Journal of Molecular Genetics 1 (2-4): 44-49, 2009
ISSN: 2070-4267

Optimizing System of SSR-PCR in *Pinus radiata* and *Pinus tabulaeformis*

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Abstract: Reaction system of SSR-PCR was optimized by orthogonal design using 21 *Pinus radiate* and 3 *Pinus tabulaeformis* originated from the different locations. The results indicated that the optimal reaction system of SSR for *P. radiata* and *P. tabulaeformis* were described as follows each DNA amplification was carried out in a volume of 25 μL containing 0.3 U Taq polymerase, 1.5 μL $Mgcl_2$ (2.0 mmol L^{-1}), 160-200 ng genomic DNA, 0.3 μL each of SSR forward and reverse primers (0.6 μmol L^{-1}) and 2.0 μL dNTPs (0.2 mmol L^{-1}). DNA amplification for SSR was performed for initial 3 min at 94°C for pre-denaturing, followed by 30 cycles at 94°C for 30 sec for denaturing, 45 sec at 45-49°C for annealing, 30 sec at 72°C for extension. The reaction was terminated with 5 min extension at 72°C. The reaction system was verified by using 24 materials. The numbers of alleles per primer were 5-10 and the range of polymorphic bands was 100-250 bp.The results suggested that the reaction system described as above exhibited high polymorphic percentage and good stability and repetition.

Key words: *Pinus radiate*, *Pinus tabulaeformis*, SSR, optimization, reaction system, China

INTRODUCTION

SSR (Simple Sequence Repeat), STMS micro-satellite (Sequence-Tagged Microsatellites) or SSRP (Simple Sequence Repeat Polymorphims) were regarded to be the best methods for studying genetic variation of genetic population and have been widely used in germplasm identification, genetic linkage map drawing marking the orientation of the characteristic gene (Zhebentyayeva *et al*., 2003). Its applications for trees are usually seen in the studies on *Pinus*, *Eucalyptus* and *Populus* (Hokanson *et al*., 1998; Streiff *et al*., 1998; Brondani *et al*., 1998). In China, we can see only the study on the genetic structure of *Castanopsis fargesii* population and on SSR-PCR primer screening and reactive system building of *P. tabulaeformis* by this method (Lian *et al*., 2001; Zhang *et al*., 2007).

Studies on the genetic relationship among tree populations need to be further expanded and especially cross-species SSR primers in the *Pinus* genus need to be further developed. In the late 1980s, *P. radiate* was introduced into the arid and semi-arid Aba Tibetan and Qiang Autonomous Prefecture (hereinafter referred to as the Aba) of the upper reaches of Minjiang River in Sichuan. *P. radiate* was the important coniferous tree species for the ecological restoration and reconstruction and *P. tabulaeformis* was the important native coniferous species in the ecological zone. Since 1998, *P. radiata* has been continuously introduced in the Aba Sichuan for the ecological restoration and reconstruction and the *P. radiate* forest stands were about 3,000 hm^2. These forest stands were developed by using commercial seeds imported from abroad at different times for seeding and afforestation. Their genetic relationships among the germplasm populations had never been studied and this may result in the great blindness in selecting new varieties and introducing germplasm resources.

SSR marker showd the characteristics of species and genuses and the studies on SSR-PCR reaction system of *P. radiata* and *P. tabulaeformis* have never been reported yet at home so optimization of the SSR-PCR reactive system of imported *P. radiata* and native *P. tabulaeformis* can provide necessary theoretic and practical supports for their genetic analysis, tree species optimization and germplasm resources research.

MATERIALS AND METHODS

Experimental materials: Young leaves of the materials from different provenances were used for extraction the

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genome DNA. Young leaves were sampled, immediately put into the ice shell for cold preservation and were brought to the laboratory and preserved in a refrigerator at -70°C. The samples were collected from the imported *P. radiata* forest and the native *P. tabulaeformis* forest of Wenchuan County and Li County of the Aba in Sichuan Province and among the samples, 21 materials were from *P. radiata* and 3 materials were from *P. tabulaeformis*.

