Chloroplast Microsatellite Markers for Pseudotaxus chienii Developed from the Whole Chloroplast Genome of Taxus chinensis var. mairei (Taxaceae)

Authors: Deng, Qi, Zhang, Hanrui, He, Yipeng, Wang, Ting, and Su, Yingjuan

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PRIMER NOTE

CHLOROPLAST MICROSATELLITE MARKERS FOR *PSEUDOTAXUS CHIENII* DEVELOPED FROM THE WHOLE CHLOROPLAST GENOME OF *TAXUS CHINENSIS* VAR. *MAIREI* (TAXACEAE)[1]

QI DENG[2,3], HANRUI ZHANG[2], YIPENG HE[2], TING WANG[4,5], AND YINGJUAN SU[2,5]

[2]School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, People's Republic of China; [3]School of Medicine, Guangxi University of Science and Technology, Liuzhou 545005, People's Republic of China; and [4]College of Life Science, South China Agricultural University, Guangzhou 510642, People's Republic of China

- *Premise of the study: Pseudotaxus chienii* (Taxaceae) is an old rare species endemic to China that has adapted well to ecological heterogeneity with high genetic diversity in its nuclear genome. However, the genetic variation in its chloroplast genome is unknown.
- *Methods and Results:* Eighteen chloroplast microsatellite markers (cpSSRs) were developed from the whole chloroplast genome of *Taxus chinensis* var. *mairei* and successfully amplified in four *P. chienii* populations and one *T. chinensis* var. *mairei* population. Of these loci, 10 were polymorphic in *P. chienii*, whereas six were polymorphic in *T. chinensis* var. *mairei*. The unbiased haploid diversity per locus ranged from 0.000 to 0.641 and 0.000 to 0.545 for *P. chienii* and *T. chinensis* var. *mairei*, respectively.
- *Conclusions:* The 18 cpSSRs will be used to further investigate the chloroplast genetic structure and adaptive evolution in *P. chienii* populations.

Key words: chloroplast microsatellite; genetic diversity; *Pseudotaxus chienii*; Taxaceae; *Taxus chinensis* var. *mairei*.

Taxus L. and *Pseudotaxus* W. C. Cheng are two closely related sister genera with similar appearance in Taxaceae (Fu et al., 1999). Their only distinction is the difference in color in the stomatal bands and aril (Fu et al., 1999). Both *T. chinensis* (Pilg.) Rehder var. *mairei* (Lemée & H. Lév.) W. C. Cheng & L. K. Fu and *P. chienii* (W. C. Cheng) W. C. Cheng are coniferous species endemic to China. *Taxus chinensis* var. *mairei*, in particular, has a high medicinal value because it contains the anticancer agent taxol (Li et al., 2008). *Pseudotaxus chienii*, the sole species in the monotypic genus, is an evergreen shrub or small tree with an average height of 4 m (Su et al., 2009). Due to overexploitation and human activities, the population size of *P. chienii* is shrinking. The species is categorized as an endangered species in the *Red List of Endangered Plants in China* (Fu and Jin, 1992). As an "old rare species," *P. chienii* has adapted well to habitat fragmentation and ecological heterogeneity across a wide range of habitats and is found in Zhejiang, Jiangxi, Hunan, and Guangxi provinces (Deng et al., 2013). The previous nuclear inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers have revealed that *P. chienii* possesses high genetic diversity, which provides a large pool of raw material for adaptive evolution (Su et al., 2009; Deng et al., 2013). However, the level of genetic variation in the *P. chienii* chloroplast genome is unknown.

Chloroplast simple sequence repeat (cpSSR) markers, which have been extensively used in population genetics, possess important and unique characteristics such as haploidy, nonrecombination, uniparental inheritance, and a low nucleotide substitution rate (Ebert and Peakall, 2009). cpSSR loci are generally distributed throughout the noncoding regions with higher sequence variations and have conservative flanking regions (Huang et al., 2015). In particular, the chloroplast genome retains ancient genetic patterns and can therefore provide unique insight into evolutionary processes (Provan et al., 2001). Therefore, cpSSR markers can be used to investigate genetic variation in small, fragmented populations and can be transferred to related species (Schaal et al., 1998; Petit et al., 2005; Pan et al., 2014). More important, because cpSSRs are paternally inherited in gymnosperms, they can be used to assess pollen-mediated gene flow, population genetic variation, and phylogeographic patterns. Information revealed by cpSSRs is complementary to that obtained from nuclear SSRs (Powell et al., 1996; Provan et al., 2001). Although no chloroplast genome sequences of *P. chienii* have been reported, the complete chloroplast genome sequence of *T. chinensis* var. *mairei* is available in the National Center for Biotechnology Information's GenBank (accession no. NC_020321.1). Thus, here we first isolated 18 cpSSRs in

