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Full Length Research Paper

DNA isolation and optimization of sequence-related amplified polymorphism-polymerase chain reaction (SRAP-PCR) condition for endangered *Polyporus umbellatus*

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To provide a fast genetic diversity survey of endangered *Polyporus umbellatus* (Pers.) Fries for immediate conservation, DNA isolation and optimization of polymerase chain reaction (PCR) assay of sequence-related amplified polymorphism (SRAP) were investigated. Due to high amount of polysaccharide contained in mycelium, three special approaches were adopted to eliminate it during DNA isolation, including adding RNAiso-mate for Plant Tissue buffer to mycelium powder, ethanol to DNA extraction buffer and 5% NaCl solution to the mixture of isoamyl alcohol and DNA deposit solution. Based on screening design, the optimal SRAP-PCR condition was a total volume of 25 µl containing 20 ng of DNA template, forward primer 0.4 µM, reverse primer 0.4 µM, 1× Taq MasterMix and the best annealing temperature was 35/50°C for each primer combination. According to our optimal SRAP-PCR condition, 49 out of 81 primer combinations were chosen for their high clarity and repetition in all samples.

Key words: DNA isolation, sequence-related amplified polymorphism (SRAP), optimal polymerase chain reaction (PCR) condition, chuling, *Polyporus umbellatus.*

INTRODUCTION

Chuling [*Polyporus umbellatus* (Pers.) Fries], a well-known medicinal fungus, is widely used in China and its sclerotium has been officially listed in China Pharmacopoeia as diuretic (National Committee of China Pharmacopoeia, 2010). However, with the quick growth of commercial demands for this medicinal material in recent years, excessive exploitation has severely shrunk its natural resources and it has been listed as endangered species in China Red Book (Li, 2008). To date, previous studies mainly focus on the resources distribution, components extraction, pharmacological properties and the relationship with its companion fungus (Xu, 1997; Xing and Guo, 2004; Yuan et al., 2004; Kikuchi and Yamaji, 2010; Zhao et al., 2010), and no effort has been made to its genetic diversity for immediate conservation of wild resources and future cultivar breeding.

Fortunately, DNA markers make a fast survey of a species' genetic diversity and the sequence-related amplified polymorphism (SRAP), a new molecular marker first introduced by Li and Quiros (2001), has several advantages such as simple, reproducible and easy isolation of fragments for sequencing. Now it has widely applied in genetic diversity analysis, genetic linkage map construction and molecular identification (Ren et al., 2010; Wang et al., 2011).

To conserve wild genetic resources of this medicinal fungus as soon as possible, a previous mycelial growth and polysaccharide content of seven wild *P. umbellatus* strains collected from seven provinces of China have been evaluated by our laboratory (Zhang et al., 2010). In present study we aimed to: (1) form an improved DNA isolation method for *P. umbellatus*; (2) obtain an efficient

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Table 1. The isolated DNA purity and yield from 100 mg fresh mycelium of *Polyporus umbellatus* by different isolation methods.

DNA isolation method	A260/A280	A260/A230	Yield (mg g^{-1})
CTAB method reported by Han 2008 [a]	1.44 ± 0.09	0.34 ± 0.03	13.03 ± 0.02
Present method with RNase treating 1 h [a]	1.92 ± 0.03	1.98 ± 0.01	149.65 ± 0.03
Present method with RNase treating 30 min [a]	2.08 ± 0.02	2.08 ± 0.01	45.45 ± 0.02

[a]Mean of eight samples from three independent experiments with standard deviation.

protocol of SRAP-PCR condition; and (3) screen primer combinations by using the optimal SRAP-PCR system.

MATERIALS AND METHODS

Sclerotium material

Eight wild sclerotium strains of *P. umbellatus* were collected from seven provinces of China, including Shaanxi, Henan, Heilongjiang, Sichuan, Yunnan, Hebei and Hubei. They were identified and preserved at local laboratory in each region after collection.

