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Primer Note

Development of nuclear and chloroplast microsatellite markers for the endangered conifer *Callitris sulcata* (Cupressaceae)[1]

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- *Premise of the study:* Microsatellite markers were developed for *Callitris sulcata* (Cupressaceae), an endangered conifer species in New Caledonia.
- *Methods and Results:* Using sequencing by synthesis (SBS) of an RNA-Seq library, 15 polymorphic nuclear and chloroplast microsatellite markers were developed. When evaluated with 48 individuals, these markers showed genetic variations ranging from two to 15 alleles and expected heterozygosity ranging from 0 to 0.881.
- *Conclusions:* These markers will be useful for examining the genetic diversity and structure of remaining wild populations and improving the genetic status of ex situ populations.

Key words: *Callitris sulcata*; Cupressaceae; microsatellites; New Caledonia; sequencing by synthesis.

Callitris sulcata (Parl.) Schltr. (Cupressaceae) is a conifer species endemic to the ultramafic massif of southern New Caledonia. This species occurs on lowland gallery rainforests, usually close to rivers, and grows as thickets or scattered with other tree species (Farjon, 2005). Total population size is estimated to be less than 2500 individuals, and the species is recognized as endangered by the International Union for Conservation of Nature (IUCN) Red List (Thomas, 2010). An ex situ propagation program is being undertaken to secure the wild populations, but there is no genetic information on how much species-wide genetic diversity the ex situ populations can capture and how one can manage plants derived from different river systems. Thus, there is an urgent need for development of genetic markers useful for conservation genetics.

METHODS AND RESULTS

Total RNA was extracted from a one-year-old seedling derived from the Comboui River (21°28′24″S, 166°14′35″E) using the Agilent Plant RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, California, USA). Sequencing by synthesis of the normalized cDNA library was performed with a genome sequencer HiSeq 2000 (Illumina, San Diego, California, USA; sequencing performed by BGI, Shenzhen, China), which produced 44,159,690 cleaned 100-bp reads. CLC Genomics Workbench 7.5.1 (CLC bio, Aarhus, Denmark) was used to run de novo assembly (parameters used: mismatch cost 3, insertion and deletion cost 2, length fraction 0.5, similarity fraction 0.9), resulting in 63,448 contigs with an N50 length of 1348 bp. Using MSATCOMMANDER (Faircloth, 2008), we screened the contigs including microsatellite regions for ≥6 dinucleotide repeats and ≥4 tri- to hexanucleotide repeats. A total of 138 primer pairs were designed using Primer3 (Rozen and Skaletsky, 1999) with an optimal annealing temperature of 60(±1)°C, GC content 30–70%, and product size ranges of 100–500 bp, 49 of which were selected based on microsatellite repeat number for the first PCR amplification trials using three individuals from each of the three known populations (n = 9; collected from Comboui River [21°47′33″S, 166°22′20″E], Dumbea River [22°7′15″S, 166°31′2″E], and Tontouta River [21°52′52″S, 166°19′12″E]). The sequence data for the remaining primer loci are available from the authors upon request. For all the loci, the forward primer was synthesized with one of three different M13 sequences

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This study was undertaken by the Institut Agronomique néo-Calédonien (IAC) as part of the action plan developed by the tripartite partnership (Convention no. 2012/49) between Thio Council, Noé Conservation, and the IAC to improve the conservation status of *Callitris sulcata*. The authors thank the traditional owners of the study area for supporting this research program. The South Province of New Caledonia gave financial support as a part of the work IAC dedicated to rare and endangered species, and authorized by exception the collection of *C. sulcata* material. Funding was also provided by a Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (JSPS KAKENHI 26850098, 13J06059) and the Environment Research and Technology Development Fund of the Ministry of the Environment (4-1403).

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TABLE 1. Characteristics of 24 microsatellite markers developed for *Callitris sulcata*.

