

Short communication

Genetic diversity of an endangered aquatic plant, *Potamogeton lucens* subspecies *sinicus*

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Abstract

The genetic diversity of the species, *Potamogeton lucens* subsp. *sinicus* var. *teganumensis*, which is critically endangered in Japan, was investigated. This species now occurs in only two known localities in Japan. One is a native population (Oitoike population), but the other (Teganuma-Okahotto population) is found in a small artificial pond that was dug in 1998. It is considered that the Teganuma-Okahotto population grew from a soil seed bank. Based on RAPD variation, we compared the genetic diversity of the two populations of *P. lucens* var. *teganumensis* in Japan and one population of *P. lucens* subsp. *sinicus* var. *sinicus* in China. The Teganuma-Okahotto population showed RAPD variation, suggesting that it may be derived from more than one seed buried in old sediments. This population also had the highest value of Shannon's Information Index among the three study populations. This finding suggests that seeds buried in sediments can contain genetic variability, and may be used to conserve the genetic diversity of rare and endangered plants.

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1. Introduction

Potamogeton lucens L. subsp. *sinicus* (Migo) Hara var. *sinicus* (*P. lucens* var. *sinicus*) is distributed in China, whereas *P. lucens* L. subsp. *sinicus* (Migo) Hara var. *teganumensis* (Makino) Hara (*P. lucens* var. *teganumensis*) is distributed in Japan. *Potamogeton lucens* L. var. *teganumensis* (Makino) Hara (hereafter referred to as *P. lucens* var. *teganumensis*) was first identified in Teganuma Lake (139°59'E–140°4'E and 35°52'–35°50'N) in central Japan, and was described as *P. lucens* L. var. *teganumensis* Makino (Makino, 1905). At that time, this species was widely distributed in Japan. However, the distribution of *P. lucens* var. *teganumensis* decreased rapidly because of the destruction of wetland environments (Otaki, 1975). Before 1958, rich aquatic flora was recorded in Teganuma Lake (Hosoya, 1993), and the lake was covered with aquatic vegetation (Otaki, 1975). Historically, the native population of *P. lucens* var. *teganumensis* grew in large

numbers in Teganuma Lake, in sufficient quantities that it was mowed and used as green manure (Makino, 1905). However, urbanization and land reclamation work between 1958 and 1968 made this lake notorious as the most polluted in Japan for nearly 30 years. All submerged and floating leaved plants became extinct in the lake before 1973 (Hosoya, 1993). Although *P. lucens* var. *teganumensis* also grew in Inbanuma Lake, about 20 km from Teganuma Lake, it disappeared from that area in 1980 (Kadono, 1983). The native population of *P. lucens* var. *teganumensis* in Teganuma Lake has been extinct since 1970 (Hosoya, 1993), and it is now found in only two locations in Japan: Oitoike Pond (130° 52'E and 33° 44'N) and Okahotto Pond (140° 3'E and 35° 51'N), an artificial pond near Teganuma Lake that was constructed in 1998. This species is now considered to be in very high danger of extinction in the near future (Anon., 2000).

Within the last decade, *P. lucens* var. *teganumensis* and several other aquatic plants that were once extinct in Teganuma Lake have been found in artificial ponds dug in reclaimed fields near Teganuma Lake (Saito, 1991; Anon., 1999). These plants appeared just after the ponds were dug, and are considered to have germinated from seeds buried in old lake sediments prior

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to the reclamation work. Plant seeds often undergo dormancy and form soil seed banks in the ground, where they can live for several years or several decades. Seeds of aquatic plants may maintain their germination capability for a very long time under anaerobic conditions at the bottom of a lake. The plants on these ponds became extinct in 2–3 years because of the deterioration of the aquatic environment. The population of *P. lucens* var. *teganumensis* in Okahotto Pond appeared following the construction of the pond.

The purpose of this study is to compare the genetic diversity of the population and discuss the effective use of seeds buried in sediment for the conservation of rare and endangered plants. We calculated the genetic diversity of three populations of *P. lucens* var. *teganumensis* and *P. lucens* var. *sinicus* using random amplification of polymorphic DNA (RAPD).

2. Methods

2.1. Plant materials

We investigated two Japanese populations of *P. lucens* var. *teganumensis*: (1) the Teganuma-Okahotto population, sampled in Okahotto Pond, an artificial pond built in 1998 near Teganuma Lake (this population may have grown from seeds buried in old sediment); (2) the Oitoike population, sampled in Oitoike Pond (this is a native population). The distance between the two populations is ca. 900 km. We also sampled a Chinese population of *P. lucens* var. *sinicus*; (3) the Erh-Hai population from Erh-Hai Lake, Yunnan, China.