DNA isolation

The activation culture of eight original sclerotium strains and their liquid culture were followed by our previous work (Zhang et al., 2010). The activated mycelium on PDA medium was transplanted to liquid medium and cultivated at 25°C for 7 days under dark condition. The mycelium was washed with ddH_2O followed by filtering with sterile gauze and then stored at -20°C for use after water absorption with sterile filter paper. Total genomic DNA was extracted from 100 mg fresh mycelium by using the modified cetyltrimethyl ammonium bromide (CTAB) method (Han, 2008).

In a pre-chilled mortar and pestle, 100 mg fresh mycelium was finely ground using liquid nitrogen, and quickly transferred to 2 ml RNAiso-mate for plant tissue buffer to primarily eliminate polysaccharide. The mixture was ground again until the solution became transparent after the powder completely dissolved in the kit buffer, followed by centrifugation of the mixture at 12,000 × g for 5 min at 4°C A 700 µl extraction buffer pre-warmed at 65°C for 5 min was added to the upper aqueous phase and the mixture was shaken slightly for 4 times when incubating at 65°C for 1 h. The 700 µl phenol : chloroform : isoamyl alcohol (25:24:1) was added to it and mixed well by invert mixing. The contents were centrifuged at 13,000 × g for 10 min at 4°C. Two microlitre RNase was added to the upper aqueous phase and the mixture was maintained at 37°C for 1 h. Another 700 µl phenol : chloroform : isoamyl alcohol was added to the mixture and mixed well, followed by centrifugation at 13,000 × g for 10 min at 4°C. Equal volume of chloroform : isoamyl alcohol (24:1) was added to the upper aqueous phase and mixed well, followed by 13,000 × g for 10 min at 4°C. The two volumes of isoamyl alcohol were added to the collected upper phase. The pellet was collected by centrifugation (12,000 × g for 2 min at 4°C) after incubating the tubes at -20°C for 30 min and then washed twice with 75% ethanol, and centrifuged at 12,000 × g for 1 min at 4°C. The pellet was re-suspended in 30 µl ddH_2O water after evaporation of ethanol.

DNA quantifications were performed by UV-spectrophotometer at 260 and 280 nm, and the purity was then determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (OD_{260}/OD_{280}). DNA concentration and purity was also determined by electrophoresis on 0.7% agarose compared with DM 15000 DNA marker. The DNA solution was then diluted in 30 µl sterile distilled water to different concentrations for screening use.

Optimization of SRAP-PCR condition

Based on the report of Li and Quirous (2001), SRAP-PCR amplification was performed with 1× Taq MasterMix (Kangwei, Beijing) in a total volume of 25 µl. Another three factors including DNA template, primer combination concentration and annealing temperature were evaluated. DNA template included five concentrations: 10, 15, 20, 25 and 30 ng. Primer combination concentration included five grads: 0.24, 0.32, 0.40, 0.48 and 0.56 µM. Annealing temperature contained four combinations: 35/48, 35/50, 32/50 and 30/50°C. The PCR was performed in a PTC200 thermocycler (MJ Research Inc, Watertown, Mass.). A denaturation period of 5 min at 94°C was followed by 5 cycles of 1 min at 94°C, 60 s at different annealing temperature and 1 min at 72°C, and then 35 cycles of 1 min at 94°C, 60 s at different annealing temperature and 1 min at 72°C with 10 min at 72°C for final extension. A 6 µL aliquot of the amplification products was separated by electrophoresis in 2.0% agarose gel in 1 × TAE buffer and the DNA fingerprints were photographed by an automatically imaging system.

RESULTS

DNA isolation and detection

White deposit pellet was obtained in all strains by using the improved CTAB method. Both A260/A230 and A260/A280 ratios were larger than 1.8, suggesting that the isolated DNA had high purity and was free of polysaccharide/polyphenol and protein contamination, respectively (Table 1 and Figure 1).

