Population genetic study of *Morus australis* Poir. in the Ogasawara and other islands of Japan

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小笠原諸島および日本の他の諸島におけるシマグワの集団遺伝学的研究（英文）

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Summary

Invasive species are serious threat to biota in the Ogasawara Islands, which are oceanic islands harboring many endemic species. The islands are now invaded by *Morus australis* that was artificially introduced from the Ryukyu and Izu Islands. Hybridization was reported to take place between this species and an endemic species *M. boninensis*.

To clarify the invasion routes and the genetic composition of founding populations, plant samples of *M. australis* were collected from 32 populations across Japan, including 12 from the Ogasawara Islands and population genetic analyses were conducted using 14 microsatellite markers. In addition, to elucidate extent of hybridization between invasive *M. australis* and native *M. boninensis*, plant sampling was performed from two wild seedling populations, two populations of *M. boninensis* and another population of *M. australis* in the Ogasawara Islands. Then, the same population genetic analyses were conducted also for the additional samples.

The UPGMA dendrogram based on Nei's genetic distance, the Principal Coordinate Analysis based on pairwise F_{ST} values, and the Bayesian Clustering using STRUCTURE software indicated that the populations of *M. australis* in the Ogasawara Islands are genetically similar to those in the Ryukyu Islands, while they are clearly differentiated from those in the Izu Islands and mainland of Japan. The level of genetic diversity in the Ogasawara Islands (A_R = 4.24; H_E = 0.60) was similar to that of the Ryukyu Islands (A_R = 4.70; H_E = 0.66), higher than the Izu Islands (A_R = 3.70; H_E = 0.51) and lower than mainland of Japan (A_R = 5.80; H_E = 0.73).

The obtained results in this study showed that the *M. australis* plants now growing in the Ogasawara Islands are descendants of those introduced from the Ryukyu Islands. The individuals from the Izu Islands might not be able to successfully expand their distribution in the Ogasawara Islands. It was also suggested that numbers of transplanted individuals from the Ryukyu Islands to the Ogasawara Islands were large because similar amount of genetic variation was observed in the two island groups. Such high genetic diversity might have enhanced invasiveness of *M. australis* in the Ogasawara Islands.

This study also revealed that *M. boninensis* is genetically differentiated from *M. australis*, and hybrids between the two species are very rare, maybe due to the difference in ploidy levels between them. Thus, a limited bad effect of *M. australis* to the endemic *M. boninensis* through hybridization was detected. However, all the seedling individuals were those of *M. australis*, suggesting regeneration of *M. boninensis* rarely occurs in the Ogasawara Islands. Meanwhile, the amount of genetic diversity within *M. boninensis* was similar to that within *M. australis*. The endangered *M. boninensis* still maintains high genetic diversity and may be able to survive if we increase the number of its individuals.

The results obtained in this study provide us basic information to prevent disturbance of the invasive *M. australis* in the Ogasawara Islands since knowing the origin of the invasive species may help to understand its ecological features.

General Introduction

Oceanic islands often have an exceptional rank in global biodiversity conservation due to their high degree of endemism (Hobohm 2000, Whittaker & Fernández-Palacios 2007). Oceanic islands are volcanic islands in the ocean such as the Hawaiian, Galapagos and Canary Islands, which have never been connected to large landmasses since the formations. They usually have unique land biota. For example, 524 endemic vascular plant species were found in the Canary Islands (Fernández-Palacios & Whittaker, 2008). Consequently, oceanic islands contribute to a disproportionately high degree to global biodiversity despite their relatively low percentage of surface area and limited number of species growing in the islands (Cowie & Holland 2006). This phenomenon is caused by adaptive radiation resulting in many species adapted to diverse ecological niches in the islands i.e. can achieve parallel speciation (Sugai *et al.* 2013, Government of Japan 2010).

The Ogasawara Islands are one of the oceanic islands in Japan with many endemic plant taxa. The islands are located about 1,000 km south from the main islands of Japan, covering surface area of 79.39 km^2, with total terrestrial area of 63.58 km^2 and belong to subtropical climatic zone (Government of Japan 2010). There are four island groups, Chichijima, Hahajima, Mukojima and Iwoto island groups in the Ogasawara Islands. They have 112 endemic land plant taxa (Kobayashi & Ono1987). Among which 40% of vascular plant species, including 70% of the tree species, are endemic (Toyoda 2003). In a recent report, the number of endemic plant taxa reached 161 (Government of Japan 2010). The Ogasawara Islands were designated as one of UNESCO World Natural Heritage sites (UNESCO 2011).

Invasive Alien Species (IAS) are problematic in the world. Invasive alien species (IAS) are species whose introduction and/or spread outside their natural habitats threaten biological diversity (Convention on Biological Diversity 2009). Sufficient evidence has emerged that IAS may now be the most significant drivers of population declines and species extinctions in oceanic islands (Veitch & Clout 2002; Donlan *et al.* 2003). IAS are most common in man-made environments, for example, urban landscapes and farmlands, broadleaved deciduous forestry plantations, or forest clearings (Chytry *et al.* 2008). Common characteristics of IAS are strong tolerance against a wide range of environmental conditions such as high temperature, salinity, and dryness, and a higher biotic potential than non-invasive ones (Devin & Beisel 2007) and higher trait plasticity (Richards *et al.* 2006). These characteristics provide IAS higher reproductive rates and make them more harmful to the native species and they have likely escaped competition/ parasitism from their native range (Torchin *et al.* 2003).

Existences of invasive plant species have been reported even in the conserved areas of the islands. For example, sixty four alien invasive plant species belonging to twenty three families were recorded at six conservation areas of West Sumatera, Indonesia (Syamsuardi *et al*. 2016). IAS tend to spread exceedingly, and thus their introduction or spreading threatens native species in various places in the world including oceanic islands (Convention on Biological Diversity 2009).

IAS are problematic especially in oceanic islands (Whittaker & Fernandez 2007, Pearson 2009). It is because oceanic islands have high endemism as mentioned above and are vulnerable to several threats, such as decrease of population sizes and fragmentation, loss of mutualisms, habitat alteration and destruction, climate change

(Caujape *et al*. 2010). Moreover, IAS are reported to be one of the largest threats in the oceanic islands because they increase the above-mentioned threats to the endemic plants (Sugiura 2016, Kawakami & Okochi 2010).

Many endemic plant species are threatened by IAS also in the Ogasawara Islands (Kawakami 2008). The ecosystem of the islands has already suffered from various negative effects by human impacts, biological invasions and others. Sugiura (2016) reported that many native species in the islands, including those of land plants, have been negatively impacted by introduced predators and herbivores (*e.g.*, lizards, rats, flatworms, goats, and others) through competitive and trophic interactions. Introduced predators and herbivores have had greater impacts on native species than introduced competitors in the Ogasawara Islands. Among the 441 vascular plant taxa, 32% (144 taxa) of them were recognized as endangered (Government of Japan 2010). In the Ogasawara Islands, many of the endemic plants are now endangered due to the effects of IAS (Tani *et al.* 2006, Environment Agency of Japan 2000), in addition to those of urban development, destructive exploitation and others. To control the effects and conserve the endemic species, several studies for establishing relevant management plans have been conducted in the Ogasawara Islands (Hata *et al.* 2006; Osawa 2019) but so far they are not effective enough.

Morus australis Poir. is one of the invasive plants now present in various places in east and southest Asia including the Ogasawara Islands of Japan (Kato *et al.* 2006). The natural distributions of this plant species are over Japan (lower montane region in the main islands of Japan and the Ryukyu Islands), Sakhalin, China, Indochina, India, and Himalayas where it inhabited in humid forests (The National Institute for Environmental Studies, Japan 2017). *Morus australis* was

introduced to the Ogasawara Islands for sericulture of silkworm *Bombyx mori* (Awasthi *et al*. 2004; The National Institute for Environmental Studies, Japan 2017, Tokyo Metropolitan Government Ogasawara Island Branch 1938). Because it recently expanded even into conserved areas in the islands where an endangered endemic plant species, *M. boninensis* is distributed, even after the cultivation of *M. australis* has been stopped, it has been recognized as IAS (The National Institute for Environmental Studies, Japan. 2017).

Morus boninensis is one of the endemic plant species in the Ogasawara Islands that need careful conservation. IAS closely related to native species such as *M. australis* are expected to have strong impacts on the native species by hybridization (Mooney & Cleland 2001). Hybridization between IAS and native species not only helps IAS to locally adapt to their new environments by incorporating beneficial alleles from the native species (Prentis *et al.* 2008) but also make species boundary between them obscure and replace pure strains of native species with admixed ones (Levin *et al.* 1996). It was reported that hybridization between *M. australis* and *M. boninensis* has occurred on the Chichijima and Hahajima Islands (Tani *et al.* 2003, 2006). Thus, invasion of *M. australis* is likely to threaten the endemic and endangered *M. boninensis* not only through competition but also through genetic disturbances or genetic pollution (Hufford & Mazer 2003).

Clarifying genetic diversity within each species is necessary and important for biological understanding of IAS. It is because population genetic analyses based on intra-specific genetic diversity give us important information, for example about reproductive mode and gene flows among populations (frequency of movements by seeds and/or pollens) of IAS. It is substantial to study the genetic diversity by using

molecular markers which can play an important role in providing information about pathways of introduction and the amount of genetic variation introduced (Durka *et al.* 2005). IAS are expected to have been adapted to the environments of the source populations, and thus knowledge about their origin may help us to estimate ecological features of the IAS.

Microsatellites seem to be one of the best DNA markers to clarify the genetic diversity within a species due to the high level of polymorphisms (Aggarwal *et al.* 2004, Mburu & Hanotte 2005, Oguri *et al.*2013). For *Morus* species, microsatellite markers have been developed for *M. indica* (Aggarwal *et al.* 2004, Krishnan *et al.* 2014) and *M. boninensis* (Tani *et al.* 2005). Using these markers, Krishnan (2014) successfully revealed the historical events of introduction and the spread of cultivated mulberry in the Indian subcontinent. Therefore, microsatellites seemed suitable molecular genetic markers for the analyses of the *Morus* plants in Japan.

In the present study, population genetic studies of invasive *Morus australis* and endemic *M. boninensis* in the populations of the Ogasawara Islands, as well as in those of other regions in Japan for the former species, were conducted for deeper understanding of present genetic situations of the two related plant species. The structure of this thesis is as follows. In Chapter I, population genetic analyses by using 14 microsatellite markers were conducted to infer the invasion routes of *M. australis* to the Ogasawara Islands and the genetic composition of its founding populations. In Chapter II, the same microsatellite markers as applied in Chapter I were used to estimate the proportion of the hybrids between endemic *M. boninensis* and invasive *M. australis* species in the Ogasawara Islands.

Chapter I

Microsatellite markers reveal genetic differentiation of an invasive mulberry species *Morus australis* Poir. among the island groups in Japan and inferring the source of introduction to the Ogasawara Islands

Introduction

Morus australis has been reported as one of invasive plant species in the Ogasawara (Bonin) Islands (The National Institute for Environmental Studies, Japan 2017) but its origin is obscure. It was historically recorded that this species has been introduced to the Ogasawara Islands, especially to Chichijima and Hahajima Islands from the Ryukyu, the Izu Islands and mainland of Japan in the 1890s and 1920s for culture of silkworm (The National Institute for Environmental Studies, Japan 2017). In 1927, the introduction of 9,000 nursery trees from across Japan to the Ogasawara Islands was recorded (Tokyo Metropolitan Government 1929, Tokyo Metropolitan Ogasawara Island Branch Office 1938). However, the origin of each population of *M. australis* now growing in the Ogasawara Islands is unknown.

Morus kagayamae Koidz., another species of the genus *Morus* in the Izu Islands might become IAS in the Ogasawara Islands while this species is morphologically not easily distinguishable from *M. australis*. *Morus kagayamae* was recorded to be introduced from Hachijojima to the Ogasawara Islands in 1926 (Tokyo Metropolitan Government Ogasawara Island Branch Office 1938). Based on AFLP analysis, *M. kagayamae* and *M. australis* are closely allied (Chumchuen &

Kanekatsu 2011). Although they are also morphologically similar, *M. kagayamae* tends to have multilobed and broad-cordate-based hairless leaves whereas *M. australis* has lanceoloate and truncate based leaves with white stiff hairs on both sides (Kitamura 1977, Katsumata 1974). *M. kagayamae* used to grow wild and was also used for feeding silkworms in the Izu Islands, especially in Hachijojima and Miyakejima (Wilson 2021). However, typical plants of *M. kagayamae* in the Izu Islands are very rare now, maybe due to intensive hybridization with *M. australis*, and it is difficult to distinguish the two species even in the Izu Islands. In the present study, these two species are not distinguished and all the mulberries examined in the Ogasawara and Izu Islands are tentatively treated as *M. australis*.

In Chapter I, population genetic studies of *M. australis* were conducted using microsatellite markers for 32 populations of *M. australis* across Japan, including 12 populations from the Ogasawara Islands. Introduced species tend to suffer from loss of genetic diversity caused by founder effects and then genetic drift due to small number of individuals in initial introduced populations (Richardson *et al.* 2000a). Since *M. australis* was scattered on both of the inhabited islands in the Ogasawara Islands: Chichijima and Hahajima, genetic analyses of its populations within each island and comparisons of the genetic diversity between the islands or with the populations of the other regions in Japan (ex, the Ryukyu Islands, the Izu Islands, or the mainland of Japan) are needed. Such analyses are necessary to clarify origin of this introduced species now growing in the islands. It would be also useful for conducting appropriate conservation managements in the Ogasawara Islands by controlling the alien plant species because knowledge about the origin of IAS can be useful to understand their ecological characteristics which cause invasiveness. In

additiion, microsatellite markers seem the best for analyzing *Morus* plants in the Ogasawara Islands as mentioned in the General Introduction. Population genetic analyses using microsatellite markers are expected to answer all the below questions.

Based on the above background, the following questions will be addressed in this chapter: 1) Where was the geographical origin of *M. australis* now growing in the Ogasawara Islands?; 2) What level of genetic differentiation and spatial genetic structure are observed in *M. australis* growing in the Ogasawara Islands and other islands in Japan?; 3) What level of genetic diversity is observed in the invasive species *M. australis* growing in the Ogasawara Islands and other islands of Japan?

Materials and Methods

Samples collections

Plant sample collection of *Morus australis* was conducted in 2016-2019 from 12 populations in the Ogasawara Islands (seven populations in Chichijima and five populations in Hahajima) and 20 populations in other islands of Japan including the Ryukyu Islands (Okinawa and Yoronjima islands), the Izu Islands (Hachijojima, Oshima and Kozushima islands) and the mainland of Japan (Honshu, Shikoku, and Kyushu including Koshikishima island) (Table I-1 and Fig. I-1). All the examined populations in this study were selected based on the observation and the availability of a sufficient number of plants with a minimum distance of 5 meters between the individuals and expectation to cover each island.

