New Microsatellite Markers for Tricyrtis macrantha (Convallariaceae) and Cross-Amplification in Closely Related Species

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Primer Note

New microsatellite markers for *Tricyrtis macrantha* (Convallariaceae) and cross-amplification in closely related species[1]

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- *Premise of the study:* Microsatellite markers were characterized in *Tricyrtis macrantha* (sect. *Brachycyrtis*, Convallariaceae), a vulnerable species endemic to Japan, to investigate its genetic diversity and population structure.
- *Methods and Results:* Eleven microsatellite markers were developed. The number of alleles per locus ranged from one to seven, and genetic diversity per locus ranged from 0.142 to 0.812. Four markers were successfully amplified in other species of sect. *Brachycyrtis* (*T. ishiiana*, *T. ishiiana* var. *surugensis*, and *T. macranthopsis*).
- *Conclusions:* The microsatellite markers can be used to investigate the genetic diversity and population structure of the vulnerable and endangered species of *Tricyrtis* sect. *Brachycyrtis*, to aid in the development of conservation strategies for each species.

Key words: Convallariaceae; cross-amplification; genetic diversity; microsatellite; population structure; *Tricyrtis macrantha.*

Tricyrtis macrantha Maxim. (sect. *Brachycyrtis*, Convallariaceae) is endemic to Kochi Prefecture, Japan. This species inhabits precipices of limestone gorges in deciduous forests. The large showy flowers make this species horticulturally valuable, and illegal removal from natural populations is a major threat to the persistence of this species. *Tricyrtis* sect. *Brachycyrtis* comprises three species and a variety: *T. macrantha*, *T. macranthopsis* Masam., *T. ishiiana* (Kitag. & T. Koyama) Ohwi & Okuyama, and *T. ishiiana* var. *surugensis* Yamazaki. *Tricyrtis macrantha* is listed as vulnerable and other species of sect. *Brachycyrtis* are listed as endangered (class IB) in the Red Data Book of plants in Japan based on IUCN criteria (Japan Society of Plant Taxonomists, 1993; Ministry of the Environment of Japan, 2000).

Nine microsatellite markers were previously developed for *T. ishiiana* (Setoguchi et al., 2011); however, only four markers could be amplified in all species of sect. *Brachycyrtis*. Development of additional microsatellite markers would contribute not only to the elucidation of the genetic structure of *T. macrantha* populations but will also allow us to compare the genetic diversity among the four taxa of *Tricyrtis* sect. *Brachycyrtis* using the same markers. Here, we report the development and characterization of microsatellite markers for *T. macrantha* and the transferability of *T. macrantha* markers to the other species of sect. *Brachycyrtis*.

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METHODS AND RESULTS

Genomic DNA was extracted from fresh leaves of one *T. macrantha* individual from the Kyoto Prefectural Botanical Garden, originally collected from a wild population at Mt. Yokokura (Ochi-cho, Takaoka-gun), in Kochi Prefecture, Japan (33°32′N, 133°12′E). The voucher specimen (*H. Setoguchi JP12005*) was deposited at the herbarium of Kyoto University (KYO).