Extraction of DNA: Total genomic DNA was extracted from young leaves of *Pinus* plant by use of a modified Cetyltrimethylammonium Bromide (CTAB) method according to the procedure of Wang (2006), with some modifications.

Primer screening: Twenty pairs of SSR primers were synthesized by Shanghai Shenggong Inc. and were selected. The sequence of these primers was downloaded from http://bioinformatics.pbcbsac.Latrobe.edu.au and from http://www.pierroton.inra.fr/genetics/ssr. Among the 20 pairs of primers, 13 pairs with strong stability and good polymorphism and high resolution response were selected as the formal primers in the experiment. About 13 pairs of primers and their amplification results were shown in Table 1. Material 14 of *P. tabulaeformis* and material 19 of *P. radiata* were randomly selected and were used for screening the primers, the results indicated that the above two materials showed high polymorphism, so the selected primers were adapted for the amplification on 24 materials under the condition after optimization.

The SSR amplification system and the optimization of reactive conditions: Primers of gi28515642 randomly selected from 13 pairs of primers with the highly-polymorphic primers were used in the experiment. TaqDNA polymerase, dNTPS, 10×Buffer and Mg^{2+} were purchased from Shanghai Biological Engineering Technology Services Limited. The volume of PCR reactive system was 25 μL, which contained 2 μL of 100 ng μL^{-1} template DNA, 2.5 μL of 10×Buffer, 2.0 mmol L^{-1}, 1.5 μL of 2.0 mmol L^{-1} Mg^{2+}, 2.0 μL of 0.2 mmol L^{-1} dNTP, 0.3 μL of each 0.6 μmol L^{-1} SSR primer (forward primer and reverse primer contained 0.3 μL, respectively), 0.3 μL of 1.0 U Tap DNA polymerase and 16.1 μL of ddH_2O. When the concentration gradient test or dosage gradient test of an ingredient was carried out, the concentrations of other ingredients remained unchanged and the effects of different treatments on the amplifications were compared. The gradient settings of ingredients were shown in Table 2.

PCR amplification procedure: Predegeneration for 3 min at 94°C; degeneration for 30 sec at 94°C, annealing for 30 sec at 45°C, extension for 30 sec at 72°C, 30 cycles for this amplification; final extension for 5 min at 72°C. The gradient annealing temperature of temperatures (37, 41, 45, 49, 53, 57 and 61°C) was set up for finding the optimum temperature and the PCR amplification was carried out in the PTC-200PCR Instrument.

Table 1: Amplified results based on 13 SSR primers with 24 *pinus* materials

Primer no.	Primer sequence (5'~3')	No. of alleles	Locus heterozygosity
gi28520065	F : TACTCCTTCCAATCGCTCT R : TCAATCTACTCACACCCACTC	9.0	0.8494
Contig1547	F : CACTCACCCCACAAATACGG R : CTCGAATTTACCTAGGCCCC	5.0	0.7695
Cn299	F : GGGGAAGGTGTTCATACCG R : AGCGCCACAGTTTACTACCC	8.0	0.8729
gi28515642	F : GAGAAAGATTCACAATGAGG R : GTATCACCACCACCAGCA	9.0	0.7524
Ctg1376	F: CGATATTATGGATTTTGCTTGTGA R: AAATGCATGCCAAACTTAAATAC	10.0	0.8876
Contig1542	F: AAATGCAGTCTTCAAAGCGG R: TACTAGCGAGAAACTGGTCGCC	7.0	0.8139
Ctg16811	F: TCCATGATGTTGCAGATTGG R: GTGTTCCCCAATGGTCTGTC	9.0	0.8489
Contig17330	F: GGACAGTCCTTACTGCCCAA R: CCCATGGTTTTCCATTGTTC	7.0	0.8072
ctg18103	F: CCTGGATTCATTTGTGGCTAA R: CATGCCAACTTCTTGCATTG	9.0	0.8719
Ctg275	F: ACGGAGATATATTGCTGGCG R: AAAGAATAACGTGAAACAAACCC	7.0	0.8469
Ctg17607	F: CGCCATTAATATGCCTACCG R: ATCTCTGCGCTGCTTGAAGT	5.0	0.6920
Ctg6390	F: ATCCACGACTTGTCGACGC R: ATCAACCAACTTAGGCAGCG	5.0	0.6963
Ctg7024	F: GGGAATTCTGAAAGACAAGGG R: AACTTACCATCGAGAGCCCC	7.0	0.8166
Average		7.5	0.8097

