Plant Diversity 40 (2018) 41–44

Contents lists available at ScienceDirect

Plant Diversity

journal homepage: http://www.keaipublishing.com/en/journals/plant-diversity/
http://journal.kib.ac.cn

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

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ARTICLE INFO

Article history:
Received 23 March 2017
Received in revised form
3 September 2017
Accepted 6 September 2017
Available online 14 September 2017

(Editor: Xun Gong)

Keywords:
Microsatellites
Polymorphism
MiSeq
Critically endangered plant
Primula reinii

ABSTRACT

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 varieties; these plants are very localized and rare in the wild. In this study, 43 microsatellite markers were developed using MiSeq sequencing to facilitate conservation genetics of these critically endangered primroses. We developed novel microsatellite markers for three varieties of *P. reinii*, and tested its polymorphism and genetic diversity using natural populations. These novel markers displayed relatively high polymorphism; the number of alleles and expected heterozygosities ranged from 2 to 6 (mean = 3.2) and 0.13 to 0.82 (mean = 0.45), respectively. All loci were in Hardy–Weinberg equilibrium. These microsatellite markers will be powerful tools to assess *P. reinii* genetic diversity and develop effective conservation and management strategies.

1. Introduction

Primula reinii Franch. et Sav., a perennial herb belonging to the *Primula* section *Reinii*, occurs on wet shaded rocky cliffs in the mountains of Japan (Richards, 2003). The species comprises four narrow endemic varieties (Fig. 1, Yamazaki, 1993): *P. reinii* var. *reinii*, *P. reinii* var. *myogiensis* Hara, *P. reinii* var. *kitadakensis* (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 phylogenic analyses using both chloroplast and nuclear DNA have shown distinct sequence divergence between vars. *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 *myogiensis*) or 'Vulnerable' (vars. *reinii* and *kitadakensis*) 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 fruition,

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Peer review under responsibility of Editorial Office of Plant Diversity.

http://dx.doi.org/10.1016/j.pld.2017.09.003

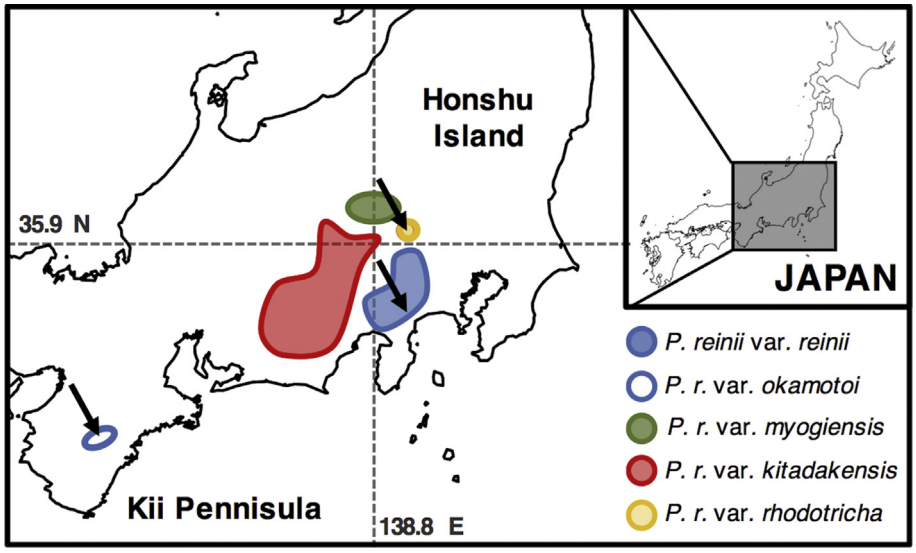

Fig. 1. Presumed range of *Primula* sect. *Reinii* species. Black arrows indicate the populations sampled.

