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Development and characterization of chloroplast microsatellite markers for *Pinus massoniana* and their application in *Pinus* (Pinaceae) species

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Abstract. *Pinus massoniana* is one of the important afforestation and pioneer tree species, which is widely distribute in southern China. Chloroplast simple sequence repeat markers (cpSSRs) have been widely used in studies of tree genetics, phylogenetic and breeding. We sequenced the whole chloroplast genome sequences of *P. massoniana* using PCR and Sanger sequencing. A total of 71 cpSSRs were identified, among which mononucleotide repeats were predominant (70.42%). Seventeen primer pairs were developed and amplification tests were conducted with 15 *P. massoniana* individuals. Also, cross-species amplification tests were conducted among 15 individuals per *Pinus* species, including *P. elliottii*, *P. bungeana*, *P. armandii*, *P. caribaea*, *P. tabulaeformis*, *P. taiwanensis* and *P. yunnanensis* which revealed polymorphic information content ranging from 0.2 to 0.8 and average of haploid diversity (*h*) ranging from 0.29 to 0.63. In addition, the polymorphic cpSSRs were useful in distinguishing the sampled pine species, and could be powerful tool in phylogenetic studies.

Keywords. chloroplast simple sequence repeat markers; transferability; genetic diversity; *Pinus massoniana*.

Introduction

The genus *Pinus* contains pioneer species characterized by tolerance to drought and poor soil. Nuclear genome simple sequence repeats (nSSRs) were widely used in studies of genetic diversity (Morinha *et al.* 2016), population structure and paternal analysis (Grattapaglia *et al.* 2014; Mansour *et al.* 2016). Chloroplast simple sequence repeat markers (cpSSR) have a great advantage in these studies because of paternal inheritance of the chloroplast genome in *Pinus* and conservation of the chloroplast genome sequence (Bilgen and Kaya 2014; Um *et al.* 2014; Wang *et al.* 2016).

The cpSSRs are widely found in chloroplasts and have characteristics common with nSSRs (e.g. dominant and multiple alleles). The cpSSRs also have some differences with nSSRs: the most common microsatellite motifs are mononucleotide in chloroplast genome and cpSSRs have higher transferability in related species because of conservation of the chloroplast genome sequence. The cpSSRs were used in studies of genetic diversity, phylogeography and genetic conservation (Park *et al.* 2016; Ueno *et al.* 2016; Wang *et al.* 2017). However, few cpSSRs were developed for gymnosperm species compared with angiosperms. Therefore, we developed 17 cpSSRs for *Pinus massoniana* and examined the transferability of cpSSRs in seven other *Pinus* species: *P. elliottii*, *P. bungeana*, *P. armandii*, *P. caribaea*, *P. tabulaeformis*, *P. taiwanensis* and *P. yunnanensis*. These markers were used in preliminary studies of phylogenetics and genetic diversity of the sampled *Pinus* species.

Materials and methods

DNA source, template amplification and primer design

The chloroplast DNA of *P. massoniana* was used in this study. The entire chloroplast genome of *P. massoniana* was amplified by 35 primer pairs (table 1) (Cronn *et al.* 2008). We obtained the sequence of these regions, which were averaged to ~3.6 kb in size using Sanger sequencing and primer walking strategy (GenBank accession number: MF564195). The microsatellite motifs were searched by MIcroSAtellite identification tool (misa.ini; http://pgrc.ipk-gatersleben.de/misa/misa.html) (Thiel *et al.* 2003)

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Table 1. Amplification primers used to amplify *Pinus* chloroplast genomes.

