Amplified fragment length polymorphism analysis of the genetic variation of an endangered plant Lysimachia tashiroi (Myrsinaceae)

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Abstract

Lysimachia tashiroi Makino (Myrsinaceae) is an endangered perennial herb growing in moist places along riverbanks in restricted localities of Kyushu, Japan. The largest population known to date is located in Gokayama and is under threat of extinction because of the planned construction of a dam. To develop a conservation plan of the riverbank population of L. tashiroi in Gokayama, we examined genetic variation in the population using amplified fragment length polymorphisms (AFLPs). A related and widespread species, L. japonica, was included in the analysis. A total of 733 AFLP fragments were obtained from 73 accessions. An AMOVA for L. tashiroi showed that within-population and between-population variability were 62% and 38%, respectively. In addition, genetic variability was higher in L. tashiroi than in L. japonica. These results are unexpected because plants of L. tashiroi propagate vegetatively and seed fertility is very low in the Gokayama population. Based on our findings, we suggest that as many genets as possible should be transplanted to appropriate habitats neighboring the dam construction area.

Keywords: amplified fragment length polymorphisms, endangered species, genetic diversity, Lysimachia tashiroi.

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Introduction

Japanese vascular flora is characterized by its high diversity and high endemism: approximately 7000 taxa (species, subspecies and varieties) of vascular plants are native to Japan among which approximately 36% are endemic. This high diversity is partly attributed to climatic and geographic complexity. In addition, an ice bed did not develop in the Japan Archipelago over the past two million years and, thus, many plants have survived through several ice ages by migrating to refugia or adapting to the new environments. Recently, however, Japanese flora has suffered from a crisis of mass extinction as in other areas of the world. Among approximately 7000 vascular taxa, 25 taxa are extinct and 1665 taxa are qualified as threatened in the Red Data Book (Environment Agency 2000). This crisis has resulted from over-collection for horticulture, habitat destruction for various land uses and succession of grasslands and secondary forests, all of which is mostly anthropogenic.

Reflecting on the on-going crisis of biodiversity, there has been a growing body of interest in the genetic diversity of threatened species because an accurate estimate of the genetic diversity of these species helps to design conservation programs (Frankham et al. 2002). Thus, studies on genetic diversity within and among populations of threatened plants have been done using various molecular markers, including random amplified polymorphic DNA (RAPD), intersimple sequence repeats (ISSR) and amplified fragment length polymorphism (AFLP) (Bouza et al. 2002; Kim et al. 2005; Kolseth & Lönn 2005; Tero et al. 2005). Among them, AFLP (Vos et al. 1995) provides particularly effective markers that are highly polymorphic and reproducible.

In this study, we apply an AFLP analysis to Lysimachia tashiroi Makino (Myrsinaceae), an endangered perennial herb endemic to Japan (Environment Agency 2000). This genus was formerly placed in Primulaceae, but was transferred to the family Myrsinaceae based on molecular and
morphological studies (Anderberg et al. 1998, 2002; Källersjö et al. 2000). The distribution of this species is restricted to three prefectures (Fukuoka, Saga and Oita) of Kyushu Island, Japan, and only a few populations have been recorded. Unfortunately the largest population previously known is located in the center of an area designated for the construction of the Gokayama Dam, and a large part of the population will be lost unless adequate conservation action is carried out. A survey of genetic diversity is needed to develop a conservation plan of the plant at the dam site. In addition to careful conservation of plants that grow outside of the dam site, transplantation of plants growing within the dam site is desired.

Plants of *L. tashiroi* in the vicinity of the Gokayama Dam site grow in moist places along riverbanks that are sometimes submerged during floods, and they propagate vegetatively by long creeping stems with nodes rooting onto the ground. In contrast, flowers rarely set fruits in the field, although there is a report of its diploid nature (Nakamura 1987). It is believed that this species is highly endangered status of *L. tashiroi*, however, there has been no population genetic study for this species. The aims of the present study were: (i) to examine how variable the riverbank population of *L. tashiroi* is in the Gokayama Dam construction area using AFLPs; and (ii) to consider how seriously the genetic variation should be taken into account in the transplantation program.

**Materials and methods**

**Sampling**

Young leaves were collected from 48 individuals of *L. tashiroi* in Gokayama, five individuals in Yahazu-toge and nine in Yabakei and from six individuals of *L. japonica* in Yahazu-toge and five in Yakushima (Table 1, Fig. 1). All samples were collected from individuals at least a few meters apart. The leaves were immediately stored in silica gel. Voucher specimens of some sampled individuals were deposited in the herbarium of Kyushu University (FU).

