JMB 2007; 26 (4) DOI: 10.2478/v10011-007-0036-2

UDK 577.1 : 61 **ISSN 1452-8258**

JMB 26: 289–293, 2007

Original paper
Originalni naučni rad

APPLICATION OF RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS IN IDENTIFYING *PHELLINUS IGNIARIUS* STRAINS

PRIMENA RAPD ZA IDENTIFIKACIJU *PHELLINUS IGNIARIUS* SOJEVA

Ping Du[1], Yan-Qiu Chen[1,2], Chang-Tian Li[2], Yu Li[2]

[1]Agricultural College of Yanbian University, Longjing, Jilin 133400, China
[2]Institute of Mycology, Jilin Agricultural University, Changchun, Jilin 130118, China

Summary: Described in this paper, the random amplified polymorphic DNA (RAPD) analysis was conducted with 20 random primers in various strains of *Phellinus igniarius* collected from different localities. The results showed that 17 of the 20 random primers were polymorphic ones. The DNA bands derived from each primer amplifying in tested strains ranged from 10 to 33. The size of the amplified DNA fragments ranged from 250 to 2000 base pairs. Of each test primer, a wide variation in banding profiles was observed among the 7 strains of *P. igniarius*. A total of 377 band positions were scored for all of the tested strains, which differed significantly among the bands from different primers. UPGMA cluster analysis subdivided the tested strains into two groups, which was helpful to find out the difference among the tested strains and to distinguish them directly.

Keywords: *Phellinus igniarius*, RAPD, genetic relationship, cluster analysis

Kratak sadržaj: U ovom radu opisana je analiza sprovedena pomoću metode RAPD na 20 nasumično odabranih prajmera iz različitih sojeva *Phellinus igniarius*-a uzetih sa različitih lokaliteta. Rezultati su pokazali da je 17 od 20 prajmera bilo polimorfno. Broj prstenova DNK dobijenih prilikom pojedinačne amplifikacije prajmera u testiranim nizovima kretao se od 10 do 33. Veličina amplifikovanih fragmenata DNK kretala se od 250 do 2000 baznih parova. Velike varijacije u profilima traka svakog testiranog prajmera uočene su kod 7 sojeva *P. igniarius*-a. Dobijeno je ukupno 377 traka za sve testirane sojeve, među kojima su se trake iz različitih prajmera značajno razlikovali. Klaster analiza UPGMA dodatno je podelila testirane nizove u dve grupe, što je pomoglo u otkrivanju razlika između testiranih nizova i njihovom brzom identifikovanju.

Ključne reči: *Phellinus igniarius*, RAPD, genetska veza, klaster analiza

Introduction

[*Phellinus igniarius* (L.ex.Fr)Quel.] is a rare medicinal fungi that is placed in the Basidiomycota, Hymenomycetes, Aphyllophorales, Hymenochaetaceae and *phellinus* (1). As a form of traditional Chinese medicine, these species have been reported to cure several diseases (2), and have also been used in Japan as a diuretic (3). *P. igniarius* contains several bioactive substances including polysaccharides, flavones and triterpenoids (4). More recent research showed that *P. igniarius* inhibited cancer cell growth, possessed immunomodulatory properties, and exerted an anti-hepatic fibrosis function (5). Fruit bodies of the genus of *P. igniarius* mostly assume horse unguis shape, and it is difficult to distinguish them from macroscopical ones, so at present *P. igniarius*'s right classification and naming have a lot of controversies (1).

In this paper, RAPD technique was used to identify *P. igniarius*'s germplasm resources, study their relationship and genetic diversity in order to explore the genetic differences of the genus on the DNA level and to provide the theory basis for classification and identification of *P. igniarius*.

Address for correspondence:

Yan-Qiu Chen
Agricultural College of Yanbian University
Longjing, Jilin 133400, China
e-mail: cyq326@yahoo.com

Materials and Methods

Tested strains

The strains tested in this study are listed (*Table I*). Seven strains were collected, or isolated from fruit bodies, in different localities. All strains growth medium was Potato Dextrose Agar (PDA) and they were incubated at 25 °C in the dark for 15 days, to be preserved at 4 °C in a refrigerator.

PCR reagent

200 bp DNA ladder marker, Taq enzyme, 10 × Buffer. dNTPs, magnesium, agarose (Spain-packing), random primers and other reagents were purchased from Beijing branch of Sino-American Biotechnology Company. Random primers and the sequences are listed (*Table II*).

DNA extraction

Method used for genomic DNA extraction of different *P. igniarius* strains was as YangHua *et al*. described (6), and improved further (DNA samples would be diluted to 15 ng × L^{-1}).