Locus[a]	Primer sequences (5′–3′)[b]	Repeat motif	Allele size range (bp)	Fluorescent dye[c]	Multiplex PCR set[d]	BLASTX top hit description [species][e]	*E*-value	GenBank accession no.
Csul_257*	F: CACGACGTTGTAAAACGACGCCAGATTGAGCAGAGGAAG	$(AT)_{12}$	206	2	3	No hit	—	FX982972
	R: GTTTCTTGGTGAAGGGATGCTTGAACC							
Csul_1067	F: CTATAGGGCACGCGTGGTGAACAATACGTCCCGCCATG	$(AT)_9$	440–444	4	3	Chloroplast genome [*Callitropsis nootkatensis*]	0	FX982962
	R: GTTTCTTTCGGAGTCCTTCATTTGCCC							
Csul_1298*	F: CTATAGGGCACGCGTGGTAGGGCTTGCTATGTACAGGG	$(AG)_{10}$	393	4	2	No hit	—	FX982964
	R: GTTTCTTAGCGGAGAACAAACAAACGC							
Csul_1566*	F: CACGACGTTGTAAAACGACCCCGAATCTTCTCACACCAC	$(AG)_{11}$	506	2	2	No hit	—	FX982968
	R: GTTTCTTTGGTAGAGCGTTGTGGAGAG							
Csul_3143*	F: CTATAGGGCACGCGTGGTTCCCATGGAGATTTGGCATG	$(AT)_{12}$	358	4	1	Unknown protein [*Picea glauca*]	0	FX982973
	R: GTTTCTTAACCAATATCGGCATGCGTG							
Csul_3144*	F: CTATAGGGCACGCGTGGTTCTCGCGGATCACATAGACG	$(AG)_{13}$	373	4	3	No hit	—	FX982974
	R: GTTTCTTGCATGGTAGGTACAGGGACC							
Csul_3288	F: CTATAGGGCACGCGTGGTGTCTATTGTGCTGCGCTCTC	$(AG)_{15}$	316–346	4	3	No hit	9.00E-42	FX982975
	R: GTTTCTTCAATCGCGAAGGATGAGGTG							
Csul_3325	F: CACGACGTTGTAAAACGACACTGTGCCTCTTTAATGGGC	$(AG)_{18}$	238–266	2	1	No hit	—	FX982976
	R: GTTTCTTTCTGCTCAGCTGCTCCATTC							
Csul_3584	F: TGTGGAATTGTGAGCGGAACATAATGGGCAGGTCTCC	$(AG)_{14}$	188–206	3	2	Ion protease homolog [*Nelumbo nucifera*]	0	FX982977
	R: GTTTCTTTTCCCATTCGGCTGTCAATG							
Csul_4758	F: CTATAGGGCACGCGTGGTCGCTATGCAACACTCACCTG	$(AT)_{11}$	198–228	4	1	No hit	—	FX982979
	R: GTTTCTTCATTGTGCAGTGAGACCTGG							
Csul_6899	F: CACGACGTTGTAAAACGACTTCTCCAGTTTCGCAGCAAC	$(AT)_{15}$	408–432	2	3	Casein kinase I isoform delta-like [*Nelumbo nucifera*]	1.00E-130	FX982981
	R: GTTTCTTCACGGGTCATGGTTAAGTGC							
Csul_7561*	F: CACGACGTTGTAAAACGACTGAGTTGTAGAGCAGCCAGG	$(AG)_{13}$	351	2	2	No hit	—	FX982982
	R: GTTTCTTGGTACAAATTCCACCCTGCAG							
Csul_8090*	F: TGTGGAATTGTGAGCGGAAACATGCCATGATCTGCCG	$(AGC)_{10}$	494	3	1	No hit	—	FX982983
	R: GTTTCTTTGCACCATTTACAGCGGTTC							
Csul_10000	F: CACGACGTTGTAAAACGACCCCACTGTCAAGAATGTTCCC	$(AG)_{11}$	217–227	2	2	Serine/threonine-protein kinase PBS1-like [*Citrus sinensis*]	4.00E-145	FX982960
	R: GTTTCTTGCTGAGGGTGCATCTGTTTC							
Csul_10591	F: CTATAGGGCACGCGTGGTGGCCACAAGTTCTTTCTGCC	$(AG)_{14}$	246–286	4	1	Unknown protein [*Picea glauca*]	0	FX982961
	R: GTTTCTTTGAAGGCTCAAGTTTGCACG							
Csul_12276	F: CACGACGTTGTAAAACGACGAGGCAGCAATTGATAGCGG	$(AAG)_{10}$	132–153	2	1	No hit	—	FX982963
	R: GTTTCTTTCTTCGTCTGTTTCAATCCCTG							
Csul_13471	F: CTATAGGGCACGCGTGGTCCTATGCATTGGGAAGAGCC	$(AG)_{13}$	290–310	4	2	No hit	—	FX982965
	R: GTTTCTTCACAACTGTCCAGCTTGCTC							
Csul_13965	F: TGTGGAATTGTGAGCGGTCGCGTTTGAGTGATATGCC	$(AG)_{22}$	319–339	3	2	No hit	—	FX982966
	R: GTTTCTTAGTACGAGATATGGCGGCTC							
Csul_14454	F: CACGACGTTGTAAAACGACTGAGGGTTTGGGTTCATGTG	$(AG)_{13}$	293–319	2	2	No hit	—	FX982967
	R: GTTTCTTTGAAATGACCTTGAGCAGTGC							
Csul_18188*	F: CACGACGTTGTAAAACGACCTGGAAATCGCTGACCATGG	$(AT)_{11}$	362	2	3	No hit	—	FX982969
	R: GTTTCTTTGTGAAGATGACGTTGCTGC							
Csul_22047	F: TGTGGAATTGTGAGCGGCGGAATGATCGGATGAACGG	$(AG)_{15}$	176–216	3	1	No hit	—	FX982970
	R: GTTTCTTTCCGCTTCTTGTTTCGACAC							
Csul_23874	F: TGTGGAATTGTGAGCGGCATGTGAATGGAAGGCGGAG	$(AT)_{14}$	320–338	3	3	RNA-binding protein 24 [*Sesamum indicum*]	3.00E-47	FX982971
	R: GTTTCTTGCTTCTGATGCTGAGCCATG							
Csul_42274*	F: CTATAGGGCACGCGTGGTGACTCTAATCTTGAGGCGTGTG	$(AC)_{11}$	301	4	3	No hit		FX982978
	R: GTTTCTTGATTTCTTCCAGGTGCCAGC							
Csul_58499	F: CTATAGGGCACGCGTGGTTGGGAGTGAAGGCCATGAAG	$(AG)_{11}$	217–253	4	2	No hit	—	FX982980
	R: GTTTCTTGATGGCCCAGAAATTCCCAG							

