Cross-Species Amplification of Microsatellite Loci for the Endangered Conifer, *Taxus chinensis* var. *mairei* (Taxaceae)

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Abstract

Being an economical and endangered species, microsatellite markers of *Taxus chinensis* var. *mairei* were very limited. We have developed a set of microsatellite markers, which was benefit for future genetic analysis of this rare species. Polymorphic loci were developed from congeneric species by cross-species amplification methods, and new primers were redesigned to test for potential null alleles. 15 loci showed polymorphism. The number of alleles per locus varied from 2 to 23 tested in 48 individuals. The observed heterozygosity (*Ho*) and expected heterozygosity (*He*) values ranged form 0.000 to 0.854 and 0.082 to 0.827, respectively. Newly redesigned primer confirmed that no null allele existed in most suspected loci. These microsatellite markers will be useful for future genetic analysis and conservation of this endangered species.

Key words: cross-amplification, microsatellites, *Taxus chinensis* var. *mairei*, population genetics, genetic diversity.

Introduction

Maire Yew (*Taxus chinensis* var. *mairei*) is an endemic *Taxus* species in China (ZHANG, et al., 1978). Its central distribution range consists of mainly scattered populations from the south of the Yellow river to the Nanling Mountain. Maire Yew has attracted much attention due to an anti-cancer drug that it contains, and due to the high aesthetic timber. However, long term over-exploitation has made this species become scarce and seriously threatened with extinction. In order to protect this rare species, the Chinese Government has listed Maire Yew among the first class protected plants since 1999 (The State of Council of the People's Republic of China, 1999), and several nature reserves have been set up in Southern China.

Conserving genetic diversity is one of the most important factors for threatened species. In order to conserve the genetic diversity, it has to be measured first. Compared with random fingerprinting approaches, microsatellites (simple sequence repeats, SSRs) are an ideal marker system for population genetics studies due to several advantages (JARNE and LAGODA, 1996). However, to develop new species-specific microsatellites demands knowledge about their flanking sequences through traditional approaches, which is costly and time-consuming (ZANE et al., 2002; SQUIRRELL et al., 2003). One shortcut strategy is cross-species amplification to develop new species microsatellite markers (BARBARÁ et al., 2007), based on the conservation of microsatellites flanking regions in closely related species. This approach has been used in different studies, either to derive nuclear SSR (ALDRICH et al., 2003: PEAKALL et al., 1998) or cpSSR (WEISING and GARDNER, 1999; CHUNG and STAUB, 2003) and EST-SSR marker (HEESACKER et al., 2008).

Until now, only a few studies focused on the genetic diversity of Maire Yew, by analyzing dominant markers, such as RAPD (ZHANG et al., 2003) and ISSR (LI et al., 2010). But no study was done based on microsatellites yet, due to the limited number of markers available. To the author's knowledge, microsatellite loci have been isolated from several *Taxus* species, including *T. baccata* (DUBREUIL et al., 2008, MAHMOODI et al., 2010), *T. yunnanensis* (MIAO et al., 2008), *T. sumatrana* (HUANG et al., 2008) and *T. wallichiana* (YANG et al., 2009). These loci are potential resources for Maire Yew in order to develop specific microsatellite markers. In the present study, we evaluated 68 candidate loci (including one mitochondrial marker) from congeneric species, and also tested 4 Maire Yew microsatellite loci developed by ZHOU et al (2009). 15 polymorphic markers were selected and their characterization were described.

Materials and Methods

In order to evaluate the polymorphism of the candidate microsatellite markers, 48 individual samples were selected from a wide range of Maire Yew's distributions in Southern China. DNA extraction from the silica dried leaves was carried out by a modified CTAB method (TSUMURA et al., 1995). A total of 72 candidate loci were used to test PCR amplification and their polymorphism, in which 8 primer pairs were from *T. yunnanensis* (MIAO et al., 2008), 12 prime pairs were from *T. sumatrana* (HUANG et al., 2008), 10 primer pairs form *T. wallichiana* (YANG et al., 2009), 7 primer pairs from (DUBREUIL et al., 2008), 4 primer pairs from (ZHOU et al., 2009) and 31 primer pairs from (MAHMOODI et al., 2010). Some of the primers were end-modified to adapt post-PCR fluorescent labeling method (KONDO et al., 2000). For each of the primer pairs, 4 individuals were used to test whether they could be successfully amplified by PCR. PCR was performed in a 6.0 µl volume according to QIAGEN® Multiplex PCR kit instructions, containing 1 × Multiplex PCR master mix (Qiagen), 0.2 µM of forward and reverse primers, and 5–10 ng of template

