Development and characterization of 43 microsatellite markers for the critically endangered primrose *Primula reinii* using MiSeq sequencing

Masaya Yamamoto a,*, Yoshihiro Handa b, Hiroki Aihara b, Hiroaki Setoguchi a

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**A B S T R A C T**

*Primula reinii* (Primulaceae), a perennial herb belonging to the *Primula* section *Reinii*, occurs on wet, shaded rocky cliffs in the mountains of Japan. This threatened species comprises four narrow endemic varieties (Fig. 1, Yamazaki, 1993): *P. reinii* var. *reinii*, *P. reinii* var. *mygiensiis* Hara, *P. reinii* var. *kitakakenesis* (Hara) Ohwi, and *P. reinii* var. *rhodotricha* (Nakai et Maek.) Yamaz. In addition, *P. reinii* var. *okamotoi* (Koidz.) Murata., which is found on the Kii Peninsula, is a synonym of var. *reinii* (Fig. 1). However, molecular phylogenetic analyses using both chloroplast and nuclear DNA have shown distinct sequence divergence between var. *reinii* and *okamotoi* (Yamamoto et al., 2017b).

*P. reinii* is the most attractive representative in sect. *Reinii* because these primrose plants have a small number of relatively large flowers just above their very dwarf emerging foliage (Richards, 2003). Furthermore, these plants, which are threatened species, are very localized and rare in the wild. Based on their rarity, and reductions in the numbers of individuals and populations, due to anthropogenic activities, all four varieties of *P. reinii* are listed on the latest Japanese Red List (Ministry of the Environment, 2017), and are assigned to the 'Critically Endangered' (vars. *rhodotricha* and *mygiensiis*) or 'Vulnerable' (vars. *reinii* and *kitakakenesis*) categories. Despite the need for conservation, little is known of the life history, reproductive system, or vegetative characteristics of these plants.

Recent ecological and genetic studies have examined *P. reinii* var. *rhodotricha*, a typical species in sect. *Reinii* that faces a risk of extinction (Yamamoto et al., 2013, 2017a). Yamamoto et al. (2017a) reported molecular evidence of population depletion of the critically endangered primrose using 11 microsatellite markers that were originally developed for *Primula sieboldii* E. Morren. Furthermore, they also revealed a relationship between genetic diversity and the population sizes of *Reinii* species, and suggested that a purge of recessive detrimental genes to increase homozygosity could prevent additional genetic degradation in their wild habitat (Yamamoto et al., 2017a). However, only six microsatellite loci were used in that study to assess the genetic diversity of these species. Therefore, additional highly polymorphic molecular markers are required to investigate genetic status more reliably and to conduct effective conservation activities for *P. reinii*. Even in var. *rhodotricha*, additional microsatellite markers are needed to measure the degree of inbreeding and inbreeding depression (e.g., pedigree analysis) to improve their low fertility (approximately 5% in fruiting,

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* Corresponding author.

E-mail address: yamamoto.masaya.73m@st.kyoto-u.ac.jp (M. Yamamoto).

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Yamamoto et al., 2017a). In this study, we isolated and characterized 43 genomic microsatellite markers for P. reinii, which will be powerful tools aiding assessment of their genetic diversity.

2. Materials and methods

To develop useful microsatellite markers for P. reinii, which comprises several narrow endemic taxa, genomic DNA from three varieties (vars. reinii, okamotoi, and rhodotricha) was extracted from leaf tissues collected from each population (Fig. 1) using a modified CTAB protocol (Doyle, 1990). Each genomic DNA sample was used for library preparation with the KAPA HyperPlus Kit (Kapa Biosystems, Wilmington, MA, USA). Sequencing analyses was performed on the MiSeq Benchtop Sequencer (Illumina, San Diego, CA, USA) using a 2 × 250-bp read length for each DNA sample. Raw reads of each sample were quality trimmed (Q > 20) using Sickle (https://github.com/najoshi/sickle). High-quality reads from the three samples, vars. reinii, okamotoi, and rhodotricha, were assembled, using Velvet (Zerbino and Birney, 2008), into 246,887, 313,719, and 285,839 contigs, respectively. Potential microsatellite regions with at least five repeats were detected in each assembled draft genome sequence using QDD ver. 2.1 (Meglécz et al., 2010). QDD was the most versatile software for estimating microsatellites based on next generation sequencing datasets in our pipeline. In total, 505, 732, and 562 microsatellite markers were predicted for each taxon, of which 73, 70, and 65 markers were selected as based on next generation sequencing datasets in our pipeline. In this study using only six loci (estimated heterozygosity (H_e), inbreeding coefficient (FIs), and deviations from Hardy–Weinberg equilibrium were calculated using Arlequin 3.5 (Excoffier and Lischer, 2010).