2.2. Study sites

2.2.1. Teganuma Lake, Okahotto Pond

Teganuma Lake is located ca. 25 km northeast of Tokyo and measures ca. 7 km × 1 km. Okahotto Pond, near Teganuma Lake, is very small (ca. 10 m × 10 m). Plants growing in this pond include *P. lucens* var. *teganumensis*, *P. malaianus*, *Monochoria korsakowii* and *Ottelia alismoides*, as well as some Cyperaceae. Twenty-six samples were collected at least 1.5 m apart to minimize sampling the same individual ramet twice.

2.2.2. Oitoike Pond

Oitoike Pond is ca. 120 m × 80 m, and surrounded by low limestone mountains. This pond was constructed several hundred years ago as a reservoir. The aquatic vegetation at this site is dominated by *P. lucens* var. *teganumensis* and *P. inbanensis*, a putative hybrid between *P. lucens* var. *teganumensis* and *P. malaianus* (Kadono, 1983). Twenty-two samples were collected at least 5 m apart to minimize sampling the same individual ramet twice.

2.2.3. Erh-Hai Lake

Erh-Hai Lake is located at 1980 m a.s.l. in northwestern Yunnan, China, near the city of Dali (100°10′–100°20′E and 25°57′–25°40′N), and measures ca. 50 km × 16 km. Aquatic vegetation can be found along the shore on the southwestern side of the lake near Dali. The dominant species in this lake is

P. malaianus, and a few *P. lucens* var. *sinicus* and *P. perfoliatus* have also been observed. In Yunnan Province, *P. lucens* var. *sinicus* has become rare because of water pollution and lake reclamation work. Lakes in and around Kunming have been heavily polluted, and most of the submerged plants have become extinct. Water pollution in the mountainous areas of northwestern Yunnan has become severe in the last 10 years. *P. lucens* var. *sinicus* populations were scattered over 4 km along the lakeshore. We sampled 16 individuals in that place.

Since the native population of *P. lucens* var. *teganumensis* in Japan has almost become extinct, the Chinese Erh-Hai population of *P. lucens* var. *sinicus* was used for comparison, though we recognized that although these species are closely related, they may show differences at the genetic level. Therefore, it was necessary to determine the genetic diversity of *P. lucens* var. *sinicus*, which occupies the same ecological niche as *P. lucens* var. *teganumensis* in Japan.

2.3. DNA analysis

Several fresh leaves were sampled from 16 to 26 individuals of each population. Total DNA was extracted from the leaves by the CTAB method (Hasebe et al., 1994). RAPD amplifications were performed using arbitrary primers according to the method of Williams et al. (1993). The following cycling profile was used for all reactions: 2 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C for 1 cycle; and 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C for 44 cycles. Eight primers from Operon Technologies were used: OPAA04 (5′-AGGACTGCTC-3′), OPAA06 (5′-GTGGGTGCCA-3′), OPAA17 (5′-GAGCCCGACT-3′), OPAA18 (5′-TGGTCCAGCC-3′), OPAA19 (5′-TGAGGCGTGT-3′), OPV07 (5′-GAAGCCAGCC-3′), OPV17 (5′-ACCGGCTTGT-3′) and OPV19 (5′-GGGTGTGCAG-3′). Each DNA sample was replicated by running repeat amplifications to confirm the banding pattern.

Amplification products were scored manually, and each fragment in the RAPD profile was treated as an independent locus with two alleles, the presence or absence of a fragment. The genetic diversity was measured using Shannon's Information Index (Lewontin, 1973) $I = -\sum p_i \log_2 p_i$, where p_i is the frequency of a given RAPD fragment. Shannon's Information Index is suited for the analysis of RAPD data because of its insensitivity to bias that can be introduced into data by the inability to detect heterozygous individuals (Dawson et al., 1995). A matrix of pairwise distances among all individuals within each species was calculated using Euclidian distance (Excoffier et al., 1992), and the matrix was applied to an AMOVA analysis (Excoffier et al., 1992; Huff et al., 1993). These calculations were performed using Arlequin ver. 2.000 (Schneider et al., 2000).