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 candidate microsatellite markers for vars. *reinii*, *okamotoi*, and *rhodotricha*, respectively. Primers were designed automatically using the Primer3 algorithm (Rozen and Skaletsky, 2000) implemented in QDD. Due possibly to low coverage (attributed to the large genome size of these plants), as well as lineage divergence among taxa (Yamamoto et al., 2017b), common microsatellite regions were not found in this study.

To assess amplification and polymorphism at all 208 candidate microsatellite loci, additional leaf tissues from 32 individuals were collected from a natural population for each taxon. PCR amplifications were conducted in 10-μL reaction mixtures containing 1.0 μL of DNA solution (0.1 ng/μL), 0.20 μL of each primer (10 μmol/L), 2.0 μL of 5 × PCR buffer, 0.80 μL of dNTP mixture (each 2.5 mM), 0.20 μL of PrimeSTAR GXL polymerase (0.25 U; Takara Bio, Kusatsu, Shiga, Japan), and 5.6 μL distilled water. Each forward primer was labeled using FAM, VIC, NED, and/or PET. Amplifications consisted of an initial denaturation at 98 °C for 5 min, 39 amplification cycles using a touchdown protocol of 98 °C for 30 s, annealing for 30 s, 68 °C for 40 s, and a final extension at 68 °C for 2 min. The annealing temperatures were 63, 62, and 61 °C for 9 cycles and 59, 58, and 53 °C for 30 cycles. Fragment analysis was performed using the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For each locus, the number of alleles (N_A), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), 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 tetranucleotides, respectively. The most common di- and trinucleotide repeats were $(AG)_n$ (25%) and $(TTA)_n$ (13%), respectively. No common motif was found among the tetranucleotide 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 F_{IS} were 4.16, 0.56, and 0.05, respectively. Meanwhile, the 10 loci for var. *rhodotricha* showed relatively low polymorphism, with values of 2.60, 0.39, and 0.08 for N_A, H_E, and F_{IS}, respectively. Similarly, the 14 loci for var. *okamotoi* showed low polymorphism, with average values for N_A, H_E, and F_{IS} 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 1799 microsatellite loci from *P. reinii* and its relatives. A total of 208 primer pairs were used for wild

Table 1
Primer specifications for the 43 polymorphic microsatellite markers developed for *P. reinii* in this study.

