Establishing and Optimizing AFLP Amplification Reaction System of Shiraia Bambusicola

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Abstract. DNA of Sharaia bambuiscola was extracted by the improved method of CTAB. The several key factors affecting the effect of DNA digestion and the PCR selective amplification such as the time of DNA digestion, the times of the per-amplified dilute products, the mount of the selective amplification primer, Taq concentration and dNTPs concentration were optimized and trialed with establishment of an optimized AFLP reaction system of S. bambusicola. The best time of digestion DNA with double endonucleases (EcoR Ⅰ and Mse Ⅰ) was 3h. The optimized selection amplification system was 25μl reaction mix containing 2.5μl the 20 times of the per-amplified dilute products, 2.5μL 10× buffer(with Mg^{2+}), 10mmol/L primer each 1.5μL, 2.5U μL^{-1} Taq polymerase 0.7μL, 10mmol/L dNTP 0.5μL, and 15.8μL ddH_2O. Stable and clean DNA finger print can be obtained and 3 pairs of AFLP primers with good genetic diversity were selected according to the optimized reaction system. The results will be an effective protocol for further studying the genetic stucture and differentiation, phylogenetic trees, host specificity and artificial cultivation of Shiraia bambusicola population.

Introduction

Shiraia bambuiscola P. Hennings has been reported as a parasitic medicinal fungus on branches of bamboo. It is widely applied in various fields such as biological technology and medicine. But the phylogeny is unclear. Recently, the research results show that a new family Shiraiaceae (Pleosporale, Dothideomycetes, Ascomycota) was established with S. bambusicola as representative gene and species[1]. Stromata of S. bambusicola has a long history as a traditional Chinese medicine, and are the raw material for extracting of hypocrellin[2]. Hypocrellin is promising in the photodynamic therapy (PDT) for anticancer treatment[3,4]. Furthermore, it has a striking function to kill tumor cells, inhibit viruses, cure diabetic retinopathy and against human immunodeficiency virus type 1(HIV-1)[5-8].

S. bambusicola is distributed in southern China and Japan[9]. Sri Lanka is hopefully one of distribution regions[10]. But the biomass of the fungus is very limited. Driven by market demand, the wild resource of S. bambusicola has been overexploited and become scarce[11]. The fungus and its genetic diversity has not been sufficiently studied.

S. bambusicola is discontinuously distributed in China and Japan[9], which may results in rich genetic diversity within the species but from different geological locations. RAPD (Random Amplified Polymorphic DNA) was used to analyze genetic differentiation of S. bambusicola populations from east China[12]. However, little has been done in the genetic diversity.

Materials and Methods

Materials

35 test materials of Shiraia bambusicola were collected from Anhui, Zhejiang, Guizhou provinces; primers and adapter (JieLi); 10U/μL of EcoRⅠ, 10U/μL of MseⅠ(Thermo), 5 U/μL of T_4 DNA ligase, 2.5U/μL of Taq DNA polymerase (buffer with Mg^{2+}), 10mmol/L each of dNTPs (Zomanbio) were applied.

Methods

DNA Extraction. DNA of Shiraia bambusicola was extracted by the modified CTAB[13-14] and detected by using 0.8% agarose gel electrophoresis.

Establishment and Optimization of AFLP Reaction System

Enzyme Digestion and Ligation. EcoRⅠand MseⅠ were used for double enzyme digestion. The enzyme digestion system is shown in Table 1. Enzyme digestion time is an important factor for the result. Enzyme digestion time was set 4 gradients for 2, 3, 4 and 5h in this research. The result was detected by using 0.8% agarose gel electrophoresis. Then the ligation reaction was conducted at 16℃ after the single stranded adapter (Table 2) become double. The time of reaction was 12h. Ligation reaction system is also shown in Table 1.

Tab. 1. Part of the AFLP reaction system

Enzyme digestion/μL		Ligation/μL		Per-amplification/μL	
Total volume	20	Total volume	20	Total volume	25
Template DNA	5	Digestion products	15	Ligase products	2.5
EcoR Ⅰ	0.5	EcoR Ⅰ adapter	1	E00 primer	1
MseⅠ	0.5	MseⅠ adapter	1	M00 primer	1
10×Tango buffer	2	T4 buffer	2	10×Taq buffer	2.5
ddH_2O	12	T4 DNA ligase	1	Taq	0.5
				dNTP	0.5
				ddH_2O	17

Amplification Reaction. After the ligation reaction, two PCR amplification were reacted. The per-amplification and the selective amplification was conducted. The per-amplification primer were E00 and M00 (Table 2), and the reaction system was shown in Table 1. Per-amplification PCR reaction was performed under the following thermal conditions: initial denaturation at 95℃ for 3min; 30 cycles of denaturation at 95℃ for 30s; annealing at 55℃ for 30s; an extension at 72℃ for 1min, and a final extension at 72℃ for 7min. The final products were stored at -20℃.

