High levels of genetic diversity in island populations of the island endemic *Suzukia luchuensis* (Labiatae)

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Genetic diversity and genetic differentiation within and among island populations was examined by allozyme electrophoresis in *Suzukia luchuensis* (Labiatae), which is endemic to four of the Ryukyu Islands, southern Japan, and one island near Taiwan. Intrapopulation allozyme diversity was very low in all the four Ryukyu Islands, probably due to the effects of random drift in small populations. In contrast, genetic diversity at the species level was high, possibly because of an ancient origin of populations and/or multiple colonization of the species on different islands. Genetic differentiation among the overall populations was high ($G_{ST} = 0.863$), while gene flow (N_m) as estimated from allozyme frequency data was 0.041, suggesting that its occurrence among populations is highly restricted. Hierarchical analysis of genetic differentiation indicated that a high proportion of the total allelic variance is attributed to variation among islands, corresponding to the fact that several alleles were fixed on only one island. However, intraisland genetic differentiation was small on all islands except Yonaguni Island, where *S. luchuensis* is relatively widely distributed. Most diversity was thus due to differences among islands.
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Introduction

Island floras are of great interest to evolutionary biologists (Stuessy and Ono, 1998). This is because they often contain many unique endemic species, which are morphologically divergent from continental relatives and are considered to have evolved in environments that differ from those of the mainland. The floras of some islands have an extremely high ratio of endemic species; for example, in Hawaii approximately 90% of the angiosperms are endemic (Groombridge, 1992). Often, gene flow from the mainland to an island is highly limited due to spatial isolation. This can result in rapid fixation of mutations and subsequent speciation (Barton, 1998). In addition, a lack of competition with other species and the possibility for colonization of new habitats may promote speciation on islands (Crawford *et al*, 1987).

Insular endemic plants are susceptible to extinction due to the negative impact of humans, competition and hybridization with alien plants, and the deterioration of their habitats (Rieseberg and Swenson, 1996; Maunder *et al*, 1998). They are thus of primary interest to conservation biologists. A major problem in this respect is that they often lack genetic variation (Frankham, 1997) and therefore will have difficulty in adapting to changing environments (Hamrick and Godt, 1995). Detailed examination of the genetic diversity of island floras has shown that insular endemic plants generally maintain a low level of genetic diversity and high genetic differentiation among populations (Francisco-Ortega *et al*, 2000; Crawford *et al*, 2001). However, levels of genetic diversity within species are substantially larger in endemics of the Canary Islands than in those of the Pacific islands, suggesting that the pattern and level of genetic diversity in endemic plants differs among different island groups (Crawford *et al*, 2001).

The Ryukyu Islands are located to the south of the Japan Archipelago and consist of ∼140 subtropical islands (Figure 1). Approximately 150 plant taxa, equivalent to 7% of the flora, are endemic to these islands (Hatusima, 1980), suggesting that the islands are not oceanic but continental in origin. For example, the proportion of endemics in the Ryukyu flora is comparable to that of several continental islands in the Mediterranean (see references in Affre and Thompson, 1997). In fact, previous geological studies showed that the islands have repeatedly had connections to the adjacent mainland (Kizaki and Oshiro, 1980). To date, genetic diversity has only been quantified in a small number of endemic plants in the Ryukyu Islands (Maki, 1999, 2001; Hiramatsu *et al*, 2001).

Suzukia luchuensis Kudo (Labiatae) is a perennial herb occurring on rocky sites near the seashore. It creeps on the ground to reach a length of a few meters, and produces racemes near the top of stems. Its flowers are pink and 12–14 mm long. Pollinators for the species are not known. *S. luchuensis* has been reported to be distributed on only four islands of the Ryukyu

Archipelago (Figure 1): Yonaguni Island, Kuroshima Island, Kume Island, and Okinawa Island and its contiguous small island (Okinawa Prefecture, 1996). It has also been recorded on Aragusuku Island (Chinen, 1993), but it is unknown whether it is still present here. Recently, the species was recorded on Lutao Island, east of Taiwan (Hsieh, 2000) (Figure 1). The species is listed in the national list of threatened plants (Red Data Book) in Japan as 'vulnerable' to extinction (Environment Agency of Japan, 2000), and the total number of extant individuals is estimated to be less than 1000. The species is subject to severe damage from trampling by humans (Environment Agency of Japan, 2000).

