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Genetic Relationships among 21 *Pinus radiate* Revealed by SSR Molecular Markers in the Upper Minjiang River

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Abstract: Genetic relationship and genetic diversity of 21 *Pinus radiate* samples originated from different geographic regions were revealed by SSR markers. The results showed that from 21 pairs of SSR primers, 13 pairs displayed obvious polymorphism and each primer could amplify 5-8 alleles' bands, genetic diversity index was 0.8076. Cluster analysis of the 21 samples was carried out and a dendrogram of genetic relatedness was developed on the basis of the SSR data. Moreover, 21 *P. radiate* samples could be clustered into 3 groups based on genetic similarity coefficient (GS= 0.804). Group 1 was composed of 17 samples of *P. radiate,* which were introduced from USA, Australia, Mexico and New Zealand. Group 2 contained 3 *P. radiate* samples from New Zealand and Group 3 included 1 *P. radiate* sample from New Zealand. Three out of 21 *P. radiate* introduced may be homegenetic. Because of high genetic diversity, extensive genetic variance and strong adaptability, the introduced New Zealand *P. radiate* can be widely used for population propagation, forestation in the dry-hot regions and restore the degenerating ecosystem in the upper Mingjiang river Basin.

Key words: *Pinus radiate*, germplasm, affinity, SSR, genetic relationships

INTRODUCTION

To restore the forest in the dry-hot regions of the upper Minjiang river Basin, *Pinus radiate* was introduced from other countries to reforest in Aba, Wenchuan and Lixian of Sichuan at the end of 1980's. *P. radiate* showed excellent characters with fast growth, high resistance and well adapted to the ecological conditions in Sichuan (Liu *et al*., 2003; Bi *et al*., 2003; Yan *et al*., 2007). The effect was significant when used *P. radiate* for ecological restoration in the dry-hot regions. The *P. radiate* seedlings were generally bred from the introduced seeds by nutrition bag. Therefore, the seeds were too expensive, the cost was so high and name of the same provenance was always different, which bewildered many researchers. Up to now, there are few studies on the identification provenance and affinity relationships among them have not been clearly defined either, which badly restrict proper assessment, exact conservation and reasonable utilization.

Recently, Simple Sequence Repeat (SSR) marker, also called microsatellite markers, has been used extensively in genetic diversity. SSR markers are chromosome-specific, highly polymorphic, co-dominant, simple and evenly distributed in the genome (Powell *et al*., 1996; Hokanson *et al*., 2001; Luis *et al*., 2001; Liebhard *et al*., 2003; Paul *et al*., 2005; Gygax *et al*., 2004). Thus, SSR was considered one robust technique for appraisal of the affinity relationships among *P. radiate* provenances. However, there were no reports on identification of the affinity relationships among *P. radiate*. The objective of this study is to identify 21 *P. radiate* samples originated from different geographic regions and their genetic relationships, which are useful to establish a robust theoretical basis for further fine preservation, utilization, selection and restoration of the degenerating ecosystem.

MATERIALS AND METHODS

Plant materials and DNA extraction: Fifty gram mixed young leaves were sampled from the nutritional branches of *P. radiate* juvenile trees in Wenchuan, Lixian of Sichuan province in April, 2007. Young leaves were freeze-dried and stored at -70°C. Number of *Pinus* germplasm samples from different geographic regions are shown in Table 1.