Tab. 2 The swquences of all adapter and primer in experiment

Sequences of adapter and primer		
Double stranded adapter	EcoR Ⅰ	5-CTC GTA GAC TGC GTA CC-3
		3-CAT CTG ACG CAT GGT TAA-5
	Mse Ⅰ	5-GAC GAT GAG TCC TGA G-3
		3-TA CTC AGG ACT CAT-5
Per-amplification primer	E00	5-GAC TGC GTA CCAAT TCA-3
	M00	5-GAT GAG TCC TGA GTA AC-3

Tab. 3 The swquences of all adapter and primer in experiment (Cont.)

Sequences of adapter and primer		
Selective amplification primer	E1-ACT	5-GACTGCGTACCAATTC**ACT**-3
	E2-AAC	5-GACTGCGTACCAATTC**AAC**-3
	E3-AAG	5-GACTGCGTACCAATTC**AAG**-3
	E4-ACA	5-GACTGCGTACCAATTC**ACA**-3
	E5-AGG	5-GACTGCGTACCAATTC**AGG**-3
	E6-ACG	5-GACTGCGTACCAATTC**ACG**-3
	E7-AGC	5-GACTGCGTACCAATTC**AGC**-3
	E8-ACC	5-GACTGCGTACCAATTC**ACC**-3
	M1-CAG	5-GATGAGTCCTGAGTAA**CAG**-3
	M2-CAC	5-GATGAGTCCTGAGTAA**CAC**-3
	M3-CTC	5-GATGAGTCCTGAGTAA**CTC**-3
	M4-CTG	5-GATGAGTCCTGAGTAA**CTG**-3
	M5-CAA	5-GATGAGTCCTGAGTAA**CAA**-3
	M6-CTA	5-GATGAGTCCTGAGTAA**CTA**-3
	M7-CAT	5-GATGAGTCCTGAGTAA**CAT**-3
	M8-CTT	5-GATGAGTCCTGAGTAA**CTT**-3

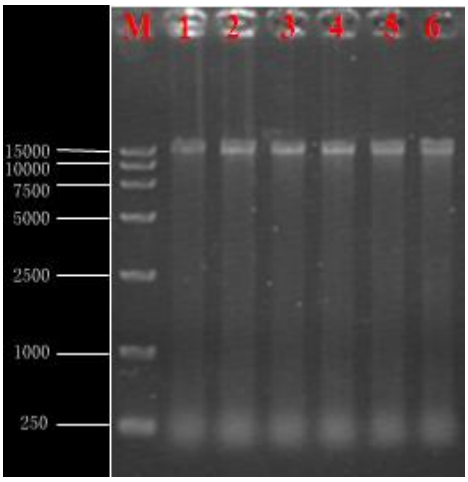
In the selective amplification reaction system, different gradients of 4 factors were set for the amount of per-amplification products, Taq, dNTP, primers as well: the total selective amplification reaction system was 25 μL, the additive amount of per-amplification products was 2.5 μL. The seven gradients of the times of per-amplification dilute products we set, including 5, 10, 20, 40,60,80 and 100 times; concentration of Taq was 2.5U/μL, and 6 gradients of additive amount we set, including 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 μL; concentration of dNTP was 10mmol/L, and 6 gradients of additive amount we set, including 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 μL; concentration of primer was 10mmol/L, and 6 gradients of additive amount we set, including 0.5, 1.0, 1.5, 2.0, 4.0 and 6.0 μL. Reaction with a single variable each time. Finally, the reaction system contained 2.5 μL of 10 × Taq buffer(with Mg^{2+}) and ddH_2O was added to the final volume. The reaction amplification has two steps, gradient cooling amplification and common amplification. The first step was initial denaturation at 95℃ for 3min; 13 cycles of denaturation at 95℃for 30s; annealing at 60℃for 30s; an extension at 72℃ for 1min, the annealing temperature of each cycle was declined 0.7℃ in this step. And the secong step was 30 cycles of denaturation at 95℃ for 30s; annealing at 53℃ for 30s; an extension at 72℃ for 1min, and a final extension at 72℃ for 7min.