Fresh-young leaves in the terminal stems were collected from each individual in order to obtain the total DNA with the best quality. The collected leaves were stored in plastic bags with silica gel to keep them dry until DNA extraction. The coordinates of collected sites were recorded using the GPS GARMIN Oregon 450TC. Voucher specimen was collected for each population, photographed using a digital camera RICOH WG-4 16 mega pixels, and deposited in the Makino Herbarium (MAK) of Tokyo Metropolitan University (Appendix I-1). Although the plant samples collected in the Izu Islands may contain *M. kagayamae*, they were also treated as *M. australis* in the present study. It is because typical plants of *M. kagayamae* are now very rare in the Izu Islands according to the personal communications of local residents and by our own observation during the sample collections.

DNA extraction and microsatellite analysis

Total DNAs of the samples were extracted from a small amount of silica gel dried leaf tissue (ca. 2 mg) using the modified CTAB method (Doyle & Doyle 1987). Quality of the obtained DNA was checked using Thermo Scientific Nanodrop Lite Spectrophotometer with higher than 1.70 A260/A280 values. The obtained DNA was stored at -20ºC until use.

Microsatellite analysis was conducted by using 14 loci. Eleven out of the 14 loci were developed previously for *Morus indica*: MESTSSR 31, MESTSSR 48, MESTSSR 73, MESTSSR 126, MulSSR 147, MulSSR 258, MulSSR 313, MulSSR 338, Mul3SSR 6 (Krishman *et al.* 2014), MulSTR 2 and MulSTR 4 (Aggarwal *et al.* 2004), and three loci for *M. boninensis*: Mos0031, Mos0157-2 and Mos0340-2 (Tani *et al.* 2005). The PCR amplifications were performed following the standard protocol of the Type-it® Microsatellite PCR Kit (Qiagen), in final volume of 5 µl, which contained 5 ng of the extracted DNA from each plant sample, 2.5 µl of 2x Type-it Microsatellite PCR Master Mix, and 0.2 µM of each primer. The PCR products were visualized with fluorescently labeled universal primers (Blacket e*t al.* 2012). The amplification was conducted using the GeneAmp PCR System 9700 (Applied Biosystems) with profiles included initial denaturation at 95ºC for 5 min; followed by 32 cycles of 30 s at 95ºC, 90 s at 58ºC and 30 s at 72ºC; then final extension at 60ºC for 30 min. The sizes of PCR products were measured by comparison wise GeneScan 500 LIZ size standard (Applied Biosystems) using the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) and GeneMapper analysis software version 4.0 (Applied Biosystems).

Data Analysis

Deviations from Hardy-Weinberg Equilibrium (HWE) for each locus at each population were tested by χ^2 tests and confirmed by Benjamini-Hochberg procedure using software GenAlEx 6.503 (Peakall & Smouse 2012) to check the relevance as genetic markers. Linkage disequilibrium (LD) between loci for each population was tested by an exact test in FSTAT 2.9.3.2. (Goudet 2002). Null allele frequencies were calculated by using software FreeNA (Chapuis & Estoup 2007) for each locus and population following the Expectation Maximization (EM) algorithm (Dempster, Laird & Rubin 1977). To evaluate the genetic diveristy within populations of *Morus australis*, number of alleles per locus (N_A), number of private allele (P_A), number of common allele (N_{CA}) based on frequency ≥ 5%, found in 50% or fewer populations, allelic richness ($A_{R;}$ El Mousadik & Petit 1966), observed heterozygosity (Ho), expected heterozygosity (H_E), and fixation index (F_{IS}, Weir& Cockerham 1984) were calculated using the software GenAlEx 6.503 and FSTAT 2.9.3.2.

To clarify relationships among the populations, the global genetic differentiation (F_{ST}) and pairwise F_{ST} (Weir& Cockerham 1984) were calculated by using FSTAT 2.9.3.2. The deviation of each pairwise F_{ST} from zero was tested based on 1000 randamization. The Analysis of Molecular Variance (AMOVA) was conducted in order to reveal partitioning of genetic differentiations based on F_{ST} and R_{ST} estimators by using GenAlEx 6.503. The Mantel tests (Mantel 1967), which were performed with 999 permutations, were conducted to detect the Isoloation by Distance (IBD) (Wright 1943) between the genetic distances through a matrix of adjusted pairwise genetic differentiation values $F_{ST}/(1-F_{ST})$ and logarithms of geographic distance (Rousset 1997) by using GenAlEx 6.503.

The UPGMA dendrogram based on Nei's genetic distance (Nei *et al.* 1983) was drawn by using POPULATIONS 1.2.3.0 (Langella 1999) and MEGA 6.06 (Tamura *et al.* 2013). The Principal Coordinate Analysis (PCoA) were also conducted by using GenAlEx 6.503 based on pairwise F_{ST} values. The Bayesian Clustering analysis was run by using the software STRUCTURE 2.3.4 (Falush *et al.* 2003, 2007; Pritchard *et al.* 2000) to clarify genetic structure of all the examined populations. Genetic structure was simulated for all the individuals using the allele frequency correlated and ancestry admixture models. For all analyses, 100,000 burn-in steps and 1,000,000 replicates were used. Twenty runs were performed for each value of *K* ranged from1 to 10, where *K* is the number of genotypic groups. The optimal *K* was chosen using the delta*K* method and the Log-likelihood (Evanno *et al.* 2005) with Structure Harvester on web (Earl & vonHoldt 2012), Distruct 1.1 (Rosenberg 2004) and CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) were used to visualize bar plots obtained by the STRUCTURE analyses.

Estimation of contemporary migration was conducted using BayesAss v3.0.4 (Wilson & Rannala 2003), which estimates the fraction of immigrants in a population using Bayesian inference. Firstly, estimation of contemporary migration for pairwise island groups as many as three runs were performed using 10,000,000 iterations, a burn-in of 1,000,000, a sampling interval of 100, and an average of the gene flow estimates was calculated. The mixing parameters Delta *A*, Delta *F* and Delta *M* were set to 0.10, 0.15 and 0.06, respectively. Convergence of the chains was validated using Tracer v1.7.1 (Rambaut *et al*. 2018). Secondly, estimation of contemporary migration for pairwise populations was also performed using 10^7 iterations, a burn-in

of 10^6 and a sampling interval of 100, with mixing parameters Delta A, Delta F and Delta M were set to 0.99, 1.00 and 1.00, respectively.

Results

Characteristics of the used microsatellite loci for Morus australis

The statistical tests for Hardy-Weinberg Equlibrium (HWE) on each single locus for each investigated population revealed that most of the cases (380 of 448 combinations) and no specific loci or populations were not significantly deviated from HWE. Significant LD was not observed in any pairs of loci. Average null allele frequencies calculated across population and loci were low enough, lower than 0.06. Thus, all the obtained data inferred from 14 loci were used for further population genetic analyses.

Genetic diversity detected in M. australis

Summary statistics describing genetic diversity within each studied population are shown in Table I-1.

The mainland of Japan had higher mean numbers of private alleles (P_A = 4.83) than those of other islands, the Ogasawara Islands (P_A = 0.42), the Ryukyu Islands (P_A = 0.75) and the Izu Islands (P_A = 2.00). The allelic richness (A_R) in the Ogasawara Islands ranged from 3.78 to 5.21 were similar to those of the Ryukyu Islands (A_R = 4.36 - 5.21). These values were lower than those of the mainland of Japan (A_R = 4.25 - 6.49), but higher than that of the Izu Islands (Hachijojima, A_R = 2.93 - 3.25) except for Oshima and Kozushima populations (A_R, 5.75 and 4.28, respectively).

The genetic diversity (H_E) in the Ogasawara Islands ranged from 0.56 to 0.68. These values were lower than those of the mainland Japan (H_E = 0.65 - 0.79) and the Ryukyu Islands (H_E = 0.64 - 0.71). However, the genetic diversity in the Ogasawara

Islands were higher than that of the Izu Islands (Hachijojima, H_E = 0.41 - 0.46) except for Oshima and Kozushima populations (H_E , 0.71 and 0.60, respectively). The mean genetic diversity (H_E) in the Ogasawara Islands (H_E = 0.60) was relatively similar to those of the Ryukyu Islands (H_E =0.66), higher than the Izu Islands (H_E = 0.51) and lower than mainland of Japan (H_E = 0.73) (Table I-1). The mean genetic diversity (H_E) with 95% confidence intervals is shown in Fig. I-2.

The highest genetic diversity in the Ogasawara Islands is in HKW population (H_E = 0.68) and the lowest in CHT and HMN populations containing similar amount of genetic diversity (H_E = 0.56). Outside of the Ogasawara Islands, the highest genetic diversity was in HFK population of Honsu Island (H_E = 0.79) and the lowest was in IHC population of the Izu Islands (H_E = 0.41) (Table I-1).

Genetic differentiation among the populations

The genetic differentiation in the Ogasawara Islands (F_{ST} = 0.03) was slighly lower than those of the Ryukyu Islands (F_{ST} = 0.08) as well as those of the Izu Islands (F_{ST} = 0.09), and much lower than the mainland of Japan (F_{ST}, 0.13). Thus, the global F_{ST} value among populations in the Ogasawara Islands was lower compared to those of other islands.

In addition, the pairwise F_{ST} values between the Ogasawara Islands and the Ryukyu Islands were low (F_{ST} = 0.01-0.16; 17 pair out of the 96 pairs are not significant). The pairwise F_{ST} values between the Ogasawara Islands and the Izu Islands (range, 0.19-0.45; all pairs are significant) were much higher than those between the Ogasawara Islands and the Ryukyu Islands. The pairwise F_{ST} values between the Ogasawara Islands and the mainland of Japan were also higher (F_{ST} =

0.13-0.30; one pair out of the 72 pairs are not significant). The pairwise F_{ST} values between the Ogasawara Islands and the Ryukyu Islands are the lowest compared to those of the other two pairs.

Furthermore, differentiation between the CMK population and the other populations of the Ogasawara Islands (range, 0.03 - 0.08) was slightly higher than those between the other pairwise populations within the Ogasawara Islands (range, -0.001 - 0.07) except for CHT population (0.08). In contrast, low differentiation was detected between the CMK population and the populations in the Ryukyu Islands (range, 0.01 - 0.04) except for RYO population (0.07). Thus, the CMK population of the Ogasawara Islands was genetically more similar to the populations in Okinawa of the Ryukyu Islands than to the other populations in the Ogasawara Islands.

The low level of genetic differentiation among populations was also shown by the results of AMOVA, which showed only 5 % of total variations were detected by both F_{ST} and R_{ST} estimators (Table I-2). In contrast, large genetic variation was detected within each population, which were 76% of total variations through F_{ST} parameter and 72% through R_{ST} parameter.

Spatial genetic structure among all populations

The patterns of spatial genetic structure were evaluated among populations over geographical distances (Fig. I-3 and Appendix I-2). Significant isolation by distance (IBD) was detected among populations across Japan (r = 0.586, P = 0.001; Fig. I-3a). Within island groups, significant IBD was not detected in the Ogasawara Islands (r = -0.111, P = 0.184; Fig. I-3b), the Ryukyu Islands (r = 0.353, P = 0.142; Appx. I-2a)**,** or the mainland of Japan (r = -0.207, P = 0.177; Appx. I-2c) except in

the Izu Islands (r = 0.915, P = 0.028; Appx. I-2b). Within islands, no significant IBD was detected in Chichijima (r = 0.278, P = 0.189; Appx. I-2d), Hahajima (r = -0.140, P = 0.423; Appx. I-2e), Okinawa (r = 0.206, P = 0.202; Appx. I-2f), and Hachijojima (r = 0.327, P = 0.334; Appx. I-2g).

Genetic relationships among the populations of Morus australis

The UPGMA dendrogram based on Nei's genetic distance (Nei et al. 1983) is shown in Fig. I-4. The three clusters were revealed in this dendrogram: cluster I comprises of all population of the Ogasawara Islands and the Ryukyu Islands, cluster II contains all the populations within mainland of Japan, and cluster III contains all the populations within the Izu Islands.

In cluster I, the genetically most differentiated population from the others in the Ogasawara Islands was the CMK population in Chichijima. In addition, the most genetically similar populations in the Ryukyu Islands were RAF and RYM populations, and the most distinct population was RYH population. The CMK population in Chichijima in the Ogasawara Islands was genetically similar to the RKT, RYM, RAF, RAH and RNK populations in the Ryukyu Islands. In cluster II, no obvious geographical pattern of genetic similarity among the populations was observed within mainland Japan. In cluster III, populations within the Izu Islands can be clearly divided into three groups based on each island (Hachijojima, Oshima or Kozushima).

The result of the Principal Coordinate Analysis (PCoA) is shown in Fig. I-5. The first axis showed 53.53 % and the second axis showed 18.11 % of the total variation. Along the first axis, the two clusters were formed. One cluster comprises

of the populations within the Ogasawara Islands and the Ryukyu Islands. Another cluster comprises of the populations belonging to the Izu Islands and mainland of Japan. Along the second axis, no obvious clusters were formed. In total, the cluster of the populations in the Ogasawara Islands and those in the Ryukyu Islands were well separated from those in the Izu Islands and the mainland of Japan.

Results of the STRUCTURE analysis

The results of the Bayesian clustering are shown in Fig. I-6. The ΔK was highest at $K = 2$, followed by $K = 4$ (Fig. I-6a). The log-likelihood values reached flattening at $K = 4$ (Fig. I-6b). The probability of each individual belonging to the inferred genetic clusters was shown in Fig. I-6c.

At $K = 2$, one cluster (green) was dominant in populations of the Ogasawara Islands and the Ryukyu Islands, while another cluster (pink) was dominant in the Izu Islands and mainland of Japan. Admixture of two clusters was observed in one population of the Ogasawara Islands (HKW).

At $K = 4$, four clusters were formed where green cluster at $K = 2$ was divided into two clusters (yellow and green). The populations in the Ryukyu Islands and the CMK population of the Ogasawara Islands belonged to the yellow cluster and the other populations in the Ogasawara islands to the green cluster. Blue and pink clusters were dominant in the Izu Islands and the mainland of Japan, respectively. Admixture of blue and pink clusters was observed in one population (IIO) of the Izu Islands.