Fresh leaf material was frozen in liquid nitrogen and then ground into a fine powder. The leaf powder was suspended in 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (pH 8.0) and centrifuged (10,000 rpm at 20°C for 5 min) to remove polysaccharides (Setoguchi and Ohba, 1995). Total DNA was then isolated from each pellet using cetyltrimethylammonium bromide (CTAB; Doyle and Doyle, 1990). The extracted DNA was dissolved in 100 µL of TE buffer. Microsatellite loci were isolated using an improved technique for isolating codominant compound microsatellite markers (Lian and Hogetsu, 2002; Lian et al., 2006). A total volume of 100 µL containing ~2.5 µg genomic DNA was digested separately with the blunt-end restriction enzymes *Ssp*I, *Eco*RV, and *Alu*I. The restricted fragments were ligated with a specific blunt adapter (consisting of the 48-mer 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and an 8-mer with the 3′-end capped with an amino residue: 5′-ACCAGCCC-NH_2-3′) using a DNA ligation kit (TaKaRa Biotechnology Co., Ohtsu, Shiga, Japan). Fragments were amplified from *Ssp*I, *Eco*RV, and *Alu*I DNA libraries using one of the compound simple sequence repeat (SSR) primers $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adapter primer (5′-CTATAGGGCACGCGTGGT-3′). PCR was performed in a total reaction volume of 50 µL containing 38.25 µL sterilized water, 0.2 mM dNTP mixture, 0.125 U AmpliTaq Gold (Applied Biosystems, Foster City, California, USA), 1.5 mM reaction buffer with $MgCl_2$ (Applied Biosystems), 0.5 µM of each primer, and 0.5 µL of template DNA (10 ng/µL). The amplification profiles included initial denaturation at 94°C for 9 min; followed by 20 cycles of 30 s at 94°C, 30 s at 66–60°C (reduced 0.3°C/cycle), and 1 min at 72°C; 20 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C; and a final extension at 72°C for 6 min. After electrophoresis on a 1.5% agarose gel, the fragments (400–800 bp) were purified using the GENECLEAN II kit (QBioGene, Solon, Ohio, USA) and cloned using the QIAGEN PCR Cloning^{plus} Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. Recombinant clones were identified using blue/white screening on Luria–Bertani agar plates containing ampicillin, X-gal, and isopropyl-β-D-1-thiogalactopyranoside (IPTG).

TABLE 1. Characteristics of 11 microsatellite markers developed in *Tricyrtis macrantha*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
TM_1	F: TGTCCATGAGTACGCCAAGC R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_{24}$	219–233	58	AB716690
TM_2	F: CGTGACGTCATAAGTGCGGC R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_5$	257–273	58	AB716691
TM_3	F: TCTAGGGCTCACTCTTTGATCTC R: <HEX>TCTCTCTCTCTCACACACACAC	$(TC)_6(AC)_8$	188–194	58	AB716692
TM_4	F: CTCCCAAATTTCTTTCATAGTGTGC R: <HEX>TCTCTCTCTCTCACACACACAC	$(TC)_6(AC)_7$	177–187	58	AB716693
TM_5	F: TGGTTCTTGTCTCCCACCCG R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_7$	175	58	AB716694
TM_6	F: TGACCACGAGTACGCCAAGC R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_{20}$	221	58	AB716695
TM_7	F: CTCGGGTGTTTTCCACCAAC R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_7$	309–311	58	AB716696
TM_8	F: AAAGCTCGGTACCACGCATG R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_{18}$	183–185	58	AB716697
TM_9	F: TTGTGCGTTGGGGCAAAGAC R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_8$	223–247	58	AB716698
TM_10	F: TCTAGTCGCCACATACACCC R: <6-FAM>ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_6$	154	58	AB716699
TM_11	F: AAGGAGAAGCAATGTCTCTCAGGC R: <HEX>TCTCTCTCTCTCACACACACAC	$(TC)_6(AC)_{11}$	134–140	58	AB716700

Note: T_a = optimal annealing temperature.
[a]The fluorochrome (6-FAM or HEX) is shown with the reverse primer sequence.

