards (2005) suggested, should be decided after careful comparison of morphology or other ecological differences. However, the genetic structure of the

Black-Footed Albatross may change in the future, depending on population trends. In 2011, the low-lying colonies in the central North Pacific were strongly damaged by the 11 March tsunami as well as by earlier storm surges that season. At least 110,000 of the Laysan and Black-Footed Albatross chicks and at least 2,000 adults were killed on Midway Atoll (U.S. Fish and Wildlife Service 2011). In contrast, the high-lying western North Pacific colonies were not damaged and continued to expand. This may promote gene flow from the western North Pacific to the central North Pacific. However, if further loss of low-lying colonies in the central North Pacific occurs due to sea level rise, as Baker *et al*. (2006) noted, the western North Pacific colonies may function as refugia for the central North Pacific population. In this case, gene flow from the central to the western North Pacific may accelerate. Rangewide and long-term monitoring of Black-Footed Albatross populations using both empirical observations and genetic data may help to characterize the complex dispersal dynamics of this highly mobile but philopatric albatross species and to craft an appropriate conservation strategy that incorporates environmental changes.

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Table A2.1 Genetic diversity of the six populations of Black-footed albatross based on microsatellite data

| | Microsatellite loci | | | | | | | | | | |
	10C5	11F3	11H1	11H7	12C8	12H8	Dc5	Dc9	Dc20	De11	Ave.
Tern (*n* = 48)											
N_A	3	1	9	11	4	1	4	3	2	7	4.5
R_S	2.69	1.00	6.64	8.27	2.64	1.00	3.21	2.61	1.65	5.54	3.52
H_E	0.26	0.00	0.77	0.84	0.52	0.00	0.50	0.27	0.08	0.62	0.39
H_O	0.27	0.00	0.73	0.92	0.54	0.00	0.42	0.31	0.08	0.56	0.38
Laysan (*n* = 44)											
N_A	3	1	9	9	2	1	3	4	2	7	4
R_S	1.83	1.00	6.81	7.15	2.00	1.00	2.69	3.31	1.83	4.48	3.21
H_E	0.09	0.00	0.78	0.80	0.49	0.00	0.44	0.45	0.13	0.55	0.37
H_O	0.09	0.00	0.75	0.73	0.57	0.00	0.39	0.41	0.14	0.64	0.37
Midway (*n* = 48)											
N_A	3	1	9	10	4	1	3	4	2	8	5
R_S	2.45	1.00	6.95	7.05	3.56	1.00	2.86	2.98	1.75	5.47	3.51
H_E	0.21	0.00	0.81	0.80	0.61	0.00	0.50	0.41	0.10	0.67	0.41
H_O	0.23	0.00	0.79	0.77	0.47	0.00	0.51	0.38	0.11	0.66	0.39
Kure (*n* = 11)											
N_A	3	1	8	5	2	1	3	3	1	4	3.1
R_S	3.00	1.00	8.00	5.00	2.00	1.00	3.00	3.00	1.00	4.00	3.10
H_E	0.24	0.00	0.84	0.65	0.46	0.00	0.42	0.17	0.00	0.63	0.34
H_O	0.27	0.00	1.00	0.64	0.36	0.00	0.45	0.18	0.00	0.55	0.35
Bonin (*n* = 77)											
N_A	3	2	9	10	2	2	5	3	2	5	4.5
R_S	2.14	1.14	6.78	7.28	1.99	1.37	3.19	2.92	1.27	3.42	3.15
H_E	0.33	0.01	0.79	0.83	0.28	0.04	0.30	0.41	0.03	0.59	0.36
H_O	0.34	0.01	0.81	0.84	0.05	0.04	0.30	0.38	0.03	0.51	0.32
Izu (*n* = 50)											
N_A	4	1	8	8	2	1	4	3	3	4	3.8

R_S	2.44	1	6.55	5.91	1.99	1	2.91	2.98	1.92	2.61	2.93
H_E	0.40	0.00	0.77	0.78	0.30	0.00	0.35	0.50	0.10	0.52	0.37
H_O	0.48	0.00	0.86	0.82	0.20	0.00	0.38	0.48	0.06	0.52	0.38
All ($n = 278$)											
N_A	4	2	10	13	5	2	6	4	3	9	5.8
R_S	2.55	1.04	6.87	7.50	2.53	1.12	3.03	2.98	1.64	4.50	3.38
H_E	0.26	0	0.79	0.78	0.44	0.01	0.42	0.37	0.07	0.6	0.38
H_O	0.28	0	0.82	0.79	0.37	0.01	0.41	0.36	0.07	0.57	0.37

number of individuals (n), number of alleles per locus (N_A), allelic richness (R_S), and expected (H_E) and observed (H_O) heterozygosities for 10 microsatellite loci in the six populations.