After reaction, 5 μL volume of denaturing sample buffer(98% of deionized formamide, 10mmol/L EDTA, 0.25% of bromophenol blue and 0.25% of xylene cyanol FF) was added into 20 μL volume of selective amplification products for 5 min of denaturing at 95℃, and then immediately placed into ice mixture to prevent the products from renaturation. The per-electrophoresis was carried out for 30min with 6% denaturing polyacrylamide gel under constant power of 70W, after sample application, electrophoresis was carried out for about 2h under constant power of 70W, and then the silver staining continued for further analysis[14,15].

Results and Analysis

DNA Detection

The electrophoresis bands had shown good with no impurities (Fig. 1), which Indicates that the genomic DNA we extracted by modified CTAB were high-quality, and suitable for analysis.

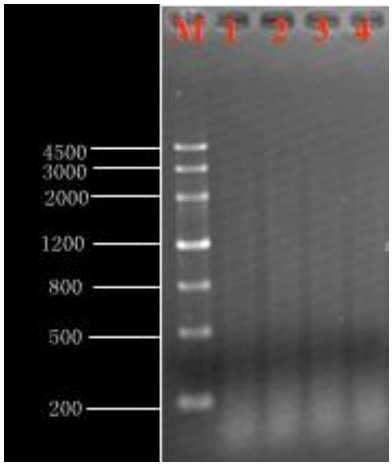


Note: M:Marker DL 15000; 1~6: Six different samples

Fig.1 The electrophoresis results of genomic DNA

Enzyme Digestion and Ligation

Double enzyme digestion was used in the enzyme and ligation reaction. The 4 gradients of enzyme digestion time were detected. As Fig. 2 shown that the electrophoresis bands were uniformly scattered without main band. This result indicates that double enzyme digestion was able to digest genomic DNA, and the complete digestion time was 3h. The enzyme digestion products were good templates for ligation reaction.



Note: M:MarkerⅢ; 1~4: Severally, the digestion time was 2, 3, 4, 5 U:h

Fig. 2 The results of different enzyme digestion time

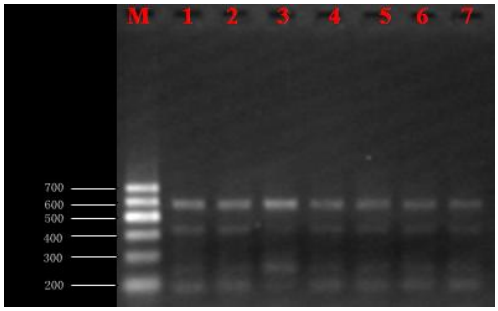
Amplification and Optimization

The pairs E00 and M00 were added into the products from Enzyme digestion and ligation reaction. After the PCR per-amplification, the small uniforml scattered bands which about from 50bp to 500bp were obtained. Not only the results of ligation reaction were detected in this process, but also the templates of next step were prepared.

In the selective amplification reaction system, different gradients of 4 key factors were set for optimizing the system. Four key factors include the times of the per-amplified dilute products, the mount of selective amplification primer, Taq concentration and dNTPs concentration. The results were detected by using 2.5% agarose gel electrophoresis, and the optimized reaction system was obtained finally.

As shown in Fig. 3, different bands were observed from different times of per-amplification dilute products. The additive amount was 2.5μL, and the band which dilute from 5 times to 20 times was better and clearer than the others. When the times was more than 20, the bands were weaker and

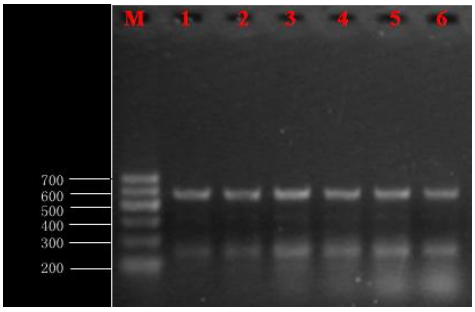
amplificated deficiently. According to the results, the selective amplification system with 20 times of per-amplification dilute products was the best times.