Total genomic DNA was extracted from young leaves of *Pinus* plant by use of a modified Cetyltrimethylammonium Bromide (CTAB) method

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Table 1: The number of *Pinus* germplasm resources in different geographic regions

Samples in different region	Afforestation time	Afforestation land	Ecological conditions: Average temperature of a year	Rainfall of a year (mm)	Vaporing value of a year (mm)	Slope ()	Altiude (m)	pH
New Zealand *P. radiata*	2003	Wenchuan Taoguan I	15.1	388	1482	25	1120	7.55
	2003	Wenchuan Taoguan II	14.9	388	1487	25	1250	7.43
	2004	Wenchuan Zaojiaotuo I	15	396	1465	25	1180	6.96
	2004	Wenchuan Zaojiaotuo II	14.9	400	1470	25	1295	7.05
	2002	Wenchuan Zaojiaotuo I	15	396	1465	25	1180	6.96
	2002	Wenchuan Zaojiaotuo II	11..9	400	1470	25	1295	7.05
	1999	Lixian Boxi I	12.1	580	1615	25	1670	8.03
	1999	Lixian Boxi II	11.8	595	1630	25	1790	7.95
	1999	Lixian Boxi III	11.7	600	1635	25	1875	8.06
	1998	Lixian Ganbao I	10.3	670	1700	25	2070	8.22
	1998	Lixian Ganbao II	10.1	670	1700	25	2235	8.24
	1998	Lixian Ganbao III	10	675	1715	25	2370	8.19
Australia *P. radiata*	2003	Lixian Ganbao II	10.1	670	1700	25	2235	8.24
New Zealand *P. radiata*	2003	Lixian Ganbao I	10.3	670	1700	25	2070	8.22
America *P. radiata*	2003	Lixian Ganbao II	10.1	670	1700	25	2235	8.24
	2003	Lixian Ganbao II	10.1	670	1700	25	2235	8.24
	2003	Lixian Ganbao I	10.3	670	1700	25	2070	8.22
Mexico *P. radiata*	2003	Lixian Ganbao II	10.1	670	1700	25	2235	8.24
New Zealand *P. radiata*	2004	Lixian Ganbao I	10.3	670	1700	25	2070	8.22
	2003	Lixian Ganbao I	10.3	670	1700	25	2070	8.22
Mexico *P. radiata*	2003	Lixian Ganbao II	10.1	670	1700	25	2235	8.24

Table 2: Amplified results of 21 *pinus radiate* samples by 13 SSR primers

Primer No.	Primer sequence (5'~3')	No. of alleles	Locus heterozygosity
gi28520065	F: TACTCCTTCCAATCGCTCT		
	R: TCAATCTACTCACACCCACTC	7	0.8139
Contig1547	F: CACTCACCCCACAAATACGG		
	R: CTCGAATTTACCTAGGCCCC	5	0.7695
Cn299	F: GGGGAAGGTGTTCATACCG		
	R: AGCGCCACAGTTTACTACCC	7	0.8072
gi28515642	F: GAGAAAGATTCACAATGAGG		
	R: GTATCACCACCACCAGCA	7	0.8469
Ctg1376	F: CGATATTATGGATTTTGCTTGTGA		
	R: AAATGCATGCCAAACTTAAATAC	8	0.8876
Contig1542	F: AAATGCAGTCTTCAAAGCGG		
	R: TACTAGCGAGAAACTGGTCGCC	6	0.8035
Ctg16811	F: TCCATGATGTTGCAGATTGG		
	R: GTGTTCCCCAATGGTCTGTC	7	0.8166
Contig17330	F: GGACAGTCCTTACTGCCCAA		
	R: CCCATGGTTTTCCATTGTTC	6	0.8072
ctg18103	F: CCTGGATTCATTTGTGGCTAA		
	R: CATGCCAACTTCTTGCATTG	8	0.8719
Ctg275	F: ACGGAGATATATTGCTGGCG		
	R: AAAGAATAACGTGAAACAAACCC	6	0.8261
Ctg17607	F: CGCCATTAATATGCCTACCG		
	R: ATCTCTGCGCTGCTTGAAGT	5	0.7531
Ctg6390	F: ATCCACGACTTGTCGACGC		
	R: ATCAACCAACTTAGGCAGCG	5	0.7428
Ctg7024	F: GGGAATTCTGAAAGACAAGGG		
	R: AACTTACCATCGAGAGCCCC	5	0.7522
Average		6.3	0.8076

according to the procedure of Wang *et al.* (2006), with some modifications. For example, in order to improve the purity of the genomic DNA, we used polyvinylpyrrolidone (PVP40) to get rid of hydroxybenzene during the process.