3. Results and discussion

Of 208 candidate microsatellite markers, 98 (47%), 71 (34%), and 39 (19%) were di-, tri-, and tetrancleotide repeats, respectively. The most common di- and trinucleotide repeats were (AG)n (25%) and (TTA)n (13%), respectively. No common motif was found among the tetrancleotide repeats. The motifs (AG)n, (CT)n, (TC)n, and (AT)n accounted for 35% of the 208 candidate microsatellite markers.

Of the 208 candidate primer pairs tested, a total of 43 loci were amplified, displayed a clear polymorphism, and were in Hardy–Weinberg equilibrium (p > 0.05). All sequences were deposited in GenBank/DDBJ/EMBL (Table 1). The 19 loci developed for P. reinii var. reinii displayed relatively high polymorphism; the average values for N_A, H_e, and FIS were 4.16, 0.56, and 0.05, respectively. Meanwhile, the 10 loci for var. rhodotricha showed relatively low polymorphism, with average values of 2.60, 0.39, and 0.08 for N_A, H_e, and FIS, respectively. Similarly, the 14 loci for var. okamotoi showed low polymorphism, with average values for N_A, H_e, and FIS of 2.14, 0.35, and 0.03, respectively.

The genetic status of var. rhodotricha determined using our newly developed microsatellite markers was nearly identical to that determined using previously established markers (Yamamoto et al., 2017a), whereas our results for var. reinii and okamotoi indicated a relatively lower genetic diversity than that in a previous study using only six loci (estimated H_e of 0.620 and 0.412 for vars. reinii and okamotoi, respectively) (Yamamoto et al., 2017a). Therefore, our results imply that the genetic diversities of vars. reinii and okamotoi were overestimated in the previous study, possibly due to an insufficient number of loci.

In this study, we isolated 1299 microsatellite loci from P. reinii and its relatives. A total of 208 primer pairs were used for wild

![Fig. 1. Presumed range of Primula sect. Reinii species. Black arrows indicate the populations sampled.](Image)
Table 1
Primer specifications for the 43 polymorphic microsatellite markers developed for P. reinnii in this study.

