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Full Length Research Paper

Allele frequency analysis of Chinese chestnut (*Castanea mollissima*) populations using fluorescent simple sequence repeats (SSR) analysis

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The aim of this study was to establish a method for allele frequency detection in bulk samples. The abundance of polymerase chain reaction (PCR) products in bulk leaf samples was detected using fluorescent labeled Simple sequence repeat (SSR) primers and an Applied biosystems (AB) automatic DNA analyzer. Compared with the conventional SSR technique based on polyacrylamide gel electrophoresis (PAGE) and silver staining, fluorescent SSR was much more sensitive. A total of 78 alleles, an average of 4.6 alleles per locus, were detected among 17 chestnut populations with the primer CmTCR10 (NED) and a total of 41 alleles, an average of 2.4 alleles per locus, were detected with the primer CmTCR24 (6-FAM). Multiplexing the PCR reaction by combining the primer pairs of CmTCR10 and CmTCR24, using different fluorescent dyes for different primers, showed that the alleles could be discriminated and the sizes of the amplified segments were similar. Furthermore, the exact sizes of the amplified fragments and the abundance of the PCR products were determined by fluorescent SSR. After data analysis with GeneScan software and allele calling and output with Genotyper software, allele frequencies were calculated for equal pooled samples in each population using the FREQS-R module in the R statistical computing language. The results indicate that it is feasible to determine allele frequencies in bulked samples based on the detection of SSR-PCR products. The advantages and additional applications of this method are also discussed. The abundance of the PCR products can be used to determine the allele frequencies in bulk samples of chestnut populations.

Key words: Fluorescent simple sequence repeats (SSR), chestnut population, bulk sampling, allele frequencies.

INTRODUCTION

The Chinese chestnut (*Castanea mollissima* BI.), which is native to China, is an important species of the genus *Castanea*. Due to good nut quality and strong resistance to blight, the Chinese chestnut was used as an important genetic resource for the improvement of the world-wide variety of the edible chestnut (Wang et al., 2008; Li et al., 2009). Therefore, the analysis of genetic diversity and allele frequency in the Chinese chestnut population will provide the basis for research on the application, evolution and relationship between closely related wild relatives (Huang et al., 1998; Bao and Huang, 2002; Tian et al., 2009; Nishio et al., 2011). Bulk sampling of individuals from each chestnut population will provide comprehensive analysis of the allele number, allele frequency, genetic structure and the occurrence of rare alleles in the population (Villani et al., 1991; Schaal et al., 1998; Ai et al., 2009). It is logistically challenging to measure allele frequencies in multiple individuals of a large number of populations for a thorough analysis of genetic diversity. Furthermore, most of the individuals in the population are heterozygous; the population-wide allele frequency may not be accurately estimated from the allele frequency analysis of individuals. Thus, it is necessary to establish a method to analyze allele frequencies based on bulk samples to improve efficiency and generalizability of the measurement.

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Table 1. Origin and sample size of landraces used in the study.

Populations number	Name	Origin	Sample size
1	Xixianggou wuhua	Tai'an, Shandong	15
2	Xinmiao wild chestnut	Junan, Shandong	17
3	Liaodan 58	Kuandian, Liaoning	17
4	Dagongshu 4	Guangyuan, Sichuan	23
5	Dongmiwu wuhua	Qian'an, Hebei	15
6	Yanchang	Changping, Beijing	19
7	Paoche 2	Xinyi, Jiangsu	19
8	Chushuhong	Yixing, Jiangsu	20
9	Guanting 10	Zunhua, Hebei	16
10	Zhahongli	Zhashui, Shanxi	24
11	Yanhong	Changping, Beijing	15
12	Zundali	Zunhua, Hebei	19
13	Liaodan 61	Kuandian, Lioaning	25
14	Laixi dayouli	Laixi, Shandong	16
15	Dazaoshu	Fengcheng, Liaoning	15
16	Duanzha	Yixing, Jiangsu	14
17	Shangfeng	Yantai, Shandong	17

