Development and Characterization of Microsatellite Primers for Chamaecyparis obtusa (Cupressaceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE PRIMERS FOR *CHAMAECYPARIS OBTUSA* (CUPRESSACEAE)[1]

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- *Premise of the study:* Although several microsatellite markers of *Chamaecyparis obtusa* (Cupressaceae) have been reported in previous studies, we newly developed and evaluated polymorphic microsatellite markers of *C. obtusa*, an economically important species in Korea as a major forestation tree species, for further genetic studies.
- *Methods and Results:* Microsatellite-enrichment libraries were constructed according to the selective hybridization method. From this output, we obtained 10 polymorphic and five monomorphic microsatellite markers for *C. obtusa*. Five of these developed markers were successfully amplified for *C. pisifera*, a related species.
- *Conclusions:* These microsatellite markers can be valuable tools for further genetic studies of *C. obtusa*, and gathered genetic information may be useful for breeding program management.

Key words: *Chamaecyparis obtusa*; conifer; Cupressaceae; microsatellite.

Chamaecyparis obtusa (Sieb. & Zucc.) Endl. (Cupressaceae), an economically important tree species frequently used for construction and native to Japan, was first introduced to Korea in 1904 and mainly planted in the southern provinces (Yoon, 1959). Recently, this species has received attention in Korea for its ability to adapt to ongoing climate change and to be used for outdoor recreation as a "healing forest" (An et al., 2009). Therefore, the demand for seeds of *C. obtusa* has increased. With this increasing demand, there has been a growing interest in breeding research on *C. obtusa* using molecular markers. Microsatellite markers, with codominance and high polymorphism, are preferred for molecular breeding programs because they allow high genetic resolution (Smouse and Chevillon, 1998; Bernatchez and Duchesne, 2000). Previous studies have developed microsatellite markers for *C. obtusa* and conducted investigations of its genetic variation and population structure. Nakao et al. (2001) and Matsumoto et al. (2006) developed nine and 15 polymorphic markers, respectively. These developed markers were employed in further genetic studies on genetic diversity and structure in a natural fragmented population of *C. obtusa* (Matsumoto et al., 2010). However, microsatellite markers have some shortcomings, such as genotyping error caused by null alleles, mutation, or mistyping, which can generate false exclusion or produce ghost individuals. To overcome such problems, likelihood-based approaches are preferred. The accuracy of estimates resulting from these analyses is dependent on (i.e., increased with) the number of loci (Wang, 2015). Accordingly, previously developed markers might be insufficient to employ various methodologies of genetic analysis used in molecular breeding and breeding population management. Therefore, to secure enough polymorphic loci, we developed and evaluated additional microsatellite markers for further genetic studies for *C. obtusa*.

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METHODS AND RESULTS

A microsatellite-enrichment library was constructed on *C. obtusa* plants collected from seed production stands throughout South Korea. Genomic DNA was extracted from fresh leaves with a QIAamp 96 DNA QIAcube HT Kit (QIAGEN, Hilden, Germany) using QIAxtractor (QIAGEN). The library was constructed according to the method of Glenn and Schable (2005). Briefly, genomic DNA from the leaves of *C. obtusa* was digested with a restriction enzyme, *Rsa*I, to obtain DNA fragments approximately ranging from 300 to 1000 bp and then ligated with a modified SNX linker incorporated with a GTTT PIG-tail. This method should facilitate that the PCR product can be cloned directly without obtaining a large proportion of small DNA fragments and have efficient A-tailing to yield good results from TA cloning. The ligation products were subjected to double enrichment steps by hybridization with 3′-biotinylated microsatellite probes. The DNA fragments containing microsatellites were ligated to pGEM-T vectors (Promega Corporation, Madison, Wisconsin, USA), and the ligation mixture was transformed into competent *Escherichia coli* DH5α cells. The resulting colonies were subjected to colony PCR to identify recombinant clones using M13 forward and reverse primer sets. The PCR products were purified and directly used for sequencing by the ABI 3730 DNA Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). After the trimming of vector and linker sequences, the nucleotide sequences were assembled to generate nonredundant contigs using Lasergene SeqMan (ver. 7.0.0; DNASTAR, Madison, Wisconsin, USA). Putative SSRs were identified by MISA software (Thiel, 2010) with the following criteria: a minimum of three repeats for dinucleotides to hexanucleotides and a gap within 100 bp for composite class. Criteria for primer design were as follows: amplicon size of 85–350 bp and annealing temperature of 57–60°C. Twenty-one primers used in this study were synthesized by Biomedic Co. Ltd. (Bucheon, Korea; www.ibiomedic.co.kr). The primer specificity was validated by routine PCR using genomic DNA as templates. For the preliminary screening of markers to amplify putative single loci, routine genomic PCR was performed using genomic DNA from five samples of *C. obtusa* as templates. The PCR