SSR analysis: SSR primers were synthesized by Sangong Inc. The sequence of these primers was downloaded from PGG (http://bioinformatics.pbcbasc.latrobe.edu.au/index.htm) and INRA (http://www.pierroton.inra.fr/) (Table 2).

The optimization PCR reactions for the amplification of SSRs were carried out in a total volume of 25 μL containing 20 ng template DNA, 10×Buffer 2.5 μL, 1.5 μL $MgCl_2$ (1.5 mmol L^{-1}), 2.0 μL dNTP (2 mmol L^{-1}), 0.3 μL (0.06 μmol L^{-1}) of forward and reverse primers, 0.45 U Tap polymerase and 16.1 μL ddH_2O.

DNA amplification was optimized with the cycling conditions: One cycle of 3 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 45°C annealing temperature and 30 sec at 72°C, with a final extension step of 5 min at 72°C. The

amplified products were electrophoresed in 3% agarose gels. If the amplified bands ranged from 100-250 bp, which showed that the experiment is successful.

The amplified products were mixed with an equal volume of loading dye and were denatured. A 5 μL mixed liquid run on a 3% agarose gels at 75 W for 1 h and then gels were photographed under illumination with UV light after ethidium bromide staining.

Data scoring and analysis: All distinctively and unambiguously polymorphic bands generated by SSR analysis were scored manually (1 for present, 0 for absent and 9 for missing). Genetic similarity coefficient was calculated by the formula 1 and software NTSYS (version 2.1).

$$GS_{ij} = 2N_{ij}/(N_i + N_j) \quad (1)$$

Where,

Gs_{ij} = Genetic Similarity,

N_{ij} = The number of fragments shared between individuals.

N_i and N_j = The number of fragments in the individual i and j, respectively (Nei, 1972).

The corresponding cluster analysis was done on the similarity matrix by use of the Unweighted Paired Group Method Using Arethmetic Averages method (UPGMA) algorithm provided by the computer program STATISTICA, version 5.0 (STAT Software). Gene heterozygosity and the average gene heterozygosity for each locus were calculated based on the formula 2 and 3, respectively (Nei, 1975).

$$h = 1 = \Sigma f^2_i \quad (2)$$

$$h = 1 = \Sigma h_i/r \quad (3)$$

Where:

h = Heterozygous degree.

f_i = Allele frequency for gene i.

h_i = Heterozygous degree for allele gene i.

r = Numbers of loci amplified.

RESULTS

Primers screened and SSR polymorphism identified: We used 13 pairs of SSR primers to amplify 21 *Pinus* samples, the results were showed in Fig. 1 and Table 2. From the polymorphic 13 SSR primers, we scored 84 polymorphic loci, the effective number of alleles ranged from 5-8, the average allele's number per locus was 6.3, the locus heterozygous degree ranged from 0.7428-0.8876 and genetic diversity index was 0.8076.

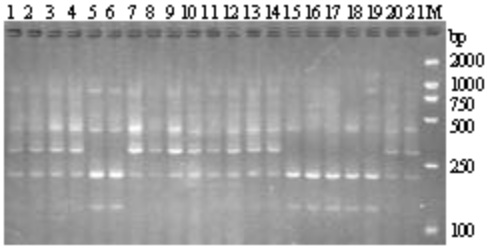

Fig. 1: SSR fingerprinting patterns of 21 *pinus radiate* using the primer Ctgl 7607