Estimated contemporary migration among the studied regions

The contemporary migration rates were estimated for the first and second generation. Among the pairwise island groups, the limited gene flow or low migration rates were detected (*m*, 0.001 – 0.071). Significant gene flow was detected between the Ryukyu Islands and the Ogasawara Islands, as well as from the mainland of Japan to the Izu Islands, as shown in Table I-3.

In addition, nine of 21 individuals were detected as first-generation immigrants based on the analysis of pairwise island groups. Based on pairwise populations, all the individuals belonging to the CMK population were detected as the first-generation immigrants from the Ryukyu Islands (Table I-4).

Discussion

The geographical origin of M. australis *growing in the Ogasawara Islands*

In Chapter I, one of our main objectives is to elucidate the geographical origin of *M. australis* now growing in the Ogasawara Islands. In this study, its origin was suggested to be from the Ryukyu Islands. Tani *et al.* (2003) had estimated that the species *M. australis* might be introduced to the Ogasawara Islands from Okinawa Island (the Ryukyu Islands) for sericulture before World War II. However, there were no scientific evidence to support the hyphotesis. Based on the results from the three genetic approaches: namely the UPGMA dendrograms (Fig. I-4), the Principal Coordinate Analysis (Fig. I-5), and the Bayesian clustering using STRUCTURE software (Fig. I-6), it was strongly suggested that the present populations within the Ogasawara Islands were originated not from the Izu Islands or the mainland of Japan, but from the Ryukyu Islands since they are genetically very similar and no historical records said that the individuals in the Ogasawara Islands were transferred to other places, such as the Ryukyu Islands, out of the Ogasawara Islands.

The CMK (Mikazuki, Chichijima) population in the Ogasawara Islands, which was genetically more similar to the populations in Okinawa Island, migh be of recent trans-planting from the Ryukyu Islands to Chichijima. In the UPGMA dendrogram, the CMK of the Ogasawara Islands was genetically close to the populations of Okinawa Island (RKT, RYM, RAF, RKW, RAH and RNK) (Fig. I-4). The Bayesian clustering also showed that the individuals of the CMK population had the genetic composition similar to those in the Ryukyu Islands (Fig. I-6c; $K = 4$). The contemporary migration analysis revealed that all the individuals within CMK population were detected as first-generation immigrant from the Ryukyu Islands

(Table I-4). These concordant results supported that geographical origin of *M. australis* in the CMK population is from the Ryukyu Islands, and the event was relatively recent. The plant samples of *M. australis* in the Ogasawara Islands, including those from CMK population (Mikazuki), are relatively young (less than 20 years old, maybe much younger), and thus it is unlikey that the individuals trans-planted before World War II (more than 80 years ago) are remaining now. They should be their offspring after mating with other individuals in the Ogasawara Islands or recently trans-planted individuals. The Mikazuki population seems the latter though it is still possible that the CMK population has been isolated in some reasons from the other populations in Chichijima. These data indicated that some of the individuals now growing in the Ogasawara Islands were surely introduced from the Ryukyu Islands.

It is also possible that the individuals from the Izu Islands could not successfully expand their distribution in the Ogasawara Islands. Tokyo Metropolitan Government, Ogasawara Island Branch (1938) reported that *M. australis* in the Ogasawara Islands have been transplanted not only from the Ryukyu Islands but also from Hachijojima Island of the Izu Islands. However, no individual having the genetic consitution of the Izu Islands (the blue culster) was detected in the Ogasawara Islands (Fig. I-6c, $K = 4$)., The plants from the Ryukyu Islands might be more adapted to the environments in the Ogasawara Islands and have been surviving untlil now though no direct evidences were obtained in this study.

As for the plant materials from the Izu Islands, their genetic constituitons were distinct from those of the Ogasawara Islands, the Ryukyu Islands or mainland of Japan, though some genetically admixture individuals were also observed in the

IIO population (Oshima) based on the result of STRUCTURE analysis, see Fig. I-6c; $K = 4$. *Morus kagayamae*, which is endemic to the Izu Islands, might be related to the results. However, our plant samples from the Izu Islands were not typical *M. kagayamae* but morphologically similar to *M. australis* as mentioned in the Materials section. Local residents of Hachijojima said to us that they have cultivated hybrid offspring between *M. australis* and *M. kagayamae* that fit the climates of Hachijojima for many years, and they rarely see typical *M. kagayamae* there now (Takeshi Kikuchi, personal communication). Still, the genetic distinctness of the *Morus* plants in the Izu Islands might be derived from the genomes of *M. kagayamae*.

Admixtures between genomes of the mainland of Japan and those of the Izu Islands, or those of the Ogasawara Islands were observed in IIO and in HKW, respectively (Fig. I-6c). Oshima is geographically most near to the mainland of Japan (Fig. I-1), and migration of *M. australis* from Honshu to Oshima and hybridization with the local *Morus* plants might occur there. Similar genetic admixture in *M. australis* was observed in HKW population in Hahajima, the Ogasawara Islands. Recent hybridization events might happen in Hahajima as well, but on a smaller scale than in Oshima.

Genetic diversity of Morus australis *in the Ogasawara Islands*

Genetic diversity (H_E , A_R) of *Morus australis* in the Ogasawara Islands was not very different from that of the Ryukyu Islands, suggesting gene flow between them (Table I-1). Based on the genetic structure clarified in this study, the plants of *M. australis* in the Ogasawara Islands was suggested to be originated from Ryukyu

Islands and thus expected to have lower genetic diversity than the latter. The results on genetic diversity suggested that a large number of juvenile plants of *M. australis* from various localities in the Ryukyu Islands might have trans-planted in the Ogasawara Islands.

Genetic differentiation and spatial genetic structure of M. australis

The genetic differentiation of *M. australis* among populations within each island group and within each island was low (Table I-2). The low level of genetic differentiation among populations of *M. australis* in Japan was also supported by the results of AMOVA analysis, which showed only 5% of total variations were among the populations based on F_{ST} as well as R_{ST} estimator (Table I-2). No significant IBD was detected on any single island including Chichijima, Hahajima, Okinawa, or Hachijojima though IBD was detected within all the populations across the Japanese archipelago. It means that there is no correlation between spatial structure and genetic differentiation within each island. Such low genetic differentiations within each islands group may be caused by high gene flow among populations (Slatkin & Barton, 1989).

The efficient seed dispersers of *M. australis* is most likely responsible for the phenomenon, especially in the Ogasawara Islands. In general, the zoochoric seeds of invasive plants are dispersed primarily by birds and mammals (Richardson *et al.* 2000b). The brown-eared bulbul *Hypsipetes amaurotis*, the Bonin Islands white-eye *Apalopteron familiare*, and the introduced Japanese white-eye *Zosterops japonicus* were known as primary seed dispersers of *M. australis* in secondary forests of the Ogasawara Islands (Kawakami *et al.*2009). The trees of introduced *M. australis* are

growing in nearly all areas of Chichijima and Hahajima and produce fruits throughout the year. In contrast, the fruiting season of the native *M. boninensis* lasts only from December to February, and its distribution range is limited to several small areas of Chichijima and Hahajima (Toyoda 2003). More productive and dispersal-efficient seeds of *M. australis* may make its populations genetically similar to each other and potentially cause the decline of the native species *M. boninensis* in the Ogasawara Islands.

Based on our results, the invasive *Morus australis* now growing in the Ogasawara Islands was originated from the Ryukyu Islands and the level of genetic diversity observed in the invasive species within the Ogasawara Islands, was similar to their source populations in the Ryukyu Islands (Table I-1, Fig. I-2). Multiple introductions might have increased genetic diversity in the Ogasawara Islands to the same extent as their populations. Such relatively high genetic diversity may have made this introduced species more invasive in the Ogasawara Islands. These results should be taken into consideration when conservation strategies are constructed to prevent wider distribution of *M. australis* in the Ogasawara Islands because knowing the origin of the invasive species may help to understand its ecological features.

Table I-1. Sampling localities and genetic diversity of *Morus australis* inferred from the 14 microsatellite markers. *N*, number of sampled individuals; N_A, number of alleles per locus; P_A, number of private alleles, N_{CA}, number of locally common alleles; A_R, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index.

Island	Location	Pop ID	Latitude (°N)	Longitude (°E)	*N*	N_A	P_A	N_{CA}	A_R	H_O	H_E	F_{IS}
The Ogasawara Islands												
Chichijima	Mikazuki	CMK	27.09862	142.18687	21	5.64	2	2.36	4.36	0.60	0.64	0.08
	Fukiagedani	CFK	27.07554	142.20870	23	5.64	0	2.57	4.09	0.59	0.59	0.02
	Siguredamu	CSG	27.05991	142.20675	16	4.64	0	1.86	3.78	0.56	0.57	0.00
	Kitafukurozawa	CCP	27.06018	142.20314	12	4.78	0	1.79	4.20	0.53	0.57	0.04
	Tatsumidoro	CTT	27.06397	142.21987	15	5.00	0	1.93	4.04	0.59	0.58	-0.02
	Yoakeyama	CYO	27.08559	142.21721	12	5.57	0	2.57	4.65	0.58	0.65	0.09
	Hatsuneura	CHT	27.07704	142.21770	11	4.71	0	2.00	4.12	0.46	0.56	0.13
Hahajima	Minamizaki	HMN	26.61139	142.17621	22	5.35	0	2.29	3.93	0.51	0.56	0.09
	Nakanotaira	HNK	26.63210	142.17363	14	5.14	0	2.07	4.13	0.59	0.59	-0.01
	Kitako	HKT	26.69634	142.14363	25	5.85	1	2.57	4.07	0.57	0.61	0.04
	Sekimon	HSK	26.67440	142.15615	34	6.35	0	3.07	4.23	0.55	0.62	0.13
	Kuwanokiyama	HKW	26.66379	142.15196	18	7.21	2	3.50	5.21	0.63	0.68	0.11
Mean					18.58	5.49	0.42	2.38	4.24	0.56	0.60	0.06
The Ryukyu Islands												
Okinawa	Katsuyama	RKT	26.62965	127.93843	20	6.14	0	0.79	4.70	0.62	0.67	0.10
	Yamagusuku	RYM	26.08380	127.68954	20	6.57	0	3.21	4.65	0.56	0.65	0.15
	Yohanayama	RYH	26.73114	128.21000	7	5.14	2	2.00	5.21	0.56	0.71	0.25
	Aha	RAH	26.71527	128.29211	21	6.35	0	2.92	4.68	0.61	0.68	0.09
	Kawata	RKW	26.64439	128.18970	20	6.64	2	3.14	4.70	0.58	0.65	0.14
	Afuso	RAF	26.50680	127.90704	20	5.57	0	2.71	4.36	0.58	0.64	0.11
	Nakadomari	RNK	26.42319	127.78805	12	5.57	0	2.50	4.71	0.66	0.66	0.00

Yoronjima	Yoronjima	RYO	27.02236	128.44975	21	5.50	2	2.57	4.56	0.62	0.64	0.02
Mean					17.63	5.93	0.75	2.48	4.70	0.59	0.66	0.11
The Izu Islands												
Hachijojima	Mitsune	IMT	33.12095	139.80899	17	4.00	0	2.50	3.02	0.43	0.45	0.05
	Hachijofuji	IHC	33.15183	139.74838	25	4.28	1	2.50	2.93	0.36	0.41	0.06
	Higashiyama	IHG	33.10169	139.82274	22	4.21	0	2.42	2.95	0.41	0.43	0.03
	Nakanogo	INK	33.07032	139.81564	21	4.71	0	2.57	3.25	0.38	0.46	0.10
Kozushima	Kozushima	IIK	34.23236	139.15797	69	8.14	9	4.50	4.28	0.50	0.60	0.14
Oshima	Oshima	IIO	34.71126	139.43777	15	7.71	2	4.71	5.75	0.59	0.71	0.16
Mean					28.16	5.50	2.00	3.20	3.70	0.45	0.51	0.09
The mainland												
Honshu	Yamagata	HYM	38.97018	140.42016	7	6.00	6	3.14	6.00	0.74	0.71	-0.03
	Fukushima	HFK	37.22404	139.80526	14	8.57	3	5.29	6.49	0.69	0.79	0.11
	Ibaraki	HKN	36.06090	140.11080	21	9.07	5	5.29	5.56	0.67	0.76	0.11
Shikoku	Kochi	SSK	33.67615	133.51610	11	6.57	2	4.14	6.00	0.59	0.69	0.18
Kyushu	Kumamoto	KKU	32.18815	130.61430	15	5.71	3	3.50	4.25	0.58	0.65	0.11
Koshikishima	Koshikishima	KKO	31.65296	129.69945	8	6.85	10	4.29	6.48	0.52	0.78	0.32
Mean					12.67	7.13	4.83	4.28	5.80	0.63	0.73	0.13

Table I-2. The results of Analysis of Molecular Variance (AMOVA) of all of the investigated populations in this study. DF, Degree of Freedom; SS, Sum of Square; MS, Mean Sum of Square.

Partition	F_{ST} estimator				
	DF	SS	MS	Variation	%
Among Regions	3	1024.555	341.518	1.111	19
Among Populations	28	397.097	14.182	0.265	5
Within Populations	1186	5250.454	4.427	4.427	76
Total	1217	6672.106		5.804	100

Partition	R_{ST} estimator				
	DF	SS	MS	Variation	%
Among Regions	3	212171.435	70723.812	232.079	23
Among Populations	28	68654.358	2451.941	47.292	5
Within Populations	1186	847167.797	714.307	714.307	72
Total	1217	1127993.589		993.678	100

Table I-3. Contemporary migration rates (*m*) among pairwise regions. The 95% confidence intervals of migration rates are in parentheses, and the gene flow values that do not cross zero (significant estimates) are in bold type. Proportions of non-migrants are on the diagonal.

	Source regions			
Destination regions	The Ogasawara Islands	The Ryukyu Islands	The Izu Islands	The mainland of Japan
The Ogasawara Islands	0.945	**0.045**	0.001	0.008
	(0.926-0.965)	(0.025-0.064)	(-0.001-0.004)	(-0.001-0.017)
The Ryukyu Islands	**0.071**	0.919	0.005	0.004
	(0.024- 0.118)	(0.873-0.967)	(-0.002-0.011)	(-0.002-0.011)
The Izu Islands	0.002	0.004	0.983	**0.012**
	(-0.002-0.006)	(-001-0.009)	(0.972-0.994)	(0.002-0.021)
The mainland of Japan	0.004	0.004	0.014	0.978
	(-0.004-0.013)	(-0.004-0.013)	(-0.004-0.031)	(0.958-0.998)

Table I-4. The first-generation immigrants identified among pairwise island groups and pairwise populations. *P*, Probability that the individuals are the first-generation migrants from the origin population.