SRAP-PCR condition

After evaluating DNA template, primer combination

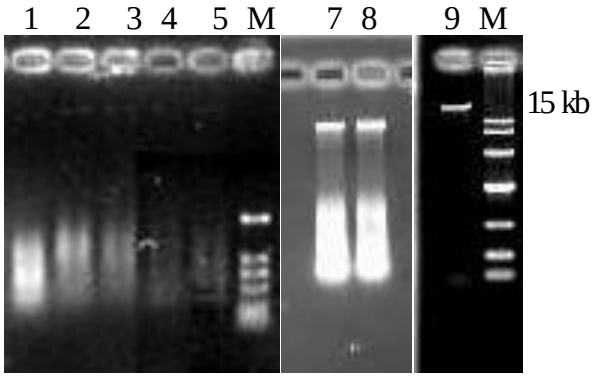

Figure 1. Electrophoresis result of genomic DNA extracted from *P. umbellatus* by using different isolation methods. M represented DM 15000 DNA marker; Lane 1 to 3: DNA extracted by the original CTAB method (Han 2008), treating with adding 35% alcohol (1), 5% NaCl solution (2 and 3), respectively; Lane 4 to 5: the original CTAB method; Lane 7 to 9: DNA extracted by the improved CTAB method, containing RNA (7 and 8) and eliminating RNA with RNase treating 30 min.

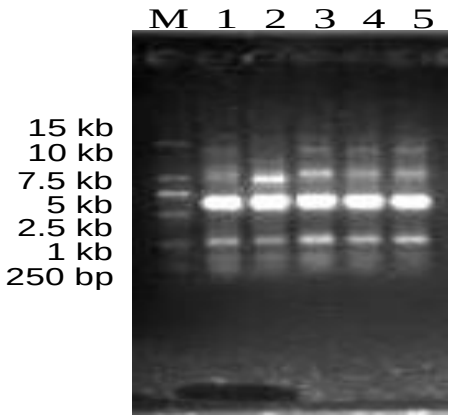

Figure 2. Electrophoresis detection of different DNA template concentrations that extracted from *P. umbellatus*. M represented DM 15000 DNA marker; Lane 1 to 5: total 25 µl reaction volume containing 10 (1), 15 (2), 20 (3), 25 (4) and 30 ng (5) DNA template, respectively.

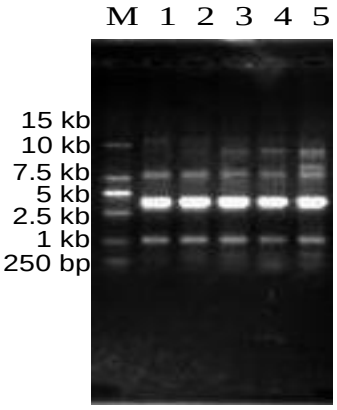

Figure 3. Electrophoresis detection of different primer combination concentrations contained in SRAP-PCR system. M represented DM 15000 DNA marker; Lane 1 to 5: 0.24 (1), 0.32 (2), 0.40 (3), 0.48 (4) and 0.56 µM (5), respectively.

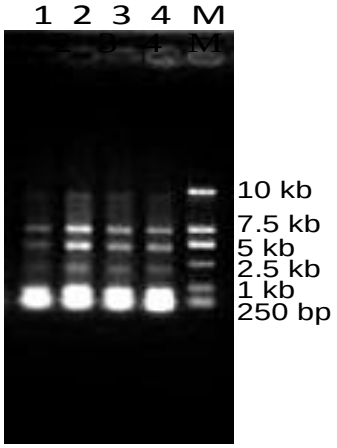

Figure 4. Electrophoresis detection of different annealing temperatures for primer combinations. M represented DM 15000 DNA marker; Lane 1 to 4: 35/48 (1), 35/50 (2), 32/50 and 30/50°C (4), respectively.