Investigations of sampling strategies for regional analysis of plant population genetics indicated that the efficiency of detection method for allele frequency analysis in bulk samples could be significantly improved (Hamrick, 1989; Fornari et al., 1999; Ai et al., 2007). Schnack et al. (2004) developed a mathematical procedure to remove stutter noise and accurately determine allele frequencies in pools. However, in the case of microsatellite markers, the overall genotype patterns of pooled samples are often distorted by polymerase chain reaction (PCR) artefacts such as stutter and preferential amplification, which prevent an accurate determination of the allele frequencies by simple procedures (Collins et al., 2000; Schnack et al., 2004). Inter-simple sequence repeat (ISSR) analysis was applied in bulk samples of the chestnut population, but it is nearly impossible to be used for analyzing the allele frequency due to the inability of polyacrylamide gel electrophoresis (PAGE) followed by silver staining to quantify conventional SSR products (Ai et al., 2007).

However, fluorescent SSR detection through an automatic DNA analyzer could analyze accurately the exact size and abundance of the amplified fragments labeled with 6-FAM, HEX and NED fluorescent primers. Quantitative image analysis was performed with GeneScan and Genotyper software. We used the R statistical computing language to calculate the allele frequency in bulk samples according to the Genotyper output, which will help to improve the efficiency and accuracy of chestnut allele frequency analysis, expand the application range of fluorescent SSR technique and screen the molecular markers linked to target loci, so as to provide the basis for marker-assisted selection in the early stages of Chinese chestnut genetic analysis.

MATERIALS AND METHODS

Plant materials and DNA extraction

Samples of 17 local varieties of the Chinese chestnut were collected from seven main growing areas of Shandong, Hebei, Beijing, Liaoning, Jiangsu, Shanxi and Sichuan provinces. When sampling, the populations with more than 20 individual trees were sampled according to the principle of uniform distribution and random sampling, while for the populations with less than 20 individual trees, every tree was sampled. In total, 14 to 25 plants were sampled from each population (Table 1). Unlabeled PCR primers were synthesized by Sangon Biotech (Shanghai) Company, Limited. SSR primers were labeled at the 5′ terminal by 6-FAM, HEX and NED (Table 2). Bulk samples of leaf tissues were composed of 1 g of leaf tissue from each of the 14 to 25 plants of each of the 17 Chinese chestnut (*C. mollissima)* populations in the Tai'an Chestnut Repository. DNA was extracted by grinding the bulk samples in liquid nitrogen, followed by phenol/chloroform (24:1, v/v) extraction and ethanol precipitation.

PCR amplification

PCR was performed in a total volume of 25 µl, composed of 10× PCR buffer (2.5 µl), 2.5 mM of each dNTP (2.0 µl), 25 mM $MgCl_2$ (2.0 µl), 0.5 µmol/L of each primer (1.0 µl), 5 units μL^{-1} *Taq* polymerase (0.2 µl), and 60 ng of DNA template. Amplifications were performed using a GeneAmp PCR system 9700 (AB) thermal cycler. Amplification cycles consisted of an initial step of 5 min at 95°C, followed by 30 cycles of 50 s at 94°C, 45 s at the annealing temperature of each primer pair, 90 s at 72°C, and a final extension step of 7 min at 72°C. Multiplex PCR was performed using DNA from Xinmiao wild chestnut population as the template and the fluorescent primers CmTCR10 and CmTCR21. The detection

Table 2. The primers used in the study and the fluorescent labels.

Primer	Sequence	Optimal annealing temperature (°C)	Fluorescent label
CmTCR10	F CACTATTTTATCATGGACGG R CGAATTGAGAGTTCATACTC	52	Non NED
CmTCR21	F CGAGGTTGTTGTTCATCATTAC R GATCTCAAGTCAAAAGGTGTC	50	Non HEX
CmTCR24	F CTGCAAGACAAGAATTACAC R GAATAACCTGCAGAAGGC	60	Non 6-FAM

channels for the primers CmTCR10 and CmTCR21 were blue and black, respectively. The PCR thermal cycler and amplification protocol for the multiplex PCR reactions were the same as that for the common PCR, with the exception of the use of two primer pairs.

Denaturation and fluorescence detection of PCR products

In total, 1 µl 6-FAM, 2 µl NED, 3 µl HEX and 22 µl distilled ultrapure water were added to each well of 96-well plates. After centrifugation for 1 min at 5000 rpm, the 96-well plate was denatured at 95°C for 5 min and then immediately placed on ice. The fluorescence detection was performed in AB 3730 DNA analyzer.