Populations	Individuals	Pairwise island groups		Pairwise populations	
		Origin	*P*	Origin	*P*
CMK	Mo_008	The Ryukyu Islands	0.61	RAF	0.99
	Mo_009		0.98		0.95
	Mo_011		0.74		0.96
	Mo_014		0.96		0.93
	Mo_015		0.93		0.98
	Mo_123		0.97		0.98
	Mo_124		0.87		0.99
	Mo_126		0.80		0.96
	Mo_129		0.59		0.93
	Mo_006		-		0.36
	Mo_007		-		0.93
	Mo_010		-		0.96
	Mo_012		-		0.93
	Mo_013		-		0.87
	Mo_119		-		0.96
	Mo_120		-		0.75
	Mo_121		-		0.94
	Mo_122		-		0.92
	Mo_125		-		0.93
	Mo_127		-		0.96
	Mo_128		-		0.94

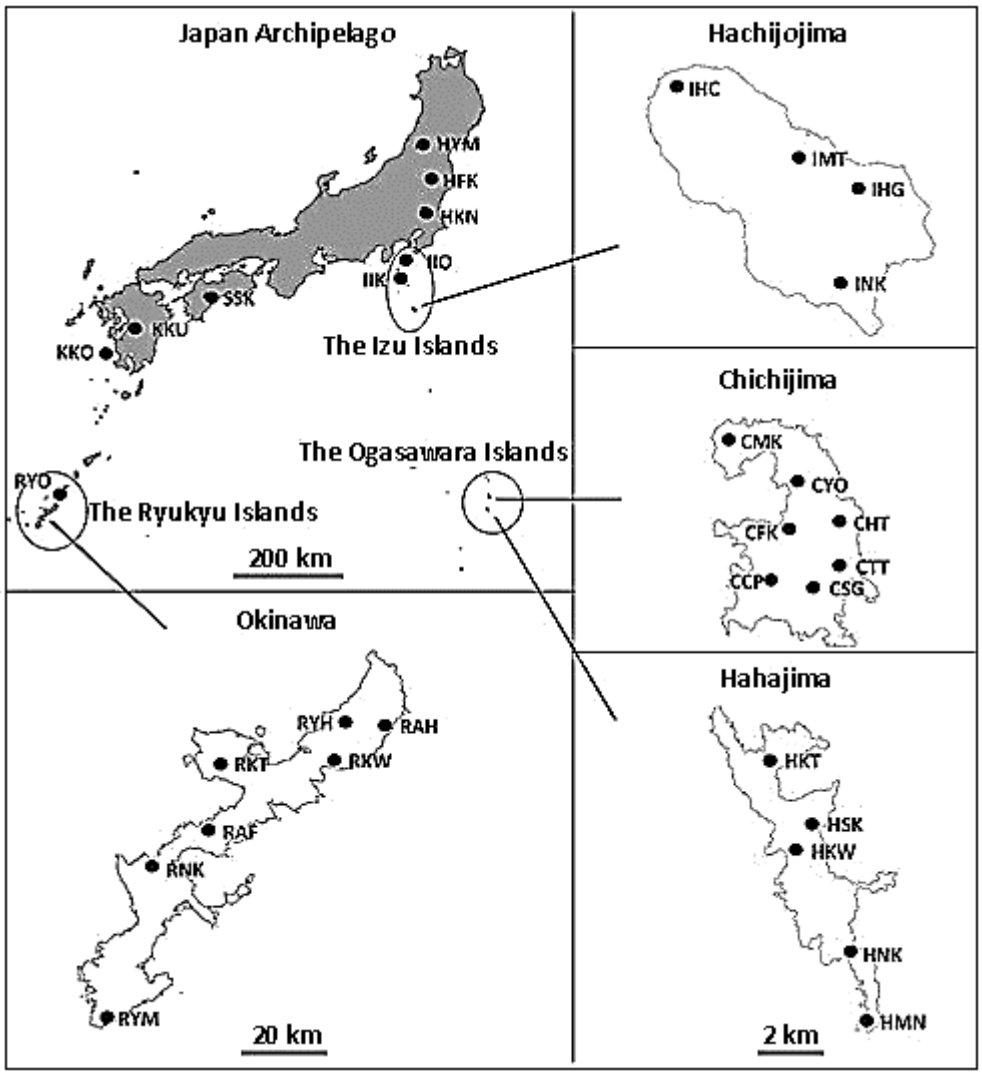


Fig. I-1. The localities of the sampled populations of *Morus australis* in Japan.

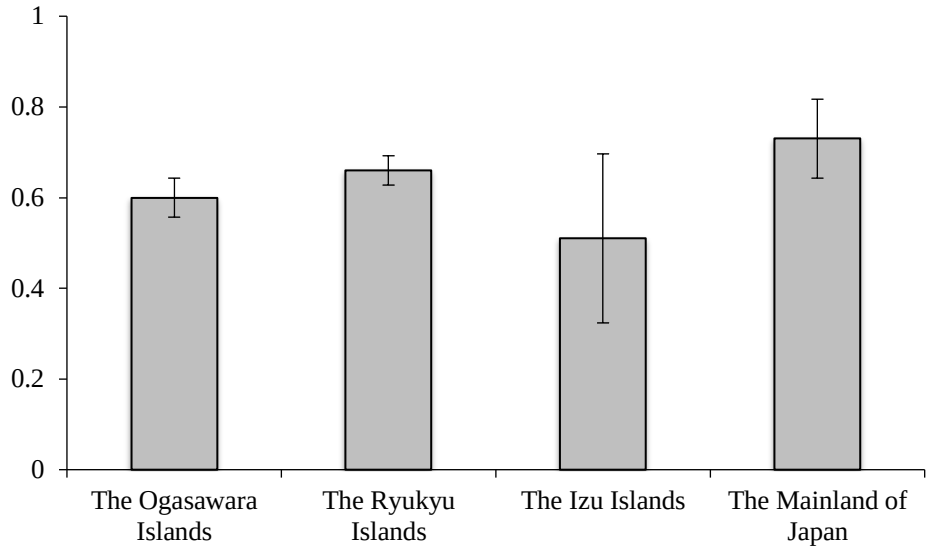


Fig. I-2. The mean genetic diversity (H_E) with 95% confidence intervals in each of the three island gorups and the mainland of Japan.

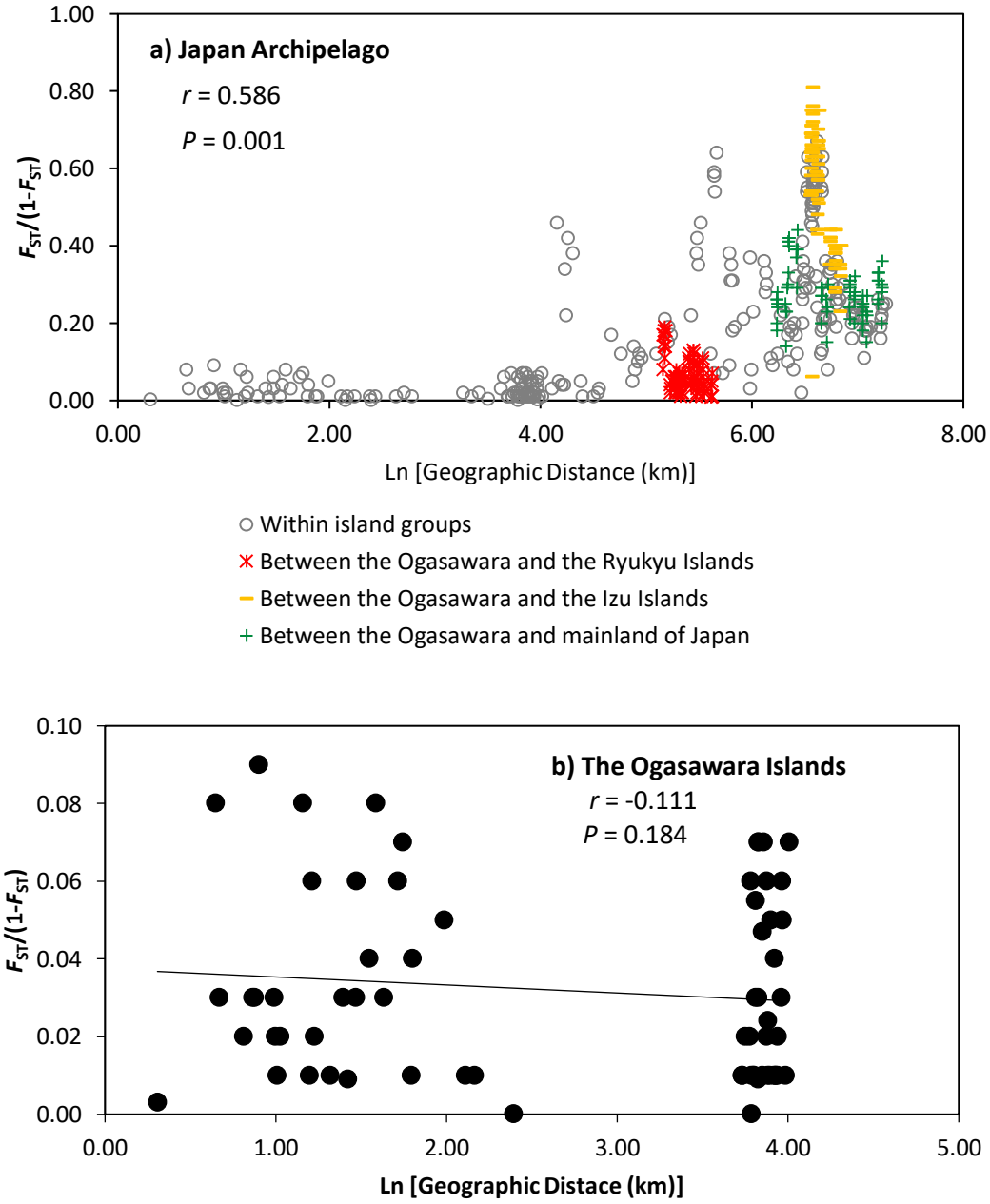


Fig. I-3. The correlations between genetic distances and natural logarithms of geographic distance for populations; a) Japan Archipelago, b) The Ogasawara Islands.

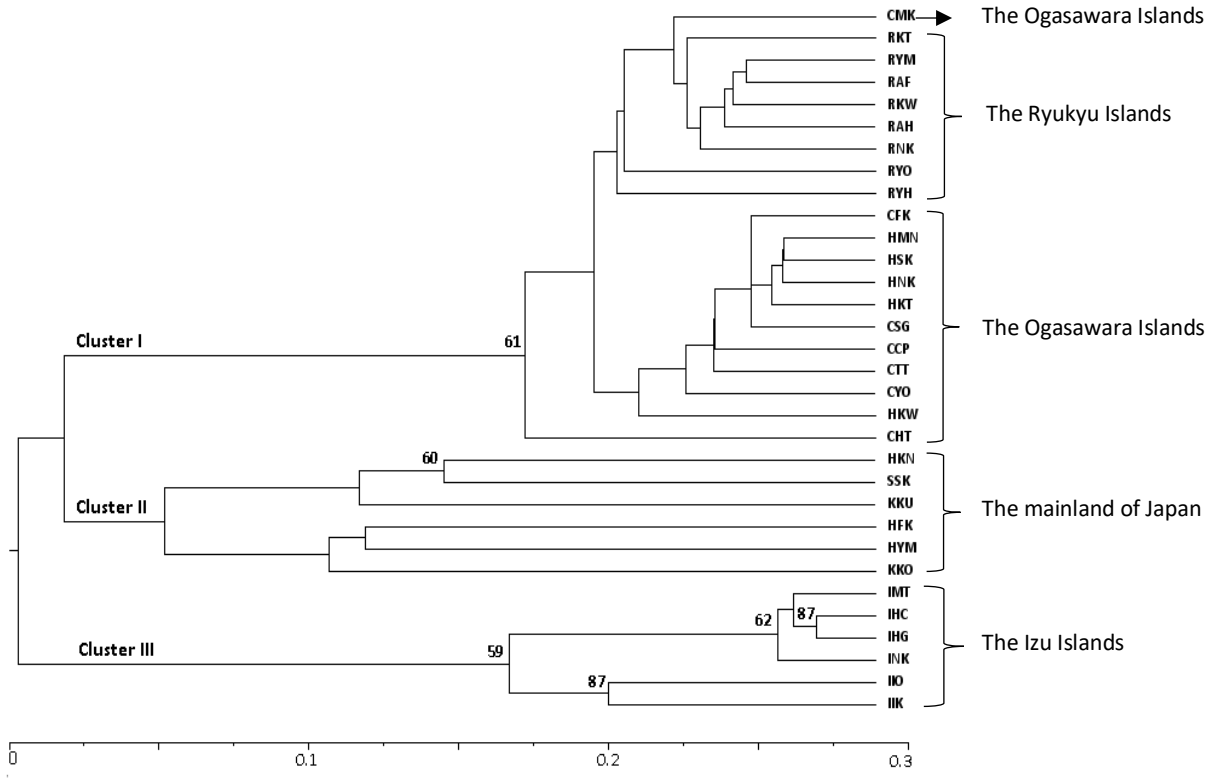


Fig. I-4. The UPGMA dendrogram among all populations across Japan based on genetic distances, D_A (Nei *et al.* 1983). Bootstrap values >50% are shown on the dendrogram.

