

High genetic variability revealed by allozymic loci in the narrow endemic fern *Polystichum otomasui* (Dryopteridaceae)

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Genetic variability was examined at 16 putative allozymic loci in the narrow endemic fern *Polystichum otomasui*. Although this species is distributed in only a few valleys within an approximately 10 km × 6 km area on the Kyusyu Island of Japan, there are a relatively large number of individuals in this area. The percentage of polymorphic loci (P), the number of alleles per locus (A), the observed heterozygosity (H_O) and the expected heterozygosity (H_E) were 61.9, 1.93, 0.166 and 0.177, respectively, at the population level, and P , A and H_E were 81.3, 2.69 and 0.199, respectively, at the species level, indicating that *P. otomasui* has extremely high allozymic variation for a fern species. The G_{ST} value among the 10 populations examined within the range was 0.108, suggesting that the interpopulation gene flow is high enough to impede genetic structuring. The mating system estimates, Wright's fixation index and the intragametophytic selfing rate, indicate that the species is outcrossing. High genetic variability, in spite of narrowness of the distribution, may have resulted from a recent rapid decline in the population and/or the large effective population size resulting from the extensive gene flow among the populations.

Keywords: allozyme, fern, gene flow, genetic variability, narrow endemic, *Polystichum otomasui*.

Introduction

Genetic variation in a population is considered to have evolutionary potential. Therefore, the level of genetic variation and the factors influencing genetic variability have been a primary concern of evolutionary geneticists. The width of distribution is one of the major factors correlated with the genetic variability of plant populations (Karron, 1987; Hamrick & Godt, 1990). Geographically restricted species tend to have lower levels of genetic variation than their more widespread congeners (e.g. Sherman-Broyles *et al.*, 1992; Purdy & Bayer, 1996). Some studies have reported no variation in allozymic loci in narrow endemic species (e.g. Waller *et al.*, 1987; Soltis *et al.*, 1992). On the other hand, a few studies have shown abundant genetic variation even in geographically restricted species (Ranker, 1994; Lewis & Crawford, 1995).

The distribution of a species is determined mainly by its adaptability to the habitat, barriers to dispersal and historical events (Cox & Moore, 1993). In

homosporous ferns, the ability to disperse is believed to be higher than for most of the seed plants because of the aerodynamic properties of their spores (Tryon, 1970; Soltis & Soltis, 1990a). In addition, a homosporous fern gametophyte may have the potential for self-fertilization because of its hermaphroditism, although most of the diploid homosporous fern species are highly outcrossing (Soltis & Soltis, 1990b). Capacity for self-fertilization seems to facilitate long-distance establishment of populations (Baker, 1955). Thus, if a fern species does not require a specific habitat, such as a high mountain area, salt marsh or a rocky site, the distribution of the species is expected to be relatively wide. Otherwise, the distribution may reflect the evolutionary history of the species.

Polystichum otomasui Sa. Kurata (Dryopteridaceae) is a perennial fern, which is distributed in an extremely restricted area of Japan (Kurata, 1963). This species occurs in the understorey of temperate evergreen forest or artificial coniferous forest and does not appear to require specific microhabitat conditions.

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In this study, we have used enzyme electrophoresis to examine genetic diversity at both the population and the species level, genetic differentiation among the populations and the mating system of *P. otomasui*.

Materials and methods

Study organism

Polystichum otomasui is distributed in only a few valleys within an approximately 10 × 6 km area on the Kyushu Islands of Japan (Fig. 1). The species is found in the moist understorey of temperate evergreen forests mainly composed of *Machilus japonica* and *Castanopsis cuspidata* and artificial forests of Sugi (*Cryptomeria japonica*). The number of individuals of *P. otomasui* in the valleys is relatively large. Curiously, however, *P. otomasui* has not been found outside this narrow area, although similar evergreen and artificial forests are common.

The chromosome number of *P. otomasui* is $2n = 82$, which is considered to be diploid (Iwatsuki, 1995). Putative natural hybridizations between *P. otomasui* and five tetraploid congeneric species (*P. makinoi*, *P. ovato-paleaceum*, *P. polybrepharum*, *P. pseudo-makinoi* and *P. tagawanum* var. *tagawanum*) have been reported (Iwatsuki, 1995). Unfortunately, at present, phylogenetic relationships between *P. otomasui* and these five tetraploid congeners are unknown.