RAPD reaction

Contents of PCR components are listed (*Table III*). The PCR program was as follows: 94 °C for 4 min; 40 circles of 94 °C for 1 min, 40 °C for 1min and 72 °C for 1.5 min, then a final extension at 72 °C for 5 min. The final temperature is 4 °C. After this, PCR was amplified, using 0.5×TBE electrophoresis buffer and 1.4% agarose gel electrophoresis (containing 0.5 μg × mL^{-1} EB). Taking 10 μL PCR products mixed with 2 μL electrophoresis buffer (100V voltage, 3~4 h), electrophoresis products were detected and photographed under GDS-8000 UV document system.

Data analysis

Records clear electrophoresis bands in the same location with presence marked as »1« and absence as »0«, and the 0.1 matrixes were input into the computer. Polymorphic loci percentage is one of the important indexes to reflect the genetic diversity within groups, according to the following formula to calculate the polymorphic loci percentage: $P=(k/n)\times 100\%$ (7), where k is the number of polymorphic loci, n is the total number of measured loci. Data were processed by MEGA2.1 software, a dendrogram was constructed based on genetic distance using unweighted pair group method average (UPGMA)(8).

Table I Tested strains of *P. igniarius*.

No.	Strains No.	Collection place	Collection time	Host
S_1	CBS01	Changbai mountain	2004	Pine
S_2	CBS02	Changbai mountain	2004	Syringa amurensis
S_3	JL01	Jilin (Yanji city)	2004	Poplar
S_4	JL02	Jilin (Antu county)	2004	White pine
S_5	YN01	Yunnan	2004	Mulberry
S_6	HLJ01	Heilongjing	2003	Mulberry
S_7	HG01	Korea	2003	Mulberry

Table III Contents of PCR components.

Component	Volume (μL)	Final concentration
ddH_2O	15.17	–
10×buffer	2	0.8 ×
dNTPs	1	100 μmol×L^{-1}
$MgCl_2$	2.5	2.5 mmol×L^{-1}
Taq enzyme (3 U×μL^{-1})	0.33	1 U
primer (5 pmol×μL^{-1})	2	10 pmol
DNA template	2	30 ng
Total	25	–

Table II Commercial numbers and nucleotide sequences of random primers tested.

No.	Primer	5,-3,	No.	Primer	5,-3,	No.	Primer	5,-3,
1	A-02	TGCCGAGCTG	8	B-11	GTAGACCCGT	15	H-17	CACTCFCCTC
2	A-03	AGTCAGCCAC	9	D-18	GAGAGCCAAC	16	M-09	GTCTTGCGGA
3	A-04	AATCGGGCTG	10	F-14	TGCTGCAGGT	17	Y-19	TGAGGGTCCC
4	A-05	AGGGGTCTTG	11	G-15	ACTGGGACTC	18	OPA-19	CAAACGTGGG
5	A-09	GGGTAACGCC	12	G-19	GTCAGGGCAA	19	RA01	AGCGCCATTG
6	A-12	TCGGCGATAG	13	H-07	CTGCATCGTG	20	RA02	CAGCACCCAC
7	A-20	GTTGCGATCC	14	H-13	GACGCCACAC			

Results

Primer screening

Among the 20 random primers, H-17, M-09 could not amplify any electrophoresis bands; A-09 could only amplify a part of the tested strains' genomic DNA, and produced amplified products, but with poor repeatability, and DNA fragments were instable; another 17 primers could amplify DNA bands of all tested strains, and bands were clear, stable and repeatable. These primers were A-02, A-03, A-04, A-05, A-12, A-20, B-11, D-18, F-14, G-15, G-19, H-07, H-13, Y-19, OPA-19, RA01, RA02.

Analysis of RAPD amplified profiles

Seventeen primers amplified clearly reproducible bands of seven strains. The DNA bands derived from each primer amplifying in tested strains ranged from 10 to 33, generally 20~27; the size of the amplified DNA fragments ranged from 250 to 2000 base pairs (*Figure 1*). From the amplified profile of primer D-18 can be seen that S_6 and S_7 had much more similar bands. S_3 and S_6, S_7; S_1 and S_2 had slight differences between the bands. But, compared with other strains, S_4, S_5 were quite different. The amplified profiles of other primers also reflected this trend. Namely, RAPD profiles can be used to reflect the genetic differences of *P. igniarius* strains. Amplification using each primer carried out in three triplicate can be stable. It was demonstrated that RAPD is a reliable method for identifying the genetic relationship between different strains of *P. igniarius*.