Table A2.2 Private alleles in each region and population

Region	Population	Locus	Allele size (bp)	Frequency
West	Midway	11H7	215	0.01
West	Midway	12C8	223	0.04
West	Tern	12C8	261	0.01
West	Tern	Dc05	176	0.02
Central	Ogasawara	11F3	240	0.01
Central	Ogasawara	11H7	194	0.01
Central	Ogasawara	12H8	167	0.02
Central	Ogasawara	Dc05	173	0.01
Central	Ogasawara	De11	200	0.01
Central	Izu	10C5	171	0.01
Central	Izu	Dc20	112	0.02

Table A2.3 AMOVA results based on F_{ST} and R_{ST}

	d.f.	SS	Var.	%	
F_{ST}					
Between regions	1	35.287	0.119	6%	F_{ST}=0.062 (*P*=0.001)
Among populations within regions	4	9.613	0.006	0%	N_{em} = 3.78
Within populations	548	1041.511	1.901	94%	
R_{ST}					
Between regions	1	1382.46	4.84	6%	R_{ST} = 0.052 (*P*=0.001)
Among populations within regions	4	240.76	0.00	0%	
Within populations	548	45442.16	82.92	94%	

d.f.: degree of freedom, SS: sum of squares, Var: genetic variability, %: percentage variability

Table A2.4 Pairwise F_{ST} (above diagonal) and R_{ST} (below diagonal) values

	Tern	Laysan	Midway	Kure	Ogasawara	Izu
Tern	_	0.004	0.000	0.012	0.073*	0.073*
Laysan	0.000	_	0.004	0.014	0.065*	0.066*
Midway	0.000	0.002	_	0.000	0.054*	0.055*
Kure	0.000	0.000	0.000	_	0.030*	0.043*
Ogasawara	0.059*	0.064*	0.042*	0.000	_	0.002
Izu	0.062*	0.065*	0.040*	0.003	0.000	_

* $P < 0.01$

Table A2.5 The number of migrants per generation between each pair of populations as estimated with the maximum likelihood estimation (MLE) with 95% CI.

		2.5%	MLE	97.5%
From	To			
Midway	Laysan	0.002	0.003	0.004
	Tern	0.034	0.037	0.042
	Kure	0.025	0.028	0.032
	Ogasawara	0.010	0.012	0.015
	Izu	0.011	0.013	0.015
Laysan	Midway	0.000	0.000	0.000
	Tern	0.000	0.000	0.000
	Kure	0.000	0.000	0.000
	Ogasawara	0.000	0.000	0.000
	Izu	0.000	0.000	0.000
Tern	Midway	0.027	0.032	0.037
	Laysan	0.012	0.015	0.019
	Kure	0.034	0.040	0.045
	Ogasawara	0.046	0.052	0.059
	Izu	0.016	0.020	0.024
Kure	Midway	0.902	1.008	1.122
	Laysan	0.655	0.746	0.846
	Tern	0.120	0.161	0.209
	Ogasawara	0.008	0.019	0.038
	Izu	1.534	1.673	1.819
Ogasawara	Midway	0.296	0.399	0.525
	Laysan	1.489	1.715	1.963
	Tern	0.481	0.611	0.764
	Kure	4.101	4.474	4.868
	Izu	1.151	1.350	1.571
Izu	Midway	4.257	4.717	5.209
	Laysan	3.880	4.318	4.789