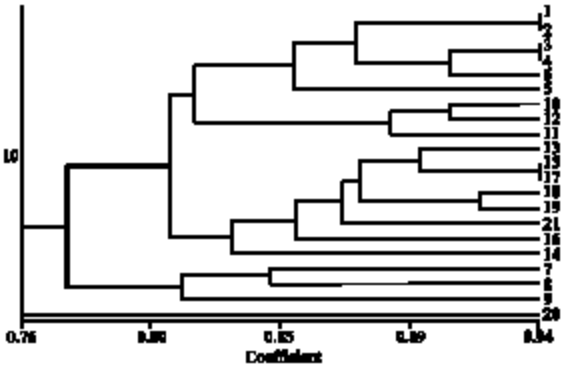

Fig. 2: The UPGMA clustering dendrogram of *pinus radiata* based on SSR data

Genetic similarity degree: Table 3 listed the sum genetic similarity coefficients of SSR among the *Pinus* samples; Genetic similarity coefficients of 21 *Pinus* samples ranged from 0.6907-0.9381 and the average was 0.8141. GS between 1 and 2, 3 and 4, 15 and 17, were 0.9381. These materials were originated from the same provenance and the same afforestation time, but from different afforestation land, thus, they could be considered homegenetic.

Genetic distance analysis: The corresponding cluster analysis of the 21 samples was carried out and a dendrogram of genetic relatedness was developed on the basis of the SSR data (Fig. 2). Moreover, 21 samples could be clustered into 3 groups based on genetic similarity coefficient (GS = 0.8041). Group 1 was composed of 17 samples of *P. radiate*, which were introduced from USA, Australia, Mexico and New Zealand (64.7%). Group 2 contained 3 *P. radiate* samples (No. 7, 8 and 9) from New Zealand and group 3 included 1 *P. radiate* sample (No. 20) from New Zealand.

Based on GS (0.8247), group 1 could be clustered into 3 subgroups. Subgroup 1 was composed of sample 1-6. Among them, No. 1-4 would be considered homegenetic. Subgroup 2 contained 3 *P. radiate* samples, which were

Table 3: The genetic similarity coefficients of 21 *pinus radiate*

Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1																				
2	0.9381	1																			
3	0.8763	0.9278	1																		
4	0.8144	0.8660	0.9381	1																	
5	0.8351	0.8866	0.8763	0.8144	1																
6	0.8557	0.9072	0.9381	0.8763	0.8557	1															
7	0.7423	0.7938	0.8454	0.7835	0.7423	0.8247	1														
8	0.6907	0.7423	0.7938	0.7320	0.6907	0.7732	0.8454	1													
9	0.7113	0.7526	0.7835	0.7216	0.7010	0.7835	0.8351	0.7938	1												
10	0.8041	0.8557	0.9072	0.8454	0.8041	0.8866	0.8866	0.8247	0.8144	1											
11	0.7423	0.7938	0.8454	0.9072	0.7216	0.8247	0.8144	0.7732	0.7526	0.8969	1										
12	0.7526	0.8041	0.8557	0.8041	0.7320	0.8557	0.8247	0.7732	0.8247	0.9072	0.8763	1									
13	0.8144	0.8557	0.8866	0.8247	0.7835	0.8660	0.8351	0.7835	0.7938	0.8763	0.8144	0.8454	1								
14	0.7526	0.7938	0.8041	0.7423	0.7216	0.8247	0.7629	0.7320	0.7526	0.8041	0.7216	0.7526	0.8351	1							
15	0.8247	0.8660	0.8763	0.8144	0.7938	0.8557	0.8041	0.7732	0.8041	0.8247	0.7835	0.7938	0.9072	0.8557	1						
16	0.7629	0.8041	0.7938	0.8557	0.7320	0.7732	0.7423	0.7113	0.7216	0.7629	0.8454	0.7423	0.8454	0.7938	0.8969	1					
17	0.8247	0.8660	0.8557	0.7938	0.7732	0.8351	0.8041	0.7526	0.8247	0.8041	0.7423	0.7732	0.8866	0.8144	0.9381	0.8557	1				
18	0.8247	0.8763	0.8454	0.7835	0.7629	0.8454	0.8041	0.7629	0.7938	0.8351	0.7526	0.7835	0.8557	0.8557	0.9072	0.8454	0.9072	1			
19	0.7938	0.8351	0.8660	0.8041	0.7629	0.8454	0.8041	0.7629	0.7526	0.8351	0.7732	0.7835	0.8763	0.8247	0.8660	0.8454	0.8454	0.9175	1		
20	0.7320	0.7732	0.7835	0.7216	0.7629	0.7526	0.7320	0.6804	0.7010	0.7732	0.7113	0.7320	0.7835	0.7629	0.8144	0.7526	0.7938	0.8041	0.7835	1	
21	0.8247	0.866	0.8763	0.8144	0.7938	0.8557	0.8041	0.7629	0.7835	0.8454	0.7835	0.8144	0.866	0.8454	0.8763	0.8351	0.8557	0.8866	0.8660	0.8351	1

