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

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**Primer Note**

**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*.

**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 JP112005*) 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 *SspI*, *EcoRV*, and *AluI*. The restricted fragments were ligated with a specific blunt adapter (consisting of the 48-mer 5′-GTAATACGACCTACTATAGGACCCGGCGCGGCGGCGGCGGGCCGCTGGTG3′ and an 8-mer with the 3′-end capped with an amino residue 5′-ACCCAGCCC-NH2-3′) using a DNA ligation kit (TaKaRa Biotechnology Co., Ohtsu, Shiga, Japan). Fragments were amplified from *Sspa*, *EcoRV*, and *AluI* DNA using one of the compound simple sequence repeat (SSR) primers (AC)64(AG)5 or (TC)64(AC)5 and an adapter primer (5′-CTAGGACCAGGCGTG-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 MgCl2 (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 55°C, 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® 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-thiogalactopyranoside (IPTG).

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Cross-amplification with primer (5) was approximately 20–25 bp. PCR amplifications were performed following the GC content with optimal annealing temperature at 58°C (Rozen and Skaletsky, 2000). Each primer was designed to have <50% of sequence at one end, primers were designed using Primer3 version 0.4.0.

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, 0.5 μM of primer, and 1 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 Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). For 48 fragments containing the (AC)₆(AG)₈ or (TC)₆(AC)₈ compound SSR sequence at one end, primers were designed using Primer3 version 4.0 (Rozen and Skeleteky, 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)₈] or (TC)₆(AC)₈] were labeled with the fluorochromes 6-FAM or HEX (Applied Biosystems). 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. Two hundred and fifty insert-positive clones were amplified using M13 for- 
ward primer (5′-GTTCATGAGTACGCGGACAC-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primer (5′-GTAA...
carry out population genetic studies to aid in the development of conservation strategies for each species.

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