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PRIMER NOTE

MICROSATELLITE PRIMERS FOR THE PACIFIC NORTHWEST CONIFER *CALLITROPSIS NOOTKATENSIS* (CUPRESSACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed for Nootka cypress (*Callitropsis nootkatensis*) to provide quantitative measures for gene conservation that can assist in guiding management decisions for a species experiencing climate-induced decline.
- *Methods and Results:* Using multiplexed massively parallel sequencing, we identified 136,785 microsatellite-containing sequences from 489,625 Illumina paired-end 80-bp reads. After stringent filtering, we selected 144 primer pairs and screened variation at these loci in five populations of *C. nootkatensis.* Loci show between three and 36 dinucleotide repeats per locus, with an average of 13. Screening of these markers in the Pacific Northwest relative *Chamaecyparis lawsoniana* demonstrated no marker transferability. This finding highlights the narrow taxonomic utility of microsatellite markers in *Callitropsis*.
- *Conclusions:* These microsatellites show high polymorphism and can be used for routine screening of natural variation in *Callitropsis nootkatensis*, and will be particularly helpful in identifying clones and inbred relatives at the stand-level.

Key words: *Callitropsis nootkatensis*; *Chamaecyparis nootkatensis*; climate change; germplasm; Pacific Northwest; Nootka cypress.

Nootka cypress or yellow-cedar (*Callitropsis nootkatensis* (D. Don) Oerst. [1864]; syn. *Chamaecyparis nootkatensis* (D. Don.) Spach [1841]) is a North American conifer with a complex phylogenetic history and affinities to New World *Cupressus* L. and Asian *Xanthocyparis* Farjon & T. H. Nguyên (Yang et al., 2012). The species spans 18° of latitude from northern California to Alaska's Kenai Peninsula, and is common in coastal ranges characterized by heavy snowfall. Nootka cypress is ecologically important in coastal forests, and economically and culturally important for its fibrous bark and decay-resistant wood.

Nootka cypress is experiencing a widespread decline, as forest health surveys have identified >250,000 ha of dead and dying Nootka cypress in the coastal temperate rainforest of southeastern Alaska and adjacent British Columbia, Canada (Hennon et al., 2005; Hennon et al., 2012). Evidence connects the decline to recent changes in climate, as reduced late season snowpack increases the likelihood of cold injury to shallow-rooted trees (Hennon et al., 2012). Identification of genotypes with slow dehardening or enhanced spring cold tolerance is key to restoration, and this requires the development of restoration germplasm. DNA markers are valuable in managing germplasm resources, as they provide unique identities for genotypes in orchards, offer insights into the frequency and distribution of clonal reproduction in wild stands, and clarify spatial differentiation across the range of the species. Five microsatellite markers were recently described for Nootka cypress from British Columbia (Bérubé et al., 2003), but these show poor transferability to populations in Alaska (Jennings, unpublished observations). Here, we describe additional microsatellite markers for Nootka cypress that are derived from an Alaskan source and are useful for Alaskan populations. When combined with existing microsatellites, these markers can be used to estimate the impact of "yellow-cedar decline" on genetic diversity of the species across its range in Alaska and adjacent British Columbia.

METHODS AND RESULTS

Genomic DNA of one tree (Tongass National Forest no. 199, Oregon State University herbarium [OSC] voucher 232031) forms the basis of this resource. Genomic DNA was extracted from one seed using the FastPrep DNA kit (MP Biomedicals, Solon, Ohio, USA). Microsatellites were isolated and sequenced using multiplexed massively parallel sequencing (MMPS) with 80-bp paired reads on the Illumina GA II (Illumina, San Diego, California, USA), as described by Jennings et al. (2011). Briefly, this approach combines microsatellite enrichment using biotinylated probes ($[CT]_{15}$, $[GT]_{15}$, $[CA]_{15}$) with MMPS to generate a library of microsatellite-containing microreads.