[a] Monomorphic loci are marked with an asterisk.

[b] Forward and reverse primer sequence with tag sequence. One of the M13 sequences (5′-CACGACGTTGTAAAACGAC-3′, 5′-TGTGGAATTGTGAGCGG-3′, or 5′-CTATAGGGCACGCGTGGT-3′) was added to the forward primer, and the reverse primer was tagged with a PIG-tail sequence (5′-GTTTCTT-3′).

[c] Fluorescent label used for the M13 sequence.

[d] Annealing temperature in PCRs is 60°C for all loci.

[e] Putative functional annotation by the NCBI nr database search.

TABLE 2. Genetic diversity of the 15 polymorphic microsatellite markers for *Callitris sulcata*.[a]

	Comboui River (n = 24)			Dumbea River (n = 24)			Total (n = 48)		
Locus	A	H_e	H_o	A	H_e	H_o[b]	A	H_e	H_o[b]
Csul_1067[c]	2	0	0	2	0	0	2	0	0
Csul_3288	7	0.675	0.625	5	0.717	0.625	8	0.778	0.625
Csul_3325	5	0.444	0.458	5	0.588	0.542	7	0.536	0.500
Csul_3584	5	0.671	0.583	5	0.677	0.750	7	0.720	0.667
Csul_4758	4	0.539	0.583	11	0.837	0.792	11	0.758	0.688
Csul_6899	6	0.612	0.542	8	0.695	0.458*	10	0.670	0.500*
Csul_10000	4	0.157	0.167	4	0.636	0.667	5	0.471	0.417
Csul_10591	5	0.664	0.667	10	0.731	0.625	12	0.771	0.646
Csul_12276	2	0.375	0.333	4	0.584	0.625	4	0.581	0.479
Csul_13471	6	0.652	0.875	7	0.745	0.542	8	0.745	0.708
Csul_13965	6	0.735	0.875	5	0.473	0.500	8	0.650	0.688
Csul_14454	8	0.802	0.875	9	0.786	0.750	11	0.847	0.813
Csul_22047	6	0.332	0.375	12	0.803	0.875	15	0.748	0.625
Csul_23874	7	0.804	0.833	9	0.859	0.792	10	0.881	0.813
Csul_58499	8	0.793	0.792	11	0.841	0.792	13	0.850	0.792
Average[d]	5.4	0.590	0.613	7.1	0.712	0.683	8.7	0.715	0.651