Denaturing polyacrylamide gel electrophoresis analysis

Loading buffer (5 µl at 6×) was added to 25 µl of the PCR product. After mixing and denaturation at 95°C for 5 min, samples were immediately placed on ice. The PCR products were loaded onto a 6% polyacrylamide gel with pBR332/MspI as a molecular size standard. The electrophoresis was performed in the Sequi-Gen® GT electrophoresis system for 50 min to 1 h at 90 W of constant power.

Data analysis

The image analysis and data collection were performed using Genotyper and GeneScan software, respectively. After scanning the gel, analyzing the original SSR data, setting the size standard and parameters, reading the peaks of each sample and counting the non-peak samples, the success ratio of each plate was computed and used for the basis of testing and correcting the electrophoresis effects of the instrument. The ratio of samples without peak should be controlled in less than 5% (Dane et al., 1999; Hao et al., 2005; Yong et al., 2009). The parameters such as starting fragment size, repeating type of primer, threshold and output items were specified in Genotyper, and a table of results was created. The Genotyper output was analyzed and allele frequency was computed through the FREQS-R module in R.

RESULTS

Comparison of AB automatic genetic analysis and PAGE silver staining

The SSR analysis with fluorescent primers by AB automatic DNA analyzer showed that this method was much more sensitive than PAGE silver staining, and read distinctly more polymorphic sites with higher polymorphic information. In this study, a total of 78 alleles from 17 test populations were detected with the primer CmTCR10 (NED), with an average of 4.6 alleles from each popu- lation. With primer CmTCR24, 41 alleles were detected, with an average of 2.4 alleles from each population. In the PAGE silver staining map of the PCR products generated with the unlabeled primer CmTCR10, only four alleles were detected from Xinmiao wild chestnut population (Figure 2 and Lane 2), while five alleles were distinctly detected from the same sample using fluorescent primers through the AB 3730 DNA Analyzer. The fluorescent detection method also allowed the calculation of the exact size of fragments and the abundance of PCR products (Table 3).

Multiplex PCR

In order to further improve the detection efficiency, we investigated the feasibility of application of multiplex PCR combined with fluorescent SSR on the allele frequency analysis of chestnut bulk samples. Following the multiplex PCR amplification using template DNA from Xinmiao wild chestnut population with the fluorescent primers CmTCR10 and CmTCR21, the product was detected using the AB 3730 DNA analyzer. When analyzing data and reading the bands according to the appropriate channel, O stands for the red peaks of the size standard and B stands for the blue peaks of the 5′ NED on the CmTCR10 primer. Thus, in the image, there will be red peaks for the size standard and blue allele peaks for the PCR product. The PCR products generated using the primer CmTCR21 appeared as black peaks for the alleles, with red peaks for the size standard. Figure 1C is the synthesized map of Figure 1A and B, which illustrates the discrimination of the PCR products from the different primers.

Fragment sizes and peak heights can be extracted from Genotyper. Among the Xinmiao wild chestnut population, multiplexing the PCR primers CmTCR10 and CmTCR21 resulted in distinct discrimination without interference. The allele fragments from the two primers overlapped: five alleles of 177, 189, 195, 206 and 212 bp were amplified

Table 3. Results from Genotyper data of multiplex PCR in Xinmiao wild chestnut in ABI3730 DNA Analyzer.

Primer	Detection color	Alleles	Size/bp	Peak height
CmTCR10	Blue B	1	177	93
		2	189	2316
		3	195	655
		4	206	146
		5	212	4350
CmTCR21	Black HEX	1	152	379
		2	158	157
		3	167	361
		4	175	74
		5	184	5348
		6	189	66
		7	201	943
		8	209	97
		9	215	1352
Molecular size standard	Red O	1	165	201
		2	169	249
		3	176	177
		4	180	246
		5	187	1452
		6	192	61
		7	194	734
		8	203	2145
		9	208	146
		10	219	457

with primer CmTCR10, and nine alleles of 152, 158, 167, 175, 184, 189, 201, 209 and 215 bp were amplified with primer CmTCR21. The peak heights of the alleles were read according to different detection channels and were correlated to the abundance of the PCR products (Table 3). Peaks could be converted to the quantity and frequency of alleles. The results show that fluorescent SSR technique combined with multiplex PCR can discriminate and quantify allele fragments and can be used for analysis of allele frequency in bulk samples of chestnut populations.