The statistical analysis of amplification showed that 17 primers produced 377 bands, of which 366 were polymorphic; percentage of polymorphic bands (PPB) accounted for 97.1% (*Table IV*). This showed a high level of genetic variation in the tested strains. Although individual primers (OPA-19) were unable to distinguish between certain strains, all strains could be separated with most primers. Another 11 amplified bands were shared by all the strains indicating that they had some genetic similarity.

Cluster analysis

Distance coefficients were calculated by MEGA2.1 software based on the statistical results of the PCR products of 17 primers. The smaller the distance

Table IV Nucleotide sequence of primers for RAPD and the numbers of amplified bands.

Prime code	Amplified bands	Polymorphic bands	Common bands	Polymorphic frequency (%)
A-02	23	23	0	100
A-03	33	33	0	100
A-04	20	20	0	100
A-05	25	25	0	100
A-12	19	19	0	100
A-20	14	10	4	71
B-11	28	28	0	100
D-18	27	27	0	100
F-14	23	23	0	100
G-15	20	20	0	100
G-19	27	26	1	96
H-07	22	22	0	100
H-13	23	23	0	100
Y-19	27	27	0	100
OPA-19	10	6	4	60
RA01	13	11	2	85
RA02	23	23	0	100

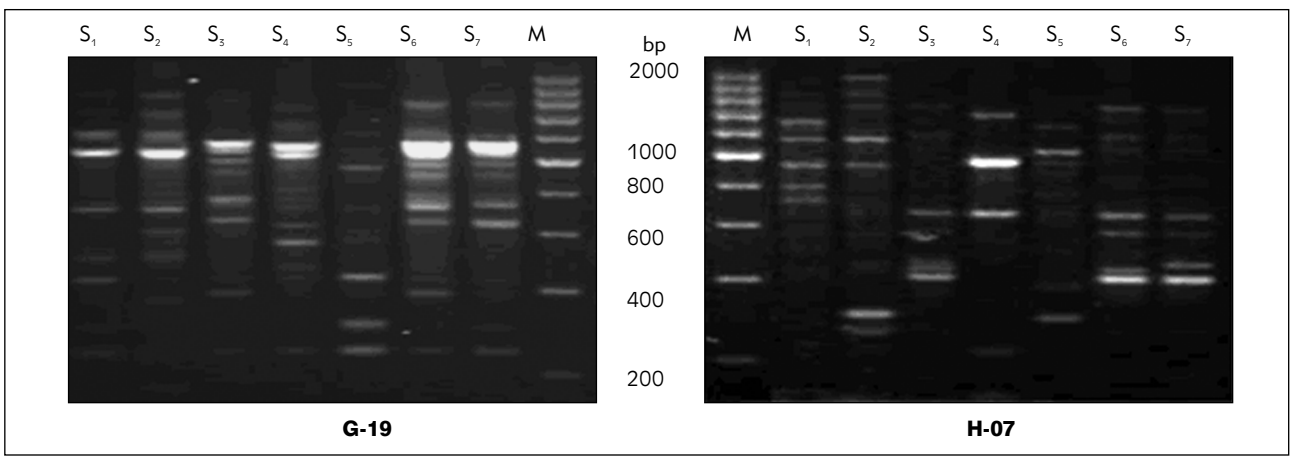

Figure 1 RAPD patterns of primers G-19 and H-07.
S_1-S_7: Strains No.; M: 200 bp DNA ladder marker

Table V Distance coefficient matrix based on RAPD of 7 strains of *P. igniarius*.

Strains	S_1	S_2	S_3	S_4	S_5	S_6	S_7
S_1	0.000						
S_2	0.303	0.000					
S_3	0.469	0.539	0.000				
S_4	0.440	0.502	0.494	0.000			
S_5	0.402	0.440	0.456	0.411	0.000		
S_6	0.477	0.506	0.166	0.510	0.456	0.000	
S_7	0.419	0.498	0.191	0.469	0.432	0.100	0.000

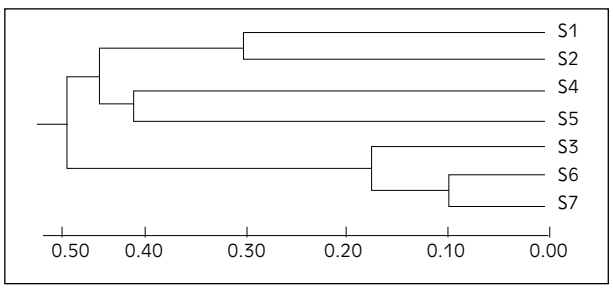

Figure 2 Dendrogram of tested *P. igniarius* strains based on RAPD data.