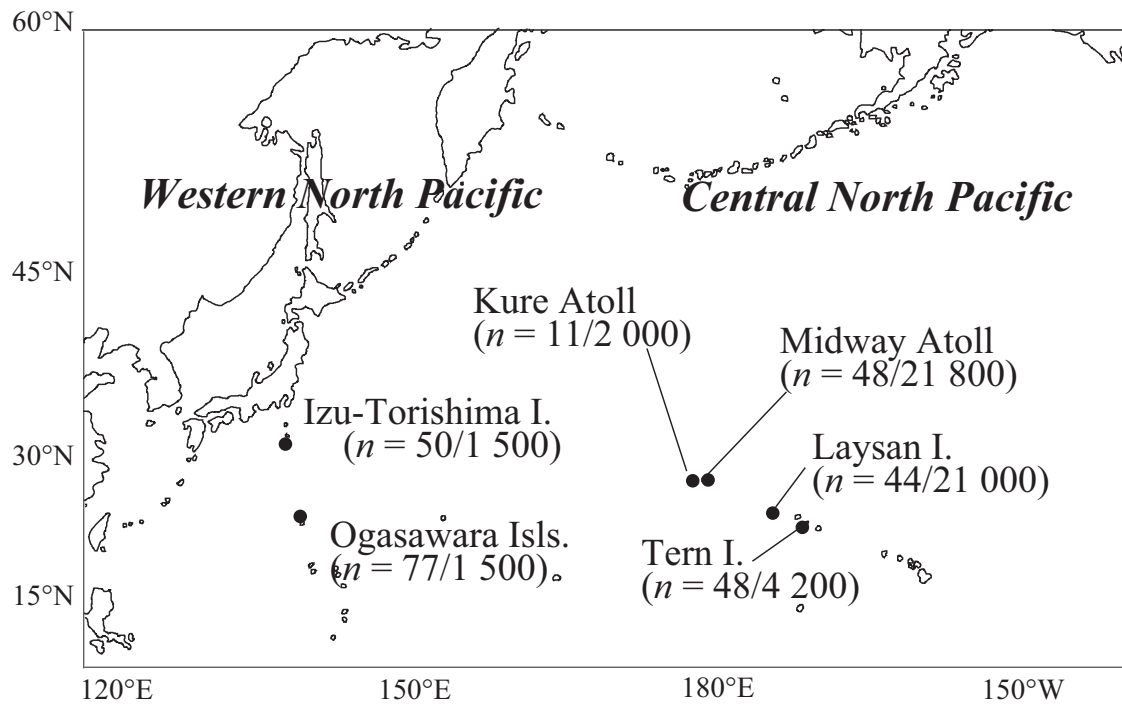|			
Tern	0.423	0.572	0.754
Kure	3.703	4.131	4.592
Ogasawara	4.021	4.468	4.946

Fig. A2.1 Sampling locations in the central and western North Pacific colonies of the Black-footed albatross. The numbers in parentheses are sample sizes / number of breeding pairs.