Two hundred and fifty insert-positive clones were amplified using M13 forward primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-AACAGCTATGACCATG-3′). The amplification profiles included initial denaturation at 95°C for 9 min; followed by 40 cycles of 30 s at 94°C, 1.5 min at 54°C, and 1 min at 72°C; and a final extension at 72°C for 4 min. PCR was performed in a total reaction volume of 10 µL containing 6.75 µL sterilized water, 0.08 mM dNTP mixture, 0.25 U TaKaRa *Ex Taq* (TaKaRa Biotechnology Co.), 1.0 mM reaction buffer (TaKaRa *Ex Taq*), 0.2 µM of each primer, and 1.0 µL of template DNA (10 ng/µL). The PCR products in a total reaction volume of 5 µL, using M13 forward primer, were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For 48 fragments containing the $(AC)_6(AG)_n$ or $(TC)_6(AC)_n$ compound SSR sequence at one end, primers were designed using Primer3 version 0.4.0 (Rozen and Skaletsky, 2000). Each primer was designed to have <50% of GC content with optimal annealing temperature at 58°C, and total size of approximately 20–25 bp. PCR amplifications were performed following the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN) in a final volume of 6 µL. Compound SSR primers $[(AC)_6(AG)_5$ or $(TC)_6(AC)_5]$ were labeled with the fluorochromes 6-FAM or HEX (Applied Biosystems). The amplification profiles included initial denaturation at 95°C for 15 min; followed by 35 cycles of 30 s at 94°C, 1.5 min at 58°C, and 1 min at 72°C; and a final extension at 60°C for 30 min. The size of the PCR products was measured using the ABI PRISM 3100 Genetic Analyzer with GeneScan 350 ROX Size Standard (Applied Biosystems) and GeneMapper analysis software (Applied Biosystems).

Eleven microsatellite loci that showed a clear and strong band on the electrophoretic gel were selected in the preliminary screening (Table 1). Each reverse primer was designed to contain six repeats of AC followed by five repeats of AG $[(AC)_6(AG)_5]$ or six repeats of TC followed by five repeats of AC $[(TC)_6(AC)_5]$. For further characterization, 20 individuals of *T. macrantha* were genotyped using the procedure described above. These plant samples were collected from a population at Mt. Yokokura in Kochi Prefecture, Japan. Three loci were monomorphic and the number of alleles per locus varied from four to seven in polymorphic loci (Table 2). The observed heterozygosity and genetic diversity per locus (H_S; Nei, 1987) ranged from 0.050 to 0.851 and 0.142 to 0.812, respectively.

Tests for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were conducted using FSTAT version 2.9.3.2 (Goudet, 1995). Significant deviations ($P < 0.05$) from HWE were detected in all polymorphic loci except TM_11. No significant linkage disequilibrium was found in the pairwise comparisons of the polymorphic loci. Of the 11 microsatellite markers developed for *T. macrantha*, eight, four, and four were successfully amplified in *T. ishiiana*, *T. ishiiana* var. *surugensis*, and *T. macranthopsis*, respectively (Table 2).

CONCLUSIONS

The microsatellite markers presented here will be useful for investigating the genetic diversity and population structure of the species of *Tricyrtis* sect. *Brachycyrtis*. The markers that amplified in all species of *Tricyrtis* sect. *Brachycyrtis*, developed by Setoguchi et al. (2011) and this study, are now being used to

TABLE 2. Results of initial primer screening in one population of *Tricyrtis macrantha* and three individuals each of *T. ishiiana*, *T. ishiiana* var. *surugensis*, and *T. macranthopsis.*

	T. macrantha ($N = 20$)			Cross-amplification		
Locus	A	H_o	H_S	I	IS	MP
TM_1*	4	0.050	0.466	—	—	+
TM_2*	5	0.158	0.740	+	+	+
TM_3*	4	0.851	0.574	+	+	+
TM_4*	4	0.800	0.733	—	—	+
TM_5	1	—	—	+	+	+
TM_6	1	—	—	—	—	+
TM_7*	2	0.150	0.142	—	—	—
TM_8*	2	0.200	0.184	—	—	+
TM_9*	7	0.550	0.812	—	—	—
TM_10	1	—	—	—	—	—
TM_11	5	0.651	0.670	+	+	+

Note: + = successful PCR amplification; — = unsuccessful PCR amplification; A = number of alleles per locus; H_o = observed heterozygosity; H_S = genetic diversity; I = *Tricyrtis ishiiana*; IS = *Tricyrtis ishiiana* var. *surugensis*; MP = *Tricyrtis macranthopsis*.
* Deviations from Hardy–Weinberg equilibrium: $P < 0.05$.

carry out population genetic studies to aid in the development of conservation strategies for each species.

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