From a pool of 489,625 reads, 117,670 contained dinucleotide and trinucleotide microsatellites (repeat length ≥4 for each). We removed duplicate

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sequences with CD-HIT-454 version 4.5.4 (Niu et al., 2010) and only used repeat-containing sequences that had a microsatellite located near the center of the sequenced fragment (Jennings et al., 2011). Filtering yielded 490 sequences, all of which were used for primer design with BatchPrimer3 version 2.3.0 (You et al., 2008). Default parameters were used for all but the following variables: product length (min = 100 bp, max = 200 bp); primer length (min = 17 bp, max = 25 bp, optimum = 19 bp); annealing temperature (min = 48°C, max = 63°C, optimum = 54°C); and primers per sequence (1). Microreads are archived in the European Bioinformatics Institute (EBI accession no. ERP000647), and filtered microsatellite-containing reads and primers are available on the DRYAD repository (accession doi:10.5061/dryad.g625b; http://dx.doi.org/10.5061/dryad.g625b).

One hundred and forty-four primer pairs were selected for screening using DNA from four Nootka cypresses from across Alaska. Primers were screened by PCR in a 10-μL reaction containing 1–10 ng of genomic DNA, 0.2 mM each dNTP, 0.4 μM primer, and 5 μL 2× Phusion Flash Taq polymerase mix (New England Biolabs, Ipswich, Massachusetts, USA). Cycling conditions used an initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 45 s, 62°C for 45 s, and 72°C for 5 s; and a final extension of 72°C for 5 min. PCR products were screened on 3% agarose/Tris-acetate-EDTA gels to identify and discard primer combinations that yielded weak amplification or multibanded phenotypes. Based on this filter, we discarded 132 loci, primarily due to multibanded phenotypes. As noted (Jennings et al., 2011), multibanded phenotypes are common in microsatellite libraries from large conifer genomes, and these likely represent repetitive elements.

For the 12 markers passing our selection criteria (Table 1), forward primers were labeled with a fluorophore (FAM or HEX; Integrated DNA Technologies, Coralville, Iowa, USA) and screened on 50 individuals (10 individuals per population). This includes four populations from Alaska (Hawkins Island, Shelter Island, Kennel Creek, and Bullhead Cove) and a geographically distant population from Oregon, USA (John Day). Detailed sample localities are provided in Appendix 1, and sample vouchers are deposited at the Oregon State University herbarium (OSC 232031). Cross-species amplification of primers was tested using four individuals of *Chamaecyparis lawsoniana* (A. Murray) Parl. from southern Oregon, USA. Microsatellite fragment sizes were determined on the ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using GeneMapper 4.0 and GeneScan 500 ROX Size Standard (Applied Biosystems). Collection information and genotypes for all individuals are deposited in the DRYAD repository (doi:10.5061/dryad.g625b; http://dx.doi.org/10.5061/dryad.g625b).

We found that 11 of 12 primer pairs yielded consistent amplification in the Alaskan populations and could be scored using a diploid locus/allele model. The number of alleles (A) and observed (H_o) and expected (H_e) heterozygosities were obtained for each locus using GenAlEx version 6.4 (Peakall and Smouse, 2006) (Table 2). In our sample of 50 individuals, the total alleles per locus varied from six to 36 for all samples, and from one to 15 within populations (Table 2). Observed and expected heterozygosities ranged from 0.0 to 1.0 and 0.0 to 0.925, respectively. Tests for deviations from expectations for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were conducted using GENEPOP version 4.2 (Raymond and Rousset, 1995; Rousset, 2008). Tests of linkage disequilibrium were not significant for all locus pairs (after Bonferroni correction). Only four primer sets (AYC36, AYC65, AYC85, AYC128) amplified targets from Oregon *C. nootkatensis*, and only two of these (AYC65, AYC85) amplified targets in all populations. Tests for deviation from HWE were significant for three loci by population combinations, two of which involved the Oregon population. Deviation from HWE in these populations may be attributable to clonal reproduction, a process that contributes repeated genotypes and increases local inbreeding (Bérubé et al., 2003). None of the 11 primers showed cross amplification with *Chamaecyparis lawsoniana*.