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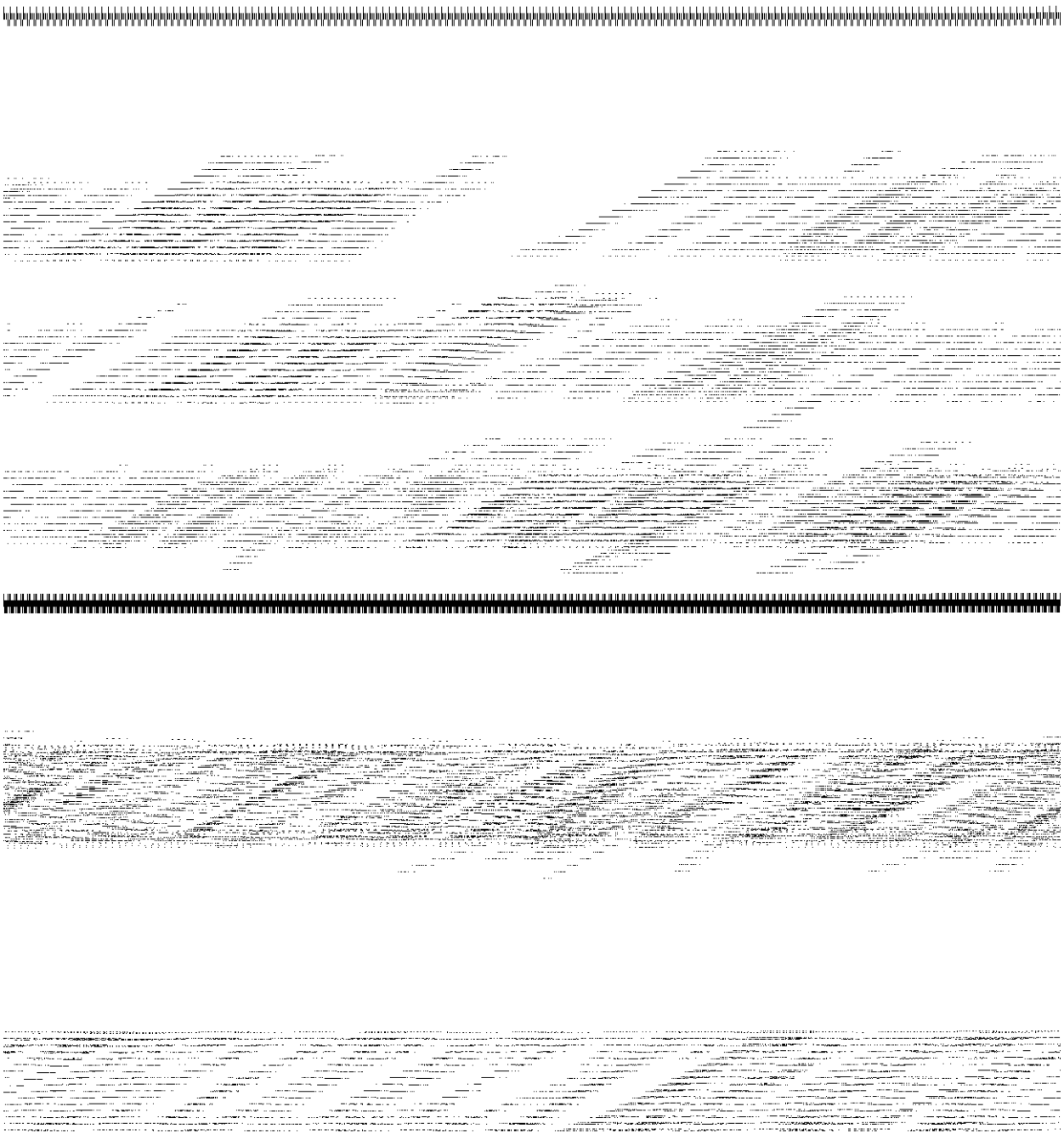
DNA. The PCR amplification conditions were: 15 min at 95 °C, then 30 cycles of 30 s at 94 °C, 90 s at the primer pair's optimized annealing temperature (*Table 1*), and 60 s at 72 °C, followed by a 30 min extension step at 60 °C. The PCR products were electrophoretically separated on 2% agarose gels stained with ethidium bromide. For successfully amplifying loci, further singleplex PCR was carried out, in order to check their polymorphism using 48 individuals. Primers were labeled at the 5' end with FAM or HEX fluorescent dyes (Applied Biosystems). PCR amplification system and conditions were the same as above. Genotypes were determined using an ABI3100 genetic analyzer and Liz 600 size standards. Population genetic parameters were estimated using GENALEX6.3 (PEAKALL and SMOUSE, 2006), including the number of alleles per locus (*Na*), effective