**Table 1** Information on the number of individuals and the locality of the five investigated populations of *Lysimachia tashiroi* and *Lysimachia japonica*

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample size</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. tashiroi</em></td>
<td>48</td>
<td>Gokayama, Fukuoka Prefecture</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Yahazu-toge, Oita Prefecture</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Yabakei, Oita Prefecture</td>
</tr>
<tr>
<td><em>L. japonica</em></td>
<td>6</td>
<td>Yahazu-toge, Oita Prefecture</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Yakushima, Kagoshima Prefecture</td>
</tr>
<tr>
<td>Total</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>

**DNA isolation**

Total genomic DNA was isolated from 100 mg of dried leaf tissue using the cetyltrimethylammonium bromide (CTAB) method (Dolye & Dolye 1987) with minor modifications (Nakazawa et al. 1997).

**Amplified fragment length polymorphism fingerprinting**

The AFLP procedures followed the protocol of Vos et al. (1995) with minor modifications. Genomic DNA (200 ng) was digested with two restriction enzymes, *Mse*I and *Eco*RI, at 37°C for 2 h. Adaptors were ligated to the digested fragments by double-stranded *Mse*I and *Eco*RI adapters with T4 ligase at 20°C for 2 h. Preamplification reactions were carried out with diluted DNA from the ligation reaction and preselective primer pairs with a single selective nucleotide (Applied Biosystems Inc., Foster City, CA, USA). The reactions were placed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems Inc.) programmed to start at 72°C for 2 min, 20 cycles, each consisting at 94°C for 20 s, at 56°C for 30 s, at 72°C for 2 min, and then at 60°C for 30 min to incubate at 4°C. Selective amplifications were carried out using diluted DNA from the preamplification reaction, and the primer combinations, *Mse*I-CTC and *Eco*RI-ACA, *Mse*I-CTG and *Eco*RI-ACA, *Mse*I-CAT and *Eco*RI-CTC and *Mse*I-CTC and *Eco*RI-CTG (Applied Biosystems Inc.). The thermal cycler was programmed to start at 94°C for 2 min, 10 cycles, each consisting at 94°C for 20 s, at 56°C for 30 s and at 72°C for 2 min. The 66°C annealing temperature of the first cycle was subsequently reduced by 1°C for the next 10 cycles and continued at 56°C for 30 min for the remaining 20 cycles, and then at 60°C for 30 min to incubate at 4°C.

These primer combinations were adopted from a test of 64 primer pair combinations in three accessions of *L. tashiroi*, for which they had given clear, easily and unambiguously scoreable results. Selective amplification products were mixed with a deionized formamide, blue loading dye and GeneScan 400HD Rox size standard (Applied Biosystems Inc.). The resulting mixtures were heated at 90°C for 3 min, quickly cooled on ice, and 1.5 uL was immediately loaded on 5% polyacrylamide gels on an ABI Prism 377 automated sequencer. Electrophoresis was carried out at a constant voltage (3000 V) at 51°C for 3 h.

**Amplified fragment length polymorphism data analysis**

Raw fragment data were analyzed using GeneScan version 3.1 (Applied Biosystems Inc.) with the following peak amplitude thresholds in effect: BLUE (50) and RED (50). The presence or absence of peaks was scored for each accession within a readable range of 50–400 bp and assembled as a binary (1/0) matrix manually.
Electrophenograms were carefully checked individually to avoid possible misinterpretations because of automated fragment scoring.

The analysis of molecular variance (AMOVA; Excoffier et al. 1992) was implemented to estimate variance components for AFLP phenotypes where total genetic diversity was partitioned into components at different hierarchical levels (i.e. among populations and within populations). We applied AMOVA using ARLEQUIN ver. 2.000 (Schneider et al. 2001). Genetic distances between pairwise combinations of populations were expressed as $F_{ST}$ values. Nei’s (1973) gene diversity statistics in different populations was estimated using POPGENE ver. 1.31 (Yeh et al. 1999) based on the assumption that each locus is a two-allele system in Hardy–Weinberg equilibrium.

Pairwise genetic distances for all accessions were calculated from the presence/absence matrix according to the complementary value of Nei and Li’s (1979) similarity coefficient implemented in PAUP4.0b10 (Swofford 2001). A neighbor-joining phenogram was then calculated based on the distance matrix and 1000 bootstrap replicate datasets using PAUP.

