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PRIMER NOTE

IDENTIFICATION AND CHARACTERIZATION OF MICROSATELLITE MARKERS IN *PINUS KESIYA* VAR. *LANGBIANENSIS* (PINACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed in *Pinus kesiya* var. *langbianensis* (Pinaceae), a species native to southwestern China, to investigate its genetic diversity and population structure in order to provide information for the conservation and management of this species.
- *Methods and Results:* Using next-generation sequencing, a total of 2349 putative simple sequence repeat primer pairs were designed. Eighteen polymorphic markers in 60 individuals belonging to four populations of *P. kesiya* var. *langbianensis* were identified and characterized with two to 11 alleles per locus. The observed and expected heterozygosity ranged from 0.000 to 0.800 and 0.000 to 0.840, respectively. Each of these loci cross-amplified in the closely related species *P. massoniana*, *P. densata*, *P. tabuliformis*, and *P. yunnanensis*, with one to seven alleles per locus.
- *Conclusions:* The new markers are promising tools to study the population genetics of *P. kesiya* var. *langbianensis* and related species.

Key words: microsatellite; next-generation sequencing; Pinaceae; *Pinus kesiya* var. *langbianensis*; population genetics.

Pinus kesiya Royle ex Gordon var. *langbianensis* (A. Chev.) Gaussen (Pinaceae) is an important forest tree species in Yunnan Province, China. It has been recorded at altitudes from 600–1800 m in the southern, semihumid climate zone of Yunnan (Editorial Committee of Flora of China, 1978; Wu, 1986) and accounts for 11% of the forest area and 1.0×10^8 m^3 of the forest volume (Jiang et al., 2007). The wood is extensively used in building, furniture, and the fiber industry. *Pinus kesiya* var. *langbianensis* is also highly valued for its high resin content, with an annual output of 179,100,000 kg (Editorial Committee of Flora of China, 1978; Wu, 1986; Dong et al., 2009). Output of gum turpentine from *P. kesiya* var. *langbianensis* accounted for more than 90% of the total output in Yunnan (Yin et al., 2005). However, germplasm resources of *P. kesiya* var. *langbianensis* have decreased in recent years as a result of overexploitation (Zhao et al., 2016).

Information on genetic diversity and spatial structure in *P. kesiya* var. *langbianensis* is important for its future conservation and can be used to help guide local forest management (Sanchez et al., 2014). No specific conservation strategy is available for this species, in part due to the limited understanding of genetic diversity and structure of the natural populations. As a primary forest tree species in southern Yunnan Province, resource conservation of *P. kesiya* var. *langbianensis* will benefit the entire ecological system in the region (Li et al., 2015). Therefore, in this study we developed novel microsatellite markers for *P. kesiya* var. *langbianensis* by applying next-generation sequencing to investigate the genetic diversity and population structure of this species at the molecular level.

METHODS AND RESULTS

Needle samples of 60 individuals from four *P. kesiya* var. *langbianensis* populations located in Yunnan Province, China, and 59 individuals from four related species (*P. massoniana* D. Don, *P. densata* Mast, *P. tabuliformis* Carrière, *P. yunnanensis* Franch.) were collected (Appendix 1). All needle samples were dried and preserved in silica gel. Total genomic DNA was isolated from dried needle samples using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Paired-end libraries were constructed on four individuals sampled from Puer City Institute of Forestry Sciences and sequenced by a customer sequencing service (Beijing Honor Tech Co. Ltd., Beijing, China) using the Illumina HiSeq 2500 Sequencing System (Illumina, San Diego, California, USA). Clean reads were assembled using Trinity version 2.2.0 (https://github.com/trinityrnaseq/trinityrnaseq/wiki; Grabherr et al., 2011; Haas et al., 2013). Data quality control was carried out with the software FastQC (Andrews, 2010). The Q30 percentage exceeded 90% and the GC contents were 45.60–46.56%, which suggests that the sequencing was highly reliable. Data filtering was carried out according to the following criteria: (1) removed reads with adapters; (2) removed reads with unknown bases >10%; and (3) removed low-quality reads (defined as reads having >50% bases with quality value ≤5). A total of 104,392 unigenes were obtained with an N50 length of 1349 bp. The data have been deposited in the Short Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI; accession no. SRP093696). Genomic microsatellite loci for *P. kesiya* var. *langbianensis* were detected using the software MicroSAtellite Identification Tool (MISA; Thiel et al., 2003). Two