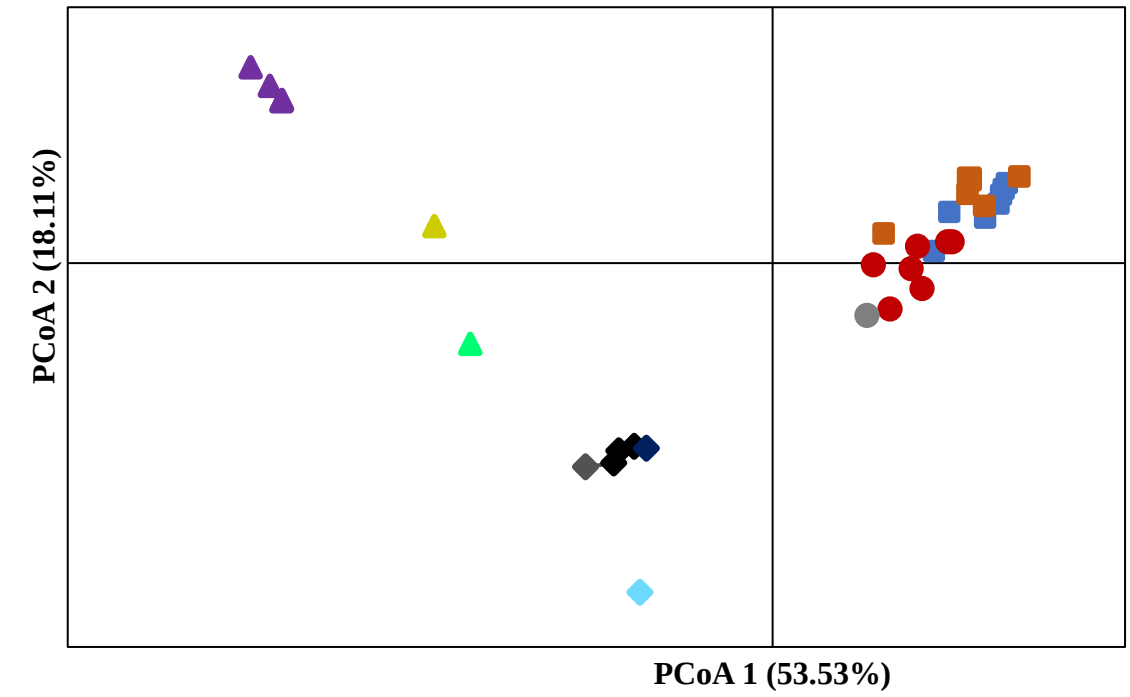
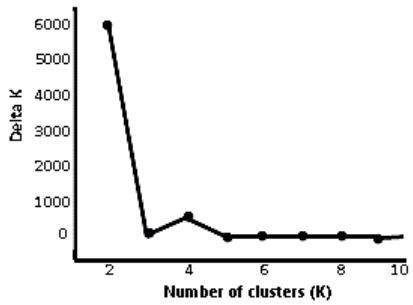
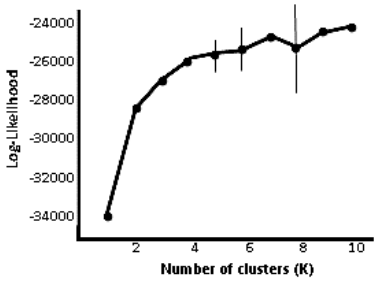


Fig. I-5. The Principal Coordinate Analysis (PCoA) based on pairwise F_{ST} values among populations.

a)



b)



c)

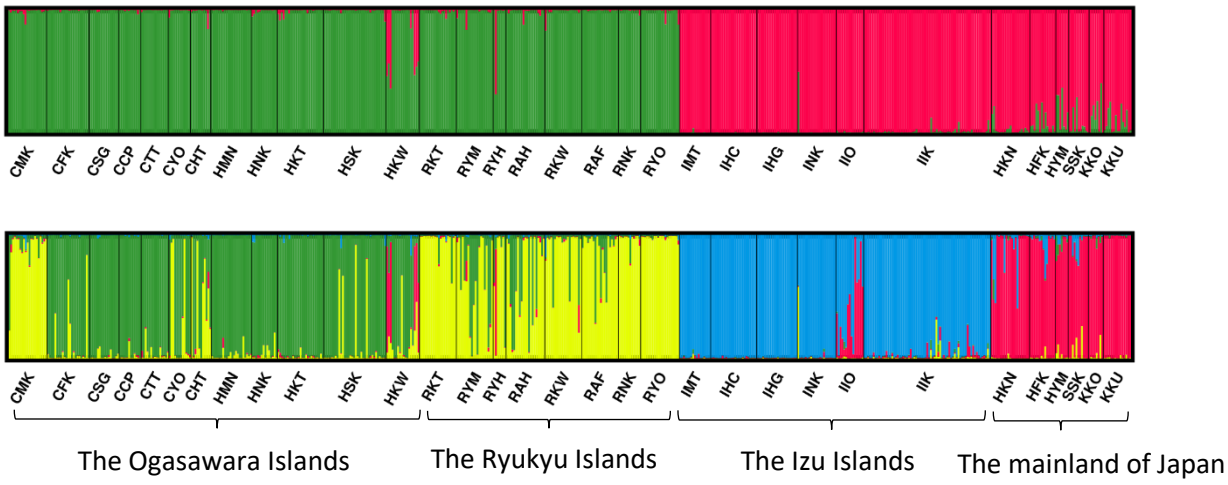
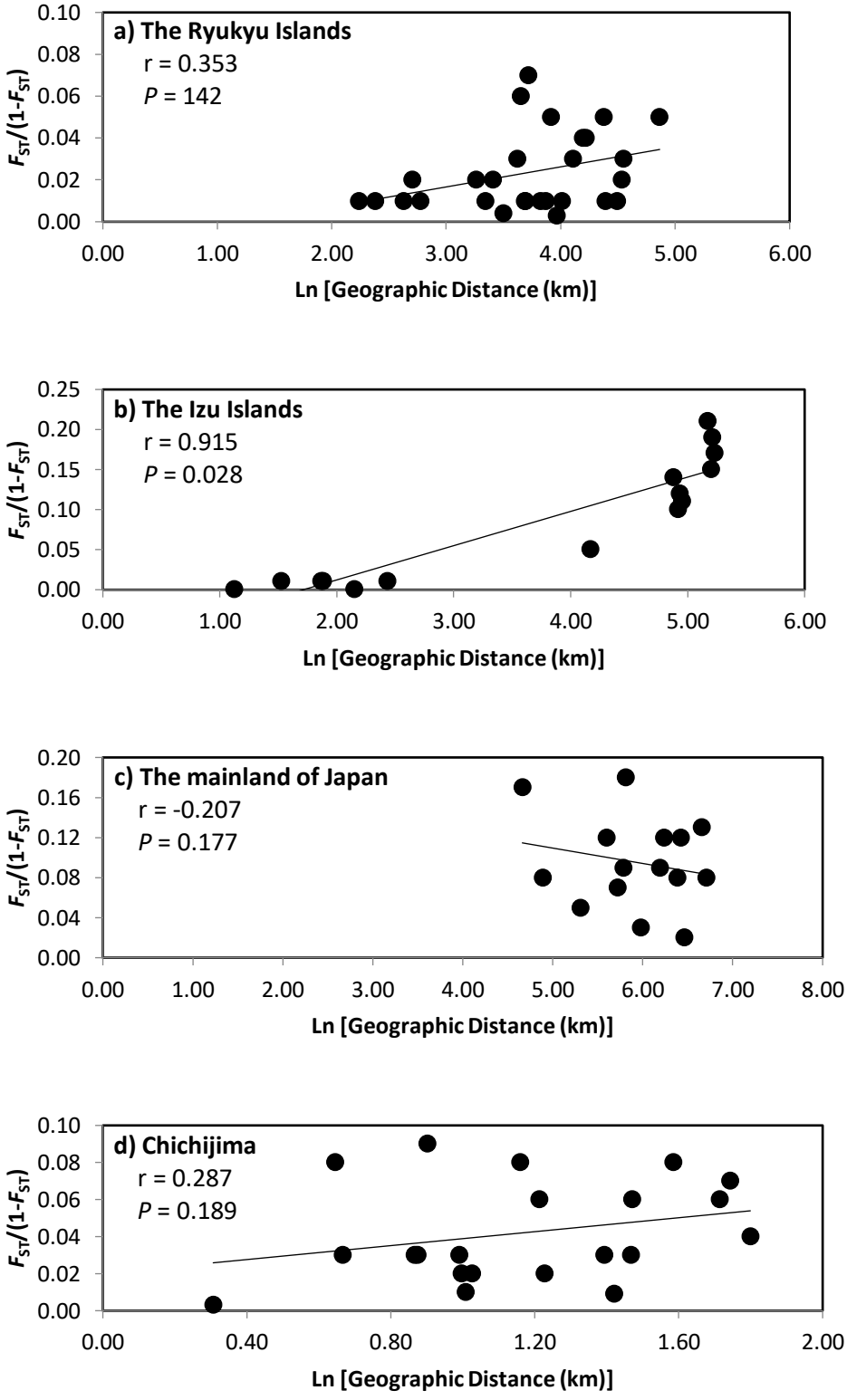


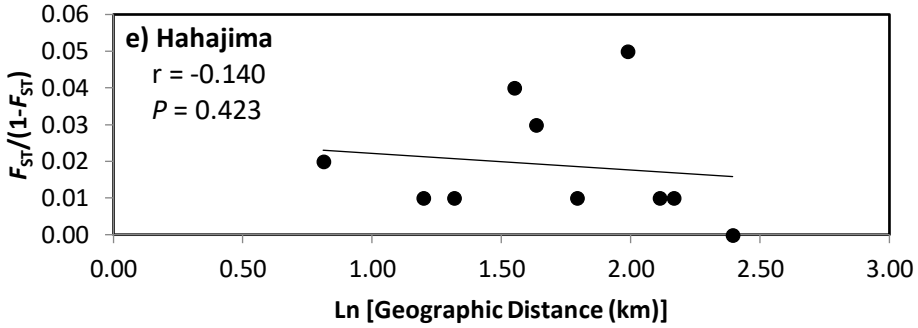
Fig. I-6. Bayesian clustering results in all examined populations a) delta *K*, b) the log-likelihood , c) bar plot for *K* = 2 and *K* = 4, respectively.

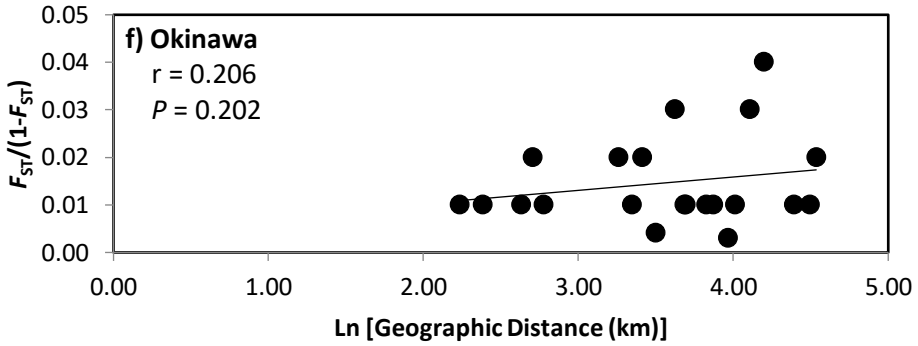
Appendix I-1. Voucher specimen ID of each population in this study.

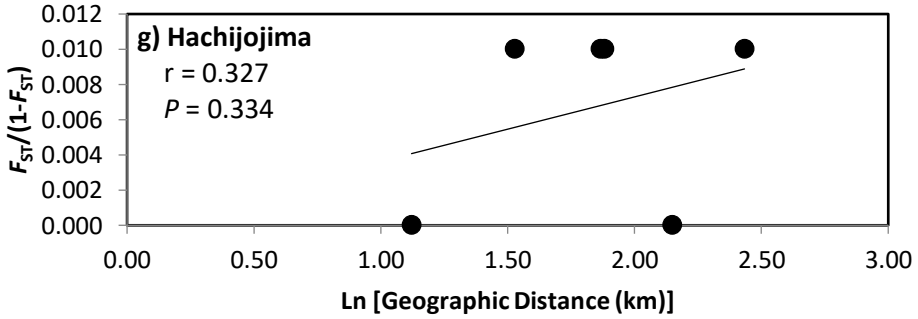
Island	Populations	Pop ID	Specimen ID
The Ogasawara Islands			
Chichijima	Mikazuki	CMK	MAK467119
	Fukiagedani	CFK	MAK467120
	Siguredamu	CSG	MAK467121
	Kitafukurozawa	CCP	MAK467122
	Tatsumidoro	CTT	MAK467123
	Yoakeyama	CYO	MAK467124
	Hatsuneura	CHT	MAK467125
Hahajima	Minamizaki	HMN	MAK467126
	Nakanotaira	HNK	MAK467127
	Kitako	HKT	MAK467128
	Sekimon	HSK	MAK467129
	Kuwanokiyama	HKW	MAK467130
The Ryukyu Islands			
Okinawa	Katsuyama	RKT	MAK467131
	Yamagusuku	RYM	MAK467132
	Yohanayama	RYH	MAK467133
	Aha	RAH	MAK467134
	Kawata	RKW	MAK467135
	Afuso	RAF	MAK467136
	Nakadomari	RNK	MAK467137
Yoronjima	Yoronjima	RYO	MAK467138
The Izu Islands			
Hachijojima	Mitsune	IMT	MAK467139
	Hachijofuji	IHC	MAK467140
	Higashiyama	IHG	MAK467141
	Nakanogo	INK	MAK467142
Kozushima	Kozushima	IIK	MAK467144
Oshima	Oshima	IIO	MAK467143
The Mainland of Japan			
Honshu	Yamagata	HYM	MAK467145
	Fukushima	HFK	MAK467146
	Ibaraki	HKN	MAK467147
Shikoku	Kochi	SSK	MAK467148
Kyushu	Kumamoto	KKU	MAK467149
Koshikishima	Koshikishima	KKO	MAK467150

Appendix. I-2. The correlations between genetic distances and natural logarithms of geographic distance for island groups and islands.




e) Hahajima
r = -0.140
P = 0.423
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.00
0.50
1.00
1.50
2.00
2.50
3.00
F_ST/(1-F_ST)
Ln [Geographic Distance (km)]


f) Okinawa
r = 0.206
P = 0.202
0.05
0.04
0.03
0.02
0.01
0.00
0.00
1.00
2.00
3.00
4.00
5.00
F_ST/(1-F_ST)
Ln [Geographic Distance (km)]


g) Hachijojima
r = 0.327
P = 0.334
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.00
0.50
1.00
1.50
2.00
2.50
3.00
F_ST/(1-F_ST)
Ln [Geographic Distance (km)]

Chapter II

The current hybridization between the endemic *Morus boninensis* Koidz. and the invasive *Morus australis* Poir. in the Ogasawara Islands

Introduction

Morus boninensis is one of the endangered endemic plant species in the Ogasawara Islands. It is dioecious tree species whose pollen and seeds are dispersed by wind and birds, respectively. It is tetraploid of *2n* = 28 while its related alian *M. austrlis* is diploid of *2n* = 28 (Hoshi 1995). Population size of *M. boninensis* has been reduced by over-exploitation before World War II (Government of Japan 2010) since the Japanese government permitted free logging to encourage immigration to the islands during the last quarter of the 19th century and early 20th century (Tani *et al*. 2003). Due to this, it is currently categorized as endangered species (Government of Japan 2010, Tani *et al*. 2006, Environment Agency of Japan 2000). Its distribution range is now limited to several small areas of Chichijima, Hahajima and Otoutojima (Toyoda 2003, 2014). Therefore, the current conservation status of *M. boninensis* needs to be investigated for establishing better conservation strategies. Especially, the main concerns about the conservation of *M. boninensis* at this moment are i) effects of *M. australis,* an Invasive Alien Species (IAS) closely related to *M.*

boninensis and ii) an expected decline of genetic diversity due to the reduction of its population size.