3. Results

We were able to amplify DNA fragments from 40 of 64 individuals (Teganuma-Okahotto population: 19; Oitoike population: 13; Erh-Hai population: 8) with all primers and obtained 59 informative amplified DNA fragments. Of these,

Table 1
Analysis of molecular variance of endangered aquatic plants

Source of variation	d.f.	Variance components	Total variance (%)	P-value
Among populations	2	7.58	58.7	<0.001
Within populations	37	5.33	41.3	

d.f.: degrees of freedom.

nine fragments were peculiar to the Erh-Hai population, four to the Teganuma-Okahotto population, and three to the Oitoike population. Several amplification fragments were seen in two populations: three in the Erh-Hai and Teganuma-Okahotto populations, one in the Erh-Hai and Oitoike populations and 20 in the Teganuma-Okahotto and Oitoike populations. Nineteen amplification fragments were observed in all three populations. Three fragments were present in all Japanese individuals but in none of the Chinese individuals. Another three fragments were

present only in all of the individuals in China, but in none of the Japanese individuals.

Based on the RAPD information, we calculated the genetic diversity of each population, Shannon's Information Index (I ; Fig. 1), and molecular variance (AMOVA; Table 1). The Teganuma-Okahotto population showed the highest value of I (0.31), followed by the Oitoike population ($I = 0.23$) and the Erh-Hai population ($I = 0.18$). Detailed data for each population are shown in Fig. 1.

We obtained Shannon's Information Index for the three study populations. This index represents genetic diversity in plant populations, and the Teganuma-Okahotto population had the highest value (Fig. 1). As a result of comparing the detailed data (Fig. 1), we found that in the Teganuma-Okahotto population, 40 of 59 loci had indices that ranged from $I = 0.2$ to 0.7. The remaining 19 loci were conservative and showed no variation ($I = 0$). In contrast, in the Erh-Hai population, which had the lowest value, only 19 of 59 loci had indices that ranged from $I = 0.3$ to 0.7. The Erh-Hai population had 40 conservative DNA fragments that showed no variation ($I = 0$). Compared with the Teganuma-Okahotto population, few DNA fragments in this population showed variation. In the Oitoike population, 29 of 59 loci had indices that ranged from $I = 0.2$ to 0.7, and 30 of 59 loci showed no variation. Thus, the Teganuma-Okahotto population had many variable loci, and the Oitoike population was intermediate between the Teganuma-Okahotto population and the Erh-Hai population.

4. Discussion

4.1. Genetic diversity

We compared the distribution of the fragments between *P. lucens* var. *teganumensis* and *P. lucens* var. *sinicus* and found that there are only six loci where no allele is shared between the two taxa. Most loci have alleles that exist in both taxa. We concluded that it is possible to compare populations of both taxa with RAPD.

These results indicate that the Teganuma-Okahotto population has high genetic variation compared with the native Oitoike and Erh-Hai populations. All individuals of the Teganuma-Okahotto population are considered to derive from seeds buried in old sediment, because 30 years have passed since the native Teganuma population disappeared. Plural genetically variable seeds originating from the former native Teganuma population were considered to be involved in the formation of the current Teganuma-Okahotto population.

Over the last several decades, Japanese wetland environments have been damaged by being drained for development and by water pollution. The living population of *Potamogeton lucens* var. *teganumensis* is considered to exist only in Okahotto Pond and Oitoike Pond (Anon., 2000). There is no refugia which supplied seeds for Teganuma-Okahotto population. The Teganuma-Okahotto population is distantly isolated (ca. 900 km) from the Oitoike population of China and Korea where the possibility of distribution exists. We considered that the seeds containing the high genetic diversity