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TABLE 1. Characteristics of 18 polymorphic microsatellite loci in *Pinus kesiya* var. *langbianensis.*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
Pkvl001	F: TTTGCAGTCTGTTGCCTTTG	$(GCC)_6$	149–179	60.0	KX519328
	R: GTGGAGGAAGATGGAAACGA				
Pkvl002	F: GCACGTTGGATTTCAGGTTT	$(GCA)_5$	134–146	60.0	KX519329
	R: GCAACTCCAGCAACTCCTTC				
Pkvl003	F: CAGGCTGTAACGCTCAATCA	$(CAG)_5$	151–163	60.0	KX519330
	R: TTGAAGGATCCCAACTTCCTT				
Pkvl004	F: CCCTGAGTTTGACCGACAGT	$(AAT)_5$	265–280	60.1	KX519331
	R: GGGCATTTTAATGCTGCTGT				
Pkvl005	F: GGGATTGGACTGGATGAGAA	$(AGA)_5$	241–256	59.9	KX519332
	R: TTTTGTAAACTGCAGCCGTG				
Pkvl006	F: GCAGTGGCTGACATTTGAGA	$(CTG)_6$	147–174	59.9	KX519333
	R: GCTTCACGCCGTTCTTTATC				
Pkvl007	F: GTAGGCACTCCGTGAAGCTC	$(CAG)_5$	240–255	60.0	KX519334
	R: ATCGGGACCTGTTGTTTCAG				
Pkvl008	F: TGGGAAGTTTGTCCATGTCA	$(TCT)_5$	258–273	60.0	KX519335
	R: GCATTGTTGGCGTTGTATTG				
Pkvl009	F: GTGGTCTGAAAATACCGCGT	$(TCT)_5$	174–180	60.0	KX519336
	R: GCAGCAGTAGCCATCATCAA				
Pkvl010	F: CGAGACGAAGCAATTCCAA	$(AT)_6$	244–262	59.9	KX519337
	R: CGAAGATCAAGAAAGGCAGG				
Pkvl011	F: AGCTCGGGATCAGGAGTATG	$(TA)_6$	169–183	59.6	KX519338
	R: TTTGGTGAAGTTTTGATTGCC				
Pkvl012	F: GTTCTGTGAGCTTGGGGAAG	$(AT)_6$	144–166	60.0	KX519339
	R: TAAGACCGATTTGGCTACGG				
Pkvl013	F: GATTGGCAGAGGCTACAAGC	$(TA)_{11}$	114–150	59.9	KX519340
	R: ATGCTTCCGCTGTTCAACTT				
Pkvl014	F: TGCCACATTTGGGTAAGAAA	$(AT)_9$	224–252	59.5	KX519341
	R: TCGGAATGATGGATAGGAGC				
Pkvl015	F: CCTTTTATGGGGGCGATAAT	$(AT)_9$	208–224	59.9	KX519342
	R: TTGACTTGAACACAAAGCCG				
Pkvl016	F: ATCCTTACGCCTGCAGAGAA	$(AT)_7$	152–158	60.2	KX519343
	R: CATCGATTGCGCTACATCAG				
Pkvl017	F: GATGCATTTGGATCAGCAAA	$(TA)_9$	226–242	59.9	KX519344
	R: ACCAATCGCTTGCATCTTTC				
Pkvl018	F: CTGGTGCATAGACCCGAGAT	$(TA)_9$	235–257	60.0	KX519345
	R: TTTTCTGCTTCAAGTGGCCT				

Note: T_a = annealing temperature.