Allele frequency analysis of population bulk samples

The quantitative analysis principle of the fluorescent SSR is to label the 5' terminal of a primer with the fluorescent dye, such as FAM, HEX and NED, then the fluorescent was inspired by the laser from the amplified products with fluorescent label; the optical density (OD) detected was in linear positive correlation with the abundance of PCR products which was used to quantify the products. The amplification results with the fluorescent primer CmTCR21 for 19 individual trees from the Zundali population are shown in Figure 3. According to the Genotyper analysis, the bulk samples from the nine populations (Xixianggou wuhua, Xinmiao wild chestnut, Liaodan 58, Dagongshu 4, Dongmiwu wuhua, Yanchang, Paoche 2, Chushuhong and Guanting 10) with primer CmTCR24 and from eight populations (Zhahongli, Yanhong, Zundali, Liaodan 61, Laixi dayouli, Dazaoshu, Duanzha and Shangfeng) with primer CmTCR10 (Table 4) were counted. For the former, the allele size ranged from 199 to 219, and the allele frequency ranged from 0.155 to 0.835. For the latter, the allele size ranged from 176 to 203, and the allele frequency ranged from 0.045 to 0.855. The rarest allele was allele 10 (199 bp) from Zhahongli, with a frequency of 0.045, and the most common allele was allele 2 (179 bp) from Zundali, with a frequency of 0.855. These results indicate that allele frequencies for bulk samples can be determined through quantitative analysis.

DISCUSSION

SSR markers have been widely recognized as powerful and informative markers due to the advantages of

Table 4. The alleles and their frequencies were analyzed with bulk sampling in chestnut populations.

Name	Primer	Allele 1		Allele 2		Allele 3		Allele 4		Allele 5		Allele 6		Allele 7		Allele 8		Allele 9		Allele 10		Allele 11	
		FS	f	FS	f	FS	f	FS	f	FS	f	FS	f	FS	f	FS	f	FS	f	FS	f	FS	f
Xixianggou wuhua	CmTCR24	-	-	204	0.375	-	-	211	0.325	-	-	214	0.3	-	-	-	-	-	-	-	-	-	-
Xinmiao wild chestnut		-	-	-	-	-	-	211	0.285	-	-	-	-	-	-	218	0.715	-	-	-	-	-	-
Liaodan 58		199	0.535	-	-	-	-	-	-	-	-	-	-	-	-	-	-	219	0.465	-	-	-	-
Dagongshu 4		-	-	204	0.815	-	-	-	-	212	0.185	-	-	-	-	-	-	-	-	-	-	-	-
Dongmiwu wuhua		-	-	-	-	207	0.165	-	-	-	-	-	-	-	-	218	0.835	-	-	-	-	-	-
Yanchang		199	0.155	-	-	-	-	-	-	212	0.425	214	0.42	-	-	-	-	-	-	-	-	-	-
Paoche 2		-	-	-	-	207	0.325	-	-	-	-	-	-	-	-	-	-	219	0.675	-	-	-	-
Chushuhong		-	-	204	0.185	207	0.56	-	-	-	-	-	-	215	0.255	-	-	-	-	-	-	-	-
Guanting 10		-	-	-	-	-	-	211	0.275	-	-	-	-	-	-	-	-	-	-	221	0.725	-	-
Zhahongli	CmTCR10	176	0.375	-	-	-	-	-	-	185	0.225	-	-	-	-	194	0.355	-	-	199	0.045	-	-
Yanhong		-	-	-	-	181	0.525	-	-	-	-	-	-	-	-	194	0.085	-	-	-	-	203	0.39
Zundali		-	-	179	0.855	-	-	-	-	-	-	-	-	-	-	-	-	195	0.145	-	-	-	-
Liaodan 61		-	-	-	-	-	-	182	0.525	-	-	-	-	-	-	-	-	195	0.475	-	-	-	-
Laixi dayouli		176	0.325	-	-	-	-	-	-	185	0.675	-	-	-	-	-	-	-	-	-	-	-	-
Dazaoshu		-	-	-	-	-	-	-	-	-	-	188	0.105	-	-	194	0.605	-	-	199	0.29	-	-
Duanzha		-	-	-	-	181	0.2	-	-	-	-	-	-	191	0.8	-	-	-	-	-	-	-	-
Shangfeng		-	-	-	-	-	-	-	-	-	-	188	0.775	-	-	-	-	195	0.225	-	-	-	-