Primer name	Sequence	T_m	Size (kb)
1F	CTCTCCCCAAACCGTGCT	57.6	3536
2R	TGGGTTACGAAGGTACTAATCAAA	53.7	
2F	GATCAGCTATTTGATTAGTACCTTCG	53.3	4474
3R	GTAGCGGGAATCGAACCCGCATC	62.1	
3F	AGCCTTCCAAGCTAACGATG	55.1	1542
4R	AATCTCCCGCTCCAGGTATT	55.7	
4F	AATACCTGGAGCGGGAGATT	55.7	3590
5R	TGTTCGAGATCCACAAACCA	54.1	
5F	TGGTTTGTGGATCTCGAACA	54.1	3632
6R	CAATGTGTGTCATGCACCTG	54.5	
6F	CAGGTGCATGACACACATTG	54.5	3427
7R	CCTAAAGGATCAAATGGAACGA	52.6	
7F	TCTCGTTCCATTTGATCCTTT	51.9	2923
8R	CACACCCCTTATCCCAATTTT	53	
8F	AAAATTGGGATAAGGGGTGTG	53	3590
9R	AGAGTACCGCCCTGTCAAGA	57.8	
9F	TCTTGACAGGGCGGTACTCT	57.8	2943
10R	TGCTGCATTGATCCGAGTAA	54.2	
10F	TACTCGGATCAATGCAGCAG	54.8	3977
11R	CAAATAACCAACCTGCAATGAA	52	
11F	TTCATTGCAGGTTGGTTATTTG	52	3558
12R	AGGTTCGTTAGCGATTCACG	55.1	
12F	TTCGTGAATCGCTAACGAAC	53	1813
13R	CCGGCAATTACAATGGTTCT	53.3	
13F	ACCATTGTAATTGCCGGAAG	53.3	2210
14R	TTGATGTGATTGATCGCTTG	51	
14F	TGGATTACAAGCGATCAATCA	51.9	3812
15R	CGATGTCAACATCTCTCTCTGC	55.3	
15F	TGGTAAATAATGCAGAGAGAGATGT	53.8	3072
16R	TTGGTCCACTTGGCTACGTC	57.2	
16F	GACGTAGCCAAGTGGACCAA	57.2	3783
17R	AAAGTAAACCAACCCCTTGGA	54.3	
17F	TCCAAGGGGTTGGTTTACTTT	54.3	3562
18R	GGATTTGCTGGTTCACCAAT	53.6	
18F	ATTGGTGAACCAGCAAATCC	53.6	4033
19R	TCGTAAACAACATAGGGGAAGAA	53.8	
19F	TCTTCCCCTATGTTGTTTACGAA	53.8	3683
20R	GGTCACAAGCGTCTGTATCG	55.6	
20F	CGATACAGACGCTTGTGACC	55.6	3050
21R	GGTTCAAGTCCCTCTATCCC	54.4	
21F	TGGGGATAGAGGGACTTGAA	54.8	3874
22R	CGCTTCAAAACCGTACATGA	53.4	
22F	GTACGGTTTTGAAGCGGAGA	55.1	3487
23R	TCCCAGGCAGATACTTGACA	55.5	
23F	TCAAGTATCTGCCTGGGATCA	55.4	3510
24R	GAGAAGATGCGGGTTCGAT	54.7	
24F	GATCGAACCCGCATCTTCT	54.7	3430
25R	GCCCTTGGAAAGTCTTCTAAA	52.6	
25F	AGGGCTATAGTCATAGCGATCC	55.5	3630
26R	CGCTCTACCGCTGAGCTAAT	56.8	
26F	GGGCTATTAGCTCAGCGGTA	56.4	3484
27R	GATCTTAGGCCCTGACTCACC	56.9	
27F	GGTGAGTCAGGGCCTAAGAT	56.2	2897
28R	GTAGAGCGGTCGGCTGTTA	56.7	
28F	TTAACAGCCGACCGCTCTAC	56.9	3331
29R	CCAGTCTTGTTAATACGGGATTT	52.8	
29F	TCCCGTATTAACAAGACTGGTG	54.4	3124
30R	TGTGGTTTTTCGTGATCCAA	52.5	
30F	TTGGATCACGAAAAACCACA	52.5	4496
31R	TGTTCTTGGAGCAGAAGCAA	54.6	