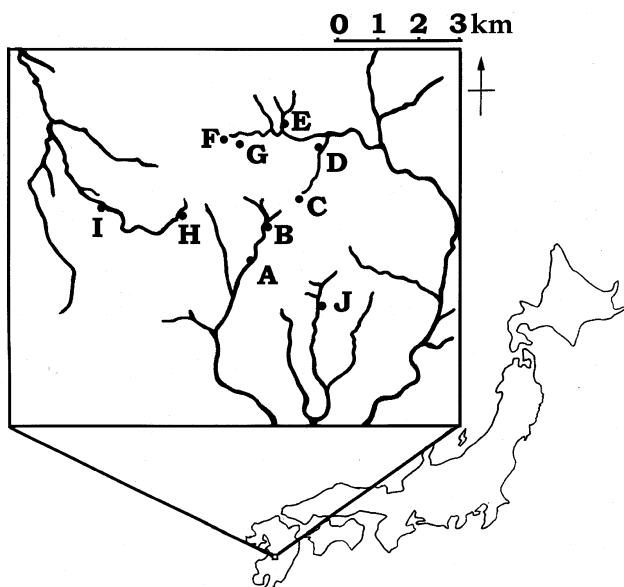


Fig. 1 Distribution of the examined populations of *Polystichum otomasui*.

Sampling

A portion of the young leaf blade was sampled from 24–35 *P. otomasui* individuals from each of 10 populations in the distribution area (Fig. 1). The samples were placed on ice and transported to the laboratory of Fukuoka University of Education, refrigerating the samples for several days until the electrophoresis was carried out.

Enzyme electrophoresis

One hundred milligrams of the leaf blade was homogenized in 1000 μL of extract buffer made up of 93 mM Tris-HCl (pH. 8.0), 23.4% glycerol, 0.6% (v/v) Tween 80, 2.8 mM EDTA, 2.3 mM NAD, 1.6 mM NADP, 11 mM dithiothreitol, 0.5% 2-mercaptoethanol, 0.08% bovine serum albumin and 7% polyvinylpyrrolidone modified from Tsumura *et al.* (1990). The homogenates were centrifuged at 20 000 g for 10 min at 4°C. Ten microlitres of the resulting supernatant was used for electrophoresis for each enzyme. Polyacrylamide vertical slab gel electrophoresis was carried out according to the procedure of Tsumura *et al.* (1990); preliminary studies using both starch and polyacrylamide gels yielded similar results, but some loci resolved better with polyacrylamide. The following 13 enzyme systems were examined: aspartate aminotransferase (AAT; EC 2.6.1.1), fructose 1,6-diphosphatase (F1,6DP; EC 3.1.3.11), glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), glutamate dehydrogenase (GDH; EC 1.4.1.2), glycerate dehydrogenase (G2DH; EC 1.1.1.29), hexokinase (HK; EC 2.7.1.1), leucine aminopeptidase (LAP; EC 3.4.11.1), menadione reductase (MR; 1.6.99.-), phosphoglucomutase (PGM; EC 5.4.2.2), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), shikimate dehydrogenase (SkDH; EC 1.1.1.25) and triose-phosphate isomerase (TPI; EC 5.3.1.1). Staining protocols followed Tsumura *et al.* (1990), except F1,6DP and G6PDH, for which we followed Soltis *et al.* (1983).

Statistical analysis

Allele frequencies in each population of *P. otomasui* were calculated for the loci encoding the 13 enzyme systems. The following indices were used to quantify the level of genetic diversity within each population: the proportion of polymorphic loci (P) at the 99% criterion; the number of alleles per locus (A); observed heterozygosity (H_O); and expected heterozygosity (H_E). All these indices except H_O were also

calculated at the species level. Following Hamrick & Godt (1990), we treated the loci polymorphic in at least one population as polymorphic at the species level. Genetic diversity of *P. otomasui* at the population and the species levels was compared with data compiled from studies examining allozyme diversity of diploid homosporous ferns (references cited in Soltis & Soltis, 1990a; Werth *et al.*, 1985; Soltis & Soltis, 1987; Haufler *et al.*, 1990; Soltis *et al.*, 1991; Werth, 1991; Ranker, 1992a,b, 1994; Li & Haufler, 1994; Korpelainen & Kolkkala, 1996). At the species level, we compiled data from the studies examining two or more populations per species, and the population-level data also included those studies examining only one population per species. For some species and/or populations, the genetic diversity indices were calculated using allele frequency tables from the literature.