FS and f denote the fragment size and allele frequency, respectively.

abundance, codominance, high levels of polymorphism, high degree of reliability and reproducibility, and ease of use with the PCR. But the conventional PAGE detection method of SSR markers has shortcomings in large-scale and multi-batch data collection and analysis, primarily with distinguishing allelic variation and processing different batches of reactions. In conventional PAGE, exact fragment size is estimated using DNA ladders (Ramana and Lon, 1997). SSR polymorphisms tend to have differences of only a few bases, which are difficult to be estimated by the naked eye in the PAGE maps, consequently, it limits the application of SSR markers. In addition, conventional SSR-PAGE is a complicated process with the possibility of error, which makes it difficult to verify the stability, consistency and repeatability of electrophoresis and silver staining (Schuelke, 2000; Liu et al., 2008). Thus, we sought to establish a more sensitive and reliable detection method.

The above mentioned problems were resolved in this study by fragment size analysis through fluorescent SSR detection based on the AB 3730 DNA analyzer. The exact size of each allele fragment was identifiable, which unified the allele analysis from different batches, furthermore, the high automatic programming simplified the processsing and saved considerable time. Programming, data collection and imaging performed on the AB 3730 DNA analyzer can precisely quantify the amplification products and assign the specific genotypes based on quantitative PCR principles. So it is possible to use PCR products and even template DNA with wide application prospects for allele frequency analysis and SSR quantification with higher efficiency and larger analysis flux.

The efficiency will be further improved by multiplex PCR. In order to establish an optimum multiplex PCR system, the following three factors should be taken into account when selecting primers (Pinar et al., 2003; Huang et al., 2003). First, the annealing temperature of primers group should be similar, and valid amplification would be obtained when single PCR was performed using each primer in the selected annealing temperature. Second, primer dimmers and competitive amplification should be avoided during grouping the