In this study, we address the following questions using enzyme electrophoresis. How much genetic diversity does *S. luchuensis* harbor at the species level and at the population level? Are levels of diversity larger or smaller than those of other insular endemic plants examined previously? To what extent does gene flow occur within and among island populations of *S. luchuensis*?

Materials and methods

Sampling populations
We sampled two populations of *S. luchuensis* from each of Kuroshima Island, Kume Island, and Okinawa Island and three populations from Yonaguni Island, where the species is more widely distributed (Figure 1). Population codes and the number of individuals sampled are given in Figure 1 and Table 1. Cuttings were collected individually from each population and samples were
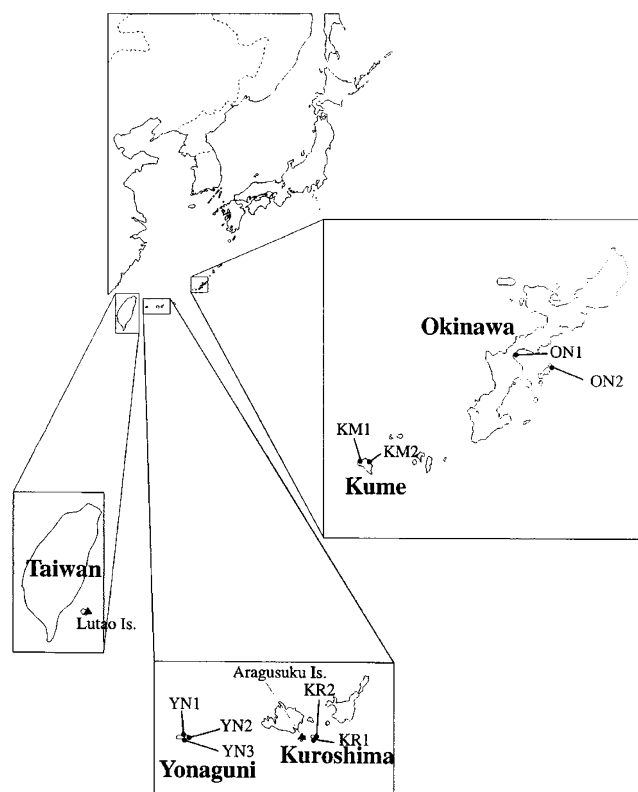
Table 1 Population codes, islands, mean sample size per locus (N), percentage of polymorphic loci (P), mean number of alleles per locus (A), and gene diversity within a population (h) at 20 loci for populations of *Suzukia luchuensis*

Population code	Island	N	P	A	h
Each population					
YN1	Yonaguni	25.8	25.0	1.25	0.087
YN2	Yonaguni	21.4	10.0	1.15	0.034
YN3	Yonaguni	35.8	20.0	1.25	0.032
KR1	Kuroshima	33.0	0	1.00	0.000
KR2	Kuroshima	15.3	0	1.00	0.000
ON1	Okinawa	16.4	0	1.00	0.000
ON2	Okinawa	16.5	5.0	1.05	0.010
KM1	Kume	26.8	15.0	1.20	0.053
KM2	Kume	30.4	15.0	1.15	0.068
Mean			10.0	1.13	0.031
Average across populations for each island					
	Yonaguni		25.0	1.90	0.108
	Kuroshima		0	1.00	0.000
	Okinawa		5.0	1.05	0.006
	Kume		25.0	1.25	0.066
At the species level			65.0	1.90	0.232

transported in plastic bags to the nursery at Tohoku University and planted in plastic pots filled with sand. Before electrophoresis, a sample of mature leaves was taken from each individual plant and transported on ice to the laboratory.