TABLE 1. Repeat motif, primer sequence, and size range for amplified microsatellite loci in *Chamaecyparis* species.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)		T_a (°C)	GenBank accession no.
			C. obtusa	*C. pisifera*		
Co2011	F: TAAAAATCGCAAGGCCAACT R: TCGACCCAATTTGGAGTTACTT	$(GAA)_3$	244	238/241	54	KF689697
Co2018*	F: GCCCTAGAAACTCGGGGTAA R: GTTTGGGGCAATACACAGCGTAATA	$(CA)_{16}...(CA)_{11}$	240–270	—	62	KF689702
Co2023*	F: CACTATCGGGATGCTTGTGA R: GTTTAGAGAATGGACTCGATGCAAA	$(CT)_{28}$	210–310	—	55	KF689706
Co2033	F: CCATTGGGCAAGGTTTCTTA R: GATGCTTCAGATGGGACCTC	$(AC)_9$	145	145	55	KF689713
Co2043*	F: GGAATTAATTACCACGCTGAAA R: CCCCAAAAGCCTATGAGACA	$(CT)_{15}TTCTC(CA)_6$	160–210	—	60	KF689719
Co2047*	F: ATGCCCAAAACACACAAACA R: GTTTCTTCCCTTTGTCTCTTTGTCA	$(CT)_{21}$	200–280	—	56	KF689720
Co2050*	F: GCCCTCAACCTCAAACCATA R: GTTTCACACTCCCTTATCTCCCTCA	$(GA)_{31}$	110–180	224/228	56	KF689723
Co3008	F: GAGAATGCACCAGGCAATAAA R: GTTTGATCTTCAATTCCCTCCCATA	$(AG)_4$	152	—	55	KF689734
Co3016	F: GGAAAAAGTTTGGCATCGAG R: GTTTCTCCTTGTGCCAACACAGAA	$(ACAG)_5$	157	157	55	KF689739
Co3026*	F: CGAAACGAAACCAATTCCTC R: GTTTACCTCCTCCCACAATTCG	$(CTAT)_{19}$	210–290	—	60	KF689746
Co3033*	F: TGTCTCACCAATGCCAAAAG R: CCACTCCCCCTTTTCAAATC	$(ATAG)_8$	220–250	—	60	KF689750
Co3038	F: TTTCTCCCTTATCCACCTTCC R: TTCAACTCATCACATCATCCA	$(TCTG)_5$	242	242	55	KF689753
Co4012*	F: CTTGCATCCCTACCTTGCAT R: TTCCACTTCCATGTCAACCA	$(CATC)_8…(CTAC)_7$	140–190	—	55	KF689767
Co4014*	F: TTCCACTTCCATGTCAACCA R: GTTTCTTGCATCCCTACCTTGCAT	$(TAGG)_7$	150–200	—	62	KF689769
Co4049*	F: TTTGTCTGTTTATCTGCCTTTCTC R: GTTTGCTCCTATTGTGATTTTGAGTG	$(TCTA)_7$	340–390	—	56	KF689781

Note: — = information not available; T_a = annealing temperature.
* Polymorphic microsatellite loci.

products were separated on a 2% agarose gel. Twenty-one candidate microsatellite primers, which amplified putative single loci, were selected to screen polymorphic markers and labeled with fluorescent dye (FAM).

To validate the applicability of the 21 candidate primers to genetic studies, PCR amplifications were conducted for 90 samples from three seed production stands of *C. obtusa* located in Jeonbuk, Jeonnam, and Gyeongnam provinces, Korea. To test the transferability of the markers to the other related species, 12 samples of *C. pisifera* (Siebold & Zucc.) Endl. were collected in Chungbuk Province and used for further analysis. All the DNA samples used in this study were deposited at the Gene Bank of the National Forest Seed and Variety Center (NFSV, Korea); accession numbers and locality information are provided in Appendix 1. PCR was performed in 11-µL reactions containing 9 ng of template DNA, 1.5 or 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of 6-FAM fluorescent dye–labeled forward primer and reverse primer, 0.75 units of NeoTherm *Taq* DNA polymerase (GeneCraft, Hulme, United Kingdom), and 1× reaction buffer (GeneCraft). The PCR cycling was conducted as follows: 5 min at 94°C for predenaturation; 34 cycles of 30 s at 94°C, 1 min at 54–62°C for each primer, and 1 min at 72°C; and a final extension for 10 min at 72°C. The fluorescent PCR products were mixed with Hi-Di formamide and GeneScan 500 ROX Size Standard (Applied Biosystems). Those were visualized and scored using an ABI 3730 Genetic Analyzer (Applied Biosystems) and GeneMapper 4.1 software (Applied Biosystems).