Genetic diversity among populations of *P. otomasui* was estimated by Nei's gene diversity statistics (Nei, 1987) for all polymorphic loci. Values for genetic identities (I) and standard genetic distance (D) (Nei, 1987) were also computed for each pairwise comparison among the 10 populations of *P. otomasui*.

The mating system of *P. otomasui* was examined by two estimators: Wright's (1951) fixation index and the intragametophytic selfing rate. For each polymorphic locus at which the frequency of the most common allele did not exceed 0.95, a fixation index (F) was estimated as $F = 1 - (n_o/n_E)$, where n_o is the observed number of heterozygotes and n_E is the expected number of heterozygotes at Hardy-Weinberg equilibrium. Deviations of F from zero were tested using χ^2 -statistics (Li & Horvitz, 1953). Intragametophytic selfing rates were estimated for each population using genotype frequency data at the polymorphic loci. Holsinger's (1987) method was applied to calculate the bootstrap estimates and confidence intervals.

Results

Genetic diversity at the population level

A total of 16 loci encoding 13 enzyme systems were resolved: *Aat*, *Fl,6dp*, *G6pdh*, *Gdh*, *Gpi-2*, *G2dh*, *Hk*, *Lap*, *Mnr*, *Pgm-1*, *Pgm-2*, *6pgdh-1*, *6pgdh-2*, *Skdh*, *Tpi-1* and *Tpi-2*. Because *Gpi-1* was not consistently scored, we excluded this locus from further analyses. Among the 16 loci, *Aat*, *Fl,6dp* and *G6pdh* were monomorphic in all populations and the other 13 loci were polymorphic in at least one of the populations examined.

Values for all genetic diversity indices (P , A , H_o and H_E) at the population level in *P. otomasui* were much higher than the mean values for diploid homosporous ferns (Table 1). Values for three indices (P , A and H_E) in *P. otomasui* were also even higher than the mean values for widespread seed plants (Table 1).

Genetic diversity at the species level

Values for the genetic diversity indices (P , A and H_E) at the species level in *P. otomasui* were much higher than the mean values for diploid homosporous ferns (Table 2). Values for two of the indices (P and A) in *P. otomasui* were also higher than the mean values for those in widespread seed plants (Table 2). The expected heterozygosity (H_E) in *P. otomasui* was similar to the mean value for widespread seed plants.

Genetic differentiation among populations

The average coefficient of gene differentiation (G_{ST}) across all the polymorphic loci was low (0.108) (Table 3). This value was similar to that of wind-pollinated outcrossing seed plants ($G_{ST} = 0.099$) (Hamrick & Godt, 1990). Means of Nei's genetic identity and standard genetic distance for each pairwise comparison of all 10 populations were 0.971 (range 0.929–0.989) and 0.030 (range 0.008–0.068), respectively, also indicating low genetic differentiation among the populations.

Mating system

Fixation indices (F) were tested for the significance of deviations from zero in 78 cases in *P. otomasui* (Table 4). Of those 78 F -values, 10 were significantly positive, indicating a deficiency of heterozygotes, and only two were significantly negative, indicating an excess of heterozygotes. The mean F -values for each population were nearly zero, indicating that, on the whole, the populations are at Hardy-Weinberg equilibrium. The rates of intragametophytic selfing were nearly zero in all populations (Table 5), as expected from the fixation indices.

Discussion

The genetic variability in *P. otomasui* is higher than expected given the tendency of geographically restricted species to have low levels of genetic variation (Karron, 1987; Hamrick & Godt, 1990; Soltis & Soltis, 1991). As in seed plants, geographical distri-

Table 1 The proportion of polymorphic loci (P), the number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) at 16 loci for examined populations of *Polystichum otonasui* and the genetic diversity at the population level of the diploid homosporous ferns and the widespread seed plants

Population	P	A	H_O	H_E
A	62.5	1.94	0.167	0.171
B	62.5	2.00	0.154	0.161
C	56.3	2.00	0.231	0.220
D	68.8	1.94	0.155	0.173
E	62.5	2.06	0.180	0.184
F	62.5	2.06	0.133	0.107
G	62.5	2.00	0.149	0.187
H	56.3	1.81	0.173	0.230
I	62.5	1.81	0.168	0.180
J	62.5	1.81	0.146	0.189
Mean	61.9	1.93	0.166	0.177
Diploid homosporous fern*	31.5 ^A (3.3)	1.52 ^B (0.07)	0.136 ^C (0.027)	0.110 ^A (0.014)
Widespread seed plants†	43.0 (3.3)	1.72 (0.07)	—	0.159 (0.013)

Values in parentheses indicate the standard errors.