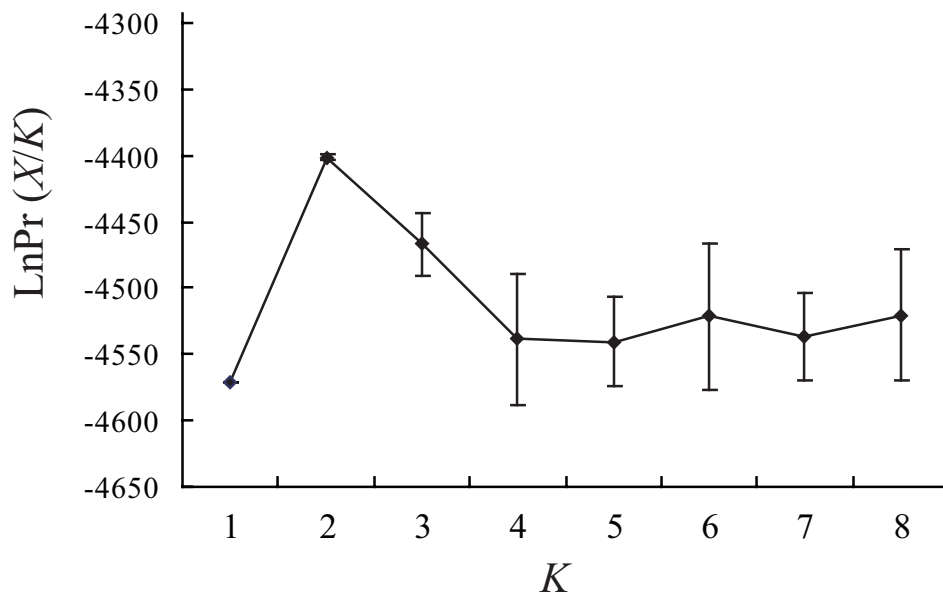**Fig. A2.2** Likelihood plot of STRUCTURE results. Ln Pr(X/K) is the log likelihood of each value of *K*, which is the number of simulated clusters. Where ln Pr(X/K) is maximized, *K* is most likely. Black squares represent the average values of ln Pr(X/K), and vertical lines represent standard deviations.
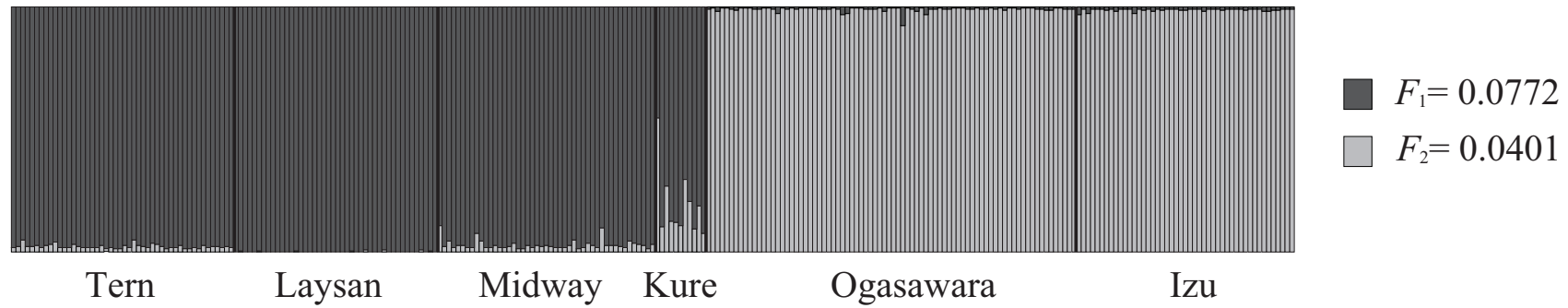
Fig. A2.3 Stacked bar chart from the results of STRUCTURE analysis with maximum likelihood $K = 2$. Each individual is represented by a single bar, broken into K colored segments. Length of each segment is propor tional to the membership fraction in each cluster. Individuals are grouped by population: Tern, Laysan, Midway, and Kure in the central North Pacific and Ogasawara and Izu in the western North Pacific. The F values of each cluster are shown to the right.

Appendix 3

Development of Microsatellite Markers for the Coastal Shrub

***Scaevola taccada* (Goodeniaceae)**

Summary

Microsatellite markers were developed for the coastal shrub species *Scaevola taccada* to estimate the population genetic structure, which may reflect different seed dispersal patterns. Thirteen microsatellite primer sets were developed for *S. taccada* using 454 pyrosequencing. The primer sets were tested on 64 individuals sampled from two populations in Japan. Fragments were amplified using the primers, with one to 10 alleles per locus, and the expected heterozygosity ranged from 0.00 to 0.85. These results indicate the utility of markers in *S. taccada* for broad estimations of the population genetic structure of this species.

Introduction

Scaevola taccada (Gaertn.) Roxb. (Goodeniaceae) is a coastal shrub species widely distributed along coastal areas of the Pacific and Indian oceans (Howarth *et al*. 2003). The white fleshy exocarp and the underlying corky layer of the fruit (Howarth *et al*. 2003) allow it to be transported in the avian gut (Kawakami et al. 2009; Emura *et al*, 2012) and by oceanic floating (Nakanishi 1988). Due to its unique seed dispersal system, *S. taccada* has been found growing in various environments. Seashores (sandy shores and cliffs, consistent with dispersal by oceanic floating and birds) represent the primary growth environment of this species, but some populations exist in island interiors (dispersal by birds) (Satake *et al*. 1989; Emura *et al*. unpublished data). Following such a distribution pattern, some isolated populations might be in the process of speciation, which may be indicated by population genetic structure. To estimate genetic differentiation caused by the different seed dispersal patterns, highly variable genetic markers are required. Here, I report 13 nuclear microsatellite loci for *S. taccada* developed using 454 next-generation sequencing, which will be useful for estimating the population genetic structure of this species.