Allozyme electrophoresis
Enzyme extraction and polyacrylamide gel electrophoresis were carried out following the method of Maki and Murata (2001). The following 12 enzyme systems were examined by polyacrylamide gel electrophoresis: alanine aminopeptidase (AAP; EC 3.4.11.1), asparate amino transferase (AAT; EC 2.6.1.1), colometric esterase (EST; EC 3.1.1.–), formate dehydrogenase (FDH; EC 1.2.1.2), glutamate dehydrogenase (GDH; EC 2.7.1.1), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (G3PDH; EC 1.2.1.9), menazion reductase (MNR; EC 1.6.99.–), phosphoglucomutase (PGM; EC 5.4.2.2), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), superoxide dismutase (SOD; EC 1.15.1.1), and triose-phosphate isomerase (TPI; EC 5.3.1.1). Staining protocols followed Tsumura *et al* (1990), except that for FDH and G3PDH, which followed Wendel and Weeden (1989).

Statistical analysis
Based on the results of allozyme electrophoresis, allele frequencies in each population of *S. luchuensis* were calculated for the loci encoding the 12 enzyme systems. The following indices were used to quantify the amount of genetic diversity within each population examined: the proportion of polymorphic loci (P) at the 95% criterion, the number of alleles per locus (A), and the expected heterozygosity (h). Genetic diversity parameters (P, A, and h) were also calculated at the species level. As in Hamrick and Godt (1989), we treated the loci

Figure 1 Distribution of the populations of *S. luchuensis*. The population codes and number of samples are shown in Table 1. Closed triangles indicate localities reported for *S. luchuensis* but not examined in this study.

Table 2 Fixation indices for individual loci and the mean values for all polymorphic loci for each population of *Suzukia luchuensis*

Population	Locus							
	Aat-1	Aat-2	Ce-1	Ce-2	Ce-4	Gdh	Sod-1	*Mean*
YN1	0.436*	0.409*	0.409*	–	0.758***	−0.155*	–	0.371
YN2	–	−0.200	–	–	0.319	−0.023	–	0.032
YN3	–	0.067	−0.067	–	0.782**	0.470*	–	0.313
KR1	–	–	–	–	–	–	–	–
KR2	–	–	–	–	–	–	–	–
ONI	–	–	–	–	–	–	–	–
ON2	–	–	–	–	−0.133	–	–	−0.133
KM1	–	–	0.614***	0.018	–	0.897***	–	0.510
KM2	–	–	0.441*	0.441*	–	–	0.384	0.422

$*P<0.05$, $**P<0.01$, $***P<0.001$.

polymorphic in at least one population as polymorphic at the species level.

Genetic differentiation among populations was estimated by gene diversity statistics corrected for errors due to small sample sizes (Nei and Chesser, 1983). The population genetic structure was analyzed by calculating G_{ST} values at each level of spatial hierarchy. G_{ST} was initially calculated among populations within an island using the genotype frequencies of each population. Following previous studies (Wright, 1978; Maki *et al*, 1996), we refer to this level as G_{PL}, denoting differentiation among populations in an island. Since no enzyme polymorphism was found in Kuroshima Island, we regarded G_{PL} for the island as zero for the following calculation. Next, the overall genotypic frequencies in each island enabled us to calculate a single G_{ST}, which is referred to as G_{LT}. The amount of gene flow among populations was estimated as $N_m = (1/G_{ST}−1)/4$ (Slatkin and Barton, 1989).

Wright's (1951) fixation index (F) was estimated at each polymorphic locus as $F = 1−n_o/n_e$, where n_o is the observed number of heterozygotes and n_e is the number of heterozygotes expected from the Hardy–Weinberg equilibrium. For loci with more than two alleles, the frequencies of the less-frequent alleles were combined into a single class. χ^2 values for each polymorphic locus in a population were calculated as NF^2, where N is the number of individuals per population (Li and Horvitz, 1953).

Standard genetic identity (I) (Nei, 1972), unbiased genetic identity ($\hat{I}$), and unbiased genetic distance ($\hat{D}$) (Nei, 1978) were calculated for each pairwise comparison of all populations examined. A phenogram based on the unbiased genetic distance was obtained using the neighbor-joining method (Saitou and Nei, 1987) with PHYLIP version 3.5c (Felsenstein, 1993).