Locus	Primer sequence (5′→3′)	Repeat motif	Size range	N_A	H_E	F_{IS}	Accession no.
For *Primula reinii* var. *reinii*							
Pre_2	F: TGGCAAATGGGAGCTTAGCA	$(TA)_9$	228–236	5	0.756	0.198	LC217340
	R: GAGGTTGTTTACGTGCCGTG						
Pre_5	F: ACACTGCTTTCTGCTAGCTCT	$(CT)_{12}$	146–158	4	0.604	0.068	LC217341
	R: CAGACAAATTATAATCAGCTCACCG						
Pre_7	F: TGACATTTGCATAATTGTTAATTTGGA	$(TC)_{11}$	144–160	5	0.699	0.240	LC217342
	R: TGTGGGTATGTAGTCCTGCA						
Pre_9	F: GGCAACCAAACAAACTCCTATAGT	$(GA)_{11}$	202–212	3	0.693	−0.173	LC217343
	R: TCCTGAGCGTTTACCAAACTCA						
Pre_10	F: CAGTTGAGAAGATCGATCAGACT	$(AG)_{11}$	149–165	5	0.696	−0.033	LC217344
	R: ATCATTTGGCTTTCTACAGCTTT						
Pre_18	F: TTGGACTTTGCGCTCATAAGC	$(TC)_{10}$	200–210	6	0.825	0.129	LC217345
	R: CTTGTTCTCTTCAACCCTTTGCT						
Pre_28	F: AGCCTTGCAGGAAGATCAAGAA	$(AG)_9$	253–263	4	0.398	−0.020	LC217346
	R: ACTCTAGCACACGTAGAGCA						
Pre_31	F: ACGGCATGAATTTGAAGAATTGGA	$(GA)_9$	277–290	3	0.305	0.179	LC217347
	R: CGGCGGATATTCAATAGGAGCT						
Pre_33	F: TGAGGGACGCCATTGCTTAT	$(AG)_9$	170–188	4	0.756	−0.033	LC217348
	R: CCATTACTTGCTCTGTTCGCT						
Pre_36	F: CTAGCGAGCGAACACAATGC	$(CA)_9$	142–152	5	0.815	−0.008	LC217349
	R: ATTGAGACTGATGGCGGGAC						
Pre_38	F: AGGCTTTCAACTGAACATAACGG	$(AG)_9$	218–226	6	0.540	−0.041	LC217350
	R: ATGGTGTTCGTCAGTCCCAC						
Pre_40	F: AGTACCTGTAGTGGAGAGGG	$(AG)_9$	202–212	5	0.687	−0.047	LC217351
	R: CCCATTAGGTTAACATTCACGGT						
Pre_43	F: GGCATTACCTTAAAGTAAGAGGGT	$(GA)_9$	304–308	3	0.392	0.433	LC217352
	R: GGCATTACCTTAAAGTAAGAGGGT						
Pre_47	F: AAGCCATCGATGAAGCTGCT	$(ATT)_8$	284–290	3	0.371	−0.262	LC217353
	R: CACTCTGCTGTGAGACTCTGA						
Pre_51	F: CCCTGTAATCTACCTCCACGG	$(TAT)_7$	152–158	3	0.595	−0.154	LC217354
	R: ATCATATCGCATTCCTAAGCATCA						
Pre_52	F: TTGCAGGGCAAGTGAACTCA	$(CAG)_7$	242–272	5	0.567	−0.047	LC217355
	R: GGCTGAGAAGGAGCAGTTGA						
Pre_57	F: TGGCTGTTTGTGGAATTAGCT	$(TCT)_7$	143–152	4	0.409	0.211	LC217356
	R: GTTTGAGTGAGAGGTGGCTCT						
Pre_61	F: TGACTGGATGGGAGGACGAT	$(TTC)_6$	266–278	4	0.489	0.148	LC217357
	R: TTGTGTACCGTACCGCAGAG						
Pre_73	F: ACGGATCTTTGTGAGGAAGGAG	$(TATG)_5$	140–148	2	0.125	−0.034	LC217358
	R: TCCGTCTGTCTGAATTTAGGGT						
For *P. reinii* var. *okamotoi*							
Pok_6	F: TGGTTCACAATTCACAACCCA	$(AG)_{11}$	160–162	2	0.474	0.116	LC217359
	R: CTGGAGCTGGGCATCTCATC						
Pok_8	F: AAGGCAGTTGAGTCCCTTTCT	$(CT)_{11}$	308–312	2	0.495	0.088	LC217360
	R: TGCGAACAAGATCTAAGGATGT						
Pok_11	F: TGAAGGATAAGTTAGTAATTTGTGCCA	$(CT)_{11}$	192–210	2	0.354	0.123	LC217361
	R: ACTCCGTATTTATTACCTGAACAAGT						
Pok_15	F: ATTTCTATGTTCTTATGCATGAACTCT	$(TA)_{10}$	206–212	2	0.497	−0.368	LC217362
	R: TGTTTGGGCCAATTGTACTACG						
Pok_24	F: ACACCATCATTCGGTTTAGTACCT	$(GA)_{10}$	151–157	2	0.382	−0.122	LC217363
	R: AACGGAGAGGCGAATAATACG						
Pok_25	F: GGTGTTCCATGAGACCAGAACA	$(GA)_{10}$	155–161	3	0.325	0.181	LC217364
	R: TGGTCTCTGGTTGCTAAGGC						
Pok_27	F: ATCCGTCTCGCATCGTCTTC	$(CT)_{10}$	156–160	2	0.542	0.135	LC217365
	R: TAGAGGCGCCATTGAAGGTC						
Pok_31	F: ACCAATTGCAGCCCAATCAAC	$(AGT)_9$	164–176	2	0.155	−0.073	LC217366
	R: CCACTAGCTCTGCAGTTCTGA						
Pok_32	F: CGAAACAATATTACCCGACCGG	$(CCA)_8$	159–162	2	0.222	−0.125	LC217367
	R: CGCTCTTCTGCCTACTCAACA						
Pok_39	F: CATCAAGATGCCACCAAGGG	$(GAA)_7$	283–286	2	0.235	0.432	LC217368
	R: CCTTTCCCTAGTTCTGGCCC						
Pok_40	F: GCAAGCAATGAGACGAGTAACT	$(TCA)_7$	277–286	3	0.194	0.288	LC217369
	R: TACGTGAGGCGCTTTGTGAA						
Pok_45	F: GGAAACACCCAAGGCTTTCA	$(TTA)_6$	167–171	2	0.487	0.079	LC217370
	R: GCTGACAAGGCTCAACTGGA						
Pok_58	F: GCCTTGTGAAACGCCGTTAA	$(ATAC)_5$	162–170	2	0.131	−0.056	LC217371
	R: ACAATCCCAGCTGAAAGATCCT						
Pok_60	F: TGCCAGGTGTATTATCCGACG	$(TTTA)_5$	189–201	2	0.354	−0.267	LC217372
	R: ACCAGACTAACACAAACCGGA						
For *P. reinii* var. *rhodotricha*							
Prh_1	F: AAACGTAGGCAGGAGCAACA	$(AT)_{12}$	254–256	4	0.514	0.089	LC217373
	R: TATGAGCGGTGGACTTAGGGT						
Prh_5	F: GCCGAAAGTGACAAATGAAAGC	$(AG)_{11}$	137–163	3	0.575	0.076	LC217374
	R: TCATGGCCAGATTCTTGTTGC						