*A, B and C are from the data available for 32, 30 and 23 species, respectively (see text).

†According to Hamrick & Godt (1990).

bution is one of the major factors determining genetic diversity in fern species (Soltis & Soltis, 1990a).

Soltis & Soltis (1991) examined the effects of species range on the genetic diversity of five North American diploid *Polystichum* species. Two geographically restricted species (*P. dudley* and *P. lemmonii*) have maintained a much lower genetic diversity than three more widespread species (*P. acrostichoides*, *P. imbricans* and *P. munitum*). The

genetic diversity of *P. otonasui* is much higher than these congeneric North American species, even though the distribution of *P. otonasui* is extremely restricted.

A few studies have shown that geographically restricted species maintain a higher genetic diversity than more widespread congeners. Ranker (1994) has shown that the very rare fern *Adenophorus periens* has much higher levels of allozymic variation than two other more widespread congeners. He hypothe-

Table 2 The proportion of polymorphic loci (P), the number of alleles per locus (A) and expected heterozygosity (H_E) at 16 loci for *Polystichum otonasui* and the genetic diversity at the species level of the diploid homosporous ferns and the widespread seed plants

	P	A	H_E
<i>P. otonasui</i>	81.3	2.69	0.199
Diploid homosporous fern*	31.3 ^A (4.2)	1.52 ^B (0.24)	0.150 ^B (0.025)
Widespread seed plants†	58.9 (3.1)	2.29 (0.16)	0.202 (0.015)

Values in parentheses indicate the standard errors.

*A and B are from the data available for 19 and eight species, respectively (see text).

†According to Hamrick & Godt (1990).

sized that, although the historical factors leading to the high genetic variability of the species are somewhat obscure, the outcrossed mating system and somatic mutations may have played a key role. In the population genetic study on 11 species of *Polystichum*, Lewis & Crawford (1995) found that the two most widespread species have a reduced within-population gene diversity with respect to their narrowly endemic congeners. He explained that these unexpected results are caused by high levels of self-fertilization for the widespread species and a large-scale migration during Pleistocene glaciation.

Table 3 Gene diversity for individual loci and the mean values for all polymorphic loci in *Polystichum otomasui*

	H_S	H_T	G_{ST}
<i>Gpi-2</i>	0.149	0.157	0.052
<i>Gdh</i>	0.253	0.273	0.075
<i>G2dh</i>	0.427	0.499	0.144
<i>Hk</i>	0.210	0.279	0.247
<i>Lap</i>	0.676	0.733	0.077
<i>Mnr</i>	0.134	0.145	0.079
<i>Pgm-1</i>	0.374	0.319	0.141
<i>Pgm-2</i>	0.099	0.130	0.235
<i>6pgdh-1</i>	0.046	0.048	0.039
<i>6pgdh-2</i>	0.121	0.133	0.092
<i>Skdh</i>	0.026	0.028	0.059
<i>Tpi-1</i>	0.425	0.440	0.035
<i>Tpi-2</i>	0.043	0.047	0.086
Mean	0.180	0.202	0.108

As with the above species, some explanations are possible for the high genetic variability of *P. otomasui*. First, *P. otomasui* may have a large effective population size at the species level, irrespective of its extremely narrow distribution. Genetic differentiation among the populations was very low, suggesting extensive gene flow among them. In the infinite island model, $G_{ST} \approx 1/(1+4Nm)$, where G_{ST} and Nm are the coefficient of gene differentiation and the number of migrants per generation respectively (Slatkin & Barton, 1989). When $Nm > 1$, genetic differentiation among populations as a result of genetic drift is highly impeded (Hartl & Clarke, 1989). In this study, G_{ST} was 0.108 among the 10 populations, giving $Nm \approx 2.072$, which is very high. Although the distribution of *P. otomasui* is extremely restricted, the individuals occur continuously in the valleys, effectively resulting in the formation of a single integrated population. In addition, mating systems profoundly influence the effective population size in plants. Theoretical considerations show that selfing reduces the effective size of subpopulations (Tachida & Yoshimaru, 1996). The outcrossing habit of *P. otomasui* may play a key role in the maintenance of genetic diversity of the species.