No. 10-12. Subgroup 3 comprised 8 *P. radiate* samples which were No. 13, 15, 17, 18, 19, 21, 16 and 14. Among them, No. 15 and 17 would be considered homegenetic.

DISCUSSION

SSR polymorphism of *Pinus* genus and its ecological significance: Allele's numbers and their heterozygous degree revealed the capacity of identification genotype by SSR markers (Zhebentyayeva *et al*., 2003). In the study, the average allele's numbers were 6.3, the highest heterozygous degree per locus was 0.8876, while the lowest was 0.7428 and genetic diversity index was 0.8076, which indicated that SSR markers could effectively reveal the population genetic diversity of *P. radiate* in the upper Minjiang river. The results, derived from UPGMA analysis, showed that all group I, II and III contained New Zealand *P. radiate*, while America *P. radiate*, Mexico *P. radiate*, Australia *P. radiate* and New Zealand *P. radiate* were clustered into group 1 and sub-group 3, which revealed that America *P. radiate*, Mexico *P. radiate* and Australia *P. radiate* were originated from New Zealand *P. radiate*. Because of high genetic diversity, extensive genetic variance and strong adaptability, the introduced New Zealand *P. radiate* can be widely used for population propagation, forestation in the dry-hot regions and restore the degenerate ecosystem in the upper Mingjiang river Basin.

Therefore, *P. radiate* was successfully introduced to afforest in the upper Mingjiang river Basin. We should pay attention to the development of population genetic characters, strengthen selection and breeding *P. radiate* species among the excellent populations and increase the diversity of *P. radiate* resources and stability of ecological communities.

Affinity relationship: The affinity relationships of 21 *P. radiate* were detected by 13 SSR primers, the results showed that 3 out of 21 *P. radiate* could not be distinguished from each other. No. 1 could not be distinguished from No. 2; No. 3 from 4 and No. 15 from 17. The reasonable explanation of this phenomenon may be that there was no genetic difference between them, or the difference was out of the SSR locus (Zhang *et al*., 2007). These *P. radiate* were originated from the same provenance but the different afforestation land, thus, they could be considered homegenetic. It is obvious that different names for the certain commodity should be paid more attention to, so that we can avoid waste resource in afforestation.

CONCLUSION

The *P. radiate* samples derived from the different geographic regions and the different afforestation times in the dry-hot regions in the upper Mingjiang river Basin of Sichuan Wenchuan, Lixian were successfully identified by SSR markers and 3 pairs of geographic provenance samples were detected, which exhibited the affinity relationships and abundance diversity among *P. radiate* samples in Sichuan Aba. The results indicated that the introduced New Zealand *P. radiate* with high genetic diversity, extensive genetic variance and strong adaptability can help to afforest in the dry-hot regions in the upper Minjiang river, which is useful to establish a robust theoretical and practice basis for further fine preservation, utilization, selection and breed and restoration of the degenerating ecosystem.

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