Tani *et al*. (2003; 2006) reported that hybridization has occurred between *M. boninensis* and invasive *M. australis*, but careful re-evaluations are still needed for the hybridization. They reported hybrids between the two *Morus* species and suggested that the hybridization potentially leads to loss of purity for endemic *M. boninensis* and the hybrids should be removed from the seedling pools to conserve *M. boninensis*. However, to evaluate actual impact of the hybridization on *M. boninensis*, further studies are necessary. It is because the previous study focused only on artificially germinated seeds collected from a single female tree of *M. boninensis* and did not check any naturally growing seedlings or mature trees in wild conditions (Tani *et al*. 2003; 2006). Thus, it is very urgent to confirm hybrids by collecting wild seedlings and mature trees in the Ogasawara Islands to clarify the present conservation status of *M. boninensis*,

In addition, evaluation of levels of genetic diversity in endemic *M. boninensis* is necessary. Genetic diversity reduction leads to reduced ability to adapt to changing environmental conditions and are susceptible to extinct even after small alterations in the environments (Dostálek *et al.* 2010; Isagi *et al.* 2020). Insular endangered endemic species are illustrated by small and isolated populations (Dostálek *et al.* 2010) and oceanic insular species often possess lower genetic diversity than their continental relatives (Frankham 1997). Moreover, population size reduction is expected to cause decreasing of the genetic diversity. Given that oceanic endemic species, *Morus boninensis* have experienced rapid declines in their individual number and population size, the species is expected to currently exhibit very low

level of genetic diversity. Therefore, investigation of the level of genetic diversity in *M. boninensis* is urgently necessary from conservation aspects. In general, a proper evaluation of genetic diversity in target species is challenging due to a lack of appropriate closely related taxon to compare. In fact, the previous study, Tani *et al.* (2006) analyzed *M. boninensis* populations using genetic markers without any congeners. In Chapter I, it was revealed that *M. australis* in Ogasawara possess similar amount of genetic diversity to the populations in its native range. This result enables us to describe and evaluate the levels of genetic diversity in *M. boninensis* by comparing with those of *M. australis* in the Ogasawara Islands and other native populations of the invasive species.

In Chapter II, for better understanding of conservation status of endemic *M. boninensis,* population genetic analysis was conducted for the two *Morus* species now growing in the Ogasawara Islands for three wild seedling populations, two populations of *M. boninensis*, and 12 populations of *M. australis.* Specifically, the following questions were addressed here: 1) To what degree is hybridization between invasive *M. australis* and native *M. boninensis* occurring in the young and matured wild populations of the two specis? 2) What level of the current genetic diversity is observed in *M. boninensis* in the Ogasawara Islands?

Materials and Methods

Samples collections

Plant materials of *Morus boninensis* and *M. australis* were collected during 2016 - 2020 from two adult populations of *M. boninensis,* 12 adult populations of *M. australis* and three wild seedling populations in the Ogasawara Islands (Table II-1 and Fig. II-1). Individuals of the seedling populations containing some juvenile trees were collected without identifying the taxa. The CMK, CFK, CSG, CCP, CTT, CYO, CHT populations on Chichijima and the HMN, HNK, HKT, HSK, HKW populations on Hahajima are the same populations that have been used in Chapter I. Fresh-young leaves in the apical stems were collected from each individual in order to obtain total DNA of the best quality. The collected leaves were stored in plastic bags with silica gel to keep them dry until DNA extraction. Voucher specimens were also collected during the sample collection and deposited in the Makino Herbarium of Tokyo Metropolitan University (MAK) (Table II-1).

DNA extraction and microsatellite analyses

Total DNA from each plant sample were extracted using the same method as reported in Chapter I. Amplification of microsatellite markers was conducted also using the same methods as reported in Chapter I. The 14 microsatellite primers developed for *M. boninensis* (Tani *et al.* 2005) and for *M. indica* (Aggarwal *et al.* 2004; Krishnan *et al*. 2014) were used for population genetic analyses. The amplified DNA was examined by using the ABI 3130xl genetic analyzer. The obtained data were processed by using the GeneMapper software version 4.0 supplied by ABI to determine the genotype of each sample.

Data Analysis

Since *M. boninensis* is a tetraploid species, the genotype determination for the species was based on the dosage of each allele that implied partial heterozygotes, following Tani *et al.* (2005) and Tsuda *et al.* (2017). For example, when only one allele is found then written as *AAAA*, when four alleles are found then written as *ABCD*, when two alleles are found then written as *AABB*, and when three alleles are found then the one allele with the highest peak written double, such as *AABC*. To show the genetic similarity among all of the population of *M. boninensis* and *M. australis*, UPGMA dendrogram based on Nei's genetic distances, D_A (Nei *et al.* 1983) was drawn by using POPULATIONS 1.2.30 (Langella 1999) and FigTree v.1.4.4 (Rambaut 2007). The Principal Coordinate Analysis (PCoA) based on pairwise F_{ST} values was conducted by using GenAleX6.503 (Peakall & Smouse 2012).

To clarify genetic differentiation of the two *Morus* species in the Ogasawara Islands and to detect hybrids between *M. boninesis* and *M. australis*, the Bayesian clustering analysis was conducted by using the software STRUCTURE version 2.3.4 (Falush *et al*. 2003, 2007; Pritchard *et al.*2000) in order to clarify assignment of each individual and one confirmed hybrid in CHK population based on the ploidy analysis to the posterior *K* grouping and to detect hybrids. All investigated population of both the species were tested together by using the setting number of burn-in 100000 and MCMC 100000 which K = 1-19 by applied 10 runs for each K value. The ΔK value was adopted through Evanno's method (Evanno *et al.* 2005) by using STRUCTURE HARVESTER on web (Earl & von Holdt 2012), and visualized by Distruct 1.1 (Rosenberg2004) and CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007).

The allele frequencies within *M. boninensis* and within *M. australis* were obtained by using SPAGeDI software (Hardy & Vekemans 2002). To do this, the putative admixed individuals were excluded at first in each population based the result of STRUCTURE analysis mentioned above. Subsequently, genetic diversity including number of alleles per locus (N_A), expected heterozygosity (H_E), allelic richness (A_R), and fixation index (F_{IS}) were also calculated by using the SPAGeDI software for *M. boninensis* and *M. australis*.

Results

Allele Frequencies within the populations of Morus *species in the Ogasawara Islands*

All of the microsatellite loci used showed polymorphisms for both the species. A total of 200 alleles were obtained from the 14 loci. The highest number of alleles was obtained from Mos0340-2 and MulSSR 258 (22 alleles), followed by MESTSSR 31 and Mos0031 (19 alleles), MulSSR 338 (18 alleles), MulSSR 313 (16 alleles), MulSSR 147 and MulSTR 2 (15 alleles), Mos0157-2 (13 alleles), MESTSSR 48, MulSTR 4 (11 alleles), MESTSSR 126 (eight alleles), and MUL3SSR6 (six alleles). The fewest number of alleles was detected by MESTSSR 73, which showed only five alleles. Thus, all of the loci were used for further analyses.

The genetic similarity among all of the population of M. boninensis *and* M. australis

The genetic similarity among all the investigated population was revealed in the UPGMA dendrogram based on Nei's genetic distances, D_A (Fig. II-2). Two clusters were revealed in this dendrogram. The first cluster (pink) comprise of all the population of *Morus boninensis*. The second cluster (green) comprise of all the population of *M. australis* and seedling/juvenile populations. The result of the Principal Coordinate Analysis (PCoA) is shown in Fig. 3. The first axis showed 68.86 % of the total variation. This axis clearly distinguished the two species, *M. boninensis* and *M. australis*. The seedling population formed a group together with *M. australis* (Fig. II-3). The second axis showed only 7.96 % of the total variation.

There was no obvious groping found along this axis either between the two species or between populations in the island groups of the Ogasawara Islands.

The Results of the STRUCTURE analysis for M. boninensis *and* M. australis

The result of the STRUCTURE analysis for *M. boninensis, M. australis* and the triploid hybrid between them confirmed by ploidy analysis is shown in Fig. II-4. The ΔK value was maximal at $K = 2$. Based on this maximal K value, two clusters corresponding to the two species, *M. boninensis* (pink cluster) and *M. australis* (green cluster) were observed. No hybrid individual was detected in seedling populations. However, nine individuals admixed between two clusters were found in the investigated populations of *M. boninensis* (HMB) and *M. australis* (CHK and HKW).

Genetic diversity of M. boninensis *and* M. australis

A relatively high level of genetic diversity was observed for all the population of *M. boninensis* and *M. australis* even after excluding all the putative.The calculated values of the common genetic diversity parameters (N_A, H_E, A_R, F_{IS}) for *M. boninensis* were also shown in Table II-1. The observed genetic diversity of *M. boninensis* ($H_E = 0.53$, $A_R = 3.27$) was almost the same as those of *M. australis* ($H_E = 0.62$, $A_R = 4.33$) and those of seedling populations ($H_E = 0.61$, $A_R = 4.29$) (Table II-1). The values of heterozygosity (H_E) for *M. boninensis* were 0.52 and 0.53, while in *M. australis*, it ranges from 0.58 to 0.68, and for seedling populations, it ranges from 0.59 to 0.65. The allelic richness (A_R) for *M. boninensis* ranges from 3.21 to 3.32.

Discussion

Hybridization between M. boninensis *and* M. australis

Genetic researches on hybridization between native species and IAS are often necessary to conserve endangered native species. As mentioned in the introduction, IAS is hypothesized to have strong impact on native closely related species by hybridization (Mooney & Cleland 2001). Such interspecific hybridization with native species not only enables IAS to expand the invaded area by introduction of the genotype adapted to the area (Prentis *et al.* 2008) but also replace pure strains of the native species with admixed ones, namely 'genetic contamination'. Therefore, an investigation of hybridization with genetic markers is needed for an endangered species threated by invasive species.

In this study, extent of hybridization between an endangered species, *Morus boninensis* and its closely related IAS, *M. australis* in natural condition of the Ogasawara Islands was investigated. Tani *et al.* (2003) investigated the extent of the hybridization between the two species by examining a proportion of hybrids in embryos of seeds collected from a mother tree of *M. boninensis*. They detected some of artificially germinated embryos as the hybrids, and concluded that hybridization with *M. australis* has strong negative impact on regeneration of native *M. boninensis*. In their study, however, seedlings and mature trees were not investigated in the natural condition and the seeds used were collected only from single female tree. Thus, the impact of hybridization in the wild still remains unclear. In this paper, naturally growing *Morus* individuals were collected by focusing on seedlings and juveniles to examine the impact of hybridization in natural condition.

No hybrid individuals were found in the wild seedling populations of the *Morus* plants in the Ogasawara Islands. Of the 71 seedlings and juveniles collected from three populations in this study, all individuals were identified as pure *M. australis* and no hybrid was detected by our STRUCTURE analysis (Fig. II-4). This result is inconsistent with the report by Tani *et al.* (2003) where a relatively high proportion of hybrids, 13% were detected in the artificially germinating seeds from a mother tree of *M. boninensis*. The hybrid seedlings might not be able to survive and/or grow in wild conditions despite that hybridization actually occurred between them (Tani *et al.* 2003). Although there is no direct evidence, natural selections against seedlings /juveniles of hybrids may act in the *Morus* species.

On the other hand, several individuals genetically admixed between the two species, possibly F_1 hybrids, were found in the populations of the mature trees (Fig. II-4). Two individuals found in HMB and one in CHK exhibited the similar proportions of genetic admixture between the *M. boninensis* cluster (Pink) and the M. *australis* cluster (Green) as close to 2:1 ratio (Q = 0.62:0.38, 0.68:0.32, 0.66:0.34, respectively). Given that *M. australis* and *M. boninensis* are diploid and tetraploid species, respectively, the proportion of genetic admixture in F_1 hybrids is expected to be 1/3 of their genetic constitution from diploid *M. australis* and 2/3 from tetraploid *M. boninensis*. Thus, these three individuals can be interpreted as triploid F_1 hybrids. In addition, the admixed individuals found in HKW population of *M. australis* exhibited various patterns of genetic admixture differentiated from that in typical F_1 hybrid mentioned above: Two showing similar proportions genetically close to pure strain of *M. boninensis* and the other four showing more admixed from *M. australis* than the expected ratio for the F_1 hybrids.

These possible hybrid individuals other than F_1 can be explained by following two hypotheses. Firstly, the two hybrids genetically close to the pure strain of *M. boninensis* could be artifacts resulting from the possession of shared alleles between the two species. They could be interpreted as pure strain of *M. boninensis.* If this is true, the other four hybrids can be also considered as F_1 hybrids with more shared alleles between *M. australis* and *M. boninensis*. Thus, the presence of the shared alleles might have caused the pattern observed in HKW population. Secondly, the hybrids could be those backcrossing with parental species. A few studies reported interploidal gene flow in angiosperms (e.g., *Betula* by Tsuda *et al.* 2017 &Wang *et al.* 2014; *Arabidopsis arenosa* by Arnold *et al*. 2015). In general, however, triploid rarely produces viable gametes due to failure in homologous chromosome pairing during meiosis. Thus, this hypothesis might be unlikely. In future study, these hypotheses may need to be tested by incorporating population genetic analysis with cytological analysis such as those using flow cytometry.

In conclusion, our study showed no hybrid seedlings in wild condition and no evidence of the expected genetic contamination in *M. boninensis* populations despite of the presence of some F_1 hybrids. Thus, the degree of hybridization between native *M. boninensis* and alien *M. australis* in the Ogasawara Islands is relatively more limited than previously expected.

