Development and Characterization of Microsatellite Loci in the Endangered Species Taxus wallichiana(Taxaceae)

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI IN THE ENDANGERED SPECIES *TAXUS WALLICHIANA* (TAXACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed in the endangered tree species *Taxus wallichiana* from Nepal to investigate regional genetic differentiation, local genetic diversity, and gene flow for the conservation of this species under climate- and land-use change scenarios in mountain regions of Nepal.
- *Methods and Results:* We developed 10 highly polymorphic microsatellite markers from 454 DNA sequencing. Characterization of the new microsatellite loci was done in 99 individuals collected from three valleys with different climatic regimes. The number of alleles per locus varied from four to 12. Observed heterozygosity of populations, averaged across loci, ranged from 0.30 to 0.59.
- *Conclusions:* The new markers provided by this study will substantially increase the resolution for detailed studies in phylogeography, population genetics, and parentage analysis.

Key words: 454 pyrosequencing; conservation genetics; Himalayan yew; Himalayas; simple sequence repeat marker.

Taxus wallichiana Zucc. (Taxaceae) is an endangered tree species distributed mainly in Nepal, North India, Bhutan, and southwestern China (Thomas and Farjon, 2011). In Nepal, populations of this species are found primarily in the hills of the Himalayas, at altitudes between 2300 and 3400 m. The species is exploited for timber, firewood, and medicinal purposes. Because of the commercial demand of *T. wallichiana*, the tree is overexploited, resulting in fragmentation of the populations in the Himalayas (Liu et al., 2011). Therefore, proper management strategies for conservation require an assessment of the genetic resources at different spatial scales (Dubreuil et al., 2008). Recently, microsatellite markers have been developed for *T. wallichiana* (Yang et al., 2009; Liu et al., 2011), *T. sumatrana* (Miq.) de Laub. (Huang et al., 2008), *T. yunnanensis* W. C. Cheng & L. K. Fu (Miao et al., 2008), *T. chinensis* var. *mairei* (Lemée & H. Lév.) W. C. Cheng & L. K. Fu (Zhou et al., 2009), and *T. baccata* L. (Dubreuil et al., 2008). However, less than 50% of the published microsatellite markers for *T. wallichiana* revealed polymorphism in recently collected populations. We therefore decided to develop additional microsatellite loci for *T. wallichiana* using 454 sequencing. In forthcoming papers, all markers will be used to study the impact of habitat fragmentation on genetic diversity at different spatial scales and, as a result from our studies, we are designing an effective conservation strategy for *T. wallichiana* in the Himalayas.

METHODS AND RESULTS

We collected leaf samples from 99 individuals belonging to three populations from the southern slopes of the Himalayas in Nepal. The populations were located in three valleys (SP: Solukhumbu District, Khumbu Valley, Sagarmatha National Park, 2550 m, 27°43.209′N, 86°42.876′E; MP: Gorkha District, Numbri Valley, Manaslu Conservation Area, Gandaki Zone, 3050 m, 28°34.153′N, 84°43.417′E; and KP: Taplejung District, Olangchungola Valley, Kanchenjunga Conservation Area, 2600 m, 27°38.946′N, 87°47.997′E). Voucher specimens from the three valleys were collected in 2011 by J.P.G. and are deposited at the Herbarium TUCH (collector numbers JPG-IZ70Z0_131338/1-SP, JPG-IZ70Z0_131338/1-MP, and JPG-IZ70Z0_131338/1-KP), and frozen specimens are archived at C.S.'s frozen herbarium at the Swiss Federal Research Institute WSL. As *T. wallichiana* populations are very fragmented and the local tree density is low, the collections were done for each tree found within a given valley. Immediately after collection, fresh leaf tissue of each individual was dried over silica gel and later stored frozen (−20°C) until DNA extraction.