| Locus | Primer sequence (5'→3') | Repeat motif | Size range | \(N_A\) | \(H_E\) | \(F_S\) | Accession no. |
|-------|--------------------------|--------------|------------|--------|-------|-------|----------------|
| **For Primula reinnii var. reinnii** |
| Pre_2 | F: TGCCCAATGGCCAGCTTACGCA (TA)\(_9\) | 228–236 | 5 | 0.756 | 0.198 | LC217340 |
|       | R: GAGGTTGTATAGCTTCGGTGG | | | | | |
| Pre_5 | F: ACACGCTTTCATGCCTGTTCTC (CT)\(_{12}\) | 146–158 | 4 | 0.604 | 0.068 | LC217341 |
|       | R: CAGACAATTTATATCAGGTATCA |
| Pre_7 | F: TGACATTTCCATATAATTGTTATACGG | (TC)\(_{11}\) | 144–160 | 5 | 0.699 | 0.240 | LC217342 |
|       | R: TGGGCTTGAGTATGGTGCA |
| Pre_9 | F: GCGAACCAAAAACAAAACCTACTGATG | (GA)\(_{11}\) | 202–212 | 3 | 0.693 | –0.173 | LC217343 |
|       | R: TCCTGAGCTTACAACCAATACTC |
| Pre_10 | F: CAGTGAGAAGACATGACTGACCT (AG)\(_{11}\) | 149–165 | 5 | 0.696 | –0.033 | LC217344 |
|       | R: ATACCTGGGTCTCTACAGGTT |
| Pre_18 | F: TTTGCTTTTCTTCTTCAACATTGCCTT | (CT)\(_{10}\) | 200–210 | 6 | 0.825 | 0.129 | LC217345 |
|       | R: CTGCTCTCCTCCAAACCCTTTCG |
| Pre_28 | F: ACCCTGCAAGCGAATCAAGGAA | (AG)\(_{9}\) | 253–263 | 4 | 0.398 | –0.020 | LC217346 |
|       | R: ACTCTGACCACAGCTAGAGCA |
| Pre_31 | F: ACCGCGATATGTTGGAATGTTGA | (GA)\(_{9}\) | 277–290 | 3 | 0.305 | 0.179 | LC217347 |
|       | R: CGCGGATATCCCIAAATAGGGAC |
| Pre_33 | F: TCGGGCCCAGACTGTTCTAT | (AG)\(_{9}\) | 170–188 | 4 | 0.756 | –0.033 | LC217348 |
|       | R: CCTGACTCTGCTGTCGAGAG |
| Pre_36 | F: CTAAGGGCCAACAACTGCG | (CA)\(_{9}\) | 142–152 | 5 | 0.815 | –0.008 | LC217349 |
|       | R: ATIGAAGACTGATGGGGGAC |
| Pre_38 | F: AGCTTTCTCAGTCAAATACAGG | (AG)\(_{9}\) | 202–212 | 3 | 0.540 | –0.041 | LC217350 |
|       | R: ATGGCTTCCTGAGTCCACAC |
| Pre_40 | F: CTCTCTCTCTCTCTCTCTCCTG | (CT)\(_{7}\) | 143–152 | 4 | 0.409 | 0.211 | LC217351 |
|       | R: CCGATCTGTAATCAATTTGACG |
| Pre_43 | F: GCGGATTTTAAATGAGAGGAG | (GA)\(_{9}\) | 304–308 | 3 | 0.392 | 0.433 | LC217352 |
|       | R: GGGATTCACCTAAAGTAAAGGAGG |
| Pre_47 | F: AGGCTATCTGAGAACTGCT | (ATT)\(_{8}\) | 284–290 | 3 | 0.371 | –0.262 | LC217353 |
|       | R: CACTCTGGTGAGCTGAGCTG |
| Pre_51 | F: ACCGTTAATCTACCTTCCACG | (TAT)\(_{7}\) | 152–158 | 3 | 0.595 | –0.154 | LC217354 |
|       | R: ATCCTATCATAGTCCACATCA |
| Pre_52 | F: TGGCGGCAAGCTGAACTCA | (CA)\(_{7}\) | 242–272 | 5 | 0.567 | –0.047 | LC217355 |
|       | R: GCGTACACAGGACAGGCTTGA |
| Pre_57 | F: TGGCTTAGTCTGTGAAATGACGT | (TCT)\(_{7}\) | 143–152 | 4 | 0.409 | 0.211 | LC217356 |
|       | R: GTCGTTAGGAGCGGCAGCAC |
| Pre_61 | F: TGCAGCTTGGGAGGAGGAT | (TTCA)\(_{6}\) | 266–278 | 4 | 0.489 | 0.148 | LC217357 |
|       | R: TTGGTACCTGACCGGGAGAGG |
| Pre_73 | F: ACAGTTCTTTGTAGGAGGAGG | (TATG)\(_{6}\) | 140–142 | 2 | 0.125 | –0.034 | LC217358 |
|       | R: TCCCCTGTCATGTAAATTGAGG |