CONCLUSIONS

These microsatellites add to the genetic resources for Nootka cypress and are especially relevant for Alaskan populations, where the impact of decline is the most severe (Hennon et al., 2012). If unabated, the decline will result in substantial economic losses and in a reduction in the abundance of this ecologically and culturally important conifer in low-elevation coastal forests. The markers described here reveal comparable per-locus allelic variability to those described previously (Bérubé et al., 2003), but reveal higher total heterozygosity (H_e averages >0.70 for polymorphic loci, vs. 0.59 for the loci described in Bérubé et al. [2003]). Eight of 12 loci could not be amplified in Nootka cypress from Oregon, a finding that highlights the regional genomic differentiation within this species, and the need for region-specific markers. The markers have immediate use in the development of conservation metrics (allelic diversity, genotypic diversity) for restoration, and are being used to

Table 1. Characteristics of 11 microsatellite primers developed for *Callitropsis nootkatensis*.[a]

Locus[b]	Primer sequences (5′–3′)	Repeat motif	Amplicon size range (bp)	T_a (°C)	A
AYC-3 [HEX]	F: AGTAAAGTTTGTGCATTTGGA R: AATCGGTGGCAGAGATATTA	$(GT)_{6+}$	253–281	62	8
AYC-17 [HEX]	F: GGTATTAATCCAGACCTCGTT R: TTTGCATACATTGTTGATGTC	$(CT)_{11+}$	290–302	62	6
AYC-36 [HEX]	F: TGTTCTATTTAAGAAACCGTCA R: TGGTTATAATGGATGATCAAGA	$(TC)_{13+}$, $(GA)_{5+}$	212–270	62	9
AYC-49 [FAM]	F: TCCCATATATTAGGGTTTTCA R: ACATCATCTGACATCTCTTGC	$(AG)_{10+}$, $(GT)_{3+}$	163–202	62	14
AYC-51 [HEX]	F: ATCCCTTCCCACTCTCTCTA R: AAACCCTAACCCTAACACAAC	$(CT)_{9+}$	157–219	62	27
AYC-53 [HEX]	F: GGAGCTTCAATCCTTTTATTG R: AAAAAGAAGAGGAGCATGAGT	$(CT)_{10+}$, $(CA)_{8+}$	297–426	62	36
AYC-65 [FAM]	F: GTGTGTTATGCTATGCGATCT R: TAAATGTGTGTTGCAGTTTTG	$(AG)_{8+}$	275–290	62	8
AYC-72 [HEX]	F: ATGTGTTGGGGAAGTCAATA R: TCGATTGTTTGTATCTCCATT	$(AC)_{6+}$	301–377	62	19
AYC-85 [FAM]	F: GTGAGAGACATGGCATAGAGA R: CCCCTTGTTACTATCATGTGC	$(GT)_{14+}$, $(AG)_{7+}$	171–197	62	11
AYC-91 [HEX]	F: TTATTTGCATAACCTTCTTGC R: TTATAATTGTCTTCTCGTGCTC	$(CA)_{6+}$	230–250	62	5
AYC-128 [FAM]	F: ACAATTCCTGGTTATAATGGA R: TTTAAGAAACCGTCATTCC	$(GA)_{36+}$	153–279	62	8

Note: A = number of alleles observed; T_a = optimal annealing temperature.

[a] Sequences are deposited in the European Molecular Biology Laboratory (EMBL) Sequence Read Archive (http://www.ebi.ac.uk/ena/data/view/ERP000647).

[b] Fluorophores used to label the forward primer are shown in brackets.