Note: M:Marker Ⅰ; 1~7: Severally, the diluted times were 5, 10, 20, 40, 80, 160, 320 U:time

Fig. 3 The electrophoresis results of selective amplification with different diluted times of pre-amplification products

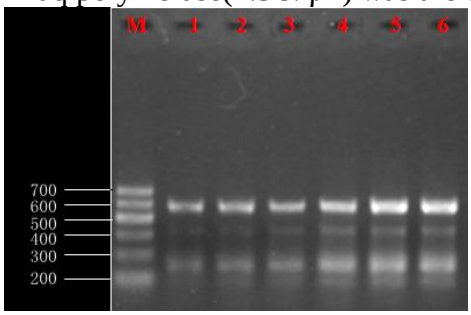
As shown in Fig. 4, with 10mmol/L concentration of primer , the bands with 1.5 μL additive amount were observed of clearer and higher polymorphism. When the additive amount was less than 1.5 μL, the bands were weaker and amplificated deficiently. When the addtive amout was more than 1.2 μL, the nonspecific amplification had significantly increased. The selective amplification system with 1.5 μL primer(10mmol/L) was the best volume.



Note: M:Marker Ⅰ; 1~6: Severally, the primer (10mmol/L) volume was 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 U: μl

Fig. 4 The electrophoresis results of selective amplification with different primer volume

As shown in Fig. 5, the concentration of Taq polymerase was 2.5U/μL, and we could observe the bands which additive amount was from 0.7 μL to 1.2 μL were better, clearer and higher polymorphism. When the additive amount was less than 0.7 μL, the bands were weaker and amplificated deficiently, there would be less or even no the products. When the addtive amout was more than 1.2 μL, the nonspecific amplification had significantly increased. According to the results, the selective amplification system with 0.7 μL Taq polymerase(2.5U/μL) was the best volume.

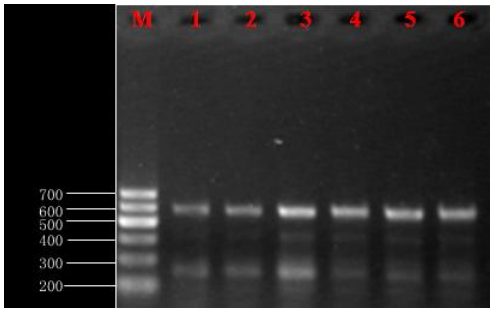


Note: M:Marker Ⅰ; 1~6: Severally, the Taq(2.5U/μl) volume was 0.1, 0.3, 0.5, 0.7, 0.9, 1.1 U: μl

Fig. 5 The electrophoresis results of selective amplification with different Taq volume

As shown in Fig. 6, with 10mmol/L the concentration of dNTP , the bands of the additive amount from 0.5 μL to 2.0 μL were o with better, clearer and higher polymorphism. When the additive amount

was less than 0.7 μL, the bands were weaker and amplificated deficiently, there would be less or even no the products. According to the results, the selective amplification system with 0.5 μL dNTP(10mmol/L) was the best volume.



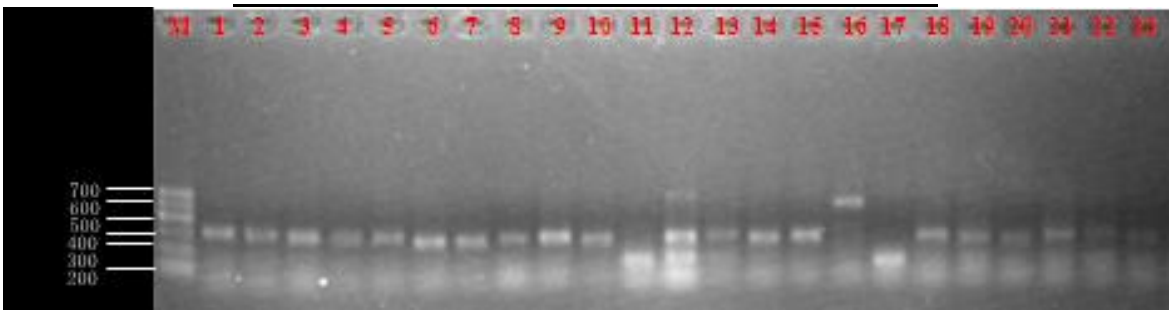
Note: M:Marker Ⅰ; 1~6: Severally, the dNTP(10mmol/L) volume was 0.1, 0.3, 0.5, 0.7, 0.9, 1.1 U:μl

Fig. 6 The electrophoresis results of selective amplification with different dNTP volume

After explored the 4 factors affecting the effect of PCR selective amplification, An optimizing AFLP reaction system could be obtained (Table 3). The detection results by using agarose gel electrophoresis primary as Fig. 7, and the results by using denaturing polyacrylamide gel are shown in Fig. 8.