concentration and annealing temperate with different treatments, the best SRAP-PCR condition was formed. The PCR reaction mixture (total 25 µl) contained DNA template 20 ng, forward primer 0.4 µM, reverse primer 0.4 µM, 1× Taq MasterMix. The best annealing temperature was 35/50°C. The isolated DNA evaluated by three factors (Figures 2, 3 and 4) were amplified by primer pair Me4/Em4 (Forward: TGAGTCCAAACCGGACC; Reverse: GACTGCGTACGAATTTGA) and an electrophoresis result of all eight strains by using Me6/Em5 and Me6/Em6 primer combinations was displayed in Figure 5. Eighty-one SRAP primer combinations were initially screened by using the best SRAP-PCR condition, of which forty-nine was selected that produced consistent and clear polymorphic fragments.

DISCUSSION

Among all molecular markers, SRAP was firstly applied in

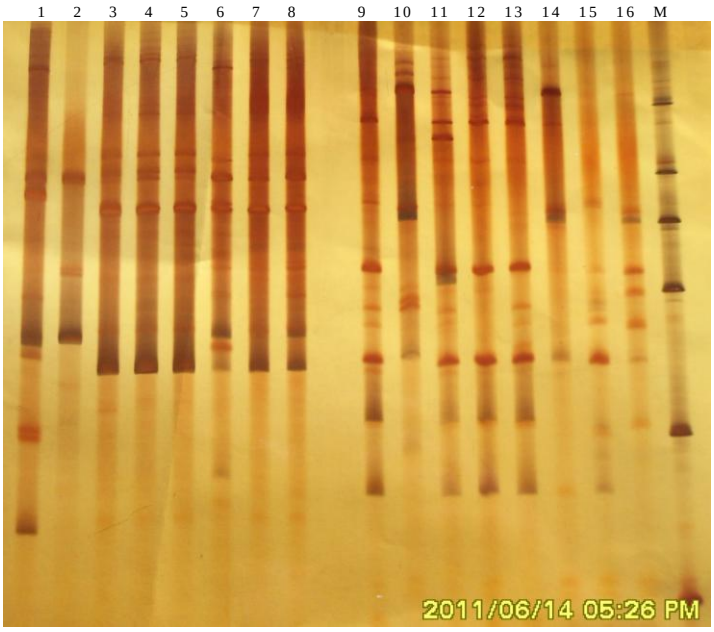

Figure 5. Polyacrylamide gel electrophoresis (PAGE) results of eight *Polyporus umbellatus* strains amplified by both primer combination Me6/Em5 (Lane 1 to 8) and Me6/em6 (Lane 9 to 16). M was DNA marker 2000.

P. umbellatus strains for subsequent resources conservation, sustainable utilization and further research. SRAP aims at the amplification of open reading frames and its markers are more consistent and repeatable than RAPD, and are less-labor-intensive and time-consuming to produce than AFLP and SSR (Li and Quiros, 2001; Ren et al., 2010; Cai et al., 2011). Budak et al. (2004) compared four marker systems in buffalo grass and found the values of revealing genetic diversity power as: SRAP>SSR>ISSR>RAPD. SRAP has been widely applied in genetic diversity analysis, molecular identification, genetic linkage map construction, gene tagging (Li and Quiros, 2001; Ren et al., 2010; Cai et al., 2011). Our protocol provides the opportunity for the genetic research in Japan or other countries for resources conservation and management in world-wide range, fragments screening of functional gene involved in polysaccharide, and molecular breeding in future, all of which are lacking.

