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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR THE CLONAL SHRUB *ORIXA JAPONICA* (RUTACEAE) USING 454 SEQUENCING[1]

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- *Premise of the study:* Microsatellite markers were developed for a dioecious shrub, *Orixa japonica* (Rutaceae). Because *O. japonica* vigorously propagates by vegetative growth, microsatellite markers can be used to identify clonal relationships among its ramets.
- *Methods and Results:* Sixteen polymorphic microsatellite markers were identified by 454 next-generation sequencing. The number of alleles and expected heterozygosity for each locus among four populations ranged from two to 10 and from 0.140 to 0.875, respectively. Five of the 16 loci showed a low null allele frequency. Because *Orixa* is a monotypic genus, cross-amplification in a consubfamilial species, *Skimmia japonica*, was tested, and only one locus showed polymorphism.
- *Conclusions:* These microsatellite markers developed for *O. japonica* contribute to clone identification for studies examining the clonal structure and true sex ratio in the wild. Moreover, five markers that have a low null allele frequency can also be used for estimating mating systems or performing parentage analysis.

Key words: clonal structure; dioecious plant; next-generation sequencing; *Orixa*; Rutaceae; sex ratio.

Orixa japonica Thunb. (Rutaceae) is a deciduous dioecious shrub known from China, Korea, and Japan; it is the only known species within the genus *Orixa* Thunb. The species propagates asexually by sprouting, layering, and root suckering and forms a large genet. Although *O. japonica* can also reproduce sexually via entomophilous flowers, its seedlings are scarce in the wild. The balance between sexual and asexual reproduction is an interesting theme related to the evolution of sexual reproduction (Obeso, 2002). In dioecious clonal plants, there are sexual differences in clonal growth, and these differences contribute to the sex ratio of the population (Escarre and Houssard, 1991). *Orixa japonica* is also an important plant for medicinal use (Kang et al., 2011). Identification and management of individual plants that are of medical benefit are important and require accurate clone identification. In this study, we developed 16 microsatellite markers to provide a useful tool for clone identification of *O. japonica*, to examine the species' clonal structure and true sex ratio in the wild, and to manage lineages within breeding programs examining the medicinal use of *O. japonica*.

METHODS AND RESULTS

Total genomic DNA of *O. japonica* was extracted from a fresh leaf collected from Yoro, Gifu, Japan, using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and a voucher specimen of this sample was deposited in the Forestry and Forest Products Research Institute herbarium, Japan (accession no. TF-K11-0215) (Appendix 1). Multiplex identifier (MID) tags were used to multiplex the extracted DNA of *O. japonica* and other species. These samples were then pooled and pyrosequenced using a 454 GS Junior System (Roche, Basel, Switzerland). The raw data were demultiplexed and MID tags were removed from the reads. The de novo sequencing produced 73,267 reads with an average length of 427 bp. The identification of microsatellite regions and design of primer pairs from the sequence data were performed with the program QDD 2.1 (Meglécz et al., 2010). Microsatellite regions bordering sequences with more than five repeats of di- to hexanucleotide motifs and a minimum sequence length of 80 bp were selected. According to these criteria, 2846 reads contained microsatellite loci. To eliminate redundancy, the similarity of sequences containing microsatellite regions was detected by all-against-all BLAST searching. Subsequently, 1168 reads were selected from the whole set of sequences containing microsatellites. PCR primer pairs were designed using Primer3 (Rozen and Skaletsky, 1999) implemented in QDD 2.1 (Meglécz et al., 2010). Finally, 803 microsatellite primer pairs were designed.

Amplification and polymorphism tests were performed for 30 selected primer pairs; we selected these primer pairs on the basis of their having single repeat motifs of di- and trinucleotides, with 10–14 repeats. All forward primers were fluorescently labeled at the 5′-end with one of four different tail sequences (A to D) shown in Table 1, according to the method by Blacket et al. (2012). All reverse primers were attached to a 5′-GTT-3′ sequence at the 5′-end of the sequence to reduce stuttering due to the addition of nontemplated adenine base pairs by *Taq* DNA polymerase (Brownstein et al., 1996).