Genomic DNA was extracted using the DNeasy Plant Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, but with a slight adjustment in volume of buffer AP1 (600 µL instead of 400 µL). The DNA quality of eight individuals from different valleys was checked by gel electrophoresis and 1 µg of pooled DNA was selected to be shotgun sequenced (1/16th run) after sonification with the Covaris S2 (BioCat, Heidelberg, Germany) using a Roche 454 Genome Sequencer FLX with the Titanium Sequencing kit XLR 70 at Microsynth AG (Balgach, Switzerland). We obtained 119 508 reads with an average read length of 337 bp. MSATCOMMANDER 0.8.2 (Faircloth, 2008) was used for screening unassembled sequences and primer design accepting dinucleotide repeats ≥10 and tri-, tetra-, and pentanucleotide repeats ≥6. A total of 110 primer pairs were found, of which 29 primer pairs were discarded from further studies due to primer dimer formation, monorepeats, compound-interrupted repeats, and duplicates. Microsatellite-containing nucleotide sequences of 20 previously published primers (Liu et al., 2011) were obtained from GenBank,

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TABLE 1. Characteristics of 10 microsatellite loci developed in *Taxus wallichiana*.[a]

Locus	Repeat motif	Primer sequences (5′–3′)	Dye	Multiplex	Size range (bp)	Primer conc. (μM)[b]	T_a (°C)	GenBank accession no.
NTWJ2	$(AG)_{11}$	F: TCAATGTTGTGCATTCACAC	VIC	2	250–380	0.2	57	JX092125
		R: TCATCAGGTGACATCTCCTC				0.2		
NTWJ3	$(AT)_{10}$	F: GTTGGAGGGAGAATTGAAG	VIC	3	150–220	0.5	57	JX092126
		R: AAATTCCACCAGTAATGACC				0.5		
NTWJ4	$(AC)_{10}$	F: CTGAATTGGGTGCTAGTGTC	FAM	3	300–400	0.3	57	JX092127
		R: TTGGTGCGGATGATATAAG				0.3		
NTWJ5	$(AC)_{18}$	F: AAACATCATCAAGCAAGTAATG	NED	3	160–260	0.4	57	JX092128
		R: GAATGTGGCTGATTTGATTC				0.4		
NTWJ6	$(AAG)_9$	F: ATAGGTGCAATCATCCTTTC	PET	3	160–260	0.5	57	JX092129
		R: TGTTGGCTATTCGTTTAAAG				0.5		
NTWJ8	$(AAG)_9$	F: GCCCTTACTGCTATTCTGAC	PET	4	200–300	0.4	57	JX092131
		R: TGGACTTACACCCACAAGTC				0.4		
NTWJ9	$(ACAT)_8$	F: CCTGCTACGTGTTTACACAC	VIC	1	200–320	0.05	57	JX092132
		R: CTTGTTAGGGCATTGAACAC				0.05		
NTWJ10	$(AGAT)_6$	F: GGGCCATAGAGATCTATAGG	VIC	3	300–400	0.3	57	JX092133
		R: TTTATGGGTTTGGGAGATAG				0.3		
NTWJ11	$(AGAT)_6$	F: CGACCGACCATATATCTGTC	FAM	4	100–190	0.2	57	JX092134
		R: TCAAGGTATGGAATGGACTG				0.2		
NTWJ12	$(AAAAC)_{12}$	F: GGAGATGGTATAGGCTCTAGG	VIC	4	139–187	0.2	57	JX092135
		R: CACGATGGAGATACCGTATC				0.2		

Note: T_a = annealing temperature.

[a] Nine loci previously described by Liu et al. (2011) that were polymorphic for the studied populations can be analyzed together with the 10 primers with the indicated dye and multiplex: TG34 (FAM, 3), TG141 (FAM, 1), TG147 (FAM, 2), TG47 (NED, 2), TY16 (FAM, 1), TWOI (VIC, 1), TS03 (VIC, 4), TC04 (VIC, 2), and Tax86 (PET, 2).

[b] End concentration of each primer.

and no matches with flanking regions of the new loci for *T. wallichiana* were found. From the 81 primer pairs, a total of 10 new loci were found to be polymorphic and to lack unspecific products when tested with the original eight individuals that were used for library preparation. The remaining loci either had unspecific products or a high number of samples with no amplification. Based on fragment length, four multiplex PCR reactions were designed for the 10 new loci using dye-labeled primers (Table 1). These multiplex PCRs were carried out for the 99 individuals belonging to three populations. PCR reactions were performed in a total volume of 10 μL containing 1 μL of ~1–5 ng genomic DNA, varying primer concentrations (Table 1), and 1× Type-it Multiplex PCR Master Mix (QIAGEN). Amplifications were run on Veriti thermocyclers (Life Technologies, Rotkreuz, Switzerland) with a 5-min cycle at 95°C; followed by 33 cycles of 30 s at 95°C, 90 s at 57°C, and 45 s at 72°C; and a final extension step at 72°C for 60 min. The PCR products were run with LIZ500 (Life Technologies) as an internal size standard on a 3130xl Genetic Analyzer (Life Technologies), and alleles were sized with GeneMapper 3.7 (Life Technologies). The data were arranged in two-column format using our own code in R 2.10.1 (R Development Core Team, 2009), and formatted to Arlequin format. Arlequin 3.11 (Excoffier et al., 2005) was used to test the linkage among microsatellite loci, to test for Hardy–Weinberg equilibrium (HWE), and to calculate polymorphism.