Like other filamentous fungi, *P. umbellatus* has rigid cell walls (Guo and Xu, 1992) which make DNA and RNA isolation more difficult. Although its DNA may be isolated before based on the report of Xing and Guo (2004), the details have not been provided. On the other hand, mycelium of *P. umbellatus* contains more polysaccharide that can severely interfere with DNA, RNA and protein purification (Sánchez-Rodríguez et al., 2008). Our past work showed that the intracellular polysaccharide contents ranged from 23.56 to 64.50 mg g^{-1} fresh mycelium weight among seven mycelial strains which sclerotium collected from seven provinces of China (Zhang et al., 2010). Therefore, three additional approaches were performed to eliminate polysaccharide step by step when extracting DNA from the mycelium. 1) Add RNAiso-mate for Plant Tissue buffer to the fine powder after ground by using liquid nitrogen; 2) Ethanol (35% volume of extraction buffer) was added to the DNA extraction buffer and mixed well immediately, making polysaccharide deposit; 3) Half volume of NaCl solution (5%) was added to the mixture of isoamyl alcohol and DNA deposit solution.

In conclusion, the protocol presented here demonstrates isolation of intact high-quality DNA and provides the best SRAP-PCR condition from *P. umbellatus*.

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REFERENCES

Budak H, Shearman RC, Parmaksiz I, Gaussoin RE, Riordan TP, Dweikat I (2004). Molecular characterization of Buffalograss germplasm using sequence-related amplified polymorphism markers. Theor. Appl. Genet., 108: 328-334.

Cai X, Feng Z, Zhang X, Xu W, Hou B, Ding X (2011). Genetic diversity and population structure of an endangered Orchid (*Dendrobium loddigesii* Rolfe) from China revealed by SRAP markers. Scient. Horticul., 129: 877-881.

Guo SX, Xu JT (1992). Origin and development of crystal and thick walled cells in sclerotia of *Grifola umbellata*. Mycosystema, 11: 49-54.

Han NF (2008). The relationship between Ramosa gene and branched spike. Master thesis, Yangling: Northwest A & F University.

Kikuchi G, Yamaji H (2010). Identification of *Armillaria* species associated with *Polyporus umbellatus* using ITS sequences of nuclear ribosomal DNA. Mycoscience, 51: 366-372.

Li G, Quiros CF (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor. Appl. Genet., 103: 455-461.

Li SQ (2008). Endangered *Polyporus umbellatus* need immediate conservation. Mod. Chin. Med., 10: 43-45.

National Committee of Pharmacopoeia. China Pharmacopoeia (2010). Beijing: Chemical Industry Press, p. 299.

Ren X, Huang J, Liao B, Zhang X, Jiang H (2010). Genomic affinities of *Arachis* genus and interspecific hybrids were revealed by SRAP markers. Genet. Resour. Crop Evol., 57: 903-913.

Sánchez-Rodríguez A, Portal O, Rojas LE, Ocaña B, Mendoza M, Acosta M, Jiménez E, Höfte M (2008). An efficient method for the extraction of high quality fungal total RNA to study the *Mycosphaerella fijiensis*-Musa spp. Interaction. Mol. Biotechnol., 40: 299-305.

Wang Z, Wang JE, Wang XM, Gao HW, Dzyubenko NI, Chapurin VF (2011). Assesssment of genetic diversity in *Galega officinalis* L. using ISSR and SRAP markers. Genet. Resour. Crop Evol., DOI 10.1007/s10722-011-9727-0.

Xing XK, Guo SX (2004). The phylogenetic relationships of *Grifola umbellate* and its companion fungus: evidence from ITS sequence analysis. Microbiology, 31: 34-38.

Xu JT (1997). Medicinal Fungi in China. United Publishing House of Beijing Medical University and Chinese Union Medical University, Beijing, China, pp 518-523.

Yuan D, Mori J, Komatsu KI, Makino T, Kano Y (2004). An anti-aldosteronic diuretic component (drain dampness) in *Polyporus Sclerotium*. Bio. Pharm. Bull., 27: 867-870.

Zhang YJ, Fan S, Liang ZS, Wang W, Guo HB, Chen DY (2010). Mycelial growth and polysaccharide content of *Polyporus umbellatus*. J. Med. Plant Res., 4: 1847-1852.

Zhao YY, Chao X, Zhang YM, Lin RC, Sun WJ (2010). Cytotoxic steroids from *Polyporus umbellatus*. Planta Med., 76: 1755-1758.