To evaluate polymorphisms in these markers using population samples, leaves were sampled from 106 individuals from four populations (Appendix 1). Because *Orixa* is a monotypic genus, to evaluate the cross-amplification potential of these markers, 16 individuals of *Skimmia japonica* Thunb., which is a consubfamilial species, were also sampled (Appendix 1). Although *Skimmia* has previously been classified into subfamily Toddalioideae, Toddalioideae is now merged into subfamily Rutoideae (Thorne, 2000), into which *O. japonica* is classified, and *Skimmia* forms a sister group to *O. japonica* on the phylogenetic trees constructed by internal transcribed spacer and/or chloroplast sequences (Poon et al., 2007; Salvo et al., 2008). Sampled leaves were dried with silica gel and stored at room temperature until DNA extraction. Leaves were pulverized using a mortar and pestle. Pulverized leaves were washed more than twice using HEPES buffer (Setoguchi and Ohba, 1995), and then the cetyltrimethylammonium bromide (CTAB) method was used to extract total DNA (Murray

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TABLE 1. Characteristics of 16 nuclear microsatellite loci isolated from *Orixa japonica*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Tail sequence[a]	Allele size range (bp)	GenBank accession no.
Oj013	F: AAATGCAACATTACATTGGTAACAT	$(AAT)_{11}$	57	Tag-A[1]	230–274	LC146669
	R: AGCAGCTTTCGATGTGTTCTAAAT					
Oj078	F: GTGAACTTCATCCACGCTCTAAA	$(AT)_{12}$	60	Tag-C[2]	210–236	LC146670
	R: CTCTGTCTTCTGGCTAGTCGCT					
Oj125	F: CTTTGTTGCAGTATATGTCGTCGT	$(AT)_{10}$	57	Tag-A[1]	212–229	LC146664
	R: GTTGGTAAGAGATCTGCAAATTGA					
Oj185	F: AGTGCATAATCACCAAACAAATGA	$(AT)_{10}$	60	Tag-A[3]	309–318	LC146673
	R: TCATAATGGTCTCCCAATAGTCCT					
Oj196	F: ATGGCCTTACATTAGTGTGTCCTC	$(AT)_{10}$	57	Tag-C[1]	222–232	LC146672
	R: TCCCTCCATTACATTTATGAAATTCT					
Oj206	F: AAGAAATAATTTATCACCGAGCCA	$(AC)_{10}$	57	Tag-B[1]	232–246	LC146661
	R: CATTTCTTATATGGACGTCTGCAA					
Oj214	F: TCGTAAGGAAAGAACTTTGGAAAC	$(AT)_{10}$	60	Tag-A[3]	160–175	LC146663
	R: CAGAATATGGTCAACCAATAGACG					
Oj365	F: TCTCAATTGCATACATTCTCATCC	$(AT)_{11}$	60	Tag-D[3]	212–247	LC146665
	R: AGTCTCCTTCACATACCCACATTC					
Oj413	F: ACCAAGGTAGTTAGCACAAAGTGG	$(AAC)_{13}$	60	Tag-B[2]	283–370	LC146666
	R: ATAGATGCGATAGAGGCATTAGGA					
Oj437	F: TGCTATATTGCAGTAACAAATAAGTGC	$(AC)_{12}$	60	Tag-B[3]	127–139	LC146671
	R: CTGCAGCAACAAACAAGTGAAA					
Oj478	F: TAAGTTGAGGATTCCCTCATTAAA	$(AT)_{10}$	60	Tag-C[3]	303–311	LC146662
	R: TTTGTCTGTCATGTGTCTGTCATATT					
Oj509	F: ATTTGCAACCCTCGGATTAGAATA	$(AG)_{13}$	60	Tag-D[2]	149–165	LC146668
	R: TAACATATTCGATCTGCTTGTCCC					
Oj549	F: GATTAATGGAAATTTGAGACGGAA	$(AG)_{10}$	60	Tag-C[2]	138–144	LC146667
	R: GTTGGATACTCTCTCCCACACACT					
Oj598	F: CTTCAAGATGAGACCATTTCAACA	$(AG)_{10}$	60	Tag-B[2]	166–182	LC146659
	R: CTTTATGAACTATTGGGCCTGAGA					
Oj609	F: ATATCCACATGACGCGTTAAGAA	$(AT)_{10}$	60	Tag-A[3]	97–114	LC146658
	R: AACCATTACAAATTAAATCTTCCAAA					
Oj661	F: AAGATATTGACCATAATTGCCCAC	$(AG)_{10}$	60	Tag-C[2]	279–280	LC146660
	R: TATGTTTGCACTAAGCACAGTCGT					

Note: T_a = annealing temperature.
[a]Tail sequences correspond to those in Blacket et al. (2012). Superscript numbers indicate the same multiplex PCR set.

and Thompson, 1980). PCR was performed in a final volume of 5 µL, containing 2.5 µL of 2× Type-it Multiplex PCR Master Mix (QIAGEN), 0.1 µM forward primers, 0.2 µM reverse primers, 0.1 µM fluorescently tagged universal primers, and 10 ng DNA template. Reactions were performed with an initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 57°C or 60°C for 90 s, and 72°C for 30 s; and finally 60°C for 30 min using a P × 2 Thermal Cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The PCR products were electrophoresed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA), and fragment sizes were determined using GeneMapper version 4.0 (Applied Biosystems).