Fifteen (71.4%) of the 21 candidate primers were successfully amplified for *C. obtusa.* Ten of these produced polymorphic DNA fragments, and the remaining five primers produced monomorphic amplicons (Table 1). The percentage of amplification for *C. pisifera* was 33.3% (5/15). Genetic properties of the 10 polymorphic primers for *C. obtusa* were evaluated (Table 2). The number of alleles (A), number of effective alleles (A_e), and observed (H_o) and expected heterozygosity (H_e) were obtained by GenAlEx version 6.41 (Peakall and Smouse, 2006), and polymorphic information content (PIC) was calculated with CERVUS version 3.0.3 (Kalinowski et al., 2007). In the total samples (90), A ranged from four to 24 per locus, A_e ranged from 1.2 to 14.2, and PIC values ranged from 0.160 to 0.927. H_o and H_e varied from 0.000 to 0.988 and from 0.165 to 0.929, respectively. The Co2047 marker, which had a high A_e value, could be a most efficient marker to infer current pollen flow using an indirect method such as TwoGener or a direct method such as parentage analysis, because high PIC values can be the result of relatively even allele frequency.

CONCLUSIONS

Ten polymorphic microsatellite markers developed for *C. obtusa* are available for genetic studies, such as analyses of current pollen flow, seed flow, mating system, and population genetic structure. Of these microsatellite markers developed in *C. obtusa*, 33.3% were successfully amplified in the related species *C. pisifera*. The genetic information gathered by these markers could be useful for breeding program management of *C. obtusa*.

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TABLE 2. Genetic properties of 10 polymorphic microsatellite loci of *Chamaecyparis obtusa*.

	Jeonbuk ($N = 30$)					Jeonnam ($N = 45$)					Gyeongnam ($N = 15$)					Total ($N = 90$)				
Locus	n	A	A_e	H_o	H_e	n	A	A_e	H_o	H_e	n	A	A_e	H_o	H_e	n	A	A_e	H_o	H_e	PIC
Co2023	29	16	5.8	0.759*	0.827	42	17	10.9	0.881*	0.908	14	9	4.8	0.857*	0.791	85	24	9.9	0.835	0.899	0.889
Co2043	30	10	2.7	0.533*	0.635	42	7	3.6	0.690*	0.720	15	10	6.5	0.933	0.847	87	13	3.8	0.678	0.734	0.704
Co2047	27	12	8.1	1.000*	0.876	43	20	12.3	1.000*	0.919	15	12	9.2	0.933*	0.891	85	22	14.2	0.988	0.929	0.927
Co2050	29	14	9.7	1.000*	0.897	44	17	9.2	0.773*	0.891	15	10	7.8	0.867*	0.871	88	22	11.3	0.864	0.911	0.904
Co3026	27	4	1.8	0.000*	0.453	41	5	1.8	0.000*	0.458	13	3	2.6	0.000*	0.615	81	5	2.0	0.000	0.496	0.439
Co3033	26	2	1.8	0.385	0.453	44	4	2.2	0.500	0.537	15	2	1.8	0.667*	0.444	85	4	2.0	0.494	0.497	0.413
Co4012	24	4	1.7	0.500	0.416	44	5	1.4	0.341	0.301	15	4	1.6	0.467	0.389	83	5	1.6	0.410	0.355	0.336
Co4014	29	3	1.2	0.172	0.160	43	3	1.1	0.116	0.111	14	3	1.4	0.071*	0.304	86	5	1.2	0.128	0.165	0.160
Co4049	30	6	3.6	0.867*	0.722	43	9	5.8	0.953	0.826	15	6	4.8	0.733*	0.791	88	9	5.1	0.886	0.802	0.775
Co2018	18	6	4.2	0.556	0.761	29	9	4.5	0.690*	0.780	12	5	3.3	0.583	0.694	59	9	4.6	0.627	0.781	0.750

Note: A = number of alleles per locus; A_e = number of effective alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; n = number of individuals genotyped; PIC = polymorphism information content.

*Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

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Appendix 1. Accession numbers and locality information of *Chamaecyparis* samples used in this study. All DNA samples are deposited at the Gene Bank of the National Forest Seed and Variety Center (NFSV), Chungju, Korea.