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[5] Authors for correspondence: suyj@mail.sysu.edu.cn; tingwang@scau.edu.cn

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TABLE 1. Characteristics of 18 cpSSR markers developed for *Taxus chinensis* var. *mairei*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Product size (bp)	Location[c]
PTC-cp01[a,b]	F: CACCATCCACTGCCTTTG R: GTGCGGTCAGAACTTGTCA	$(AT)_9$	54	168	*trnH*-GUG-*trnI*-CAU (337–354)
PTC-cp02[b]	F: TGCGTGGCTGTGAGATG R: GCGGAACCCGTAGTGAA	$(AC)_5$	54	213	*trnT-trnE* (21,944–21,953)
PTC-cp03	F: AAGCCGCCCTGTTTTA R: ATCTCATCGCATTGGAAGT	$(TA)_5(AT)_5$	54	232	*trnC*-GCA-*rpoB* (25,696–25,715)
PTC-cp04[b]	F: TGTGGCACTATCCAAGGTC R: GCGTGGCAATACATCTCC	$(AT)_5$	54	135	*rpoC2* (32,846–32,855)
PTC-cp05	F: TGAACAGGTCCGACAGCA R: CCATCCCATCTCCTACTTGA	$(A)_{11}TACAA(AT)_5$	55	376	*atpF-atpA* (39,353–39,378)
PTC-cp08	F: TGATGAGTGCCGCCTAAT R: CGGGGAACTAATCTTCTTGT	$(AT)_5AGAAGTATACTTC(TA)_5$	54	173	*rpl36-rps11* (57,373–57,405)
PTC-cp09[a]	F: TGTATCAACCAATGCTTCC R: ATTCATAGATGTTTTCGCTG	$(TC)_6$	54	273	*psbT-psbB* (62,807 – 62,818)
PTC-cp13[b]	F: AGTCCAAAATCTCCCACAC R: CTTCTTCAATGCTTCTATGCT	$(AT)_5$	54	180	*ndhF* (104,893–104,902)
PTC-cp15	F: GCTTGGACCCATTGTTGAA R: CATACTTTAGGTGGCGTTGTTA	$(A)_{10}$	55	279	*rpoC1-rpoC2* (32,043–32,052)
PTC-cp16[a,b]	F: CCCATACTCCCATTTCATAACTT R: AGCACTTGCCCAGGACTAACT	$(A)_{10}$	55	237	*rpoC2* (34,060–34,069)
PTC-cp18[a,b]	F: TCCAGGTGCTGATGCTACTAA R: TCGTGCTGCTTCTTTCTTTG	$(A)_{10}$	55	186	*rpoC2-atpI* (35,652–35,661)
PTC-cp21[b]	F: GGTGGGGTGGGAACG R: TTGGGTGAGCCATAGAAAT	$(A)_{10}$	55	306	*rpl32* (106,169–106,178)
PTC-cp22[a,b]	F: AGCAATGTTTGGAAGGGAA R: GGTGTAGTCTATTTGGTGGTGTT	$(A)_{10}$	55	130	*rpl32-trnP*-GGG (106,257–106,266)
PTC-cp23[b]	F: AACTAATCCCAATGGCTTCA R: CCCTATGCGTGCCTATCA	$(T)_{10}$	55	301	*ycf3* intron (9378–9387)
PTC-cp26[a,b]	F: TGGATAGGACCCATAACAGG R: AAACTACGGCGATTTCTTC	$(T)_{10}$	55	326	*ycf4* (65,483–65,492)
PTC-cp28	F: TGTAGTTTGCCGAGTGGTT R: AATAATAGTAGACATTGGAAGGAC	$(T)_{11}$	55	336	*psbE-petL* (70,723–70,733)
PTC-cp29[a,b]	F: AATAGGTTCTGGAGCGGTTA R: AGATTTAGTTCGTCACGGGTA	$(T)_{11}$	55	254	*rps8* (75,439–75,449)
PTC-cp32[a,b]	F: CCTCGTGCGGATAACTAAA R: TGGCAAAGATTCCCTGG	$(TCTTCC)_7$	55	284	*rps15-chlN* (121,797–121,838)