Results

Genetic diversity
A total of 20 putative loci were scored: *Aap-1*, *Aap-2*, *Aat-1*, *Aat-2*, *Est-1*, *Est-2*, *Est-4*, *Fdh*, *Gdh*, *Gpi*, *G3pdh*, *Mnr-1*, *Mnr-2*, *6pgdh*, *Pgm-1*, *Pgm-2*, *Sod-1*, *Sod-2*, *Tpi-1*, and *Tpi-2*. In all populations, *Fdh*, *Gpi*, *6pgdh*, *Sod-2*, *Tpi-1*, and *Tpi-2* were monomorphic, and the other loci were polymorphic in at least one population.

Table 3 Genetic diversity within population (H_S), total genetic diversity (H_T), proportion of the total diversity among populations (G_{ST}), and amount of gene flow (N_m) on each island and at species level in *Suzukia luchuensis*

Source	H_S	H_T	G_{ST}	N_m
Yonaguni Island	0.043	0.078	0.440	0.318
Kuroshima Island	0.000	0.000	–	–
Okinawa Island	0.005	0.006	0.049	4.852
Kume Island	0.052	0.055	0.048	4.897
Species level	0.032	0.232	0.863	0.041

Table 1 summarizes values of P, A, and h for each population and each island, and also at the species level. At the population level, the mean values of P, A, and h were 10.0, 1.13, and 0.031, respectively. The Kuroshima Island population contained no allozyme variation, while the Yonaguni Island population maintains the largest allozyme diversity.

The percentage of polymorphic loci ($P = 65.0$) at the species level was over six times that of the mean population level ($P = 10.0$), while gene diversity at the species level ($h = 0.232$) was over seven times that of gene diversity at the population level ($h = 0.031$). The mean allele number per locus at the species level ($A = 1.90$) was also higher than that at the population level ($A = 1.13$) (Table 1).

Population genetic structure
The mean values of the fixation indices across the polymorphic loci for the populations ranged from −0.133 in population ON2 to 0.510 in population KM1 (Table 2). Approximately half of the fixation index (F) values (11 of 19) for each polymorphic locus deviated significantly from zero.

The total genetic diversity (H_T) and the coefficient of gene differentiation (G_{ST}) overall for all populations were 0.232 and 0.863, respectively (Table 3). The latter value indicates that only 14% of the total genetic variation resides within a population, and the remaining 86% resides across populations. Gene flow among all populations per generation was extremely low ($N_m = 0.041$). The G_{ST} value on Yonaguni Island ($G_{ST} = 0.440$) suggests that genetic differentiation occurs among populations on this

Table 4 Estimated values of Nei's (1972) genetic identity (*I*) and Nei's (1978) unbiased genetic identity (*Î*) within and between islands

	I *(range)*	Î *(range)*
Within islands		
Yonaguni Island	0.951 (0.940–0.968)	0.882 (0.8749–0.8963)
Kuroshima Island	1.000 (–)	0.927 (–)
Okinawa Island	0.993 (–)	0.904 (–)
Kume Island	0.993 (–)	0.933 (–)
Between islands		
Yonaguni × Kusohima	0.750 (0.740–0.763)	0.696 (0.682–0.710)
Yonaguni × Okinawa	0.837 (0.880–0.867)	0.777 (0.751–0.801)
Yonaguni × Kume	0.615 (0.587–0.648)	0.575 (0.537–0.608)
Kuro × Okinawa	0.742 (0.737–0.747)	0.701 (0.687–0.715)
Kuro × Kume	0.566 (0.550–0.582)	0.529 (0.514–0.543)
Okinawa × Kume	0.731 (0.726–0.737)	0.673 (0.672–0.688)

island. In contrast, little genetic differentiation occurs among populations on other islands. Consequently, gene flow among the populations per generation (N_m) was estimated to be larger in both Okinawa Island ($N_m = 4.852$) and Kumejima Island ($N_m = 4.897$) than in Yonaguni Island ($N_m = 0.318$).