Table 1. – Characterization of 15 polymorphic microsatellite loci in Maire Yew (*Taxus chinensis* var. *mairei*).



[*] TB01 is mtSSR locus; [a] from MIAO et al. (2008); [b] from HUANG et al. (2008); [c] from YANG et al. (2009); [d] from DUBREUIL et al. (2008); [e] from ZHOU et al. (2009).

Notes: For loci TS03 and TS10, DNA sequences were not submitted to GenBank by the authors.

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number of alleles per locus (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and fixation index (F_{IS}). Hardy-Weinberg equilibrium (*HWE*) and linkage disequilibrium (*LD*) were assessed using GENEPOP (RAYMOND and ROUSSET, 1995) online version 4.0.10 (http://genepop.curtin.edu.au/index.html). Null alleles were detected by Micro-checker 2.2.3 (VAN OOSTERHOUT et al., 2004).

New primers were designed to confirm null alleles of the loci suspected by the results of Micro-checker 2.2.3. Direct sequencing of Maire Yew PCR products was according to the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Ethanol purification products were run on an ABI3100 automatic sequencer. Forward and reverse nucleotide sequences of each locus were assembled by Sequencher 4.10, and then aligned using MEGA 5.05. New primers were designed for 7 loci by Primer3Plus online (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). PCR amplification and genotyping procedures with these new primers were the same as those of the original primers. According to the level of allele matching between the two primer sets, we tried to confirm the existence of null alleles at each locus.

Results and Discussion

Cross-species transfer success

Among the total of 72 candidate loci (one was a mtSSR marker), 38 (52.8%) produced clear PCR fragment patterns (one or two bands), 3 (4.2%) showed multiple bands (more than 2 bands); while 31 (43.0%) did not produce any fragments. Out of 38 successfully amplifying loci, 15 (20.8%) showed evidence for polymorphisms (*Table 1*); the others were monomorphic. The overall average cross-amplification success was 48.6%, this data was less than conifer plants (66%, BARBARÁ et al., 2007). When markers were compared in terms of species in origin, the rate of cross-amplification success and polymorphic loci were distinct among 4 congeneric species. Primers derived from *T. yunnanensis*, *T. wallichiana* and *T. sumatrana* had high success rate. Their success rate was 100%, 90% and 75%, respectively, and polymorphic loci from these species were 66.7%, 40% and 16.7%, respectively. However, those from *T. baccata* were 23.7% and 2.6%. DUBREUIL et al. (2008) reported transferring 50% (4 of 8) microsatellite markers from *T. sumatrana* to *T. baccata*, and only one showed polymorphic banding pattern. MIAO et al. (2008) tested the transferability of 12 *T. sumatrana* markers in *T. yunnanensis*, also only one loci worked.

According to several authors (PRIMMER et al., 1996; GUO et al., 2006; VINYALLONAGA et al., 2011) that cross-amplification is except to work better for phylogenetically close species. Our data also support this idea. On the terms of phylogenetic relationship among species of *Taxus*, Marie Yew and *T. yunnanensis* has been recognized as a variety of *T. wallichiana* (FU et al., 1999); it had relatively far relationship with *T. baccata* (HAO et al., 2008).

Table 2. – Results of primer screening in 48 individuals of *Taxus chinensis* var. *mairei*.

N: number of samples, *Na*: number of alleles, *Ne*: effective number of alleles, *Ho*: observed heterozygosity, *He*: expected heterozygosity, *Fis*: fixation index; *HWE*: Hardy-Weinberg equilibrium test.

ns: not significant departure from Hardy-Weinberg equilibrium (*HWE*); **, ***: significant departure from Hardy-Weinberg equilibrium (*HWE*) ($P<0.01$, $P<0.001$, respectively).