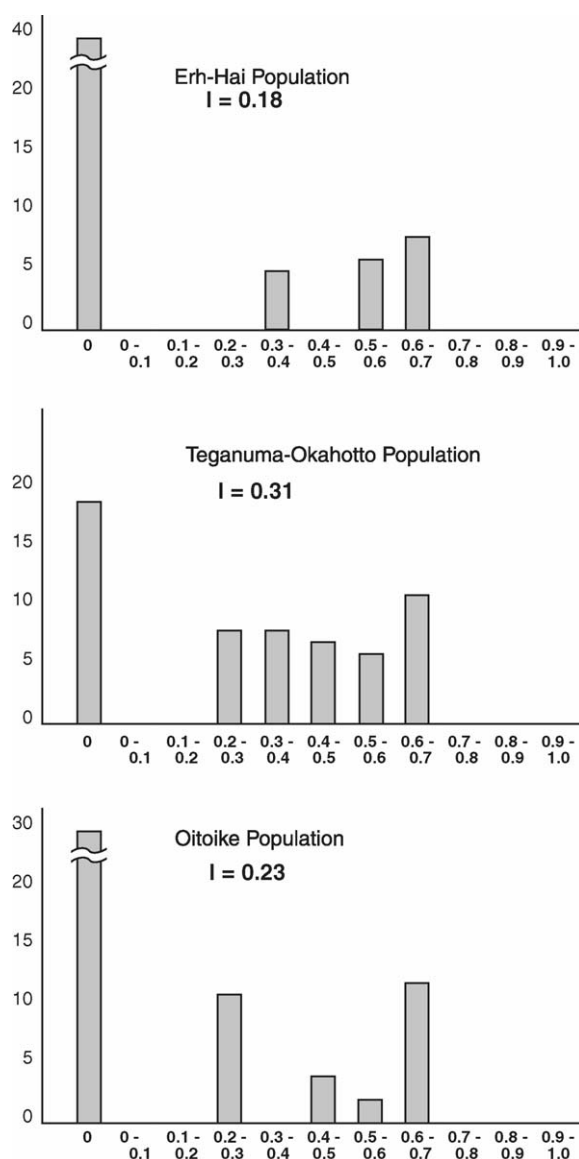


Fig. 1. Shannon's Information Index (I) of three populations of endangered aquatic plants.

of the former Teganuma population may remain in the soil seed bank and restore the population in Okahotto Pond. We found no individuals whose RAPD fragment patterns agreed with each other completely. The high genetic variation of the Teganuma-Okahotto population suggests that the former native Teganuma population was large and had rich genetic diversity.

The results of AMOVA analysis are shown in Table 1. Variation among populations (58.7%) was higher than within populations (41.3%). Bussell (1999) found that the AMOVA value of outbreeding species is 15.5% on average, and that of inbreeding species is ca. 59.6% on average in seed plants. In this study, we found a value near 60% among the populations. Inbreeding species are frequently characterized by a high degree of population genetic differentiation and relative uniformity within populations, while outbreeding species tend to be more diverse within populations with less genetic differentiation between populations.

Thus, *P. lucens* var. *teganumensis* may be an outbreeding species; however, the AMOVA value of this species is almost the same as the value of inbreeding species. Fischer et al. (2000) suggested that low gene flow among populations is in accordance with the geographic isolation of the populations. The gene flow of *P. lucens* var. *teganumensis* among populations is restricted. In fact, because these populations are geographically separated, it is difficult to consider gene flow; thus, the high AMOVA value among populations of this species is influenced by geographic isolation.

4.2. Conservation and restoration

The seeds of aquatic plants form seed banks in the anoxic environments of freshwater sediments, and it is possible for dormant seeds to be preserved alive for a long period of time. Many similar reports of the conservation and germination of seeds exist. Harrington (1972) reported that many kinds of seeds retained their ability to germinate even after several decades. Ødum (1965) reported that seeds buried in archaeologically dated soil samples germinated. He considered that the seeds of many weed species may remain viable in the soil for at least 100–600 years. Ohga (1923) found living seeds of *Nelumbo nucifera* in a peat layer in Manchuria. After he filed the hard seed coat, the most of seeds germinated. Libby (1951) tested Ohga's *Nelumbo nucifera* seeds using carbon-14 methods. These seeds were dated at 1040 ± 210 years of age (Libby, 1951). The growth of *P. lucens* var. *teganumensis* in Okahotto Pond illustrates the survival capability of the buried seeds of an aquatic plant.

The present Teganuma-Okahotto population near Teganuma Lake is protected and maintained by a civic environmental protection organization. In this pond, plants that have been extirpated from the neighboring Teganuma Lake have germinated from seeds buried in old sediment. We searched for germinable seeds in the sediments of Teganuma Lake. This seed bank includes many seeds of 19 species of submerged and floating-leaved plants. The seeds of *Potamogeton maackianus* germinated from these buried seeds (Momohara et al., 2001).

Moreover, an endangered species of *Chara* has also sprouted from the soil near Teganuma Lake (Tanaka et al., 2003).

The Teganuma-Okahotto population may have germinated from seeds buried over 50 years ago, and shows higher genetic diversity than the present native Erh-Hai and Oitoike populations. Thus, the mass of seeds buried in the sediment conserves abundant genetic diversity, which become evident when the seeds germinate.

Barrett and Kohn (1991) indicated that one of the goals of many conservation programs is to maintain existing levels of genetic variation in species that are rare or threatened. We suggest that buried seeds can contain high genetic variability, and may be effective in conserving the genetic diversity of rare plants.

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