The results of hierarchical *G*-statistics showed that G_{PL} and G_{LT} are 0.134 and 0.848, respectively. This indicates that population differentiation within islands is low compared to that among islands. Indeed, with the exception of Yonaguni Isand, values of standard genetic identity (*I*) and unbiased genetic identity (*Î*) indicate that populations on the same island are not genetically differentiated, whereas genetic differentiation among islands is high (Table 4). The standard genetic identity (*I*) and the unbiased genetic identity (*Î*) for pairwise comparisons of the island populations are 0.566–0.837 and 0.529–0.777, respectively. Each island has at least one unique fixed allele not found on others (Figure 2). The Kume Island population has two fixed unique alleles at *Aap-2* and *Aat-1*. In a neighbor-joining phenogram (Saitou and Nei, 1987) based on Nei's (1978) unbiased genetic distance, populations from the same island clustered together (Figure 3).

Discussion

Genetic diversity of *S. luchuensis* at the species level
The level of total allozyme genetic diversity in *S. luchuensis* ($H_T = 0.232$) is higher than has been estimated for many other plants endemic to oceanic islands. Crawford *et al* (2001) reported a mean total genetic diversity of 0.065 for 29 endemic plants in the Juan Fernandez Archipelago. Only one of the 29 species, *Robisonia gracilis*, contained a higher level of total genetic diversity than *S. luchuensis*. The mean total genetic diversity of 69 endemic plant taxa to the Pacific Islands is also very low ($H_T = 0.064$) (de Joode and Wendel, 1992). In contrast, Francisco-Ortega *et al* (2000) reported higher values of total genetic diversity for 69 Canary Island endemics ($H_T = 0.186$, range $= 0.047–0.456$). However, only nine of these taxa had greater genetic diversity than *S. luchuensis*.