Sixteen loci out of 30 showed clear amplification, with a single band for each allele. For each of these 16 loci, the number of alleles (*A*), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{IS}), and null allele frequency were calculated using INEst 1.1 (Chybicki and Burczyk, 2009). Deviation from Hardy–Weinberg equilibrium (HWE) was tested at each locus by a randomization test implemented in FSTAT 2.9.3 (Goudet, 1995). The HWE significance level was evaluated after Bonferroni correction for each population.

Among those 16 loci across the four populations, 132 alleles were detected. Excluding the Oj661 locus from the Mugi population, all other loci in each of the four populations showed polymorphism (Table 2). For these polymorphic loci, *A* ranged from two to 10, H_o from 0.000 to 0.767, H_e from 0.140 to 0.875, F_{IS} from −0.260 to 1.000, and the null allele frequency from 0.029 to 0.637. Five loci (Oj125, Oj437, Oj509, Oj549, and Oj598) did not significantly deviate from HWE over all the populations. Seven out of 16 loci did not amplify in *S. japonica* (Table 2). Among the remaining nine loci, eight loci were monomorphic and thus only one locus, Oj598, showed polymorphism.

CONCLUSIONS

We developed the first set of microsatellite markers for *O. japonica*. These 16 microsatellite markers showed a high level of polymorphism and can be used to identify clones. Moreover, because five of 16 markers (Oj125, Oj437, Oj509, Oj549, and Oj598) did not significantly deviate from HWE and their null allele frequencies were relatively low, these markers can also be used for estimating mating systems or performing parentage analysis. These markers will help examine the clonal structure and true sex ratio in the wild and manage lineages in the breeding program for medicinal use of *O. japonica*.

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TABLE 2. Genetic properties of 16 polymorphic nuclear microsatellite loci in four populations of *Orixa japonica* and one population of *Skimmia japonica*.