thousand three hundred forty-nine simple sequence repeat (SSR) loci were designed with Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). Among them, 192 SSR loci with dinucleotide or trinucleotide SSR motifs were randomly chosen to screen using four individuals from four populations. One hundred fifty-nine out of 192 SSR loci were amplified successfully, and 79 out of 159 SSR loci were polymorphic. Eighteen polymorphic SSR loci (Table 1) were then randomly selected for characterization using 60 individuals from four populations (Appendix 1). The SSR amplifications were multiplexed in a 10-μL reaction containing 30 ng of genomic DNA, 0.15 μM of each primer, 5 μL Mix (0.05 units/μL *Taq* DNA Polymerase, 0.4 mM dNTPs, 4.0 mM $MgCl_2$; Beijing Ruibio Biotech Co. Ltd., Beijing, China), and 1× PCR Buffer. The amplification protocol was: 95°C for 5 min; followed by 30 cycles at 95°C for 30 s, the annealing temperature for each primer (Table 1) for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 7 min.

Amplification products were resolved using capillary electrophoresis on an ABI 3730xl DNA Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). The electropherograms were analyzed using GeneMarker version 2.2.0 with GeneScan 500 LIZ as a size standard (Applied Biosystems). CONVERT version 1.3.1 (Glaubitz, 2004) was used to convert input files for analysis in subsequent software. The genetic diversity parameters of polymorphic loci, including the number of alleles, observed heterozygosity, and expected heterozygosity were calculated by GenAlEx 6.4 (Peakall and Smouse, 2006), and Hardy–Weinberg equilibrium (HWE) for each locus and the presence of linkage disequilibria for all pairwise loci in each population were tested with POPGENE 1.32 (Yeh et al., 1997). Among the 60 genotyped individuals, the number of alleles per locus varied from one to 11 (Table 2). The observed heterozygosity and expected heterozygosity ranged from 0.000 to 0.800 and from 0.000 to 0.840, respectively. There were no significant departures from HWE over all loci for any populations, but some populations deviated from HWE at up to 15 loci ($P < 0.05$) (Table 2). A significant linkage disequilibrium ($P < 0.01$) was detected in three pairwise SSR loci in two (Populations 1 and 2) out of four populations (Population 1: Pkvl007 and Pkvl010, Pkvl008 and Pkvl010; Population 2: Pkvl006 and Pkvl014). Furthermore, 16 out of 18 SSR loci were successfully amplified in 59 individuals of four related species (14–15 individuals for each species; Appendix 1). Most of these loci were polymorphic (Table 3).

CONCLUSIONS

The set of 18 novel SSR markers reported in this study will be helpful for population genetic analysis in *P. kesiya* var. *langbianensis*, which will offer valuable information for the formulation of the rational utilization and conservation strategies of this species in the future. Furthermore, the successful cross-species amplification of these SSR markers in *Pinus* (Pinaceae) suggests their potential to be used in studies of genetic variation for related pine species.

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TABLE 2. Genetic properties of 18 polymorphic microsatellite markers in four *Pinus kesiya* var. *langbianensis* populations.[a]