Table 2. Locus-specific measures of genetic diversity of five populations of *Callitropsis nootkatensis*.[a]

	Hawkins Island, Alaska			Shelter Island, Alaska			Bullhead Cove, Alaska			Kennel Creek, Alaska			John Day, Oregon		
Locus	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e[b]	A	H_o	H_e[b]	A
AYC-3	0.700	0.735	5	0.700	0.745	6	0.700	0.880	10	0.900	0.800	7	0.000	0.000	0
AYC-17	0.800	0.620	3	0.700	0.645	3	0.800	0.715	4	0.700	0.655	4	0.000	0.000	0
AYC-36	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0	0.900	0.870*	9
AYC-49	0.778	0.759	7	0.900	0.835	9	0.800	0.765	7	1.000	0.750	7	0.000	0.000	0
AYC-51	1.000	0.925	15	1.000	0.905	13	1.000	0.880	13	1.000	0.905	13	0.000	0.000	0
AYC-53	0.900	0.845	9	0.800	0.885	13	0.900	0.870	11	0.900	0.890**	12	0.000	0.000	0
AYC-65	1.000	0.741	6	0.900	0.665	4	0.800	0.800	7	1.000	0.685	5	0.500	0.760	7
AYC-72	0.700	0.740	8	1.000	0.840	11	0.900	0.795	8	0.800	0.770	9	0.000	0.000	0
AYC-85	0.800	0.515	3	0.700	0.705	6	0.667	0.735	5	0.400	0.535	3	0.500	0.565*	3
AYC-91	0.600	0.445	3	0.400	0.465	4	0.300	0.485	3	0.600	0.480	4	0.000	0.000	0
AYC-128	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0	0.600	0.775	8

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a]The sample size for all populations is 10.

[b]Asterisks indicate significant departures from Hardy–Weinberg equilibrium based on Fisher exact tests; * = $P < 0.05$; ** = $P < 0.01$.

examine diversity at multiple spatial scales spanning the range in coastal Alaska and British Columbia.

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Appendix 1. Geographic localities of *Callitropsis nootkatensis* and *Chamaecyparis lawsoniana* samples used in this study. Specimen vouchers for *C. nootkatensis* samples were deposited at the Oregon State University herbarium (collection no. OSC 232031).

Species	Population	Locality	Geographic coordinates (decimal degrees)	Notes
Callitropsis nootkatensis	Tongass National Forest Seed Lot 199	Tongass National Forest, Chichagof Island, Alaska, USA	57.825°N, 135.167°W	1 seed
Callitropsis nootkatensis	Fox Point	Chugach National Forest, Hawkins Island, Alaska, USA	60.5817°N, 145.9495°W	$n = 10$
Callitropsis nootkatensis	Shelter Island	Tongass National Forest, South Shelter Island, Alaska, USA	58.3729°N, 134.8288°W	$n = 10$
Callitropsis nootkatensis	Kennel Creek	Tongass National Forest, Chichagof Island, Alaska, USA	57.9100°N, 135.1720°W	$n = 10$
Callitropsis nootkatensis	Bullhead Cove	Tongass National Forest, Boca de Quadra, Alaska, USA	55.0625°N, 130.9860°W	$n = 10$
Callitropsis nootkatensis	John Day	Cedar Grove Botanical Area, Malheur National Forest, Oregon, USA	44.3382°N, 119.3384°W	$n = 10$
Chamaecyparis lawsoniana	Shore Acres	Shore Acres State Park, Oregon, USA	43.3142°N, 124.3930°W	$n = 1$
Chamaecyparis lawsoniana	Coos County	Coos County, Oregon, USA	43.2571°N, 124.3099°W	$n = 1$
Chamaecyparis lawsoniana	Weaver-Sitkum	Weaver Creek, Oregon, USA	43.1078°N, 123.8607°W	$n = 1$
Chamaecyparis lawsoniana	Johnson Mountain	Johnson Mountain, Oregon, USA	42.8228°N, 124.0765°W	$n = 1$

Note: n = sample size.