In contrast to oceanic islands such as the Juan Fernandez and Canary Islands, continental islands have
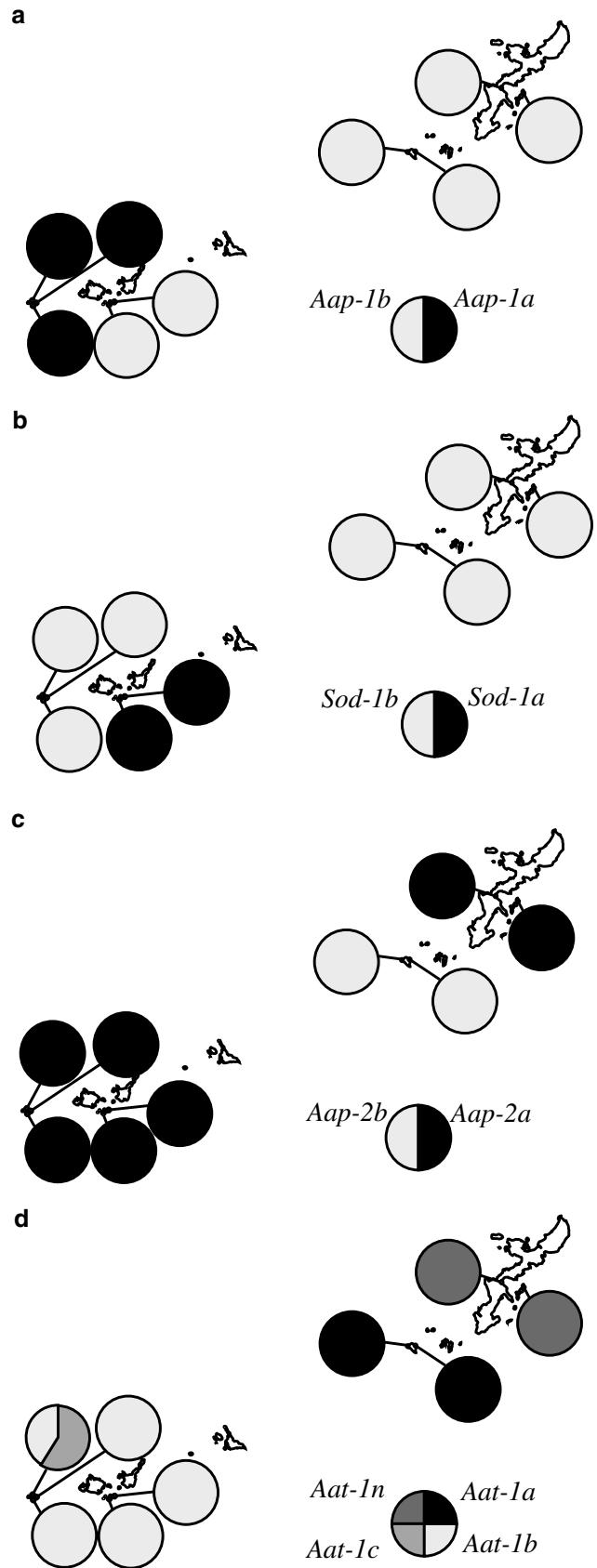
Figure 2 Allele frequency variation at *Aap-1* (**a**), *Sod-1* (**b**), *Aap-2* (**c**), and *Aat-1* (**d**) among the examined populations of *S. luchuensis*.
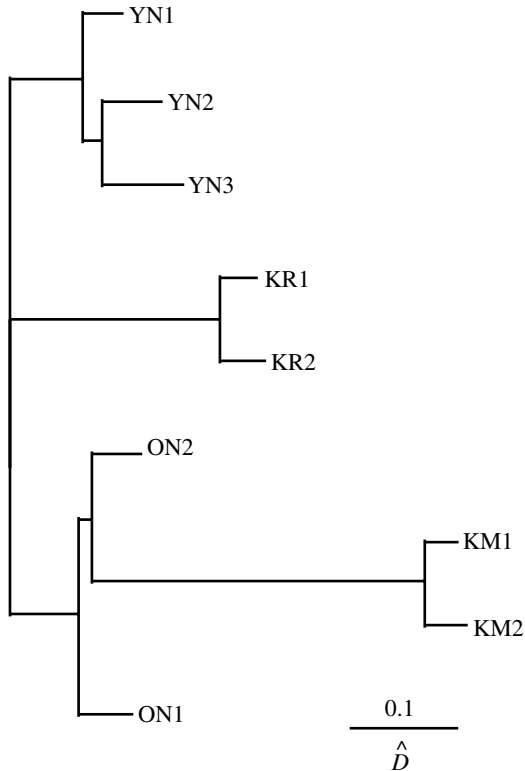
Figure 3 Phenogram for studied populations of *S. luchuensis* using the neighbor-joining method (Saitou and Nei, 1987) and Nei's (1978) unbiased genetic distance ($\hat{D}$).

a smaller proportion of endemic species. Genetic variation has been examined in only a few of these species, such as *Cyclamen creticum*, endemic to Crete and Karpathos in the Mediterranean, which contains a high total allozyme diversity ($H_T = 0.216$ for all nine loci examined) (Affre and Thompson, 1997). This value is comparable to that of *S. luchuensis* ($H_T = 0.232$). Other examples such as *Hosta venusta* endemic to Cheju Island, Korea ($H_T = 0.315$), and *H. tsushimensis* endemic to Tsushima Island, Japan ($H_T = 0.207$) (Chung, 1995), provide similar results. On the Ryukyu Islands, three other endemic plant species have been examined, yielding H_T values of 0.134 in *Aster asa-grayi* (Maki, 1999), 0.188 in *Lilium longiflorum* (Hiramatsu *et al*, 2001), and 0.148 in *Aster miyagii* (Maki, 2001). Genetic diversity at the species level in *S. luchuensis* is thus more similar to those of plant species endemic to continental islands than those endemic to oceanic islands.