* TB01 is mtSSR locus; † calculated using the method described by BROOKFIELD (1996).

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Table 3. – New redesigned primers and the result of screening in 48 individuals of *Taxus chinensis* var. MAIREI.

Locus	New primer sequence (5'- 3')	Ta/°C	Size range	PCR amplification	Proportion of matching genotypes (%)	Accession no
N-TY24	F: GTT CTC TAC CCA TAG CGT TCA TTC AG	60	115-175	Polymorphic	48/48 (100%)	AB725363
	R: ATT CTG TCC TCC CCA TAG ATC TCC					
N-TY05	F: <FAM> GGG GGA ACA TTC CTA CTC TAT CA	60	273-288	Polymorphic	48/48(100%)	AB725361
	R: GTT TAC AGT ACA GTT CGG AGT GAG					
N-TY16	F: GTG ACA GAT CTA CCA CAT CGT GA	60	148-169	Polymorphic	48/48(100%)	AB725362
	R: <FAM>TGG TAG TTG GAG CCC CTA TAC AT					
N-TC3	F: <FAM> TGC TAT GGA ATG AAG AAT CCA A	55	157-165	Polymorphic	39/45(86.7%)	AB725358
	R: GTT TCC GTG TGT GTT GTG TGT TTT					
N-TC4	F: <HEX> ACA TGG TGG CTA CAC TAG AGC AC	55	99-109	Polymorphic	31/45(68.9%)	AB725359
	R: CAA CCT AGT GAG GAT CAT ACT TTC A					
N-TG126	F: ATT GCA TAG GGG GAG AGA TTC TAT T	55	182	Monomorphic		AB725360
	R: GTT CTT ATC ACA GAG TGT CGC ACC A					
N-TA114	F: AAA TGT TCA GCC TCA AAA TCG	55		No bands		
	R: GTT TGT TCT GGC TTC CTC TAG CA					

Notes: For loci TY08 and TB86, new primers were not redesigned due to sequence failure.

Characterization of selected microsatellite loci

Among the 15 polymorphic loci, the number of alleles per locus varied from 2 to 23 when tested on the 48 individuals. The observed heterozygosity (*Ho*) and expected heterozygosity (*He*) values ranged form 0.000 to 0.854 and 0.082 to 0.827, respectively (*Table 2*). 10 loci were significantly deviated from Hardy-Weinberg equilibrium (*HWE*) due to a large heterozygote deficiency ($P < 0.01$). Twenty-four out of 91 possible pairwise comparisons between the 14 nuclear microsatellite loci showed significant linkage disequilibrium ($p < 0.05$). Numerous factors might cause the deviation of Hardy-Weinberg equilibrium (*HWE*), including inbreeding, null alleles and Wahlund effect. In our ongoing study among 26 populations using these nuclear markers, we have confirmed the influence of null alleles in most loci, which mean that inbreeding was the main reason for the large heterozygote deficiency and lead to deviation of Hardy-Weinberg equilibrium (*HWE*). Another factor such as Wahlund effect might be one of the reasons, because 48 samples were selected from a wide range of Maire Yew's distributions in Southern China.

Null allele's confirmation

Micro-checker detection indicated no apparent evidence for scoring errors due to stuttering or large allele dropout in the 14 nuclear SSR loci. Possible null alleles were suggested by this software in 9 loci (*Table 2*). Our approach to design new primer for confirmation of null alleles suggested that no such null alleles existed in the 3 loci TY24, TY16 and TY05 (*Table 3*), as the agreement between the two primer sets was 100%. For loci TC3 and TC4, the proportion of matching genotypes was 86.7% and 68.9%, respectively. This suggests there could be null alleles in these two loci. In our ongoing study among 26 populations using these 14 nuclear microsatellite loci (data not shown), TA114 and TG126 loci showed Hardy-Weinberg equilibrium (*HWE*) in most populations, which we take as an indication that no null alleles are present in them. For loci TY08 and TB86, new primers were not redesigned due to sequencing failure, they need more cautious confirmation in a future study. We have used these 15 microsatellite markers in ongoing genetic analysis of Maire Yew, which will be benefit for the conservation of this endangered species.

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