coefficient, the closer the genetic relationship and vice versa. The genetic distances among tested strains, ranging from 0.100 to 0.539, changed greatly. The lowest genetic distance based on RAPD markers was found between S_6 and S_7; distance coefficient was 0.100 (*Table V*). Namely, 90% of the genetic composition was the same, which showed the closest relationship. In fact, S_6 comes from Heilongjiang, S_7 from Korea, and they are often viewed as the same strain according to the morphological, biochemical and physiological classification. The relationship of S_6 and S_7 was very close, but they are still different strains according to the results of RAPD analysis. In addition, the distance coefficients of S_3 and S_6, S_3 and S_7 were 0.166 and 0.191 respectively, and they may be derived from the same strain or variations which have closer genetic relationship. The distance coefficient between S_1 and S_2 was 0.303, and they had close genetic relationship, whereas the highest genetic distance (0.539) was found between S_2 and S_3, so therefore they had the farthest genetic relationship.

In order to identify the tested *P. igniarius* strains more directly and accurately, a dendrogram was constructed based on genetic distance using UPGMA of MEGA2.1 software. At the genetic distance of 0.477, tested strains were classified into two groups (*Figure 2*). The first group included S_1, S_2, S_4, S_5, the second group included S_3, S_6, S_7. However, at the genetic distance of 0.440, tested strains were classified into three groups. The first group included S_1, S_2, the second group included S_4, S_5, the third group included S_3, S_6, S_7.

Discussion

Using DNA as the genetic material of RAPD analysis could reflect the genetic relationship among strains objectively and truthfully. Compared with traditional molecular markers, the advantage of RAPD lies in its ability to detect a slight difference between DNA samples, and the fact that the results are reliable, stable and repeatable (9–10) and can be used for identifying *P. igniarius* strains. Meanwhile, it has overcome the deficiency of traditional methods in identifying strains of intraspecies or interspecies, which show slight differences, and RAPD provides a new way to detect the strains of edible and medicinal fungi.

This study selected 17 primers, which produced 366 polymorphic bands; PPB accounted for 97.1% (*Table IV*), indicating that RAPD technique may be described as one of the effective methods to distinguish *P. igniarius* strains, which has great significance. It showed a high level of genetic diversity in the tested strains, and the abundant genetic diversity supplies us with a broad genetic background and plenty of genetic resources.

In addition, most of the 17 primers could amplify the specific bands among tested *P. igniarius* strains. One distinct polymorphic band of about 400 bp amplified by H-07 was clearly present in S_3, S_6, S_7, but absent from other strains. However, further research involving a large sample size is needed to establish if the degree of specificity is sufficient for these primers to be used in the routine identification of *P. igniarius* strains, and whether these bands can be used as a genetic marker of different *P. igniarius* strains, further transformed into SCAR marker and certified.

Acknowledgements. This project was supported by the Education Department of Jilin Province (No.2003-6).

References

1. Dai Y-C. New know to medical basidiomycete *phellinus igniarius (L.ex Fx.) Quel*. Chinese Traditional and Herbal Drugs, 2003; 34 (1): 94–5 (in Chinese).
2. Liu B. Chinese medical fungi [M]. Taiyuan: Shanxi People Press: 1974; 71–2 (in Chinese).
3. Yang YP, Yue DC. *Chinese medical fungi [M]. Heilongjiang:* Heilongjiang Science and Technology Publishing House, 1981; 44 (in Chinese).
4. Zheng LJ, Wang Q, JI JQ, Shen YS. The advances in studies on medical fungi-*Phellinus* [J]. Research and Practice of Chinese Medicine, 2005; 19 (3): 61–4 (in Chinese).
5. Zhou CS, Ma HL. *Phellinus igniarius* and the advances in studies on its pharmacological effect [J]. Edible Fungi, 2005; 27 (2): 50–51 (in Chinese).
6. Yang H, Li LT. Research on edible fungi DNA extraction methods. Edible Fungi of China, 2003; 22 (1): 19–22 (in Chinese).
7. Li ZB. Study on genetic relationship among 4 kinds of abalone using RAPD technique. Chinese Journal of Eco-Agriculture, 2004; 12(4): 60–3 (in Chinese).
8. He XQ, Liu QC, Wang YP, Zhai H. Analysis of genetic diversity among Sweetpotato Landraces in China. Agricultural Sciences in China, 2004; 3 (12): 888–95.
9. Huang DB, Yang Q, Chen MZ. Distinguishing strains of *Lentinula edodes* with RAPD. Edible Fungi, 2003; (6): 9–10 (in Chinese).
10. He SP, Wang D, Wang W, Chen DP, Zhao QZ, Gong WM. Genetic diversity in the Yangtze finless porpoise by RAPD analysis. High Technology Letters, 2005; 11 (2): 207–10.

Received: May 15, 2007

Accepted: October 5, 2007