Methods and results

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A fresh leaf sample of S. taccada was collected from an individual growing in a coastal area of the Ogasawara Islands, Japan, and genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). The GS Junior Titanium Series Kit (Roche) and the SPRIworks Fragment Library System (Beckman Coulter, Brea, California, USA) were used for construction of a DNA library for *S. taccada*. A 500-ng aliquot of genomic DNA was nebulized at 0.24 MPa for 1 min, purifi ed, end-repaired, and A-tailed using the SPRIworks Fragment Library Kit II (Beckman Coulter) and ligated to the Rapid Library Adapter (Roche) using RL Ligase (Roche). Suitably sized DNA fragments were selected by removing short fragments using SPRIworks Fragment Library Kit II (Beckman Coulter). Emulsion PCR (emPCR) was constructed for the desired fragments mixed with capture beads using the GS Junior Titanium emPCR Kit (Roche). After emPCR, the beads capturing the DNA library were enriched to selectively capture beads with suffi cient amounts of template DNA for sequencing. The enriched beads were annealed with sequencing primers, and the amplifi ed fragments were sequenced using the GS Junior Benchtop System (Roche). In the GS Junior sequencing, 30,497 DNA sequences were obtained. The sequences were screened to fi nd potential microsatellite loci using the MSATCOMMANDER program (Faircloth 2008). After screening, 423 repeat regions containing eight or more dinucleotide repeats were identifi ed using Primer3 software (Rozen & Skaletsky 2000) embedded in MSATCOMMANDER, and primers were successfully designed for a total of 72 repeats.

Twenty-three primer pairs were selected for amplification trials in eight *S. taccada* individuals, based on the repeat structure and avoiding sequences containing mononucleotide repeats. All of the forward primers designed from the selected loci were synthesized with a tag sequence (Boutin-Ganache et al. 2001) for fl uorescent labeling. The sequence of each tag is indicated in the notes for Table A3.1. A modified protocol for the QIAGEN Multiplex PCR Kit (Qiagen) was used for PCR amplifi cation. The fi nal volume of the PCR reaction mixture was 5 μL and contained the following: 16 ng extracted DNA, 2.5 μL Multiplex PCR Master Mix, 0.01 μM forward primer, 0.2 μM reverse primer, and 0.1 μM M13 (fl uorescently labeled) primer. The PCR program was as follows: an initial denaturation at 95 °C for 15 min; 30 cycles at 94 °C for 30 s, 57 °C for 1.5 min, and 72 °C for 1 min; and a final extension at 60 °C for 30 min. The PCR product size was determined using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and GeneMapper software (Applied Biosystems). Thirteen primer pairs amplified well and could be

scored easily and unambiguously (Table A3.1). These primer pairs were used for amplifi cation in 64 individuals sampled from two populations located on the Ogasawara Islands (26.61184 °N, 142.17543 °E) and the Okinawa Islands (26.13975 °N, 127.79644 °E), using the PCR program used for amplifi cation described above. One specimen from each population was collected and deposited at the Kyoto University Museum herbarium (accession numbers: KYO 00037380 and KYO 00037381).

The number of alleles for a given locus ranged from one to 10 (mean: 2.5). The observed and expected heterozygosities were 0.00–0.91 (mean: 0.23) and 0.00–0.85 (mean: 0.27), respectively (Table A3.2). Deviation from Hardy–Weinberg equilibrium (HWE) and the linkage disequilibrium (LD) between loci were tested using FSTAT version 2.9.3 (Goudet, 1995). Two loci (Stac21 and Stac24) exhibited a signifi cant deviation from HWE ($P < 0.05$) in the Ogasawara population. There was no evidence of LD for any loci pairs. Fixation index was calculated using FSTAT and its values were −0.18–1.00.

Conclusions

I have characterized 13 microsatellite loci for *S. taccada*. These microsatellite loci will be useful for estimating population genetic structure possibly resulting from the various seed dispersal patterns of *S. taccada*.