Crawford *et al* (2001) have discussed the reasons for the reduced level of genetic diversity of plants endemic to the Juan Fernandez Islands relative to those endemic to the Canary Islands. These authors suggest that Canary Island endemics are older lineages and therefore have accumulated many mutations since their origin. The Ryukyu Island endemics are also likely to be composed of older lineages, as the islands have a long geographic history of more than one period of landbridge connection with adjacent land masses. In the early Pleistocene (1.5–2.0 MYA), a long landbridge from central Ryukyu to Taiwan had been formed (Kizaki and Oshiro, 1980),

although during the late Pleistocene (0.4–1.0 MYA) the sea level was 200 m higher than that at present, and lower islands were submerged and pushed upward thereafter (Kimura, 1996). However, many high-elevation islands have not been submerged since the Oligocene (Kizaki and Oshiro, 1980). The ancestor of *S. luchuensis* may have moved to the Ryukyu Archipelago during the early Pleistocene across the landbridge from Taiwan, and populations became isolated due to rising of sea level in the late Pleistocene. In *S. luchuensis*, the fact that each island population has at least one unique fixed allele that was not found in other island populations supports the older lineage hypothesis (Figure 2).

An alternative hypothesis to explain differences in genetic diversity of endemic plants between island groups is that multiple introductions from an adjacent continent are common for some island groups but not others (Crawford *et al*, 2001). Multiple introductions may be frequent in the Ryukyu Archipelago. For example, a close relative of *S. luchuensis*, namely *S. shikikunensis*, occurs in Taiwan, which is located in the southwest of Yonaguni Island. *S. shikikunensis* or its ancestral species may have colonized the Ryukyu Archipelago multiple times. However, it is unlikely that repeated independent speciation events have occurred on separate islands. This hypothesis thus has little support.

Genetic diversity within and among populations of *S. luchuensis*

Mean allozymic diversity within populations of *S. luchuensis* ($H_S = 0.031$) is comparable to the mean values recorded previously for insular endemic plants ($H_S = 0.044$ for 29 endemic species on the Juan Fernandez Islands, Crawford *et al*, 2001). Barrett (1996) found that genetic variation at allozyme loci is often reduced in island populations compared to those of mainland populations of the same or related species. The fact that insular endemic plants generally maintain low levels of within-population genetic diversity is probably due to random drift in small populations following a genetic bottleneck at the time of colonization. Except on the Yonaguhi Island, most populations of *S. luchuenis* are small and often consist of less than 50 individuals, and therefore may have been severely affected by random drift. A genetic bottleneck at the time of population establishment may have contributed to a low genetic diversity within populations; each island population of *S. luchuensis* would appear considered to have established sufficiently long ago in addition to allowing fixation of a unique allele on each island (Figure 2). A comparative genetic study of *S. luchuensis* and its Taiwanese relative *S. shikikunensis* would provide interesting data on this context.

Interpopulation genetic differentiation was found to be low within islands but high among islands. Compared to the findings presented in the previous reviews on insular endemic plants (Francisco-Ortega *et al*, 2000; Crawford *et al*, 2001), interpopulation genetic differentiation is extremely high in *S. luchuensis* ($G_{ST} = 0.863$). In general, genetic differentiation among populations is larger in selfing species than outcrossing species (Hamrick and Godt, 1989). Fixation indices suggested that *S. luchuensis* is mainly a mixed mating or selfing species.

In addition to the mating system, interisland geographic isolation probably plays a major role in causing the extremely high genetic differentiation among the islands, as the hierarchical analysis of population genetic stucure in *S. luchuensis* ($G_{LT} = 0.848$) would suggest. Although pollinators of *S. luchuensis* are unknown at present, it seems unlikely that pollinators move between the islands, because the islands are isolated by long distances and populations are small and few. Accordingly, gene flow via pollen is less likely between islands. Since *S. luchuensis* is not found on any adjacent islands, long-distance migration via seeds is also likely to be a very rare event. Seeds of *S. luchuensis* do not have mechanisms specialized for wind dispersal such as wings, but may migrate by ocean currents. The genetic identities between pairs of island populations of *S. luchuensis* are nevertheless larger than most of those estimated for species pairs (Table 4) (Crawford, 1990). A study of ecological and morphological differentiation among island populations of *S. luchuensis* would help to obtain a better understanding of the ecology and evolution of the species in this archipelago.

Acknowledgements

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