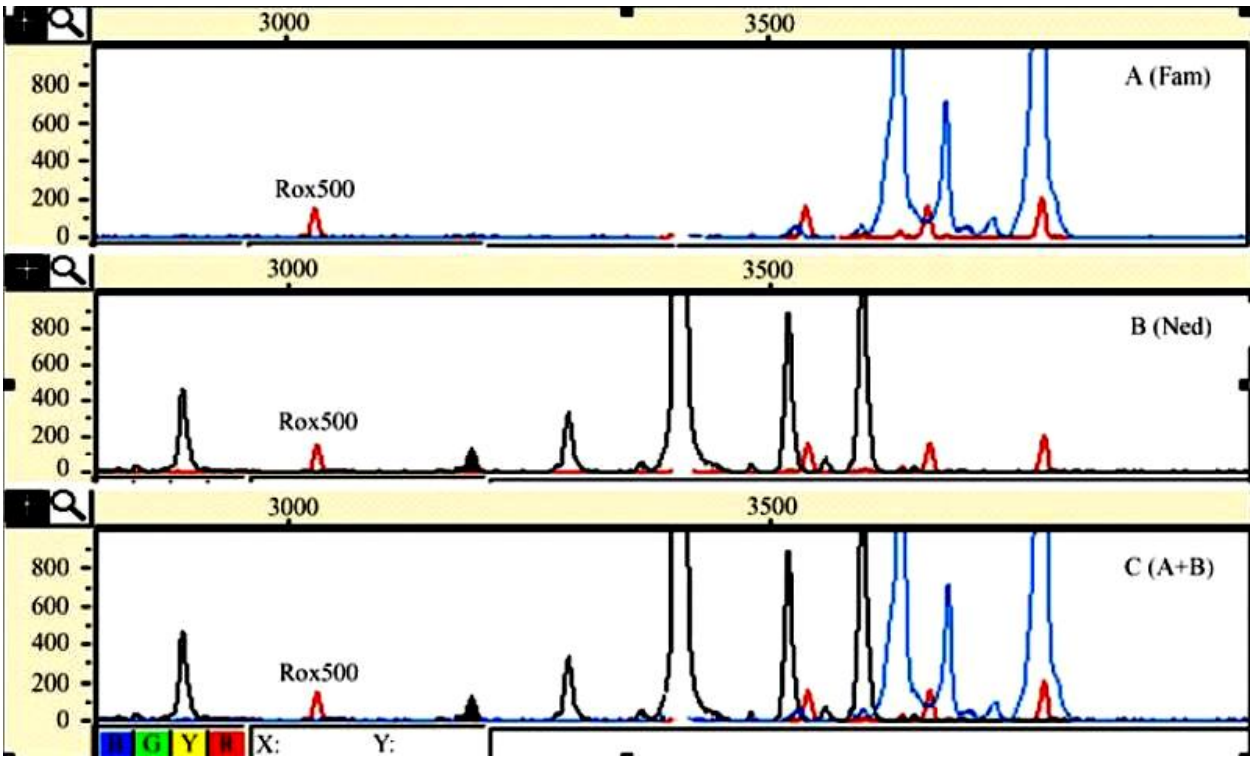

Figure 1. GeneScan profile of the multiplex PCR in Xinmiao wild chestnut with fluorescent primer CmTCR10 (NED) and CmTCR21 (HEX) in ABI3730 DNA analyzer. A, CmTCR10 (Blue NED + red size standard ROX500); B, CmTCR21 (black HEX + red size standard ROX500); C, CmTCR10 + CmTCR21 + size standard ROX500 (multi-profile).

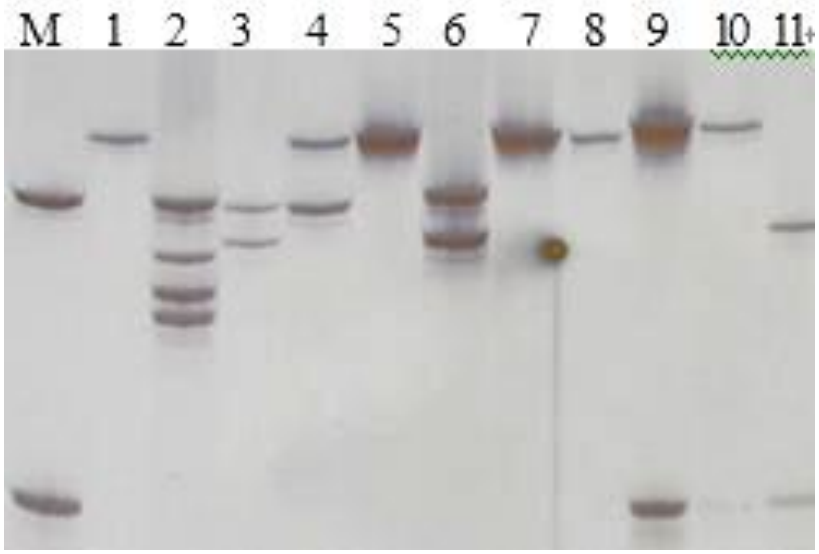

Figure 2. Polyacrylamide gel electrophoresis (PAGE) and silver staining profile of PCR amplification with primer CmTCR10 in 11 chestnut populations. M, marker (pBR322/Msp I); 1 to 11 is the number of materials (as in the Table 1).

primers. Third and final, it is advisable to use primers with different fluorescent label, respectively, but the same fluorescent label could be used in the primers with different amplification range. The annealing temperature of fluorescent primers CmTCR10 and CmTCR21 were similar (52°C for CmTCR10 and 50°C for CmTCR21),