Table 2: The orthogonal design of PCR [$L_{25}(5^5)$]

Factors				
Concentration of dNTPs (mmol L^{-1})	Concentration of primers (μmol L^{-1})	Concentration of template DNA (ng μL^{-1})	Amount of *Taq*E (U)	Mg^{2+} Concentration (mmol L^{-1})
0.05	0.2 (0.1+0.1)	40	0.25	0.50
0.05	0.4 (0.2+0.2)	60	0.50	1.00
0.05	0.6 (0.3+0.3)	80	1.00	1.50
0.05	0.8 (0.4+0.4)	100	1.50	2.00
0.05	1.0 (0.5+0.5)	120	2.00	2.50
0.10	0.2 (0.1+0.1)	40	0.25	0.50
0.10	0.4 (0.2+0.2)	60	0.50	1.00
0.10	0.6 (0.3+0.3)	80	1.00	1.50
0.10	0.8 (0.4+0.4)	100	1.50	2.00
0.10	1.0 (0.5+0.5)	120	2.00	2.50
0.20	0.2 (0.1+0.1)	40	0.25	0.50
0.20	0.4 (0.2+0.2)	60	0.50	1.00
0.20	0.6 (0.3+0.3)	80	1.00	1.50
0.20	0.8 (0.4+0.4)	100	1.50	2.00
0.20	1.0 (0.5+0.5)	120	2.00	2.50
0.30	0.2 (0.1+0.1)	40	0.25	0.50
0.30	0.4 (0.2+0.2)	60	0.50	1.00
0.30	0.6 (0.3+0.3)	80	1.00	1.50
0.30	0.8 (0.4+0.4)	100	1.50	2.00
0.30	1.0 (0.5+0.5)	120	2.00	2.50
0.40	0.2 (0.1+0.1)	40	0.25	0.50
0.40	0.4 (0.2+0.2)	60	0.50	1.00
0.40	0.6 (0.3+0.3)	80	1.00	1.50
0.40	0.8 (0.4+0.4)	100	1.50	2.00
0.40	1.0 (0.5+0.5)	120	2.00	2.50

Detection of PCR products: After the amplification, the amplified products were electrophoresed in 3% agarose gel for 1 h under the condition of stabilized 180 v voltage with the medium of 1×TBE buffer and finally, the products were observed and shot in the Gel Doc 1000 imaging system.

Verification of the SSR reactive system: Three *P. tabulaeformis* materials and 21 *P. radiata* materials from different geographical provenances were used for the verification of the SSR-PCR reactive system with the foregoing method.

RESULTS

Effects of different annealing temperatures on the SSR reaction: Material 14 (taken as A) of *P. tabulaeformis* and material 19 (taken as B) of *P. radiata* were amplified using the Primers gi28515642 under different annealing temperatures.

Figure 1 showed the amplified patterns of primer gi28515642 under the conditions of different annealing temperatures. From the Fig. 1, it could find that the bands were blurry under the conditions of different annealing temperatures of 37, 41, 53, 57 and 61°C which revealed that the rimers could not have good combinations with the template when the annealing temperature was too high or too low. When the annealing temperatures were in the scope of 45~49°C, there were more products and clear bands in the test.

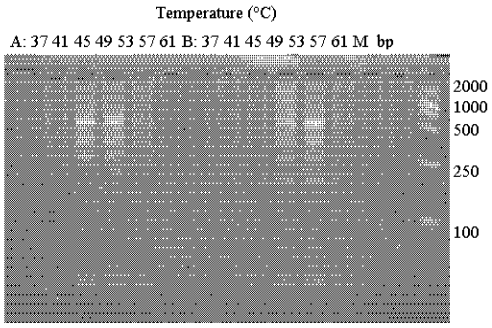

Fig. 1: SSR fingerprinting patterns of *P. radiata* and *P. tabulaeformis* using the primer gi28515642 under different annealing temperature

So the optimum annealing temperature of gi28515642 was in the scope of 45~49°C and 45°C was usually selected.