Secondly, the current high level of genetic diversity may reflect the former large population size of the species. Recent rapid decline of the populations is possible. Although the explicit causes are obscure at present, some ecological causes may be speculated on for the rapid population decline. Competition between *P. otomasui* and the tetraploid congeners in the same habitat may have caused the

Table 4 Fixation indices for individual loci and the mean values for all polymorphic loci for each population of *Polystichum otomasui*

	A	B	C	D	E	F	G	H	I	J
<i>Gdh</i>	—	—	0.280	-0.091	0.167	0.252	0.139	-0.174	0.267	-0.210
<i>Gpi-2</i>	0.345	—	0.156	—	0.191	-0.156	0.191	-0.071	—	—
<i>G2dh</i>	-0.394*	0.190	-0.222	-0.167	-0.292	0.105	0.083	-0.068	-0.142	-0.244
<i>Hk</i>	—	—	-0.069	-0.176	-0.094	—	—	-0.333	-0.087	-0.142
<i>Lap</i>	0.414*	0.380*	0.600**	0.602**	0.400*	0.214	0.200	0.399*	0.354*	0.371
<i>Mnr</i>	—	—	-0.071	-0.071	—	—	—	0.108	0.319	0.330
<i>Pgm-1</i>	0.096	0.111	0.028	0.200	0.090	-0.077	—	—	—	-0.043
<i>Pgm-2</i>	0.270*	—	-0.612	—	-0.098	—	—	—	—	—
<i>6pgdh-1</i>	—	—	—	—	-0.055	—	-0.055	—	0.267	—
<i>6pgdh-2</i>	—	0.352*	—	0.130	—	-0.052	-0.016	-0.052	-0.022	—
<i>Skdh</i>	—	—	—	—	-0.087	—	—	—	-0.065	—
<i>Tpi-1</i>	-0.232	-0.482**	-0.199	0.333	-0.033	0.314	-0.416	-0.155	-0.095	-0.008
<i>Tpi-2</i>	—	0.059	—	—	—	—	—	—	—	0.498*
Mean	0.100	0.082	-0.047	0.058	0.019	0.086	0.035	0.043	0.066	0.069

* $P < 0.05$; ** $P < 0.01$.

Table 5 Estimates of intragametophytic selfing rates for *Polystichum otomasui*

Population	Bootstrap-1000 estimate	95% confidence interval
A	0.080	0.000–0.248
B	0.085	0.000–0.254
C	0.000	0.000–0.097
D	0.039	0.000–0.194
E	0.000	0.000–0.111
F	0.105	0.000–0.260
G	0.094	0.000–0.248
H	0.000	0.000–0.115
I	0.049	0.000–0.180
J	0.063	0.000–0.237

population decline of the former species. In the habitat of *P. otomasui*, several tetraploid *Polystichum* species frequently co-occur. Polyploid species are often more vigorous than their diploid congeners (Levin, 1983). In fact, in contrast to *P. otomasui*, the tetraploid congeners are widespread in Japan (Iwatsuki, 1995). In addition, *P. otomasui* often hybridizes with these tetraploid species, especially with *P. tagawanum* var. *tagawanum*, generating sterile but more vigorous hybrid individuals (Kurata, 1963), which may outcompete *P. otomasui*. At several sites, individuals generated by the hybridization of *P. otomasui* and *P. tagawanum* var. *tagawanum* are more abundant than the parent species (M. Maki, pers. obs.). As a result of perenniality, the hybrids will occupy the same habitat for a long time, reducing the opportunity for the regeneration of *P. otomasui*.

Another possible cause of the population decline in *P. otomasui* is climate change. In the past, *P. otomasui* may have been more widely distributed but is now confined to the current restricted area following a recent climate change. Considering the dispersal ability of its spores, however, *P. otomasui* should extend its distribution to adjacent areas, which appear to be similar environments. Therefore, it is difficult to explain the narrow distribution of the species solely by climate change. Although it is difficult to examine the effects of historical events on the present distribution of a plant species, we are now testing the hypothesis that both competition and natural hybridization with the congeneric tetraploids influence the population dynamics of *P. otomasui*.

Polystichum otomasui is not listed in the Red Data Book of Japanese plants (JPRDBC, 1989), probably because relatively large numbers of individuals are

found in the area. From the point of view of conservation genetics, an urgent conservation plan does not appear to be necessary for *P. otomasui*. However, the natural evergreen forests in this area are now quickly being cut for artificial forest. Moreover, the artificial forests are also being felled for lumber production. Because *P. otomasui* yellows and wilts under direct exposure to the sun (M. Maki, pers. obs.), it is necessary to keep part of the forests in the area as at least a temporary refuge.

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