Development of microsatellite markers for an endangered fern in the Ryukyus, *Plagiogyria koidzumii* (Plagiogyriaceae)

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We developed 10 microsatellite markers for *Plagiogyria koidzumii*, a critically endangered fern species found on Iriomotejima Island in the Ryukyu Archipelago, Japan and in Taiwan. These markers showed polymorphism among 65 wild individuals from Iriomotejima Island; the number of alleles per locus was 2–14, and mean observed and expected heterozygosity in the largest population were 0.276 and 0.277, respectively. A genetic structure analysis using these markers indicated clear genetic differentiation even within the narrow geographic range (ca. 10 × 8 km) on Iriomotejima Island. These microsatellite markers should be valuable for measuring genetic diversity and comparing genetic structure within and between populations.

**Key words:** genetic structure, Iriomotejima Island, management unit, national endangered species, Ryukyu Archipelago

*Plagiogyria koidzumii* Tagawa is an evergreen fern species of Plagiogyriaceae, which was originally described from Iriomotejima Island in the Ryukyu Archipelago, Japan (Tagawa, 1933) and is now recorded also from Taiwan (DeVol, 1972). Owing to its restricted habitats and scarcity in Japan, this species is now classified as endangered in the Red List of Japan (https://www.env.go.jp/press/files/jp/109278.pdf) and prescribed as a National Endangered Species by the Act on Conservation of Endangered Species of Wild Fauna and Flora (https://www.env.go.jp/nature/kisho/domestic/list.html). The situation of *P. koidzumii* is also serious in Taiwan, where it is listed as critically endangered in the Red List of Taiwan Plants, 2017.

Within Iriomotejima Island (289 km²), several populations of *P. koidzumii* occur in separate locations. The critical situation for this species indicates the need to assess its genetic diversity and population structure in order to establish management units under an appropriate and efficient conservation strategy. However, there are currently no useful genetic markers to obtain fine-scale genetic information on this species. Therefore, we here developed and characterized 10 polymorphic microsatellite primers for *P. koidzumii*, and further assessed the population structure within Iriomotejima Island using samples from representative localities.

We used leaf tissue of an individual from Iriomotejima Island. Genomic DNA was extracted by a modified version of the CTAB method of Milligan (1992). A DNA fragment library was constructed using the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA), amplified using the Ion PGM Template OT2 400 Kit (Thermo Fisher Scientific), and then sequenced using the Ion PGM Sequencing 400 Kit (Thermo Fisher Scientific) with an Ion 318 Chip v2 (Thermo Fisher Scientific) and an Ion Torrent PGM System (Thermo Fisher Scientific).

We identified microsatellite loci including more than eight dinucleotide or seven trinucleotide repeats from sequence data obtained, using MSATCOMMANDER 0.8.2 (Faircloth, 2008). Non-redundant target sequences

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were selected by a tree-based clustering method using ClustalX 2.1 (Larkin et al., 2007) and TreeExplorer 2.12 (provided by K. Tamura), and primers for PCR amplification were designed using Primer3 4.0.0 with the default settings (Rozen and Skaletsky, 2000). We identified a total of 626 microsatellite loci and designed 40 primer sets.

We conducted amplification tests for primers designed using fluorescently labeled M13 tag sequences (FAM [5'-CACGACGTTGTAACACGAC-3'], NED [5'-CTATAGGGCAAGCTGTTG-3'], PET [5'-CGGAGAGC-GAGAGGTTG-3']; Boutin-Ganache et al., 2001) with the Multiplex PCR Kit (Qiagen, Hilden, Germany). PCR was carried out in a 5-μl reaction volume containing 5–20 ng of extracted DNA, 2.5 μl of Multiplex PCR Master Mix, 0.01 μM forward tailed primer, 0.2 μM reverse primer, and 0.1 μM fluorescently labeled M13 primer. The PCR conditions were as follows: 15 min at 95 °C; 35 cycles of 94 °C for 30 s, 57 °C for 90 s, and 72 °C for 90 s; followed by 10 min at 72 °C. Fragment sizes were determined using an ABI Prism 3130 Genetic Analyzer and GeneMapper software (Applied Biosystems, Foster City, CA, USA). Finally, we genotyped 65 individuals from seven locations following the protocol described above.

Observed (H₀) and expected (Hₑ) heterozygosity and inbreeding coefficient (Fᵢₑ) were calculated using GenAlEx 6.503 (Peakall and Smouse, 2006, 2012), and deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using Genepop 4.7.0 (Rousset, 2008).

We used STRUCTURE 2.3.4 (Pritchard et al., 2000) to infer the number of genetically subdivided clusters (K) without predefined populations and to assign individuals to such clusters. We assumed an admixture model with correlated allele frequencies and performed 10 independent runs for each value of K (ranging from 1 to 10) with 5 × 10⁵ Markov chain Monte Carlo iterations after a burn-in period of 1 × 10⁴ iterations. The value of K with the highest average “log probability of data” was accepted and that of the membership coefficients for each individual (Q) was selected with the highest “log probability of data” of 10 runs in the accepted K value. The results of the STRUCTURE analysis were visualized using DISTRUCT (Rosenberg, 2004) after aligning the cluster membership coefficients using CLUMPP (Jakobsson and Rosenberg, 2007).