Note: T_a = annealing temperature.

[a] Monomorphic loci for *Pseudotaxus chienii*.

[b] Monomorphic loci for *T. chinensis* var. *mairei*.

[c] Locus location (genic or intergenic region); the position amplified by the primers in the *T. chinensis* var. *mairei* chloroplast genome is given in parentheses.

T. chinensis var. *mairei*, then applied 10 polymorphic markers to evaluate the genetic diversity of *P. chienii.* These markers will be further applied to survey the chloroplast genetic variation in *P. chienii*.

METHODS AND RESULTS

In this study, a total of 109 individuals from four populations of *P. chienii* were collected throughout its natural distribution range, including Shuimenjian (Zjsmj) in Zhejiang Province, Zhangjiajie (Hnzjj) in Hunan Province, Zizhuba (Jxzzb) in Jiangxi Province, and Damingshan (Gxdms) in Guangxi Zhuang Autonomous Region, China (Appendix 1). One *T. chinensis* var. *mairei* population was gathered from Fenshui (Jxfs) in Jiangxi Province. Due to its rare and endangered properties, only 11 individuals were sampled. Young leaves were collected and dried in silica gel immediately. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987).

From the complete chloroplast genome sequence for *T. chinensis* var. *mairei* (GenBank accession no. NC_020321.1), 32 cpSSR loci were identified with the repeat threshold settings of 10 repeats for mononucleotides and five repeats for di-, tri-, tetra-, penta-, and hexanucleotide cpSSRs. Based on their flanking regions, we designed 27 primers using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, California, USA). One individual (SMJ27) from Zjsmj population for *P. chienii* and one individual (FS7) from Jxfs population for *T. chinensis* var. *mairei* were selected to screen these primers. PCR was performed in a total volume of 20 µL containing 20 ng of genomic DNA, 1× PCR buffer, 5 mM $MgCl_2$, 0.2 mM dNTPs mixture, 0.25 µM of each primer, and 1 unit *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China). Reaction conditions included initial denaturation at 94°C for 3 min; followed by 35 cycles at 94°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min; with a final extension at 72°C for 10 min. The annealing temperature was optimized by gradient PCR (Table 1). Amplified products were separated by 6% denaturing polyacrylamide gel electrophoresis and visualized by silver staining. The allele sizes were estimated with a 50-bp DNA ladder (TaKaRa Biotechnology Co.) as size standard. Eighteen of 27 primers (approximately 67%) could produce clear bands in both *P. chienii* and *T. chinensis* var. *mairei*. The 18 cpSSRs were divided into three categories in terms of motif structure: 15 perfect, one imperfect, and two compound repeats. The high frequency of perfect repeats was in accordance with Ebert's description (Ebert and Peakall, 2009).

The utility of these 18 cpSSR primers was further examined in 109 and 11 individuals of *P. chienii* and *T. chinensis* var. *mairei*, respectively. The PCR reactions were conducted as described above. Among these loci, 10 (PTC-cp02, PTC-cp03, PTC-cp04, PTC-cp05, PTC-cp08, PTC-cp13, PTC-cp15, PTC-cp21, PTC-cp23, and PTC-cp28) showed polymorphisms in *P. chienii*, whereas six (PTC-cp03, PTC-cp05, PTC-cp08, PTC-cp09, PTC-cp15, and PTC-cp28) were polymorphic in *T. chinensis* var. *mairei* (Table 1). The genetic parameters, including the number of alleles (A), haploid diversity (h), and unbiased haploid diversity (h_{unb}) for each population, were evaluated with GenAlEx version 6.41 (Peakall and Smouse, 2006). Ten polymorphic cpSSR loci for *P. chienii* and six polymorphic loci for *T. chinensis* var. *mairei* were used. For *P. chienii*, A was between one and four, h ranged from 0.000 to 0.620, and h_{unb} varied from 0.000 to 0.641 (Table 2). Population Zjsmj revealed obviously higher diversity than