Genetic diversity within endemic Morus boninenis *in the Ogasawara Islands*

The level of genetic diversity within *Morus boninensis*, an endangered endemic plant species in the Ogasawara Islands, was observed almost same as that within its widely distributed relative, *M. australis*. Reductions of genetic diversity in

insular endemics compared to continental counterparts (*ex*. related taxa in the mainland) have been discussed in several previous studies (Frankham 1997; Stuessy *et al*. 2014; García-Verdugo *et al*. 2015). The island endemics are assumed to display low levels of genetic diversity (Stuessy *et al*. 2014). However, the mean level of allelic richness and heterozygosity of *M. boninensis* (A_R = 3.27*;* H_E = 0.53) was only slightly lower than those of mature *M. australis* populations in the Ogasawara islands (A_R = 4.33*;* H_E = 0.62) in this study (Table II-1). *Morus australis* is widely distributed from Himalayas to Japanese archipelagos including the Ryukyu and Izu Islands of Japan. Intra-populational genetic diversity of *M. australis* in other areas of Japan was in a range from 3.70 to 5.80 (A_R), and from 0.51 to 0.73 (H_E) (Chapter I). Frankham (1997) investigated genetic diversity reductions by comparing heterozygosity rations (1-H_{IS}/H_M) in island populations to those in mainland populations. The study showed that the reduction rates in insular endemic plants for allozyme were indicated as 0.46, and although no plants taxa was included, for DNA markers were 0.22 to 0.62. In this study, genetic diversity reduction in each population of *M. boninensis* ranged from 0.20 to 0.27, comparing to *M. australis* populations except to those in the Izu islands that have very low genetic diversity. It suggests that *M. boninensis* maintains high levels of genetic diversity. Thus, *M. boninensis* does not seem to follow tendency of genetic diversity reducing in insular endemics.

No reduction of genetic diversity in *M. boninensis* may be attributed to biological characteristics of this species. Stuessy *et al.* (2014) suggested biological characteristics influencing levels of genetic variation in insular endemics. For example, the generation time (Stuessy *et al.* 2014) regulate the development of

genetic variation within populations by influencing the rates of recombination. Another possible reason is outcrossing of dioecious plant might be suitable for maintenance of higher levels of genetic diversity (Paschoa *et al.* 2018 & Government of Japan 2010). On the other hand, small population size was thought as negatively affecting to maintain genetic diversity. *Morus boninensis* is dioecious and produces many seeds which dispersed by birds, which suggest that this species is predominantly outbreeder. Additionally, the generation time of *M. boninensis* seems very long. A stump which annual rings over 2000 years was reported in the Ogasawara Islands (Toyoda 2003). These characteristics are suitable to accumulate and maintain genetic diversity within the species. However, on the other hand, the population size of *M. boninensis* rapidly declined because the trees had been heavily logged during the last quarter of the 19th century and the start of 20th century with encouragement by Japanese government (Tani *et al.* 2003). This reduction in population size is likely to lead to the loss of genetic diversity within *M. boninensis* in near future.

Tani *et al.* (2006) also reported that there was no observation of seedling recruitment since 1995. In this study, any pure *M. boninensis* were not detected in seedling and juvenile populations either in Chichijima or Hahajima (Fig. II-4). This suggests that *M. boninensis* failed any regenerations even after 20 years in spite of intensive effort to the conservation of the species. In current condition, although *M. boninensis* maintained relatively high amount of genetic diversity compatible to successful IAS, *M. australis*, *M. boninensis* will decline if no seedling recruitment will occur in future.

Table II-1. Sampling localities and genetic diversity of *Morus boninensis* and *M. australis* in the Ogasawara Islands. Populations of *M. australis* are cited from Chapter I except some new populations marked by asterisk. *N*, number of individuals; N_A, number of alleles; H_E, expected heterozygosity (corrected for sample size, Nei 1978); A_R, Allelic richness (k = 16) F_{IS}, fixation index.

Island	Location	Specimen ID	Pop ID	Latitude (°N)	Longitude (°E)	*N*	N_A	H_E	A_R	F_{IS}
M. boninensis										
Chichijima	Higashi-Kaigan	MAK467151	CMB*	27.06016	142.2273	17	4.43	0.53	3.32	0.06
Hahajima	Sekimon	MAK467152	HMB*	26.68412	142.1614	8	3.71	0.52	3.21	-0.03
					Mean	12.50	4.07	0.53	3.27	0.02
M. australis										
Chichijima	Higashi-Kaigan	MAK467153	CHK*	27.06491	142.22758	15	4.21	0.58	3.69	0.14
	Mikazuki	MAK467119	CMK	27.09862	142.18687	21	5.86	0.66	4.55	0.08
	Fukiagedani	MAK467120	CFK	27.07554	142.20870	23	5.64	0.61	4.28	0.04
	Siguredamu	MAK467121	CSG	27.05991	142.20675	16	4.57	0.59	3.93	0.06
	Kitafukurozawa	MAK467122	CCP	27.06018	142.20314	12	4.86	0.60	4.39	0.11
	Tatsumidoro	MAK467123	CTT	27.06397	142.21987	15	5.07	0.60	4.24	0.02
	Yoakeyama	MAK467124	CYO	27.08559	142.21721	12	5.64	0.68	4.89	0.14
Hahajima	Minamizaki	MAK467126	HMN	26.61139	142.17621	22	5.36	0.58	4.07	0.11
	Nakanotaira	MAK467127	HNK	26.63210	142.17363	14	5.14	0.61	4.33	0.06
	Kitako	MAK467128	HKT	26.69634	142.14363	25	5.93	0.63	4.26	0.08
	Sekimon	MAK467129	HSK	26.67440	142.15615	33	6.21	0.63	4.40	0.14
	Kuwanokiyama	MAK467130	HKW	26.66379	142.15196	13	5.93	0.65	4.98	0.11
					Mean	18.42	5.37	0.62	4.33	0.09
Seedling										
Chichijima	Hatsuneura	MAK467125	CHT	27.07704	142.21770	11	4.71	0.59	4.30	0.21
Hahajima	Sekimon	MAK467154	HSE*	26.68455	142.16138	52	7.21	0.65	4.56	0.04
	Uchusawa	MAK467155	HAU*	26.66491	142.15655	8	4.00	0.60	4.00	-0.05
					Mean	23.67	5.31	0.61	4.29	0.07

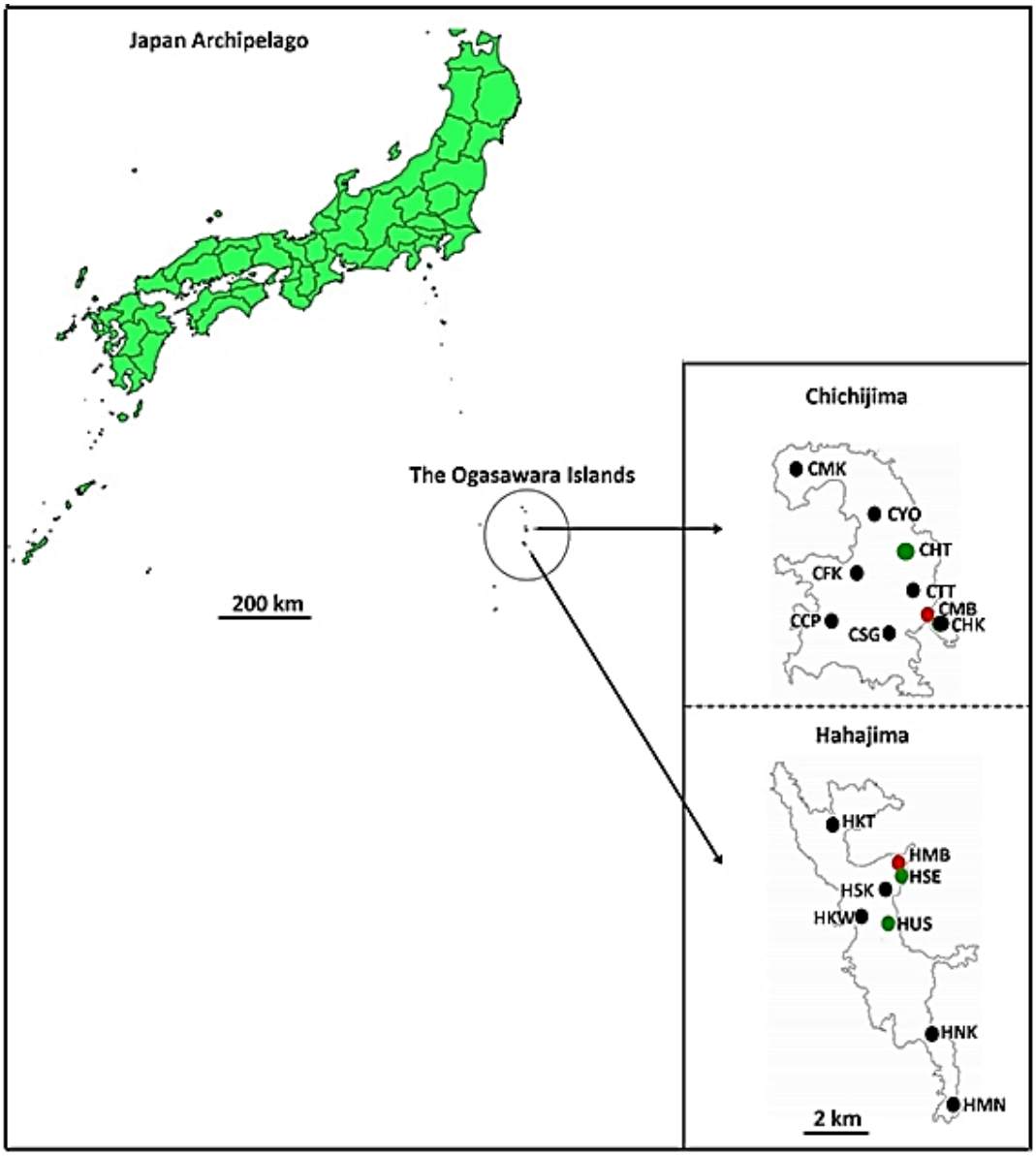


Fig. II-1. Map of sampling localities for *M. boninensis* populations ●, *M. australis* populations ● , and wild seedling populations ● , in the Ogasawara Islands. The CMK, CFK, CSG, CCP, CTT, CYO, CHT populations of Chichijima and the HMN, HNK, HKT, HSK, HKW populations of Hahajima are the populations that have been used in Chapter I.

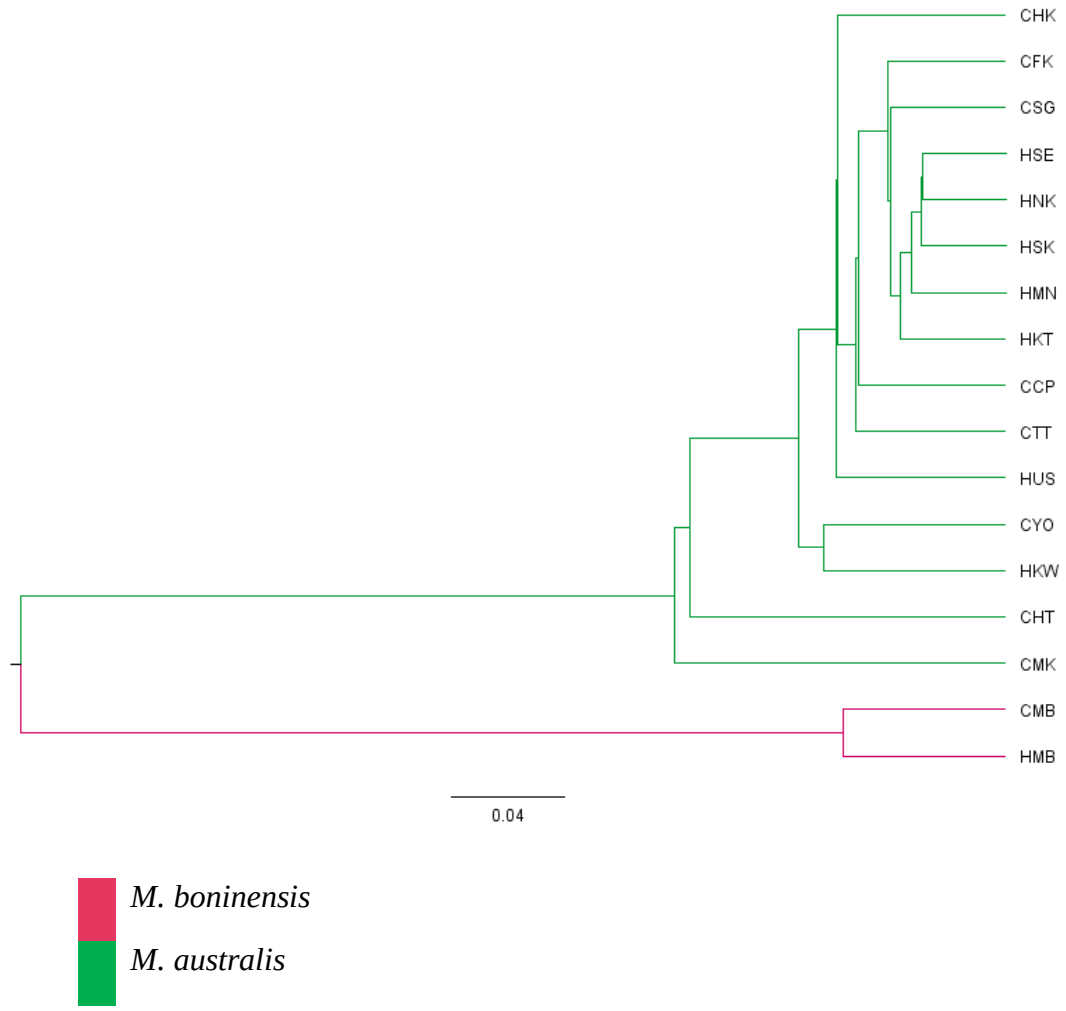


Fig. II-2. The UPGMA dendrogram among all populations of *M. boninensis and M. australis* in the Ogasawara Islands based on Nei's genetic distance (Nei *et al*. 1983).