Table 1 (*contd*)

Primer name	Sequence	T_m	Size (kb)
31F	TTGCTTCTGCTCCAAGAACA	54.6	3778
32R	TCCTCTGGATCATCCGAATC	53.6	
32F	GATTCGGATGATCCAGAGGA	53.6	3217
33R	GGCTCGATAAAGAATTCGATAAG	51.4	
33F	TTGGCTTATCGAATTCTTTATCG	51.1	3051
34R	CCATATTTTGGGTTGCTTGG	52.2	
34F	GCCAAGCAACCCAAAATATG	52.7	3285
35R	GGAATTTGAATGTGTACAAATGGT	52.2	
35F	TTTGTACACATTCAAATTCCGATT	51.6	
1R	CACGGTTTGGGGAGAGATT	54.7	

Table 2. Sampling sites of eight *Pinus* species in China.

Species	No. of individuals	Collection locality[a]	Geographic coordinates
P. massoniana	15	Zhangping, Fujian	25°13.78′N, 117°32.25′E
P. taiwanensis	15	Huangshan, Anhui	30°13.23′N, 118°10.13′E
P. yunnanensis	15	Nanning, Guangxi	22°84.13′N, 108°29.32′E
P. tabulaeformis	15	Hanzhong, Shanxi	32°83.32′N, 106°25.65′E
P. bungeana	15	Hanzhong, Shanxi	32°83.32′N, 106°25.65′E
P. armandii	15	Hanzhong, Shanxi	32°83.32′N, 106°25.65′E
P. elliottii	15	Zhangping, Fujian	25°13.78′N, 117°32.25′E
P. caribaea	15	Zhangping, Fujian	25°13.78′N, 117°32.25′E

[a]All collection localities are from China.

based on the chloroplast genome sequences of *P. massoniana*. Primer pairs were designed using Primer3 (http://sourceforge.net/projects/primer3/) (Koressaar and Remm 2007). The parameters of each primer pair complied with the following criteria: (i) amplification products of 100–400 bp, (ii) primer size of 18–24 bp, (iii) GC content of 40–60%, (iv) annealing temperature (T_a) of 50–60°C, (v) excluded hairpin, dimer, false priming and cross dimer.

Amplification tests

Amplification tests were conducted using 15 *P. massoniana* individuals (table 2). The total genomic DNA of sampled *Pinus* species was extracted from fresh leaves using Plant Genomic DNA kit (ZomanBio, Beijing, China) and quantified by NanoDrop 2000c (ThermoFisher Scientific, Waltham, USA). Polymerase chain reaction (PCR) was performed in 10 μL of the reaction mixture containing 50 ng of DNA template, 1.0 μL of 10× PCR buffer (10 mM Tris-HCl), 2.5 mM $MgCl_2$, 0.25 mM dNTP, 0.3 μM primer and 0.5 U of *Taq* polymerase (Takara, Dalian, China). The PCR programme was as follows: an initial denaturation at 94°C for 5 min; 25 cycles of 30 s at 94°C, 30 s at the annealing temperature (table 3) and 40 s at 72°C with a final extension at 72°C for 5 min. PCR products length polymorphism was detected on an ABI 3730 DNA Analyzer with GeneScan 500 ROX Size Standard (Applied Biosystems, Waltham, USA).

Statistical analysis

Peak data of PCR products length polymorphism were analysed using PeakScanner ver. 1.0 (Applied Biosystems). The genetic parameters, including number of alleles (N_a) per locus, Nei's gene diversity (h) and polymorphism information content (PIC) were estimated with Popgene32 ver. 1.32 (Yeh 1999) and PowerMarker ver. 3.25 (Liu and Muse 2005).