	Population 1 (N = 15)				Population 2 (N = 15)				Population 3 (N = 15)				Population 4 (N = 15)			
Locus	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]
Pkvl001	3	0.267	0.371	ns	6	0.400	0.564	**	3	0.133	0.338	***	4	0.133	0.187	***
Pkvl002	5	0.333	0.658	ns	2	0.000	0.391	***	4	0.200	0.296	*	3	0.267	0.338	**
Pkvl003	3	0.267	0.287	ns	4	0.267	0.293	ns	2	0.067	0.358	**	3	0.067	0.127	***
Pkvl004	4	0.000	0.516	***	5	0.067	0.482	***	4	0.133	0.293	*	3	0.067	0.184	**
Pkvl005	5	0.200	0.396	*	4	0.071	0.365	***	2	0.067	0.064	ns	2	0.067	0.064	ns
Pkvl006	7	0.400	0.491	ns	5	0.267	0.349	***	3	0.067	0.127	***	3	0.067	0.127	***
Pkvl007	3	0.133	0.240	**	3	0.133	0.291	*	1	0.000	0.000	—	2	0.067	0.064	ns
Pkvl008	4	0.200	0.393	***	4	0.133	0.242	*	3	0.133	0.127	ns	2	0.067	0.064	ns
Pkvl009	2	0.733	0.464	*	3	0.400	0.418	**	2	0.667	0.444	ns	2	0.800	0.498	*
Pkvl010	5	0.154	0.393	**	5	0.333	0.549	***	3	0.200	0.331	ns	1	0.000	0.000	—
Pkvl011	4	0.533	0.613	***	3	0.667	0.531	ns	5	0.667	0.638	***	4	0.667	0.544	**
Pkvl012	3	0.133	0.438	*	4	0.333	0.607	***	5	0.467	0.609	**	3	0.133	0.240	**
Pkvl013	6	0.133	0.551	***	11	0.333	0.840	***	4	0.000	0.459	***	6	0.143	0.561	***
Pkvl014	9	0.077	0.837	***	5	0.231	0.615	*	6	0.400	0.487	ns	9	0.333	0.616	**
Pkvl015	1	0.000	0.000	—	3	0.267	0.240	ns	1	0.000	0.000	—	1	0.000	0.000	—
Pkvl016	3	0.000	0.418	***	3	0.133	0.338	***	3	0.133	0.291	*	3	0.200	0.184	ns
Pkvl017	4	0.214	0.625	***	4	0.357	0.574	**	6	0.200	0.744	***	5	0.333	0.629	**
Pkvl018	5	0.600	0.671	*	7	0.533	0.820	***	4	0.467	0.656	***	6	0.800	0.631	***

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = number of individuals sampled.

[a] See Appendix 1 for locality and voucher information.

[b] Deviation from Hardy–Weinberg equilibrium using χ^2 tests: ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, — = monomorphic.

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TABLE 3. Cross-amplification results showing the number of alleles detected in 18 loci from *Pinus kesiya* var. *langbianensis* in four related species.[a]

Locus	*Pinus massoniana*	*Pinus densata*	*Pinus tabuliformis*	*Pinus yunnanensis*
Pkvl001	2	2	2	2
Pkvl002	4	4	2	4
Pkvl003	1	2	2	1
Pkvl004	4	2	2	3
Pkvl005	3	2	1	2
Pkvl006	1	1	1	4
Pkvl007	3	1	1	1
Pkvl008	1	1	2	3
Pkvl009	3	2	2	2
Pkvl010	3	5	2	5
Pkvl011	3	4	3	2
Pkvl012	5	3	3	3
Pkvl013	—	—	—	—
Pkvl014	—	—	—	—
Pkvl015	5	7	5	4
Pkvl016	6	2	1	3
Pkvl017	2	5	6	2
Pkvl018	2	5	3	3

[a] See Appendix 1 for locality and voucher information.

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Appendix 1. Voucher information for *Pinus kesiya* var. *langbianensis* and its four related species used in this study.[a]

Species	Collection locality	Geographic coordinates	*N*
Pinus kesiya Royle ex Gordon var. *langbianensis* (A. Chev.) Gaussen	Ninger, Yunnan, China (Population 1)	22°57′N, 101°03′E	15
Pinus kesiya var. *langbianensis*	Zhenyuan, Yunnan, China (Population 2)	23°51′N, 100°53′E	15
Pinus kesiya var. *langbianensis*	Jiangcheng, Yunnan, China (Population 3)	22°35′N, 101°51′E	15
Pinus kesiya var. *langbianensis*	Lanchang, Yunnan, China (Population 4)	22°33′N, 99°50′E	15
Pinus massoniana D. Don	Yuping, Guizhou, China	27°30′N, 109°11′E	14
Pinus densata Mast	Xianggelila, Yunnan, China	28°29′N, 99°37′E	15
Pinus tabuliformis Carrière	Lushi, Henan, China	33°44′N, 110°49′E	15
Pinus yunnanensis Franch.	Yilang, Yunnan, China	24°35′N, 103°08′E	15

Note: *N* = number of individuals sampled.

[a]Silica gel–dried needle samples of all vouchers were deposited in the Key Laboratory for Forest Genetic and Tree Improvement and Propagation in Universities of Yunnan Province, Southwest Forestry University, Kunming, Yunnan, China.

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