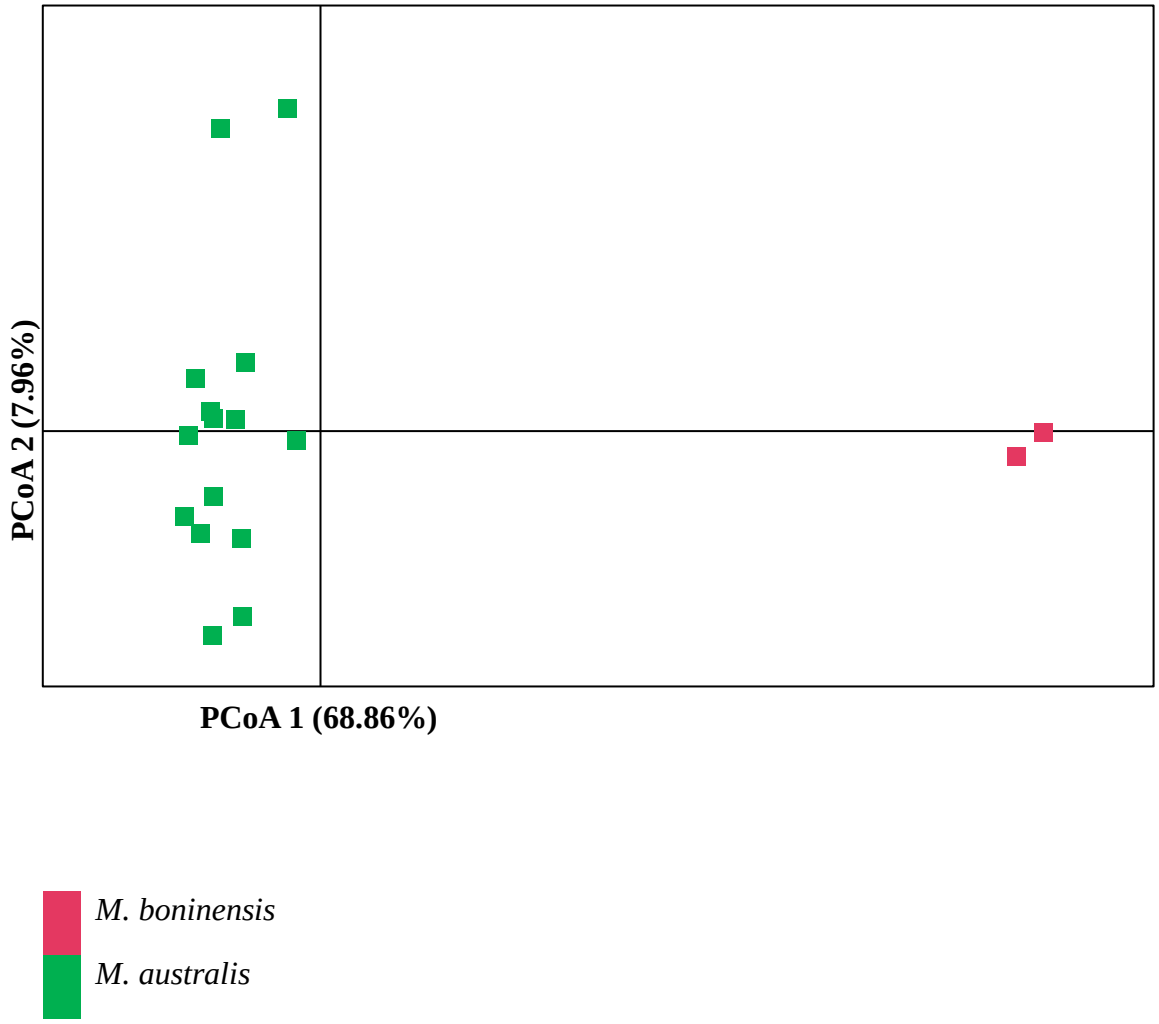
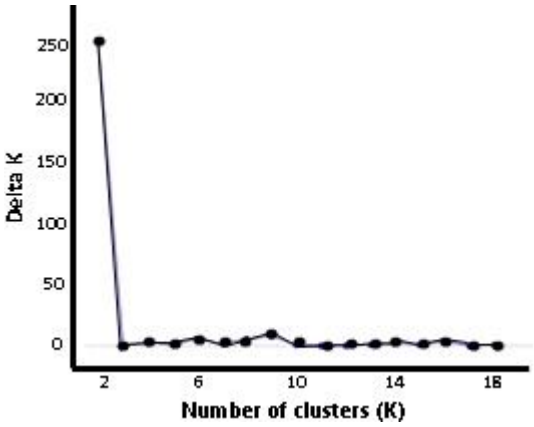


Fig. II-3. Result of the PCoA among all the populations of *M. boninensis* and *M. australis* in the Ogasawara Islands based on pairwise F_{RT} values.

a)



b)

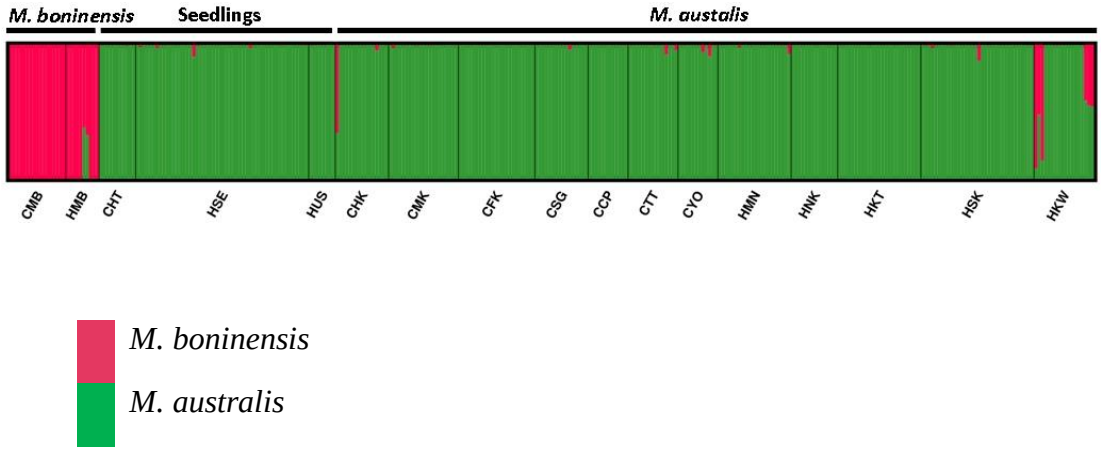


Fig. II-4. Population genetic structures; a) delta *K* values, b) all population of *M. boninensis* and *M. australis* species and one confirmed hybrid.

General Discussion

Invasive Alien Species (IAS) now threaten endemic species of the Ogasawara Islands. IAS exhibit strong tolerance against a wide range of environmental conditions (Devin & Beisel 2007) that promotes higher reproductive rates. It makes IAS more competitive to the native species (Convention on Biological Diversity 2009). Ultimately, they may expel native fauna and flora. The IAS are currently problematic also in oceanic islands with high degree of endemism (Caujape *et al.* 2010, Whittaker & Fernández-Palacios 2007, Hobohm 2000). The island endemism is vulnerable to introductions and the increase of IAS. The Ogasawara Islands that I focus in this study are one of the oceanic islands with many endemic species and now seriously threatened by IAS (Kawakami 2008, Sugiura 2016, Kawakami & Okochi 2010).

For controlling IAS, understanding genetic basis of them is quite essential. Population genetic studies using molecular markers provide us information about the introduction routes of IAS and the amount of genetic variation introduced (Durka *et al.* 2005). The knowledge about the origins of IAS may help us to estimate ecological features of them because IAS are expected to have been pre-adapted to the environments of the source populations. Additionally, the genetic markers are also useful to detect hybridization between IAS and endemic species that sometimes causes serious threat of endemic species thorough genetic contamination (Mooney & Cleland 2001 & Levin *et al.* 1996). In this study, microsatellites markers were chosen to conduct the population genetic analyses for *Morus* species in the Ogasawara islands due to their high level of polymorphisms (Krishnan *et al.* 2014,

Aggarwal *et al.* 2004, Mburu & Hanotte 2005, Oguri *et al.*2013, Tani *et al.* 2006; 2005). The population genetic studies by using microsatellite markers are useful to understand genetic basis of the IAS in the Ogasawara Islands.

Study on route of introductions and source populations of invasive *Morus australis* is necessary to be carried in the Ogasawara Islands. *Morus australis* is one of IAS that was recorded as introduced from the Ryukyu Islands and Hachijojima Island of the Izu Islands to the Ogasawara Islands for sericulture of silkworm *Bombyx mori* (Awasthi *et al*. 2004; The National Institute for Environmental Studies, Japan 2017, Tokyo Metropolitan Government Ogasawara Island Branch 1938). Although the sericulture has been declined, the species has feralized and spread even into conserved areas in the islands. However, there have been no genetic studies conducted for the alien species in the Ogasawara Islands. Thus, origin of the trees of the species now growing in the Ogasawara Islands and extent of their genetic diversity remains to be explored.

Hybridization between alien *M. australis* and native endemic *M. boninensis* in the Ogasawara Islands should be also carefully investigated using molecular genetic markers. As consequence of feralization, *M. australis* is ongoing threat to a closely related endemic species in the island, *M. boninensis.* *Morus boninensis* is designated as an endangered plant species in the Ogasawara Islands (Environment Agency of Japan 2000). IAS such as *M. australis* are expected to have strong impacts on the native species by hybridization (Mooney & Cleland 2001 & Levin *et al.* 1996). This is also true for *Morus* in the Ogasawara Islands. In fact, Tani *et al.* (2003) have reported that hybridization between *M. australis* and *M. boninensis* and suggested a possible genetic contamination to *M. boninensis*. Therefore, invasion of

M. australis is likely to threat endemic *M. boninensis* not only through spreading but also through genetic disturbances or genetic pollution (Hufford & Mazer 2003). However, the study by Tani *et al*. (2003) was only based on artificial germinated seeds collected from a female tree of *M. boninensis*. Thus, it remains unclear to what extent hybridization occurs between them in nature.

The two main questions in this thesis have been addressed in the Chapter I and Chapter II. In Chapter I, population genetic analyses were conducted to investigate the origin of *M. australis* in the Ogasawara Islands and its genetic diversity. In Chapter II, the population genetic study focusing on the impact of hybridization between endemic *M. boninensis* and invasive *M. australis* as well as the genetic diversity now observed within *M. boninensis*.

In Chapter I, the study on invasive *M. australis* was performed by using microsatellite markers focusing not only the populations in the Ogasawara Islands but also those from the Ryukyu Islands, the Izu Islands as well as the mainland of Japan. Genetic-distance based analyses and STRUCTURE analysis revealed that the Ogasawara populations of *M. australis* recurrently originated from Ryukyu Islands. The genetic statistics demonstrated that the extent of genetic diversity of the Ogasawara populations was similar to that of the Ryukyu populations. This result suggested that the similar subtropical environments both in the Ryukyu Islands and the Ogasawara Islands might support the establishment of *M. australis* in the Ogasawara Islands and the high genetic diversity might enhance the invasiveness of the species in the oceanic islands.

In Chapter II, to infer the degree of hybridization between alien *M. australis* and native *M. boninensis* in nature and to estimate the extent of genetic diversity

maintained in *M. boninensis*, population genetic analysis was performed for naturally grown seedlings and mature populations of both the species. STRUCTURE analysis revealed that all the examined seedling populations did not include any F_1 hybrids and were identified as *M. australis*. However, a few F_1 hybrid was detected in mature populations of both the species. Genetic diversity *of M. boninensis* was observed almost in the same level as that within its widely distributed relative, *M. australis* in the Ogasawara Islands. This study suggested a limited genetic impact of alien *M. australis* on endemic *M. boninensis* through hybridization and relatively higher genetic diversity of *M. boninensis* than ever expected.

High genetic diversity within the populations of *M. australis* in the Ogasawara Islands may enable the invasive alien species to continue to expand its populations and threaten native *M. boninensis*. In this study, it was revealed that both *M. australis* and *M. boninensis* exhibit relatively high genetic diversity. Although high genetic diversity of *M. australis* in the Ogasawara Islands can be attributed to the multiple introductions from the Ryukyu Islands to the Ogasawara Islands as shown in Chapter 1, the maintenance of genetic diversity might be under the common mechanism shared between the two *Morus* species. One of the possible mechanisms is dioecy in their sex expression forcing them to be predominantly outcrossing (Paschoa 2018). The observed high genetic diversity in *M. australis* suggested that the populations now growing in the Ogasawara Islands are successful and expected to persist for longer periods. Because high genetic diversity enhances invasiveness of this alien species and also contributes to the robustness to environmental differences between its source populations in the Ryukyu Islands and introduce populations in the Ogasawara Islands. Thus, careful observations on

additional expansion of *M. australis* in the Ogasawara Islands are necessary because such expansion may prevent regeneration of endemic *M. boninensis* in the islands.

On the other hand, a contrasting pattern of regeneration were also found between *M. australis* and *M. boninensis*. In Chapter 2, seedlings were collected and all the collected seedlings were genetically identified as those of *M. australis.* These results suggested that the regeneration of *M. australis* apparently outnumbered that of *M. boninensis*. This could be partly explained by the difference in flowering and fruiting period between them. *Morus australi*s produces fruits in several times in a year (June & November Toyoda, 2003), while the fruiting season of *M. boninensis* is once in a year and lasts only from December to January (Toyoda 2014). The difference in seed production between the two species might generate this pattern and the higher reproduction ability of *M. australis* could deprive the opportunity of reproduction of *M. boninensis*.

This study also suggested that hybridization between native *M. boninensis* and alien *M. australis* has a limited impact on endemic *M. boninensis*. The possible reason is because the two species have different ploidy levels from each other. *Morus boninensis* is tetraploid while *M. australis* is diploids. Even if hybrids are produced between them, they will be triploid and cannot reproduce due to unbalanced chromosome pairing in triploid cytotypes. In Chapter 2, hybrid individuals were found not in seedlings but in a few mature tree populations suggesting hybrids are rarely viable in wild conditions. Additionally, despite of the concern that introgression from alien *M. australis* to native *M. boninensis* might occurs (Tani *et al*. 2003), no clear evidences of introgression were detected based on the result of STRUCTURE analysis in Chapter 2.

Nevertheless, hybridization with *M. australis* is still likely to cause negative effects on reproduction of *M. boninensis.* It is because hybrid seeds were reported from a female tree of *M. boninensis* (Tani *et al.* 2003). Even if produced hybrids are all sterile, hybridization may cause reproductive interference that substantially reduce fitness in one species by obligating the species spend a large cost on producing sterile hybrids (Kyogoku 2015). Thus, *M. boninensis* could reduce its fitness through reproductive interference by alien *M. australis*. Especially, given that *M. australis* and *M. boninensis* are diploid and tetraploid, respectively and the difference in regeneration could result in a gap in population density between them, this situation of *Morus* in Ogasawara Islands might fit with the minority cytotype exclusion (Levin 1975). According to this theory, minority cytotype (*M. boninensis* in this case) reduce its fitness by hybridization with majority cytotype (*M. australis* in this case), and ultimately may go extinct.

In conclusion, *M. australis* in the Ogasawara Islands are quite successful and is expected to persist for longer periods probably because of the similar climate condition to its source populations in the Ryukyu Islands and the high genetic diversity within the populations of the Ogasawara Islands. As to the threats of the invasive species to local endemism in the Ogasawara Islands, although the limited genetic impacts of hybridization on the endemic species *M. boninensis* was expected, the hybridization may reduce the fitness by preventing regeneration of *M. boninensis*. Given the rapid decline of *M. boninensis,* a further expansion of *M. australis* could be a strong obstacle for *M. boninensis* to recover their populations. Therefore, efforts to reduce number of the individuals of the invasive species *M. australis* and increase those of endemic *M. boninensis* in the Ogasawara Islands are indispensable.

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