Cross-species amplification tests and phylogenetic analysis

The transferability of developed markers was tested in 15 individuals per *Pinus* species (table 2), including *P. elliottii*, *P. bungeana*, *P. armandii*, *P. caribaea*, *P. tabulaeformis*, *P. taiwanensis* and *P. yunnanensis*. Phylogenetic analysis was conducted using seven polymorphic markers (PMacp005–PMacp011). Genetic distances were estimated using Nei's (1978) by PowerMarker ver. 3.25 (Liu and Muse 2005) and the phylogenetic tree was constructed using UPGMA in MEGA7 (Kumar *et al.* 2016).

Table 3. Characters of 17 cpSSRs and transferability in seven sampled pine species.

Locus	Sequence	T_m (°C)	PCR product size range in pines (bp)								GenBank accession no.
			P. massoniana	*P. yunnanensis*	*P. taiwanensis*	*P. elliottii*	*P. bungeana*	*P. armandii*	*P. caribaea*	*P. tabulaeformis*	
PMacp001	F: CCACCCATTTGATTCCT R: ACCATCGGTAAAGTTCG	53	165	161–166	166	166	166	158	166	166	KY196977
PMacp002	F: ATTTCCCACCCATTTGAT R: TTCGGAAGACTACGACTGAT	53	157	157	157	157	157	148–149	156–157	157	KY196978
PMacp003	F: ATCCACTCCCTTCCCTG R: TTGCGAATCCCTTTGTT	53.5	192	192	191–192	191	195	193	191	192	KY196979
PMacp004	F: ATCCACTCCCTTCCCTG R:ATTTGCGAATCCCTTTG	53.5	193–194	191–194	194	193	197	194–195	193	194	KY196980
PMacp005	F: ATTTGTCCTCTTCCTCTATTACCAC R: ATCTTTATTTTCAGAACTATCGTCC	55.5	181–182	181–182	181–182	181–182	181–182	181	181–182	177–181	KY196981
PMacp006	F: GGTAAATGTTCCCTCCCA R: TGATGCTTCAATCCTTCG	56	256–260	254–258	251–256	253–254	256–259	261	253	256	KY196982
PMacp007	F: ATAGTTGGAGTCGGCGG R: CCTGGATGTCTTTGGCA	55	215–216	215–217	214–216	212–214	213	213	213	215	KY196983
PMacp008	F: GGGGTAGAGAAAATGCCT R: TTGCCGAGTTCCTTAGAG	53.5	188	185–188	187–188	187–188	188	188	188	188	KY196984
PMacp009	F: AATCCCTTCCTTTTGG R: ATGCTGGTTACTCTCG	54	264–269	263–269	264	263	261–265	262–269	263	263–264	KY196985
PMacp010	F: GTTCAATACAAATGATGGGAGTCG R: GGTCGGATTCTTCCTATCTTCTTG	55	262–263	262–263	263	261–262	261–262	261–270	262	263	KY196986
PMacp011	F: GGGTCTTCTTCTTTTTTTCATT R: CGTTGTCATTTTCCTTCCTATT	54	189–193	188–192	189–192	189–193	185–195	193–198	189	193	KY196987
PMacp012	F: CAACGCAATTCTGGAACC R: CTCGCTCTTACTGATACTGA	53	261	260–263	262	261	260–261	263–267	261	261	KY196988
PMacp013	F: ATAGTGGACGAACGGAGA R: CTTGGAGAGATGGCTGAG	53	100	96–100	100	100	100	100	100	100	KY196989
PMacp014	F: CTGATTCTGGATTGTTGATGG R: CTGGAGCGACTATGATACG	53	341–355	341–352	355	341	343	343	341–342	355	KY196990
PMacp015	F: CTATCTCCCTTCAACCCTTT R: CTAATGCGTTCTCCACTGT	53	166	165–168	165	162	161–162	164–169	162	165	KY196991
PMacp016	F: GAGCCAATGTCCGAGTAC R: TGCCGAGTTCCTTAGAGA	53	167	167–168	166–167	166–168	167	167	167	167	KY196992
PMacp017	F: CGACACAGGTAGGTAGGT R: TTACGACTTTGCGGAGAC	53	167	167	167	167	167	167	167	167	KY196993

Size ranges are based on 15 individuals per sampled *Pinus* species.