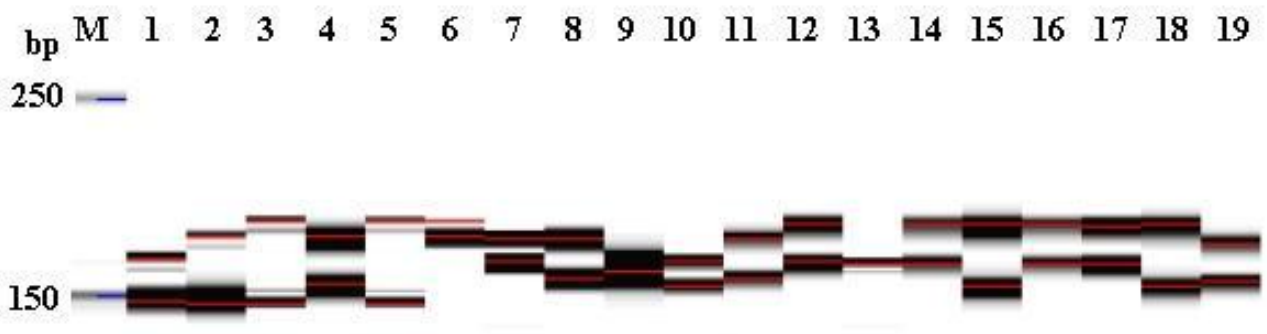

Figure 3. SSR fingerprint map with primer CmTCR21 in 19 indivduals from zundali population. M, marker; 1 to 19 is the 19 individuals from zundali population.

while the difference in temperature between other primers was much larger, thus the primer pairs of CmTCR10 and CmTCR21 were selected as the optimum primers for multiplex PCR in this research.

In the conventional method of SSR-PAGE followed by silver staining, DNA must be extracted from each individual and amplified individually. The intra-population allele frequency must be analyzed before comparing the inter-population allele frequency (Pinar et al., 2003; Silfverberg et al., 2006), while bulk sampling got much less work with higher efficiency. For example, with 15 populations, 15 individuals in each population and 60 primer pairs, 225 DNA samples must be extracted, 13,500 SSR-PCR reactions must be performed, and a great deal of work is required for PAGE and silver staining. When using bulk sampling, just 15 DNA samples need to be extracted and 900 SSR-PCR reactions need to be performed. The workload for bulk sampling is just 6.67% of that for individual sampling. The efficiency can be improved 30 times with the use of the AB 3730 DNA analyzer, and the efficiency can be further improved with the application of multiplexed PCR. Most importantly, the fluorescent SSR technique has the advantages of precise band reading, consistent data format and no data integration barriers.

Previous researches showed that 15 to 20 individuals are needed for a strong representation of a group (Dubreuil et al., 1999; Ai et al., 2007; Yong et al., 2009). Skalski et al. (2006) evaluated procedures for estimating allele frequencies by pooling DNA from multiple individuals, a method suggested as cost-effective relative to individual genotyping. Silfverberg et al. (2006) analyzed 178 apple samples and 142 alleles by multiplex PCR and fluorescent SSR and compared that to conventional denaturing PAGE followed by silver staining. The results show that the costs of 178 × 142 reactions using each of these two methods were roughly equal without regarding to the instrument cost, but the efficiency of the fluorescent SSR technique was significantly higher than that of the PAGE method. Pinar et al. (2003) analyzed 1000 alleles from 198 samples within 50 h. The automatic DNA detection method with high throughput, high efficiency and high accuracy was based on fluorescent SSR technology and was especially suitable for large-scale materials analysis research.

We would like to widen the range of the application of fluorescent SSR technique by screening the molecular markers closely linked to target traits, to further shorten the breeding cycle of fruit trees. At present, the fluorescent SSR technique was used by the *C. mollissima* Tai'an Chestnut Repository to carry out SSR analysis, population allele frequency analysis and quantitative SSR analysis for wild chestnut populations.

Conclusion

In this study, we established a method for allele frequency detection in bulk samples by detecting the abundance of PCR products using fluorescence labeled SSR primers. Compared with the conventional SSR technique based on PAGE and silver staining, fluorescent SSR was much more sensitive. As a result, a total of 78 alleles, an average of 4.6 alleles per locus, were detected with the primer CmTCR10 (NED) among 17 chestnut populations, and a total of 41 alleles, an average of 2.4 alleles per locus, were detected with the primer CmTCR24 (6-FAM). Multiplexing the PCR reaction by combining the primer pairs of CmTCR10 and CmTCR24 showed that the alleles could be discriminated and the sizes of the amplified segments were similar. The results of this study indicate that it is feasible to calculate the allele frequency of bulk samples according to the abundance of fluorescent SSR-PCR products based on AB automatic DNA analyzer. The fluorescent SSR is more sensitive and more efficient in allele frequency detection than conventional SSR, thus the detection efficiency could be further improved using multiplex PCR combined with fluorescent SSR.

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