**Results**

In the 73 accessions studied, the four primer combinations amplified a total of 733 unambiguously scoreable fragments, 726 of which were polymorphic (Table 2). Of those, 628 were shared by two or more accessions and, thus, were potentially informative, whereas 98 were either unique to a single accession or present in all except one. An identical fingerprint was not detected from the accessions. The selective primer pair combinations varied in their ability to detect AFLP across Lysimachia, with
numbers of detected polymorphic fragments ranging from 166 (MseI-CTC/EcoRI-ACT) to 201 (MseI-CAT/EcoRI-ACT). The percentage of potentially informative fragments generated by each selective primer combination varied between 83.3% (MseI-CAT/EcoRI-ACT) and 88.7% (MseI-CTC/EcoRI-ACA).

AMOVA showed that 55% of the molecular variance was found between species, 16% was among populations within species and 29% was within populations (Table 3). For *L. tashiroi*, 38% of the molecular variance was attributable to the between-population component and 62% to the within-population component.

According to Nei’s (1973) gene diversity statistic, 22% of total gene diversity was attributable to the between-population component (*G*~ST~ = 0.22). For *L. japonica*, 32% of the molecular variance was attributable to the between-population component and 68% to the within-population component. The corresponding figure in Nei’s (1973) gene diversity statistics was *G*~ST~ = 0.27. Total gene diversity in each species (*H*~T~) was 0.20 in *L. tashiroi* and 0.12 in *L. japonica*, and the average gene diversity within populations (*H*~S~) was 0.15 in *L. tashiroi* and 0.09 in *L. japonica* (Table 4). Thus, *H*~T~ and *H*~S~ in *L. tashiroi* exceeded the values of *L. japonica* by 61% and 57%, respectively.

Figure 2 showed the neighbor-joining tree based on Nei and Li (1979) genetic distance between all pairwise combinations of the 73 AFLP phenotypes. The values above branches in parentheses were bootstrap values of 1000 replicates. By using *L. japonica* as an outgroup, monophyly of *L. tashiroi* was supported by 100% of the bootstrap value. Monophyly of Gokayama and Yahazuto populations was supported by 76% and 64% of the bootstrap value, respectively.

**Discussion**

As is shown by the high bootstrap value in the AFLP tree (Fig. 2), *L. tashiroi* is a species clearly differentiated from *L. japonica*, and approximately half of the molecular genetic variance observed in this study was found between species. Remarkably, both total gene diversity among populations (*H*~T~) and average gene diversity within populations (*H*~S~) were higher in *L. tashiroi* than in *L. japonica* (Table 4), despite the fact that *L. tashiroi* is an endangered species found in limited localities and

### Table 2

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>CTC/ACA</th>
<th>CTG/ACA</th>
<th>CAT/ACT</th>
<th>CTC/ACT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fragments</td>
<td>186</td>
<td>174</td>
<td>203</td>
<td>170</td>
<td>733</td>
</tr>
<tr>
<td>No. present in all</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>No. unique to one</td>
<td>20</td>
<td>21</td>
<td>31</td>
<td>22</td>
<td>94</td>
</tr>
<tr>
<td>No. present in all except one</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>No. present in two or more</td>
<td>165</td>
<td>151</td>
<td>169</td>
<td>143</td>
<td>628</td>
</tr>
<tr>
<td>Potentially informative fragments (%)</td>
<td>88.7</td>
<td>86.8</td>
<td>83.3</td>
<td>84.1</td>
<td>85.7</td>
</tr>
</tbody>
</table>

*These variations were generated by four primer combinations (MseI-**/EcoRI-***) in the AFLP analysis of 73 accessions of *Lysimachia tashiroi* and *Lysimachia japonica*. Fragments were scored for each accession within a readable range of 50–400 bp.*