Table 4. Characterization of developed cpSSR markers in *P. massoniana* and their cross-species amplification in related pine species.

	P. massoniana			*P. yunnanensis*			*P. taiwanensis*			*P. elliottii*			*P. bungeana*			*P. armandii*			*P. caribaea*			*P. tabulaeformis*		
Locus	N_a	*h*	PIC	N_a	*h*	PIC	N_a	*h*	PIC	N_a	*h*	PIC	N_a	*h*	PIC	N_a	*h*	PIC	N_a	*h*	PIC	N_a	*h*	PIC
PMacp001	1	–	–	3	0.56	0.5	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–
PMacp002	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–	2	0.39	0.31	2	0.23	0.2	1	–	–
PMacp003	1	–	–	1	–	–	2	0.23	0.2	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–
PMacp004	2	0.44	0.35	3	0.59	0.51	1	–	–	1	–	–	1	–	–	2	0.23	0.2	1	–	–	1	–	–
PMacp005	2	0.5	0.37	2	0.44	0.35	2	0.48	0.36	2	0.5	0.37	2	0.5	0.37	1	–	–	2	0.48	0.36	2	0.41	0.32
PMacp006	3	0.43	0.39	3	0.56	0.5	2	0.32	0.27	2	0.5	0.37	3	0.5	0.44	1	–	–	1	–	–	2	0.23	0.2
PMacp007	2	0.39	0.31	3	0.59	0.51	3	0.5	0.44	3	0.5	0.44	1	–	–	1	–	–	1	–	–	1	–	–
PMacp008	1	–	–	3	0.6	0.52	2	0.32	0.27	2	0.23	0.2	1	–	–	1	–	–	1	–	–	1	–	–
PMacp009	2	0.44	0.35	4	0.71	0.66	1	–	–	1			4	0.68	0.64	5	0.75	0.71	1	–	–	2	0.23	0.2
PMacp010	2	0.32	0.27	2	0.48	0.36	1	–	–	2	0.23	0.2	2	0.5	0.37	6	0.83	0.8	1	–	–	1	–	–
PMacp011	2	0.44	0.35	3	0.65	0.58	3	0.63	0.56	2	0.32	0.27	4	0.74	0.69	5	0.79	0.76	1	–	–	1	–	–
PMacp012	1	–	–	4	0.71	0.66	1	–	–	1	–	–	2	0.28	0.24	3	0.64	0.56	1	–	–	1	–	–
PMacp013	1	–	–	4	0.71	0.66	3	0.5	0.44	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–
PMacp014	3	0.63	0.56	4	0.74	0.69	1	–	–	1	–	–	1	–	–	1	–	–	2	0.23	0.2	1	–	–
PMacp015	1	–	–	3	0.5	0.44	1	–	–	1	–	–	2	0.44	0.35	5	0.79	0.76	1	–	–	1	–	–
PMacp016	1	–	–	2	0.44	0.35	2	0.44	0.35	3	0.5	0.44	1	–	–	1	–	–	1	–	–	1	–	–
PMacp017	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–	1	–	–
Mean	1.59	0.45	0.37	2.71	0.59	0.52	1.65	0.43	0.36	1.53	0.4	0.33	1.71	0.52	0.44	2.24	0.63	0.59	1.18	0.31	0.26	1.18	0.29	0.25

N_a, number of alleles for each locus; *h*, haploid diversity; PIC, polymorphism information content; all genetic parameters were estimated based on 15 individuals per sampled *Pinus* species; – amplification without polymorphism.