Species	Accession no.	Collection locality[a]	Geographic coordinates
Chamaecyparis obtusa (Siebold & Zucc.) Endl.	0300-03-00041555	Jeonbuk	35°43′01.83″N, 127°13′29.72″E
	0300-03-00041556	Jeonbuk	35°43′01.67″N, 127°13′29.56″E
	0300-03-00041557	Jeonbuk	35°43′01.74″N, 127°13′29.91″E
	0300-03-00041558	Jeonbuk	35°43′01.83″N, 127°13′29.87″E
	0300-03-00041559	Jeonbuk	35°43′01.61″N, 127°13′29.67″E
	0300-03-00041560	Jeonbuk	35°43′01.64″N, 127°13′29.48″E
	0300-03-00041561	Jeonbuk	35°43′01.48″N, 127°13′29.04″E
	0300-03-00041562	Jeonbuk	35°43′01.41″N, 127°13′29.32″E
	0300-03-00041567	Jeonbuk	35°43′01.54″N, 127°13′29.75″E
	0300-03-00041568	Jeonbuk	35°43′01.48″N, 127°13′29.75″E
	0300-03-00041569	Jeonbuk	35°43′01.48″N, 127°13′30.03″E
	0300-03-00041637	Jeonbuk	35°43′00.02″N, 127°13′30.51″E
	0300-03-00041661	Jeonbuk	35°42′59.56″N, 127°13′30.31″E
	0300-03-00041693	Jeonbuk	35°42′59.14″N, 127°13′31.26″E
	0300-03-00041725	Jeonbuk	35°42′58.39″N, 127°13′32.17″E
	0300-03-00039814	Jeonbuk	35°24′14.47″N, 126°43′23.35″E
	0300-03-00039838	Jeonbuk	35°24′14.96″N, 126°43′24.42″E
	0300-03-00039870	Jeonbuk	35°24′14.64″N, 126°43′26.56″E
	0300-03-00039902	Jeonbuk	35°24′14.05″N, 126°43′24.58″E
	0300-03-00039916	Jeonbuk	35°24′14.38″N, 126°43′25.84″E
	0300-03-00039917	Jeonbuk	35°24′14.38″N, 126°43′26.24″E
	0300-03-00039919	Jeonbuk	35°24′14.25″N, 126°43′26.04″E
	0300-03-00039922	Jeonbuk	35°24′14.38″N, 126°43′26.76″E
	0300-03-00039923	Jeonbuk	35°24′14.29″N, 126°43′26.88″E
	0300-03-00039924	Jeonbuk	35°24′14.22″N, 126°43′26.68″E
	0300-03-00039925	Jeonbuk	35°24′14.16″N, 126°43′26.84″E
	0300-03-00039928	Jeonbuk	35°24′14.26″N, 126°43′27.23″E
	0300-03-00039929	Jeonbuk	35°24′14.19″N, 126°43′27.11″E
	0300-03-00039930	Jeonbuk	35°24′14.09″N, 126°43′27.15″E
	0300-03-00039931	Jeonbuk	35°24′13.96″N, 126°43′27.03″E
	0300-03-00040129	Jeonnam	35°21′22.27″N, 126°53′33.46″E
	0300-03-00040161	Jeonnam	35°21′22.85″N, 126°53′31.88″E
	0300-03-00040177	Jeonnam	35°21′23.28″N, 126°53′32.43″E
	0300-03-00040185	Jeonnam	35°21′22.92″N, 126°53′32.87″E
	0300-03-00040495	Jeonnam	35°21′20.72″N, 126°53′35.68″E
	0300-03-00040498	Jeonnam	35°21′20.88″N, 126°53′35.96″E
	0300-03-00040502	Jeonnam	35°21′20.59″N, 126°53′35.76″E
	0300-03-00040505	Jeonnam	35°21′20.68″N, 126°53′36.00″E
	0300-03-00042405	Jeonnam	35°00′45.27″N, 127°10′51.45″E
	0300-03-00042429	Jeonnam	35°00′45.17″N, 127°10′52.08″E
	0300-03-00042461	Jeonnam	35°00′45.66″N, 127°10′52.32″E
	0300-03-00042485	Jeonnam	35°00′46.11″N, 127°10′51.03″E
	0300-03-00042299	Jeonnam	35°00′44.72″N, 127°10′48.85″E
	0300-03-00042300	Jeonnam	35°00′44.75″N, 127°10′48.97″E
	0300-03-00042303	Jeonnam	35°00′44.88″N, 127°10′49.08″E
	0300-03-00041744	Jeonnam	35°00′45.82″N, 127°10′48.18″E
	0300-03-00039118	Jeonnam	34°45′37.04″N, 126°44′05.36″E
	0300-03-00039174	Jeonnam	34°45′36.27″N, 126°44′07.17″E
	0300-03-00039200	Jeonnam	34°45′37.96″N, 126°44′08.66″E
	0300-03-00039201	Jeonnam	34°45′37.89″N, 126°44′08.81″E
	0300-03-00039206	Jeonnam	34°45′36.56″N, 126°44′08.05″E
	0300-03-00039210	Jeonnam	34°45′37.31″N, 126°44′08.42″E
	0300-03-00039211	Jeonnam	34°45′37.47″N, 126°44′08.05″E
	0300-03-00042486	Jeonnam	34°40′08.12″N, 126°56′06.41″E
	0300-03-00042487	Jeonnam	34°40′07.99″N, 126°56′06.37″E
	0300-03-00042488	Jeonnam	34°40′08.02″N, 126°56′06.29″E
	0300-03-00042489	Jeonnam	34°40′08.06″N, 126°56′06.13″E
	0300-03-00042501	Jeonnam	34°40′08.09″N, 126°56′05.43″E
	0300-03-00042525	Jeonnam	34°40′07.54″N, 126°56′05.07″E
	0300-03-00042557	Jeonnam	34°40′07.05″N, 126°56′05.27″E
	0300-03-00042589	Jeonnam	34°40′06.03″N, 126°56′03.15″E
	0300-03-00045093	Jeonnam	34°54′29.34″N, 126°49′36.74″E
	0300-03-00045101	Jeonnam	34°54′30.38″N, 126°49′37.96″E
	0300-03-00045102	Jeonnam	34°54′30.12″N, 126°49′37.92″E
	0300-03-00045104	Jeonnam	34°54′30.06″N, 126°49′38.28″E
	0300-03-00045117	Jeonnam	34°54′29.67″N, 126°49′38.95″E
	0300-03-00045149	Jeonnam	34°54′30.81″N, 126°49′40.09″E
	0300-03-00045155	Jeonnam	34°54′30.61″N, 126°49′40.44″E