Effect of the concentration of DNA template on the SSR reaction: The PCR amplification results under the conditions of different concentrations of the DNA template were shown in Fig. 2. From the Fig. 2, it could be find that within the set scope of concentrations, there were no product under the conditions of 40 and 60 ng μL^{-1}, when the concentration of the DNA template was between 80~120 ng μL^{-1}, clear and special bands occurred. So the optimum concentration of the DNA template is 100 ng μL^{-1} in the SSR reactive system of *P. tabulaeformis* and *P. radiate*.

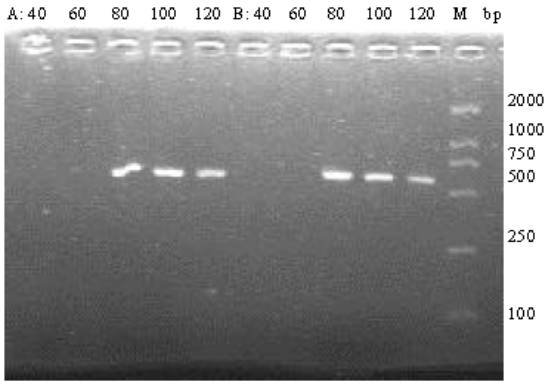

Fig. 2: The amplified patterns of SSR based on the different concentration DNA. DNA concentration unit is ng μL^{-1}

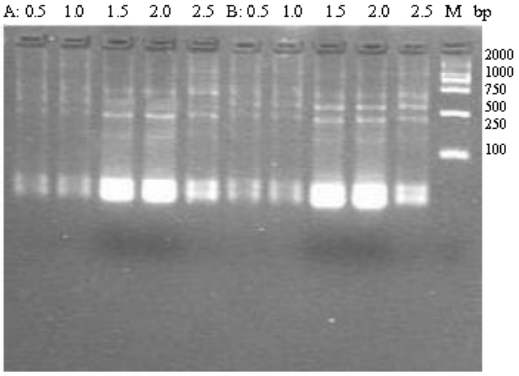

Fig. 3: The amplified patterns of SSR based on different Mg^{2+} concentration Note:Mg^{2+} concentration unit is μmol L^{-1}

Effect of the Mg^{2+} concentration on the SSR reaction: Under the conditions of different Mg^{2+} concentrations, PCR amplification results were shown in Fig. 3. The results showed that in *P. tabulaeformis* and *P. radiate,* 0.5 and 1.0 mmol L^{-1} Mg^{2+} concentrations were too low, which affected the activity of DNA polymerase and no product was found but under the conditions of 1.5, 2.0 and 2.5 mmol L^{-1}, many amplified bands were produced. Therefore, 2.0 mmol L^{-1} Mg^{2+} concentration was the optimum concentration for the SSR reactive system of *P. tabulaeformis* and *P. radiate*.

Effect of dNTPs concentration on the SSR reaction: In Fig. 4, it shown that under the conditions of dNTPs concentrations of 0.05, 0.1, 0.2, 0.3 and 0.4 mmol L^{-1}, bands were all found. The bands were not clear under the conditions of 0.05, 0.1 and 0.4 mmol L^{-1} but the PCR results were clear under the conditions of 0.2 and

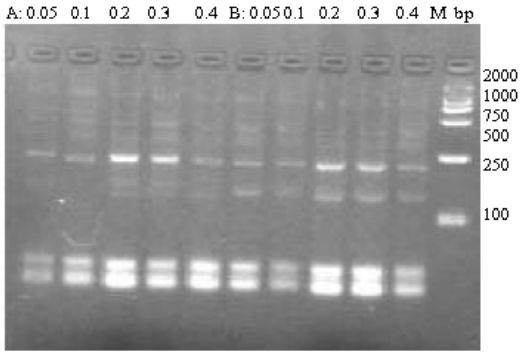

Fig. 4: The amplified patterns of SSR based on different dNTPs concentration Note:dNTPs concentration unit is μmol L^{-1}