**For P. reinnii var. okamotoi**

| Pok_6 | F: TGTTTCAACATTCAAACAACCA (AG)\(_{11}\) | 160–162 | 2 | 0.474 | 0.116 | LC217359 |
|       | R: CTGGAGCTGGTGCCTCACCTCT | | | | | |
| Pok_8 | F: AAGGAGCTGGAGTTGTCCCTTCTT | (CT)\(_{11}\) | 308–312 | 2 | 0.495 | 0.088 | LC217360 |
| Pok_11 | F: TGCCCAAAGCAAGTACTGGCATG | (CT)\(_{11}\) | 192–210 | 2 | 0.354 | 0.123 | LC217361 |
| Pok_15 | F: ATTCTATGTATTCTTTGATCAACATCT | (TA)\(_{10}\) | 206–212 | 2 | 0.497 | –0.368 | LC217362 |
| Pok_24 | F: ACACCATATCTGCTGGTTTGACCT | (GA)\(_{10}\) | 151–157 | 2 | 0.382 | –0.122 | LC217363 |
| Pok_25 | F: GGGTGGCAGTAGACAGACAAACA | (GA)\(_{10}\) | 155–161 | 3 | 0.325 | 0.181 | LC217364 |
| Pok_27 | F: ATCCTGTCTGCTCCATCGTCTCT | (CTT)\(_{10}\) | 156–160 | 2 | 0.542 | 0.135 | LC217365 |
| Pok_31 | F: ACCATGGAGCTGGCCCAATCAC | (AGT)\(_{9}\) | 164–176 | 2 | 0.155 | –0.073 | LC217366 |
| Pok_32 | F: CGGAAATATTACCCGGCGCGG | (CCA)\(_{9}\) | 159–162 | 2 | 0.222 | –0.125 | LC217367 |
| Pok_39 | F: CCTCTCTCTGCCATTCAACA | (GAA)\(_{7}\) | 283–286 | 2 | 0.235 | 0.432 | LC217368 |
| Pok_40 | F: CCATGAGAATGACAGTCACT | (TCA)\(_{7}\) | 277–286 | 3 | 0.194 | 0.288 | LC217369 |
| Pok_45 | F: GCAGAATGCGAGGAGTACTTCA | (TTA)\(_{6}\) | 167–171 | 2 | 0.487 | 0.079 | LC217370 |
| Pok_58 | F: CTTCTCTGCTTGTGCCG | (ATAC)\(_{6}\) | 162–170 | 2 | 0.131 | –0.056 | LC217371 |
| Pok_60 | F: TCGAGGTGTTTTATCCGCAAG | (TTTAA)\(_{6}\) | 189–201 | 2 | 0.354 | –0.267 | LC217372 |
|       | R: ACCAGACTAACACAAACACAGG |

**For P. reinnii var. rhodotricha**

| Prh_1 | F: AAGCGCGAGCGGCGAGGACA | (AT)\(_{12}\) | 254–256 | 4 | 0.514 | 0.089 | LC217373 |
|       | R: TATAGGCGTGATCTGCTTGGG |
| Prh_5 | F: GCCGAAGCTGACCAAAATGACGGA | (AG)\(_{11}\) | 137–163 | 3 | 0.573 | 0.076 | LC217374 |

(continued on next page)
populations of these critically endangered plants, and 43 microsatellite markers were used to assess the genetic diversity of critically endangered primroses and develop effective conservation and management strategies.

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References

Doyle, J.J., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 13–15.
Excoffier, L., Lischer, H.E., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567.

Table 1 (continued)

| Locus | Primer sequence (5’–3’) | Repeat motif | Size range | N_A | H_E | F_IS | Accession no. |
|-------|-------------------------|--------------|------------|-----|-----|------|---------------|
| Prh_6 | F: ACGCAACGGCAATACTCTTT<br>R: ACACGGACCAATTTGGAATCTTT | (CT)_{11} | 165–167 | 2 | 0.146 | –0.068 | LC217375 |
| Prh_17 | F: CAGGGTGTATCGAGATCTCT<br>R: TGCGATGGTGTAATCTCTG GT | (CT)_{10} | 212–218 | 2 | 0.418 | –0.078 | LC217376 |
| Prh_22 | F: AGCCGCGTGGTAGAAACCG<br>R: CCACCAGTCGCAGATAGAACC | (AG)_{10} | 254–256 | 2 | 0.253 | –0.151 | LC217377 |
| Prh_30 | F: GAGGCAGGATCATACCAAC | (CCA)_{9} | 220–229 | 2 | 0.275 | 0.296 | LC217378 |
| Prh_35 | F: TGCTCTGAGATACATCGG<br>R: CCAGAGCTCCAGAGGCGGAGGAA<br>F: GGGCGTATCCATATCCGCA<br>R: CCAACTCGTTGATCTACG | (GAT)_{8} | 158–167 | 3 | 0.468 | –0.002 | LC217379 |
| Prh_46 | F: AGGGCGGGTGTGATAACCG<br>R: CCAACTCGTTGATCTACG | (AG)_{10} | 253–259 | 2 | 0.490 | 0.107 | LC217380 |
| Prh_60 | F: CGTGATATCACTGTTCCGAG<br>R: TCGATTGCAACCTATCGGA | (TGT)_{12} | 130–154 | 3 | 0.352 | 0.337 | LC217381 |
| Prh_64 | F: TGGTGAGAATGGGAGAAG<br>R: CCCCCCTGCTCCAGCTAAAGC | (TTT)_{5} | 261–270 | 3 | 0.454 | 0.243 | LC217382 |

N_A, number of alleles; H_E, expected heterozygosity; F_IS, inbreeding coefficient.