	Orixa japonica																						*Skimmia japonica*				
	Ibuki (N = 30)					Neo (N = 30)					Miyama (N = 19)					Mugi (N = 27)										
Locus	A	H_o	H_e	F_{IS}[a]	P_{ID}	Null	A	H_o	H_e	F_{IS}[a]	P_{ID}	Null	A	H_o	H_e	F_{IS}[a]	P_{ID}	Null	A	H_o	H_e	F_{IS}[a]	P_{ID}	Null	A	H_o	H_e	F_{IS}	P_{ID}	Null
Oj013	9	0.333	0.875	0.619***	0.069	0.300 ± 0.066	10	0.633	0.870	0.272**	0.070	0.127 ± 0.050	8	0.368	0.807	0.543***	0.171	0.262 ± 0.121	7	0.222	0.781	0.716***	0.151	0.423 ± 0.124	—	—	—	—	—	—
Oj078	8	0.667	0.842	0.209	0.094	0.072 ± 0.046	5	0.300	0.755	0.603***	0.163	0.315 ± 0.071	6	0.737	0.720	−0.024	0.276	0.041 ± 0.037	7	0.630	0.787	0.200	0.147	0.075 ± 0.048	—	—	—	—	—	—
Oj125	6	0.667	0.671	0.006	0.253	0.038 ± 0.033	8	0.767	0.829	0.075	0.103	0.045 ± 0.033	6	0.368	0.573	0.357	0.437	0.105 ± 0.070	8	0.667	0.798	0.165	0.137	0.055 ± 0.042	1	0.000	0.000	—	—	—
Oj185	5	0.067	0.703	0.905***	0.218	0.611 ± 0.079	3	0.167	0.647	0.742***	0.302	0.403 ± 0.083	4	0.053	0.772	0.932***	0.224	0.476 ± 0.086	5	0.037	0.781	0.953***	0.158	0.495 ± 0.072	—	—	—	—	—	—
Oj196	3	0.100	0.358	0.720**	0.647	0.192 ± 0.081	2	0.200	0.427	0.532	0.597	0.161 ± 0.078	3	0.000	0.489	1.000***	0.589	0.309 ± 0.098	3	0.111	0.565	0.804***	0.391	0.376 ± 0.082	1	0.000	0.000	—	—	—
Oj206	6	0.300	0.663	0.548***	0.256	0.158 ± 0.072	6	0.567	0.628	0.098	0.285	0.065 ± 0.045	6	0.158	0.703	0.775***	0.294	0.295 ± 0.085	5	0.482	0.721	0.332	0.224	0.102 ± 0.062	1	0.000	0.000	—	—	—
Oj214	8	0.367	0.833	0.560***	0.100	0.206 ± 0.063	6	0.233	0.394	0.408	0.570	0.126 ± 0.067	4	0.368	0.442	0.167	0.629	0.085 ± 0.065	4	0.593	0.690	0.142	0.255	0.071 ± 0.049	1	0.000	0.000	—	—	—
Oj365	3	0.000	0.246	1.000***	0.802	0.277 ± 0.086	7	0.233	0.525	0.555***	0.370	0.236 ± 0.070	4	0.263	0.460	0.427	0.630	0.149 ± 0.082	6	0.148	0.512	0.711***	0.419	0.245 ± 0.076	1	0.000	0.000	—	—	—
Oj413	6	0.033	0.767	0.957***	0.153	0.592 ± 0.069	6	0.033	0.570	0.942***	0.335	0.637 ± 0.147	5	0.000	0.814	1.000***	0.172	0.527 ± 0.082	4	0.111	0.723	0.846***	0.230	0.522 ± 0.079	—	—	—	—	—	—
Oj437	7	0.667	0.743	0.103	0.179	0.042 ± 0.035	4	0.567	0.567	0.001	0.377	0.053 ± 0.041	4	0.737	0.636	−0.159	0.398	0.041 ± 0.037	4	0.407	0.448	0.091	0.528	0.078 ± 0.054	1	0.000	0.000	—	—	—
Oj478	4	0.233	0.648	0.640***	0.263	0.312 ± 0.077	5	0.367	0.666	0.449***	0.230	0.220 ± 0.067	4	0.316	0.750	0.579***	0.239	0.326 ± 0.105	3	0.148	0.328	0.548*	0.695	0.226 ± 0.085	—	—	—	—	—	—
Oj509	7	0.733	0.732	−0.002	0.176	0.033 ± 0.029	6	0.700	0.589	−0.189	0.315	0.029 ± 0.026	5	0.474	0.478	0.009	0.573	0.055 ± 0.049	7	0.741	0.728	−0.018	0.197	0.032 ± 0.029	—	—	—	—	—	—
Oj549	4	0.467	0.515	0.093	0.420	0.054 ± 0.044	3	0.333	0.377	0.117	0.623	0.087 ± 0.057	3	0.263	0.364	0.277	0.769	0.108 ± 0.076	2	0.148	0.140	−0.060	1.055	0.075 ± 0.062	—	—	—	—	—	—
Oj598	5	0.500	0.684	0.269	0.247	0.091 ± 0.056	6	0.600	0.584	−0.028	0.356	0.039 ± 0.034	4	0.579	0.460	−0.260	0.630	0.045 ± 0.041	4	0.593	0.704	0.159	0.239	0.061 ± 0.046	3	0.500	0.675	0.260	0.425	0.089 ± 0.069
Oj609	5	0.267	0.538	0.504***	0.364	0.238 ± 0.077	4	0.300	0.576	0.479**	0.343	0.191 ± 0.133	4	0.316	0.370	0.146	0.748	0.093 ± 0.068	2	0.037	0.171	0.784	0.993	0.160 ± 0.086	1	0.000	0.000	—	—	—
Oj661	2	0.033	0.440	0.924***	0.585	0.260 ± 0.087	2	0.000	0.488	1.000***	0.546	0.311 ± 0.085	2	0.000	0.398	1.000***	0.777	0.278 ± 0.105	1	0.000	0.000	—	—	—	1	0.000	0.000	—	—	—

Note: — = no amplification or values cannot be calculated due to monomorphic loci; A = number of alleles; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; Null = null allele frequency; P_{ID} = probability of genetic identity.

[a] Significance of departure from Hardy–Weinberg equilibrium: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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Appendix 1. Voucher and location information for the *Orixa japonica* and *Skimmia japonica* populations used in this study. All vouchers were deposited in the herbarium of the Forestry and Forest Products Research Institute, Japan.

Species	Population name	Voucher no.	No. of vouchers collected	No. of individuals sampled	Latitude (°N)	Longitude (°E)
Orixa japonica Thunb.	Yoro[a]	TF-K11-0215	1	1	35.27800	136.52940
O. japonica	Ibuki	TF-HDT-00001	2	30	35.39852	136.39745
		TF-HDT-00002				
O. japonica	Neo	TF-HDT-00003	1	30	35.66922	136.54450
O. japonica	Miyama	TF-HDT-00004	2	19	35.63680	136.68852
		TF-HDT-00005				
O. japonica	Mugi	TF-HDT-00006	3	27	35.59874	137.00420
		TF-HDT-00007				
		TF-HDT-00008				
Skimmia japonica Thunb.	Fukube	TF-HDT-000010	2	16	35.64427	136.89011
		TF-HDT-000011				

[a]This sample was used only for designing the primer sequences and was not included in the population analysis.