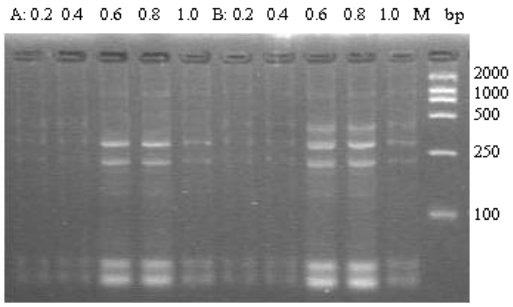

Fig. 5: The amplified patterns of SSR based on different primers concentration. Note Primers concentratin unit is μmol L^{-1}

0.3 mmol L^{-1} in *P. tabulaeformis* and the PCR results were also clear under the conditions of 0.2 and 0.3 mmol L^{-1} in *P. radiate* and the result under the condition of 0.2 mmol L^{-1} was clearer than that under the condition of 0.3 mmol L^{-1}. So 0.2 mmol L^{-1} dNTPs concentration was the optimum concentration for the SSR reactive system of *P. tabulaeformis* and *P. radiate*.

Effects of concentrations of primers on the SSR reaction: In Fig. 5, it is shown that considerable amount of products were obtained from amplification when primer concentrations were 0.6, 0.8 and 1.0 μmol L^{-1}. The bands under the conditions of 0.6 and 0.8 μmol L^{-1} were more clear in *P. tabulaeformis* and *P. radiate*, while almost no product was found under the conditions of 0.2 and 0.4 μmol L^{-1} of primer concentrations. In addition, high concentration of primer was considered to be likely to produce dual polymer, so the concentration scope of 0.6~0.8 μmol L^{-1} was optimal for *P. tabulaeformis* and *P. radiate* and 0.6 μmol L^{-1} was usually selected.

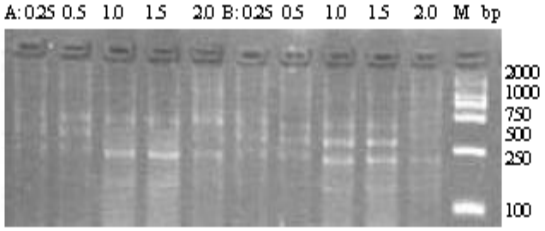

Fig. 6: The amplified patterns of SSR based on different amount of TaqE. The unit of TaqDNA is U

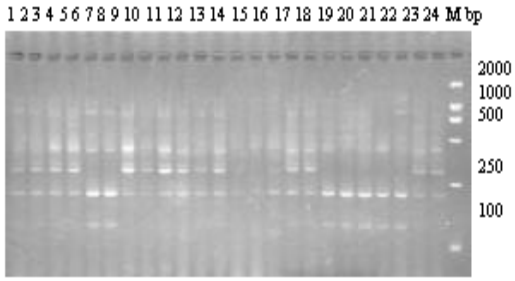

Fig. 7: SSR fingerprinting patterns of *Pirus radiata* and *Pirus tabulaeformis* No, 13, 14, 15 are *Pirus tabulaeformis* and remaining is *Pirus radiata*

Effect of TaqDNA polymerase on the SSR reaction: In Fig. 6, it shown that no product was obtained from amplification with the dosage of 0.25U TaqE, the products obtained from amplification with the dosages of 0.5 and 2.0 U were unclear, best amplification effects were obtained with the usages of 1.0 and 1.5 U for *P. tabulaeformis* and *P. radiate* and therefore, the optimum amount of TaqE was 1.0 U when the economy was considered.

SSR polymorphism validation of experimental materials: Three *P. tabulaeformis* materials and 21 *P. radiate* materials from different geographical provenances were carried out using the before mentioned optimal conditions and were adopted to detect the effects of the built SSR reactive system. The results in Fig. 7, amplified bands of 5-10 were clear and high polymorphism. The products were 100-250 bp. Furthermore, the reactive system had good stability and repeatability, which showed that the optimized reactive system could effectively make SSR amplification actions for the gene groups of *P. tabulaeformis* and *P. radiate* from different provenances.