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals genotyped.

[a]The vouchers representing the two populations are deposited at Kyoto University herbarium (accession no. KYO 00020000 for the Comboui River population and KYO 00019999 for the Dumbea River population).

[b]Asterisk denotes significant departure from Hardy–Weinberg equilibrium ($P < 0.05$).

[c]A locus located on the chloroplast genome.

[d]Average values were calculated based on nuclear markers.

(5′-CACGACGTTGTAAAACGAC-3′, 5′-TGTGGAATTGTGAGCGG-3′, or 5′-CTATAGGGCACGCGTGGT-3′), and the reverse primer was tagged with a PIG-tail sequence (5′-GTTTCTT-3′). A similarity search of the contigs against the National Center for Biotechnology Information (NCBI) nr database was conducted by BLASTX algorithm (Altschul et al., 1990) with an *E*-value cutoff of 1.0E-5. A PCR reaction was performed following the protocol of the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany), in a final volume of 10 µL, containing approximately 5 ng of DNA, 5 µL of 2× Multiplex PCR Master Mix, 0.01 µM of forward primer, 0.2 µM of reverse primer, and 0.1 µM of fluorescently labeled M13 primer. The PCR thermal profile was as follows: denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 60°C for 3 min, 68°C for 1 min; and a final 20-min extension step at 68°C. PCR product was loaded with DNA Size Standard 600 (Beckman Coulter, Brea, California, USA) onto the GenomeLab GeXP Genetic Analysis System (Beckman Coulter), and fragment size was determined by CEQ Fragment Analysis Software (Beckman Coulter). For the 24 primer pairs that showed clear microsatellite peaks (Table 1), the DNA of each of 24 individuals from Comboui and Dumbea rivers (n = 48) was used for multiplex PCR amplification to evaluate genetic polymorphism. Due to the limited number of samples currently available, we did not use the Tontouta River population sample for the second PCR trials. To characterize each marker, the following summary statistics were calculated using FSTAT 2.9.3 (Goudet, 1995): number of alleles per locus (A), expected heterozygosity (H_e), and observed heterozygosity (H_o). The significance of Hardy–Weinberg equilibrium and genotypic equilibrium were tested by randomization procedures using FSTAT 2.9.3.

Among the 24 loci tested in the second PCR trials, genetic polymorphism was detected in 15, which included 14 nuclear markers and one chloroplast marker. Identification of a chloroplast locus (Csul_1067) was based on significantly high similarity with chloroplast genome sequences of multiple conifer species. The allelic variation in 14 nuclear microsatellite loci ranged from four to 15 alleles, while H_e and H_o ranged from 0.471 to 0.881 and 0.417 to 0.813, respectively (Table 2). No significant departures from Hardy–Weinberg equilibrium were detected in each population, except for one locus (Csul_6899) in the Dumbea River population. No evidence for genotypic equilibrium for any pair of loci was detected ($P > 0.01$). In the chloroplast locus (Csul_1067), which is known to be paternally inherited in this genus (Sakaguchi et al., 2014), two alleles were detected across two populations (Table 2).

CONCLUSIONS

The markers developed in this study will be useful for population genetic research to investigate effective population size and levels of gene flow between the geographically isolated populations. Genotyping will be performed also for ex situ populations to evaluate whether they represent genetic diversity retained in each population. Understanding the genetic diversity and structure in both wild and ex situ populations will help to prioritize conservation efforts and improve genetic status of ex situ populations of this endangered species.

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