PRIMER NOTE

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

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• 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 haplotype 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 over-exploitation 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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PTC-cp02

| Locus | Primer sequences (5′−3′) | Repeat motif | Product size (bp) | Location |
|-------|--------------------------|--------------|------------------|----------|
| PTC-cp01 | F: CACCATCACCTGCTTTTG | (AT)\(_9\) | 54 | 168 | trnH-GUG-trnF-CAU (337–354) |
| R: GTGGGCTGAGACTGTTCA | (AC)\(_3\) | 54 | 213 | trnF-trnE (21,944–21,953) |
| PTC-cp02 | F: GGAGGCTCTGTTGAGAGT | (AC)\(_3\) | 54 | 232 | trnF-GCA-psbO (25,696–25,715) |
| R: AATGCTGAATGCTGTTGAAGT | (TA)\(_2\)(AT)\(_3\) | 54 | 135 | rpoC2 (32,846–32,855) |
| PTC-cp03 | F: AGGCCCTCTGTGTTTA | (AT)\(_3\) | 54 | 376 | atpF-atpH (39,353–39,378) |
| R: ATCTCATGGCATTGGAGAGT | (AT)\(_5\) | 54 | 173 | rpl36-rps15 (57,373–57,405) |
| PTC-cp04 | F: TGGGGAATCTAACCAGTCGTC | (AT)\(_3\) | 54 | 279 | rpoC1-rpoC2 (32,043–32,052) |
| R: GGTGTAGTCTATTTGGTGGTT | (T)\(_{10}\) | 55 | 180 | ndhF (104,893–104,902) |
| PTC-cp05 | F: CGGGCACTAACTCTGTTGTA | (A)\(_9\) | 55 | 237 | rpoC2 (34,060–34,069) |
| R: AGACATGGCCAGACTAACTT | (TA)\(_5\)(AT)\(_3\) | 55 | 186 | rpoC2-apl (35,652–35,661) |
| PTC-cp06 | F: GCTTGCGCTGTTGTTTG | (A)\(_10\) | 55 | 306 | rpl32 (106,169–106,178) |
| R: ATGCTGCTTCTCTTCTTGG | (A)\(_10\) | 55 | 306 | rpl32-trnF-PSB (106,257–106,266) |
| PTC-cp07 | F: AATCTTACCATACCTGTTTG | (AT)\(_9\) | 55 | 301 | ycf3 intron (9378–9387) |
| R: CCTATGCGCTTCTCTTGGTT | (T)\(_9\) | 55 | 301 | ycf4 (65,483–65,492) |
| PTC-cp08 | F: GTGGGTTGGCAGAGC | (T)\(_9\) | 55 | 326 | ycf4 (65,483–65,492) |
| R: CGGGGAAGTACCTCCTCGGT | (TA)\(_5\)(AT)\(_3\) | 55 | 326 | psbE-psbL (70,723–70,733) |
| PTC-cp09 | F: AAGAGTCTTGGAGAGGAAA | (T)\(_9\) | 55 | 254 | rps8 (75,439–75,449) |
| R: GGTGAGTCTTATGTTGGTGT | (A)\(_10\) | 55 | 254 | rps8 (75,439–75,449) |

Note: \(T_a\) = annealing temperature.

*Monomorphic loci for Pseudotaxus chienii.

#Monomorphic loci for T. chinensis var. mairei.

Locus location (genic or intergenic region); the position amplified by the primers in the chloroplast genome is given in parentheses.

### METHODS AND RESULTS

In this study, a total of 109 individuals from four populations of P. chinensis were collected throughout its natural distribution range, including Shuimenjian (Zjsmj) in Zhejiang Province, Zhangjiajie (Hnzjj) in Hunan Province, Zizhuba (Jxzzb) in Jiangxi Province, and its sibling species P. mairei in 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 (SM27) from Zjsmj population for P. chinensis and one individual (FST) from Xfs 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. chinensis 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. chinensis 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. chinensis, 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 (\(A\)) and unbiased haploid diversity (\(h_{ub}\)) for each population, were evaluated with GenAlEx version 6.41 (Peakall and Smouse, 2000). Ten polymorphic cpSSR loci for P. chinensis and six polymorphic loci for T. chinensis var. mairei were used. For P. chinensis, \(A\) was between one and four, \(h\) ranged from 0.000 to 0.620, and \(h_{ub}\) varied from 0.000 to 0.641 (Table 2). Population Zjsmj revealed obviously higher diversity than P. chinensis var. mairei.

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TABLE 2. Genetic properties of 10 polymorphic cpSSR loci for *Pseudotaxus chienii* and six polymorphic loci for *Taxus chinensis* var. *mairei*.

| Locus       | *Pseudotaxus chienii* | *Taxus chinensis* var. *mairei* |
|-------------|-----------------------|---------------------------------|
|             | Zjsmj (*n* = 30)      | Gxdms (*n* = 30)                | Hnzj (*n* = 19) |
|             | Jxzzb (*n* = 30)      |                                 | Jxfs (*n* = 11) |
|             |                       |                                 |                |
| PTC-cp02    | 1                     | 0.000                           | 2.000          |
|             |                       | 0.000                           | 0.000          |
| PTC-cp03    | 2                     | 0.180                           | 0.188          |
|             |                       | 0.186                           | 0.188          |
| PTC-cp04    | 2                     | 0.180                           | 0.188          |
|             |                       | 0.186                           | 0.188          |
| PTC-cp05    | 3                     | 0.127                           | 0.127          |
|             |                       | 0.131                           | 0.131          |
| PTC-cp08    | 2                     | 0.498                           | 0.504          |
|             |                       | 0.515                           | 0.522          |
| PTC-cp09    | NA<sup>a</sup>        | NA<sup>a</sup>                  | NA<sup>a</sup> |
|             |                       | NA<sup>a</sup>                  | NA<sup>a</sup> |
| PTC-cp13    | 3                     | 0.504                           | 0.522          |
|             |                       | 0.000                           | 0.000          |
| PTC-cp15    | 3                     | 0.418                           | 0.432          |
|             |                       | 0.000                           | 0.000          |
| PTC-cp21    | 1                     | 0.000                           | 0.000          |
|             |                       | 0.124                           | 0.129          |
| PTC-cp23    | 2                     | 0.124                           | 0.129          |
|             |                       | 0.124                           | 0.129          |
| PTC-cp28    | 4                     | 0.620                           | 0.641          |
|             |                       | 0.184                           | 0.191          |

**Note**: *A* = number of alleles; *h* = haplotype diversity; *h*<sub>unb</sub> = unbiased haplotype diversity; *n* = number of individuals sampled.

<sup>a</sup>Voucher and locality information are provided in Appendix 1.

<sup>b</sup>No analysis performed because PTC-cp09 was monomorphic in *P. chienii*.

<sup>c</sup>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*<sub>unb</sub> 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*<sub>ST</sub> = 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.

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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.

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### 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   |
|--------------------------|-----------------|--------------------------------------|------------------------|--------------------|
| *Pseudotaxus chienii*    | Zjsnj           | Shuimenjian, Zhejiang Province       | 28°43'42"N, 118°57'32"E | YJ Su 201303, SMJ27 |
| (W. C. Cheng) W. C. Cheng|                 |                                      |                        |                    |
| *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*    | Gxsdms          | 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  |

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