DISCUSSION

Optimization of the SSR reactive system of *P. tabulaeformis* and *P. radiate* from different provenances by using the orthogonal design is an effective, practical and simply method and the key factors of the SSR reactive system can be identified well by this method. The results of this study showed that the built SSR technical reactive system suitable for genetic analyses on *P. radiata* and *P. tabulaeformis* was as follows: in the 25 μL reactive system, the volume of 1.0 U TaqDNA polymerase was 0.3 μL, Mg^{2+} of 2.0 mmol L^{-1} was 1.5 μL, DNA template of 100 ng μL^{-1} was 2 μL, each primer of 0.6 μmol L^{-1} was 0.3 μL and dNTPs of 0.2 mmol L^{-1} was 2.0 μL.

SSR reaction involves a number of factors and each reactive parameter can have a great impact on the whole system and selection of appropriate reactive parameter is the prerequisite for SSR analyses. The main factors affecting the SSR reaction are template concentration, dNTP, TaqDNA polymerase, Mg^{2+} concentration and etc. The template is the key of PCR and in order to ensure that there are sufficient products in the PCR system, the template volume can not be too much and otherwise, non-specific products might be obtained from amplification.

In addition, impurities in DNA will also affect the efficiency of PCR. dNTPs is the important substrate of the PCR reaction, low content of dNTPs will directly affect the output of PCR amplification and excess dNTPs will compete with the polymerase for linking Mg^{2+} and hence inhibit PCR reaction. The dosage of TaqDNA polymerase affects the amplification efficiency and excess dosage will produce a high mismatch rate, while low dosage can not make a good combination of the enzyme and the primer, so 1.0-1.5U can be chosen as the optimal dosage scope.

Considering it in terms of economic aspect, the optimum amount of TaqE is 1.0 U. If the dosage of Mg^{2+} is too low, dNTPs and the template will compete with the enzyme for linking Mg^{2+} and the Mg^{2+} for linking the enzyme will decrease if the dosage of Mg^{2+} is too high, non-specific products will occurred. Because Mg^{2+} is the activator of Taq DNA polymerase, its concentration affects not only the enzyme activity and the synthetic reliability but also the combination efficiency of primer and template as well as the product specificity (Lin, 1993).

As the SSR reactive system contains a number of components (ingredients), each component may have effects on the sensitivity, stability and yield of amplification. Therefore, joint optimization of multifactor orthogonal experiments using appropriate orthogonal table with balanced dispersion and good comparability, can effectively resolve the contradictions between the number of tests theoretically needed to do and the practical number of tests as well as the contradictions between number-limited experiments actually done and the need to fully grasp the inherent laws of things (Jiang, 1985). The genome of *Pinus* genus is considerable conservative and in a certain extent, SSR loci also shows

a fairly conservative characteristics (Krutovsky *et al*., 2004; Brown *et al*., 2001), which lays a genetic foundation for developing cross-species SSR primers.

CONCLUSION

For the plants of *Pinus* genus, especially *Pinus radiata* and *Pinus tabulaeformis*, their primers need to be developed. Building the optimized SSR-PCR reactive system of *Pinus radiata* and *Pinus tabulaeformis* is only the preliminary research of SSR analyses, which can provide a strong practical support for further developing economic, efficient and viable *Pinus* SSR primers and has more important scientific value and practical significance for future studies on genetic relationship analyses of *Pinus* population, correct evaluation of Pinus germplasm resources, rational use or conservation of *Pinus* germplasm resources and etc.

ACKNOWLEDGEMENTS

This research was supported by the Tenth Five-Year Plan for Key Scientific and Technological Project in the Education Department of Sichuan Province (Project No. 2002A011), the Conduct Research Project in Sate Forestry Bureau (Project No. 2006065) and the Eleventh Five-Year Plan for National Science and Technology Supporting Item (Project No. 2006BAC01A11).

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