Appendix 1. Continued.

Species	Accession no.	Collection locality[a]	Geographic coordinates
	0300-03-00045157	Jeonnam	34°54′30.52″N, 126°49′40.76″E
	0300-03-00045163	Jeonnam	34°54′29.64″N, 126°49′39.07″E
	0300-03-00045164	Jeonnam	34°54′29.57″N, 126°49′39.58″E
	0300-03-00045170	Jeonnam	34°54′29.74″N, 126°49′40.96″E
	0300-03-00045171	Jeonnam	34°54′29.74″N, 126°49′40.08″E
	0300-03-00045172	Jeonnam	34°54′29.08″N, 126°49′41.35″E
	0300-03-00045173	Jeonnam	34°54′29.93″N, 126°49′40.99″E
	0300-03-00047493	Gyeongnam	34°46′58.18″N, 127°58′19.49″E
	0300-03-00047517	Gyeongnam	34°46′57.89″N, 127°58′20.39″E
	0300-03-00047549	Gyeongnam	34°46′57.17″N, 127°58′20.07″E
	0300-03-00047581	Gyeongnam	34°46′57.07″N, 127°58′20.31″E
	0300-03-00046437	Gyeongnam	34°46′59.12″N, 127°58′04.09″E
	0300-03-00046438	Gyeongnam	34°46′59.12″N, 127°58′04.28″E
	0300-03-00046439	Gyeongnam	34°46′58.99″N, 127°58′04.16″E
	0300-03-00046440	Gyeongnam	34°46′58.86″N, 127°58′04.12″E
	0300-03-00046441	Gyeongnam	34°46′58.83″N, 127°58′04.28″E
	0300-03-00046442	Gyeongnam	34°46′58.73″N, 127°58′04.16″E
	0300-03-00046443	Gyeongnam	34°46′58.73″N, 127°58′04.28″E
	0300-03-00046444	Gyeongnam	34°46′58.79″N, 127°58′04.36″E
	0300-03-00046445	Gyeongnam	34°46′58.92″N, 127°58′04.04″E
	0300-03-00046446	Gyeongnam	34°46′58.95″N, 127°58′04.56″E
	0300-03-00046447	Gyeongnam	34°46′58.82″N, 127°58′04.56″E
Chamaecyparis pisifera (Siebold & Zucc.) Endl.	0300-03-00085617–0300-03-00085628	Chungbuk	36°52′37.02″N, 127°58′25.80″E

[a] Province in Korea.