Of the 40 primer sets tested, 10 sets showed successful amplification and polymorphism for 65 P. koidzumii

| Locus (Accession no.) | Primer sequence (5′–3′) | Repeat motif | Fluorescent label | Size range (bp) | A | Population 6 (n = 33) |
|-----------------------|-------------------------|--------------|------------------|----------------|---|-----------------------|
|                       |                         |              |                  |                |   | H₀ | Hₑ | Fᵢₑ |
| Pko003 (LC433746)     | F: AAAGCCCGTATAGAGGTCG (TG)₁₂ | FAM | 199–201         | 2              | 0.000 | 0.000 | NA |
|                       | R: GGGGTATACAGTTTTGGGC | (AT)₉ | FAM | 218–228         | 4 | 0.455 | 0.471 | 0.034 |
| Pko004 (LC433747)     | F: GATGCGTCTCTGATGACG     | (AG)₁₂ | FAM | 218–234         | 0.845 | 0.443 | −0.094 |
|                       | R: GCAAGGCTTGGTAACACCGACCA | (GA)₉ | FAM | 247–253         | 3 | 0.000 | 0.000 | NA |
| Pko008 (LC433749)     | F: CTGTGGACGGATGACAGGAACCAG | (AC)₁₇ | FAM | 288–316         | 11 | 0.394 | 0.374 | −0.053 |
|                       | R: GCGACATGTCGTGCTGAGT    | (AC)₃ | FAM | 247–253         | 3 | 0.000 | 0.000 | NA |
| Pko022 (LC433750)     | F: CAAGGTACGCTGTCTGTC     | (GA)₉ | NED | 212–214         | 2 | 0.091 | 0.087 | −0.048 |
|                       | R: GAATTGACGATGCATGTCCA   | (GA)₉ | NED | 265–296         | 14 | 0.606 | 0.729 | 0.169 |
| Pko025 (LC433752)     | F: CTGTGGTGGTCCATATTTGGGC | (TG)₁₉ | NED | 265–296         | 14 | 0.606 | 0.729 | 0.169 |
|                       | R: GCAACCATCGATGAGAAGT    | (CA)₉ | PET | 188–194         | 4 | 0.000 | 0.000 | NA |
| Pko030 (LC433753)     | F: CCACTTCGTTGGGTTTATAC   | (CTT)₉ | NED | 175–187         | 0.976 | 0.638 | −0.093 |
|                       | R: GTGCAGACTCTCTTGTGGT    | (CTT)₉ | NED | 175–187         | 0.976 | 0.638 | −0.093 |
| Pko035 (LC433754)     | F: AGCGAGTCGCGAACATCCAGCG | (CA)₉ | PET | 188–194         | 4 | 0.000 | 0.000 | NA |
|                       | R: CATCTTGTAGCTCAGCTGGTGA | (GA)₁₈ | PET | 227–255         | 0.030 | 0.030 | −0.015 |
| Pko040 (LC433755)     | F: TCCATTGGATGACAGGCCAAGATC | (GA)₁₈ | PET | 227–255         | 0.030 | 0.030 | −0.015 |
|                       | R: GCACCTTCGAGTCTAGCTAGAAGA | (GA)₁₈ | PET | 227–255         | 0.030 | 0.030 | −0.015 |

A, number of alleles for all populations; H₀, observed heterozygosity; Hₑ, expected heterozygosity, Fᵢₑ, inbreeding coefficient; NA, not available due to monomorphic locus.
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individuals (Table 1). The number of alleles varied from two (Pko003 and Pko022) to 14 (Pko025) for all individuals. The mean $H_0$ and $H_E$ in the largest population (i.e., population 6; $n = 33$) were 0.276 and 0.277, respectively, and $F_{IS}$ values ranged from $-0.094$ (Pko007) to $0.169$ (Pko025). Three loci showed significant deviation from HWE: Pko003, Pko007 and Pko022 ($P < 0.05$ after sequential Bonferroni correction; Rice, 1989). Significant LD was observed at 18 pairs of loci: Pko004 vs. Pko008, Pko009, Pko030 and Pko035; Pko007 vs. Pko008, Pko025 vs. Pko022.

![Fig. 1](image-url)

**Fig. 1.** Results of STRUCTURE analysis ($K = 6$) for the genetic structure of *Plagiogyria koidzumii* on Iriomotejima Island. (A) Plot of mean likelihood $L(K)$ and variance per $K$ value from STRUCTURE HARVESTER (Earl and vonHoldt, 2012). (B) Bar plot showing each individual from the seven locations and clusters by a single vertical line and a different color, respectively. Numbers below the bar plot refer to location codes and the numbers of samples are in parentheses. (C) Relative location within the island with pie charts showing the proportions of cluster assignment from the STRUCTURE results. Numbers and colors correspond to those in (B). Detailed geographic information is not shown to prevent illegal digging. (D) Neighbor-joining tree showing divergence among clusters based on the net nucleotide distances along with its scale bar. The numbers near the colored circles show the mean values of $F_{ST}$, indicating the degree of divergence by genetic drift from the ancestral population.
and Pko040; Pko008 vs. Pko009 and Pko035; Pko009 vs. Pko030, Pko035 and Pko040; Pko030 vs. Pko035 and Pko040; and Pko035 vs. Pko040 (P < 0.05 after sequential Bonferroni correction). These results are probably due to remarkable population subdivision (see below), because no significant deviation from HWE or significant LD was detected in population 6 at all loci (6 loci and 21 pairs of loci, respectively) that could be tested.

The STRUCTURE analysis showed that the most likely number of clusters was six (Fig. 1A). Each cluster was represented by a single or several nearby populations with little admixture (Fig. 1B, 1C). The mean values of $F_{ST}$ for each cluster ranged from 0.13 to 0.56, indicating relatively large divergence from the ancestral population (Fig. 1D). These results suggest that *P. koidzumii* is genetically differentiated even within its restricted distribution range (ca. 10 × 8 km) on Iriomotejima Island and individual conservation efforts for each cluster are recommended. Further investigation of *P. koidzumii* from the entire distribution range including Taiwan will be required to assess the total genetic diversity maintained within the species, genetic differentiation between regions, and appropriate management units.

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