High levels of polymorphism were found in the newly developed 10 loci with a range of allele numbers from four to 12 across populations and a PCR success ranging from 96% to 100% (Table 1). These values are higher than those mentioned in Liu et al. (2011). In population MP, locus NTWJ2 was monomorphic, and in population KP, loci NTWJ3 and NTWJ11 were monomorphic, respectively. NTWJ12 was not analyzed in population SP. The number of alleles ranged from two to eight (mean: 3.9) in SP, one to 10 (mean: 4.4) in MP, and one to seven (mean: 2.6) in KP (Table 2). After Bonferroni correction with

TABLE 2. Results of microsatellite screening in 99 individuals of *Taxus wallichiana* belonging to three populations in different climatic regimes of the Himalayas, Nepal.[a]

	SP (N = 68)				MP (N = 16)				KP (N = 15)				Total (N = 99)
Locus	PCR product[b]	A	H_o	H_e	PCR product[b]	A	H_o	H_e	PCR product[b]	A	H_o	H_e	A
NTWJ2	67	8	0.328*	0.736	16	1	0.000*	0.000	15	7	0.400*	0.740	10
NTWJ3	67	2	0.179	0.164	16	7	0.875*	0.762	15	1	0.000*	0.000	9
NTWJ4	68	3	0.647*	0.461	16	3	0.250	0.417	15	2	0.400	0.331	4
NTWJ5	67	5	0.731*	0.571	16	3	0.688	0.627	15	4	0.533	0.641	8
NTWJ6	65	4	0.031	0.061	16	5	0.688	0.770	15	2	0.067	0.067	9
NTWJ8	68	3	0.529	0.570	16	3	0.375	0.637	15	3	0.533	0.503	5
NTWJ9	66	5	0.727*	0.541	16	4	0.438	0.490	15	2	0.267	0.405	9
NTWJ10	67	2	0.597	0.444	16	4	0.375*	0.694	15	2	0.067	0.067	6
NTWJ11	68	3	0.971*	0.579	16	4	0.000*	0.573	15	1	0.000*	0.000	6
NTWJ12[c]	0	—	—	—	16	10	0.000*	0.895	15	2	0.000	0.239	12
Mean		3.9	0.527	0.459		4.4	0.369	0.587		2.6	0.227	0.299	7.8

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

[a] SP = Solukhumbu District, Khumbu Valley, Sagarmatha National Park; MP = Gorkha District, Numbri Valley, Manaslu Conservation Area, Gandaki Zone; KP = Taplejung District, Olangchungola Valley, Kanchenjunga Conservation Area.

[b] PCR products obtained for each locus.

[c] Locus NTWJ12 from population SP was omitted.

* Indicates significant deviation ($P \leq 0.05$) from HWE after Bonferroni correction for multiple tests.

the function p.adjust as implemented in R 2.10.1 (R Development Core Team, 2009), eight loci deviated from HWE in at least one of the populations studied. The Bonferroni corrected test for linkage disequilibrium showed there were several spurious significant linkages between microsatellite loci that were not consistent across populations. Frequent deviations from HWE and spurious linkage disequilibrium indicate possible assortative mating and genetic drift in the small and highly fragmented populations of *T. wallichiana*, processes that will be investigated in detail in forthcoming studies.

CONCLUSIONS

The new technique for microsatellite development was successful, economic, and time efficient. It produced 10 new and informative microsatellite primer pairs, adding to the toolbox of markers available for genetic analyses of this species. With the newly developed loci, a sufficiently high number of highly variable microsatellite markers are now available for studies involving the characterization of the fine-scale population structure. Our future research targeting the species' genetic diversity, landscape genetics, and gene flow at various spatial scales will help to develop strategies for the conservation and management of the endangered tree species *T. wallichiana*.

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