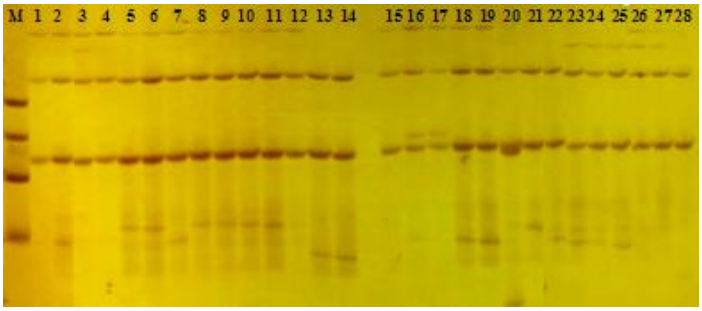
Tab. 3 Optimizing selection amplification of Shiraia bambusicola

Total volume	25[μL]
Per-amplification dilute products(20 times)	2.5
PrimerE(10 μM)	1.5
PrimerM(10 μM)	1.5
10×Taq buffer(with Mg^{2+})	2.5
Taq(2.5U/μl)	0.7
dNTP(10 μM)	0.5
ddH_2O	15.8



Note: M:Marker Ⅰ; 1-23: Twenty-three different samples

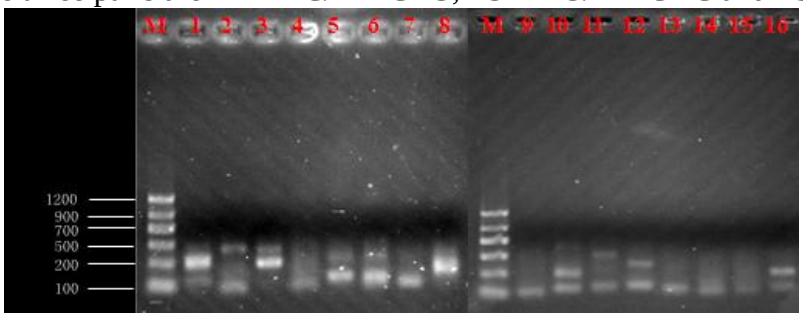
Fig. 7 The agarose electrophoresis results of the optimized selective amplification reaction system



Note: M:Marker Ⅰ; 1-28: Twenty-eight different samples

Fig.8 The polyacrylamide gel electrophoresis results of the optimized selective amplification

Screening Primer Primary. Different amplification effect were obtained through different pairs of primers between different genomic DNA[16]. Screening the suitable pairs of primers was the key to conclude polymorphism. Three pairs of primers from 64 pairs by using 2.5% agarose gel electrophoresis through observed bands were screened. The bands of 3 pairs primers were more and clearer (Fig. 9). The three pairs are E2-AAC/M4-CTG, E3-AAG/M2-CAC and E3-AAG/M4-CTG.



Note: M:Marker Ⅱ; 1~16: Severally, the primer combination was 11, 12, 13, 14, 21, 22, 23, 24, 31, 32, 33, 34, 41, 42, 43, 44

Fig. 9 Detection of selective amplification with different pairs of primers

Discussion

An AFLP reaction system of S. bambusicola was first established in this study. The several key factors affecting the effect of DNA digestion and the PCR selective amplification were explored and optimized. The results showed that extracted DNA by the improved method of CTAB is suitable for the AFLP system with the high quality DNA. Three hours of enzyme digestion was digested fully with the desired results of enzyme digestion and ligation reaction. The optimizing selection amplification system was 25 μL reaction mix containing 2.5 μL the 20 times of the per-amplified dilute products, 2.5 μL 10× buffer(with Mg^{2+}), 10mmol/L primer each 1.5 μL, 2.5U/μL Taq polymerase 0.7 μL, 10mmol L^{-1} dNTP 0.5 μL, and 15.8 μL ddH_2O.

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