Table 2. Genetic properties of 10 polymorphic cpSSR loci for *Pseudotaxus chienii* and six polymorphic loci for *Taxus chinensis* var. *mairei*.[a]

	Pseudotaxus chienii												*Taxus chinensis* var. *mairei*		
	Zjsmj ($n = 30$)			Gxdms ($n = 30$)			Hnzjj ($n = 19$)			Jxzzb ($n = 30$)			Jxfs ($n = 11$)		
Locus	A	h	h_{unb}	A	h	h_{unb}	A	h	h_{unb}	A	h	h_{unb}	A	h	h_{unb}
PTC-cp02	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.320	0.331	NA[c]	NA[c]	NA[c]
PTC-cp03	2	0.180	0.186	1	0.000	0.000	2	0.188	0.199	1	0.000	0.000	2	0.165	0.182
PTC-cp04	2	0.180	0.186	1	0.000	0.000	2	0.100	0.105	2	0.064	0.067	NA[c]	NA[c]	NA[c]
PTC-cp05	3	0.127	0.131	3	0.127	0.131	2	0.100	0.105	1	0.000	0.000	3	0.430	0.473
PTC-cp08	2	0.498	0.515	3	0.504	0.522	1	0.000	0.000	1	0.000	0.000	2	0.463	0.509
PTC-cp09	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	2	0.463	0.509
PTC-cp13	3	0.504	0.522	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	NA[c]	NA[c]	NA[c]
PTC-cp15	3	0.418	0.432	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.496	0.545
PTC-cp21	1	0.000	0.000	2	0.124	0.129	1	0.000	0.000	1	0.000	0.000	NA[c]	NA[c]	NA[c]
PTC-cp23	2	0.124	0.129	2	0.064	0.067	2	0.100	0.105	2	0.124	0.129	NA[c]	NA[c]	NA[c]
PTC-cp28	4	0.620	0.641	3	0.184	0.191	3	0.421	0.444	2	0.320	0.331	2	0.463	0.509

Note: A = number of alleles; h = haploid diversity; h_{unb} = unbiased haploid diversity; n = number of individuals sampled.

[a] Voucher and locality information are provided in Appendix 1.

[b] No analysis performed because PTC-cp09 was monomorphic in *P. chienii*.

[c] No analysis performed because PTC-cp02, PTC-cp04, PTC-cp13, PTC-cp21, and PTC-cp23 were monomorphic in *T. chinensis* var. *mairei*.

other populations. For *T. chinensis* var. *mairei*, A, h, and h_{unb} were one to three, 0.000–0.496, and 0.000–0.545, respectively (Table 2).

Analysis of molecular variance (AMOVA) was performed to measure genetic differentiation and the ratio of genetic variations within and among *P. chienii* populations in Arlequin version 3.5 (Excoffier and Lischer, 2010). The results revealed significant difference in partitioning of variation among and within populations (29.03% and 70.97%, respectively; Table 3) and uncovered significant genetic differentiation among all populations ($F_{\mathrm{ST}} = 0.2903$).

CONCLUSIONS

The polymorphic chloroplast SSR loci developed from *T. chinensis* var. *mairei* in this study were verified to be reliable for assessing genetic variation of *P. chienii* populations. Combined with the nuclear SSR loci previously developed (Deng et al., 2013), the 18 cpSSRs will contribute to further exploration of whether the adaptation of *P. chienii* to environmental heterogeneity is driven through nuclear or chloroplast loci. In addition, the conservative nature of cpDNA may allow these markers to be used in other conifers.

LITERATURE CITED

Deng, Q., Y. J. Su, and T. Wang. 2013. Microsatellite loci for an old rare species, *Pseudotaxus chienii*, and transferability in *Taxus wallichiana* var. *mairei* (Taxaceae). *Applications in Plant Sciences* 1: 1200456.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Ebert, D., and R. Peakall. 2009. Chloroplast simple sequence repeats (cpSSRs): Technical resources and recommendations for expanding cpSSR discovery and applications to a wide array of plant species. *Molecular Ecology Resources* 9: 673–690.

Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564–567.

Fu, L. G., and J. M. Jin. 1992. Red list of endangered plants in China. Science Press, Beijing, China.

Fu, L. G., N. Li, and R. R. Mill. 1999. Taxaceae. *In* Z. Y. Wu and P. H. Raven [eds.], Flora of China, vol. 4, 89–98. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

Huang, J., X. T. Yang, C. M. Zhang, X. Yin, S. P. Liu, and X. G. Li. 2015. Development of chloroplast microsatellite markers and analysis of chloroplast diversity in Chinese jujube (*Ziziphus jujuba* Mill.) and wild jujube (*Ziziphus acidojujuba* Mill.). *PLoS ONE* 10: e0134519.

Li, C. F., C. H. Huo, M. L. Zhang, and Q. W. Shi. 2008. Chemistry of Chinese yew, *Taxus chinensis* var. *mairei*. *Biochemical Systematics and Ecology* 36: 266–282.

Pan, L., Y. Li, R. Guo, H. Wu, Z. H. Hu, and C. Y. Chen. 2014. Development of 12 chloroplast microsatellite markers in *Vigna unguiculata* (Fabaceae) and amplification in *Phaseolus vulgaris*. *Applications in Plant Sciences* 2: 1300075.

Peakall, R., and P. E. Smouse. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

Petit, R. J., J. Duminil, S. Fineschi, A. Hampe, D. Salvini, and G. G. Vendramin. 2005. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molecular Ecology* 14: 689–701.

Powell, W., G. C. Machray, and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* 1: 215–222.

Provan, J., W. Powell, and P. M. Hollingsworth. 2001. Chloroplast microsatellites: New tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution* 16: 142–147.

Schaal, B. A., D. A. Hayworth, K. M. Olsen, J. T. Rauscher, and W. A. Smith. 1998. Phylogeographic studies in plants: Problems and prospects. *Molecular Ecology* 7: 465–474.

Su, Y. J., T. Wang, and P. Y. Ouyang. 2009. High genetic differentiation and variation as revealed by ISSR marker in *Pseudotaxus chienii* (Taxaceae), an old rare conifer endemic to China. *Biochemical Systematics and Ecology* 37: 579–588.

Table 3. The analysis of molecular variance (AMOVA) within and among populations based on 10 polymorphic cpSSRs in *Pseudotaxus chienii*.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	P value
Among populations	3	22.880	0.25926	29.03%	<0.0001
Within populations	105	66.542	0.63373	70.97%	<0.0001
Total	108	89.422	0.893	100.00%	

Note: df = degrees of freedom.

Appendix 1. Collection locality and voucher information for *Pseudotaxus chienii* and *Taxus chinensis* var. *mairei* populations used in this study.

Species	Population code	Collection locality	Geographic coordinates	Voucher specimen[a]
Pseudotaxus chienii (W. C. Cheng) W. C. Cheng	Zjsmj	Shuimenjian, Zhejiang Province	28°43′42″N, 118°57′32″E	*YJ Su 201303, SMJ27*
Pseudotaxus chienii	Jxzzb	Zizhuba, Jiangxi Province	26°27′18″N, 114°06′22″E	*YJ Su 201303, ZZB12*
Pseudotaxus chienii	Hnzjj	Zhangjiajie, Hunan Province	29°23′12″N, 110°28′56″E	*YJ Su 201303, ZJJ09*
Pseudotaxus chienii	Gxdms	Damingshan, Guangxi Zhuang Autonomous Region	23°29′54″N, 108°26′12″E	*YJ Su 201303, DMS17*
Taxus chinensis (Pilg.) Rehder var. *mairei* (Lemée & H. Lév.) W. C. Cheng & L. K. Fu	Jxfs	Fenshui, Jiangxi Province	28°56′31″N, 108°02′12″E	*WB Liao 201108, FS1*

[a] Voucher specimens are deposited at the herbarium of Sun Yat-sen University (SYSU).