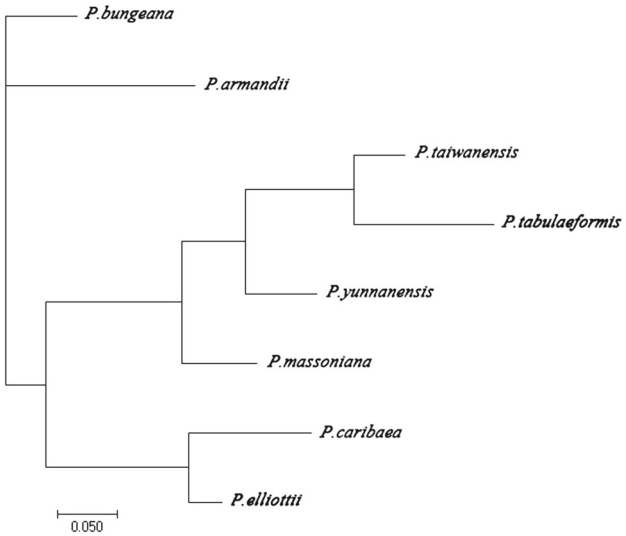

Figure 1. The phylogenetic tree of eight sampled pine species.

Results and discussion

Development of polymorphic microsatellites

In this study, a total of 71 chloroplast motifs were identified in the chloroplast genome, including mononucleotides (50, 70.42%); dinucleotides (2, 2.82%); tetranucleotides, pentanucleotides and hexanucleotides (9, 12.68%) and combination motifs (10, 14.08%). In total, 17 primer pairs were developed (table 3), of which eight could amplify polymorphic loci in 15 *P. massoniana* individuals.

Population genetic analysis

The characteristics of polymorphic cpSSRs were tested in *P. massoniana* and seven related species (table 4). The number of alleles of polymorphic cpSSRs were in the range of 2–6 in sampled *Pinus* species and differed in species (PMacp010 had the most alleles in *P. armandii*). The average of haploid diversity (*h*) ranged from 0.29 (*P. tabulaeformis*) to 0.63 (*P. armandii*). All these indicated that these polymorphic cpSSRs could produce more haplotypes (Ueno *et al.* 2016). Haploid diversity was similar to that of cpSSRs in *P. strobus*, and a decreasing trend of genetic diversity was observed for both nSSRs and cpSSRs (Zinck and Rajora 2016). In addition, PIC of polymorphic cpSSRs ranged from 0.20 to 0.80 (for PMacp010 in *P. armandii*). cpSSRs had similar PICs to those for nSSRs (Bai *et al.* 2014). According to Botstein *et al.* (1980), codominant loci can be highly informative (PIC $>$ 0.5), reasonably informative (0.5 $>$ PIC $>$ 0.25) and slightly informative (PIC $<$ 0.25). Thus, most of the polymorphic cpSSRs were highly or reasonably informative and could be useful in study of genetic diversity and paternal analysis.

Cross-amplification in related species and phylogenetic analysis

In the related species, the developed markers had different transferability: 14 in *P. yunnanensis*, eight in *P. taiwanensis*, seven in *P. elliottii*, *P. bungeana* and *P. armandii*, and three in *P. caribaea* and *P. tabulaeformis* were polymorphic (table 3). Although, the sequences of intergenic regions (*psbA-trnH*) and genes (*matK* and *rps16*) in the chloroplast genome are often used in phylogenetic analysis (Gizaw *et al.* 2016), cpSSRs are also used. The phylogenetic tree of eight sampled *Pinus* species was based on seven polymorphic markers (PMacp005–PMacp011) (figure 1). *P. bungeana* and *P. armandii* were distinguished into two separate groups (sect. *Parrya* and sect. *Cembra*), and the other species were formed into one group (sect. *Pinus*). Also, sampled pine species originating from the Eurasian continent (*P. massoniana*, *P. taiwanensis*, *P. tabulaeformis* and *P. yunnanensis*) could be distinguished from those originating from the Americas (*P. elliottii* and *P. caribaea*). Thus, cpSSRs could be useful tool in phylogenetic studies.

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