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Appendix 3

Table A3.1. Characteristics of 13 microsatellite primers developed for *Scaevola taccada* (Gaertn.) Roxb. All values are based on 64 samples from two populations on the Okinawa and Ogasawara Islands in Japan

Locus	Primer sequences	Repeat motief	Fluorescent label[a]	Ta (℃)	Size range (bp)	GenBank Accession no.
Stac05	F: TGGTGGTTAACAAGTGGCAAG	$(AG)_8$	FAM	57	175 - 179	AB872256
	R: GTTACTCAAGTCTCTTATTAGCAGTTC					
Stac06	F : GGCACCAGCTTCTGTAACC	$(AG)_9$	FAM	57	270 - 274	AB872257
	R: GCAGTGGTGGATGCATTCTG					
Stac09	F: CATGAGCCAAGGAAACGTCC	$(AG)_9$	VIC	57	325 - 335	AB872258
	R: GCTTCGGCTCTTCACAAGG					
Stac10	F: CAGCAGGAATGCACTAAGACC	$(AT)_{11}$	VIC	57	223 - 253	AB872259
	R: GGAGGGATGGAAAGGTCCG					
Stac11	F: TTTCGCAAGATCCCGGC	$(AT)_8$	NED	57	254	AB872260
	R: TGGAAGTTGGTAAATGGGTCAG					
Stac13	F: TCTTAACGACTCCTGTTCACC	$(AC)_9$	FAM	57	209	AB872261
	R: CCGTCTCCATCCCTTCGTG					
Stac15	F: ACTCATCAGACAAGGTAACGG	$(AC)_{10}$	VIC	57	368 - 372	AB872262
	R: AGCAGTCTACTTCCCACGC					

Stac17	F: CTGCAACGGTCATTCGTCC	$(AT)_9$	NED	57	289 - 303	AB872263
	R: TGCAACCTATATGCTATGTGCTC					
Stac18	F: GAGGGAAGGTCAGAGGTGC	$(AG)_9$	FAM	57	189 - 193	AB872264
	R: TCAGACAAGTGTAATAGAAGGTCATC					
Stac19	F: AAAGTCGGGCAGTAGGTGC	$(AG)_8$	FAM	57	250	AB872265
	R: GGAGTTGATGAATGCTCGGC					
Stac21	F: CCCTCAAGGCTACTGTCGG	$(CT)_{13}$	FAM	57	297 - 304	AB872266
	R: TTGCGTTCTTCCGCAATCC					
Stac24	F: ATTGATTAGTAGGTGAGAATCGTG	$(AT)_{10}$	NED	57	191 - 193	AB872267
	R: TCGGTATTGTCCTAATCTTCCG					
Stac27	F: ATACATCATCGTACCCAAATTCC	$(AT)_8$	NED	57	199 - 203	AB872268
	R: GAGCTCCTTGAAATGTCGCC					

Annealing temperature (T_a), Sequence of the fluorescent labels: FAM = 5'-CACGACGTTGTAAAACGAC-3'; NED = 5'-CTATAGGGCACGCGTGGT-3'; VIC = 5'-TGTGGAATTGTGAGCGG-3'

Table A3.2. Results for primer screening of all samples for 13 microsatellite loci in two populations of *Scaevola taccada*.

Locus	Okinawa ($N = 32$)				Ogasawara ($N = 32$)			
	A	H_O	H_E	F_{IS}	*A*	H_O	H_E	F_{IS}
Stac05	2	0.22	0.28	0.25	3	0.53	0.60	0.13
Stac06	3	0.44	0.46	0.07	2	0.41	0.36	-0.12
Stac09	4	0.22	0.20	-0.07	1	0.00	0.00	NA
Stac10	10	0.91	0.85	-0.05	4	0.44	0.48	0.11
Stac11	1	0.00	0.00	NA	1	0.00	0.00	NA
Stac13	1	0.00	0.00	NA	1	0.00	0.00	NA
Stac15	3	0.47	0.52	0.11	3	0.47	0.53	0.13
Stac17	2	0.03	0.03	0.00	5	0.53	0.65	0.20
Stac18	3	0.53	0.60	0.13	3	0.50	0.42	-0.18
Stac19	1	0.00	0.00	NA	1	0.00	0.00	NA
Stac21	3	**0.08**	0.47	0.84	3	0.06	0.15	0.58
Stac24	2	**0.00**	0.12	1.00	1	0.00	0.00	NA
Stac27	2	0.25	0.38	0.35	2	0.03	0.03	0.00

Number of alleles (*A*); expected heterozygosity (H_E); Observed heterozygosity (H_O); Sample size (*N*); Numbers in bold show deviations from HWE