### Table 3

<table>
<thead>
<tr>
<th>Species</th>
<th>SV</th>
<th>d.f.</th>
<th>SS</th>
<th>VC</th>
<th>%</th>
<th><em>F</em><del>ST</del></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. tashiroi</em></td>
<td>AP</td>
<td>2</td>
<td>804.95</td>
<td>30.43</td>
<td>37.60</td>
<td>0.38*</td>
</tr>
<tr>
<td></td>
<td>WP</td>
<td>59</td>
<td>2980.31</td>
<td>50.51</td>
<td>62.40</td>
<td></td>
</tr>
<tr>
<td><em>L. japonica</em></td>
<td>AP</td>
<td>1</td>
<td>143.14</td>
<td>18.71</td>
<td>31.29</td>
<td>0.31*</td>
</tr>
<tr>
<td></td>
<td>WP</td>
<td>9</td>
<td>369.77</td>
<td>41.09</td>
<td>67.71</td>
<td></td>
</tr>
<tr>
<td><em>L. tashiroi</em> and <em>L. japonica</em></td>
<td>AG</td>
<td>1</td>
<td>2126.41</td>
<td>95.33</td>
<td>55.23</td>
<td>0.71*</td>
</tr>
<tr>
<td><em>L. japonica</em></td>
<td>APWG</td>
<td>3</td>
<td>948.09</td>
<td>28.00</td>
<td>16.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WP</td>
<td>68</td>
<td>3350.08</td>
<td>49.27</td>
<td>28.54</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.001. Significance levels are based on 1023 permutations. Partitioning of the overall genetic variation on two levels, among and within populations and group. A, among; W, within; P, population; G, group; SV, source of variation; d.f., degrees of freedom; SS, sum of squares; VC, variance components; %, percentage of variation.*

### Table 4

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th><em>H</em><del>T</del></th>
<th><em>H</em><del>S</del></th>
<th><em>G</em><del>ST</del></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lysimachia tashiroi</em></td>
<td>62</td>
<td>0.1979</td>
<td>0.1540</td>
<td>0.2221</td>
</tr>
<tr>
<td><em>Lysimachia japonica</em></td>
<td>11</td>
<td>0.1201</td>
<td>0.0876</td>
<td>0.2705</td>
</tr>
<tr>
<td><em>L. tashiroi</em> and <em>L. japonica</em></td>
<td>73</td>
<td>0.2643</td>
<td>0.1668</td>
<td>0.3688</td>
</tr>
</tbody>
</table>

*N, survey size of the population; *H*~T~, total gene diversity within population; *H*~S~, average gene diversity among populations; *G*~ST~, coefficient of gene differentiation.*
`L. japonica` is widespread in the main islands of Japan. This result may be partly explained by the primer combinations chosen through the preliminary screening within three accessions of `L. tashiroi`, or by the fact that the set of samples examined for `L. japonica` was limited. However, the two populations of `L. japonica` sampled are isolated by long distance and the Yakushima population is often distinguished as a variety, `L. japonica` var. `minutissima`. Thus, it is plausible that `L. tashiroi` maintains a relatively higher gene diversity than widespread `L. japonica`. A number of researchers have argued that geographic range is not a good predictor of genetic structure because of the influence of historical factors and habitat heterogeneity (Loveless & Hamrick 1984; Schonswetter et al. 2003).

According to a recent review, rare and widespread species do not differ significantly in total genetic diversity (Gitzendanner & Soltis 2000).

The AFLP tree (Fig. 2) shows that the Gokayama and Yahazu-toge populations of `L. tashiroi` cluster to two distinct clades and are sister groups connected to each other, indicating that these populations have been isolated during generations sufficient to develop genetic differentiation. The AFLP tree also shows that the Gokayama population and the two other populations of `L. tashiroi` are not clonal, but rather are composed of many genets. The reason why clonal plants were not detected is that the population might be old or they might occur somatic mutation. Both AMOVA and the analysis using gene diversity
statistics showed that within-population variability is much higher than between-population variability.

This finding is unexpected because field observations revealed that plants of *L. tashiroi* propagate vegetatively by creeping stems with nodes frequently rooting onto the ground and bear few capsules even with showy flowers. To examine the cause of this low seed fertility, pollen grains were stained with acetocarmine solution and observed using a microscope Olympus BX50. As is described by Nakamura (1989), most pollen grains exhibited abnormal morphology without nuclei, but a few were normal in appearance even though it is diploid. Low seed fertility may result from the sterility of most pollen grains, although its genetic background is uncertain. The fact that the fertility of most pollen grains is low means that selfing should be low and rare outcrossing might occur within the population, so that within population genetic variability is relatively high.

Our findings suggest that we should not assume low genetic variability even in apparently clonal plants. Genetic variability should be taken seriously when developing a conservation program even in apparently clonal plants, and this is the case in the *L. tashiroi* population in the Gokayama Dam construction area. For the conservation management of this species, we suggest that as many genets as possible, samples at least a few meters apart from the nearest one, should be transplanted to appropriate habitats outside of, but neighboring, the dam construction area. This is because the conservation of genetic diversity within an individual species is an important factor in its long-term survival in the face of environmental changes and disease (Amos & Balmford 2001; Frankham et al. 2002).

**Acknowledgments**

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**References**