(*continued on next page*)

Table 1 (*continued*)

Locus	Primer sequence (5′→3′)	Repeat motif	Size range	N_A	H_E	F_{IS}	Accession no.
Prh_6	F: ACGCAACGGCAAACTTCTTT R: ACAGGGACCAAATTGAAACTATTG	$(CT)_{11}$	165–167	2	0.146	−0.068	LC217375
Prh_17	F: GAGGGTGTATCTGAAGATTACTCT R: TCGGATTGGGTTAAATTCTGGGT	$(CT)_{10}$	212–218	2	0.418	−0.078	LC217376
Prh_22	F: AGGCGGGTGTGATAAACCG R: GGGACCTGTTTGAGTAGAGGC	$(AG)_{10}$	254–256	2	0.253	−0.151	LC217377
Prh_30	F: GAGCCAGGTCATCAACACCC R: TGGATTATTCACGCTGTGAGTGA	$(CCA)_7$	220–229	2	0.275	0.296	LC217378
Prh_35	F: TGGTCTGAGGATCAACTGCG R: CACGAATTCCCAGAGGCGAA	$(GAT)_6$	158–167	3	0.468	−0.002	LC217379
Prh_46	F: GGCCGATCCACATATTCATCA R: CCAACTCGGTTTGATCCAGT	$(GAA)_6$	253–259	2	0.490	0.107	LC217380
Prh_60	F: CGTTGATCTACTGTTTCGGCAG R: TCGATTGGCACACGTATGGA	$(TGTA)_5$	130–154	3	0.352	0.337	LC217381
Prh_64	F: TGGGTGAAGAATTGGAGAAACT R: CCCTCGGTCCAGCTTAAAGC	$(TTTG)_5$	261–270	3	0.454	0.243	LC217382

N_A, number of alleles; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient.

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.

Acknowledgments

This research was financially and technically supported by FASMAC Co., Ltd. (Kanagawa, Japan) and The Environment Research and Technology Development Fund (#4-1403).

We are grateful to Chichibu Taiheiyo Cement Co. and Ryoko Lime Industry Co., Ltd. for their help with sample collection.

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