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GENETIC ANALYSIS OF *INONOTUS OBLIQUUS* STRAINS BY RAPD

GENETSKA ANALIZA NIZOVA *INONOTUS OBLIQUUSA* POMOĆU METODE RAPD

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Summary: RAPD profiling of eight *Inonotus obliquus* strains isolated from sclerotia collected from different areas of China was conducted to determine the genetic variability within this important medicinal fungus and to better define relationships between the genotype and geographical origins of isolation. Twelve 10-mer primers generated a total of 167 stable and reproducible DNA fragments, of which 101 (60.5%) were polymorphic. DNA fingerprints revealed genetic diversity among the strains tested, but there was the little intraspecific difference between the fingerprints of individual strains. A phenogram constructed based on UPGMA analysis of genetic distances calculated from RAPD fragment data identified three distinct groupings: (1) BCX01 and BCX02, (2) JL01, JL02, JL03, JL04 and JL05, (3) HLJ01. Our data confirm that the genetic variability among different strains may be a useful ancillary tool for identifying *I. obliquus* sclerotia of different geographical origins.

Keywords: DNA fingerprint, genetic distance, *Inonotus obliquus*, sclerotium

Kratak sadržaj: Pomoću metode RAPD urađeno je profilisanje 8 nizova *Inonotus obliquusa* izolovanih iz sklerocije donesene iz raznih delova Kine kako bi se utvrdila genetska varijabilnost te važne medicinske gljive i kako bi se bolje definisali odnosi između genotipa i mesta izolacije. Od 12 prajmera 10-mera generisano je ukupno 167 stabilnih i reproducibilnih DNK fragmenata od kojih je 101 (60,5%) bilo polimorfno. DNK otisci pokazali su genetsku raznovrsnost testiranih nizova, ali među otiscima pojedinačnih nizova nije bilo mnogo intraspecifičnih razlika. Fenogram sastavljen na osnovu UPGMA analize genetskih udaljenosti izračunatih na osnovu podataka RAPD fragmenata identifikovao je tri različite grupacije: (1) BCX01 i BCX02, (2) JL01, JL02, JL03, JL04 i JL05, (3) HLJ01. Naši podaci potvrđuju da genetska varijabilnost između različitih nizova može biti korisna za identifikaciju sklerocije *I. obliquus* različitog geografskog porekla.

Ključne reči: DNK otisak, genetska udaljenost, Inonotus obliquus, sklerocija

Introduction

Inonotus obliquus (Pers.:Fr.) J. Schroet is a rare and highly valuable basidiomycete fungus assigned to the family Hymenochaetaceae that has been used in Russia and other European Slavic countries as a traditional folk medicine to treat gastrointestinal disorders and various cancers (1). It is a parasitic fungus growing on birch, alder, beech and other hardwood trees throughout North America and Europe. Studies on the relationships between different strains of *Inonotus obliquus* are difficult. Zhao et al. reported that (2), while other microstructures were similar, spores obtained from sclerotial specimens collected from the Changbai Mountains, Russia and other European countries differed in size and concluded that the differences were the result of species variation or geographical distribution.

In the present study, Random Amplification of Polymorphic DNA (RAPD) was used to investigate strain heterogeneity within a population of eight *Inonotus obliquus* strains isolated from sclerotia. RAPD was chosen instead of restriction fragment length polymorphism or minisatellite DNA analysis because the procedure can be performed without any previous knowledge of specific DNA sequences of the species under study, only small amount of DNA is required, and it is faster, less costly and less labour-in-

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tensive than the other two DNA-based techniques (3, 4). RAPD analysis has been successfully used to study genetic structure in plants, birds and snails (5). Treatment of RAPD markers by analysis of molecular variance (6) has been shown to be adequate for demonstrating genetic variation among individuals/within sample sites, among sites/within regions, and among regions (7, 8).

Material and Methods

Plant species and sampling sites

I. obliquus samples were isolated from sclerotia of *Betula platyphylla* collected from Changbai Mountain Biosphere Reserve and remote mountain areas away from cultivation area. Samples were confirmed as wild *I. obliquus* sclerotia by Professor Yu Li, Jilin Agricultural University.

Isolation and culture of Inonotus obliquus

Mycelium of each of the eight *I. obliquus* samples was isolated using potato dextrose agar (PDA) medium and maintained at the Mycology Laboratory, Agricultural College, Yanbian University (*Table I*).

Table I *Inonotus obliquus* strains used in this study.

Strain code	Origin	Date of isolation
JL01	Wangqing, China	08–2004
JL02	Yanji, China	08–2005
HLJ01	Heilongjiang, China	08–2005
JL03	Songjianghe, China	08–2004
JL04	Dunhua, China	08–2004
JL05	Fusong, China	08–2005
BCX01	Rajin, DPRK*	08–2005
BCX02	Namyangjin, DPRK	08–2005

*Democratic People's Republic of Korea

Extraction of genomic DNA

Genomic DNA was extracted from fungal mycelium using the cationic detergent cetyltrimethylammonium bromide (CTAB) method. Samples were diluted 20-fold with TE buffer, and the OD values at 260 and 280 nm determined in triplicate (Hitachi U-3010 Spectrophotometer). DNA purity was calculated from the OD_{260}/OD_{280} value, and the concentration of the original DNA solution using C (ng/μL) = $OD_{260}\times 50\times$dilution ratio. The quality of the DNA samples was determined by electrophoresis (0.5× TBE buffer, 60V for 30 min) on 0.7% (w/v) agarose gels containing 0.5 μg/mL ethidium bromide (9). Samples were diluted to 10–100 ng/μL and stored at –20 °C prior to use.

Primer screening

DNA samples were amplified using 12 primers (see *Table II*) selected from a total of 50 random primers found to be effective in generating amplification products. All primers were from the Sino-American Biotechnology Company, Beijing.

Table II Nucleotide sequences of primers used for RAPD amplification.

No.	Primers	Sequence (5'–3')		Primers	Sequence (5'–3')
1	H-13	GACGCCACAC	7	A-04	AATCGGGCTG
2	OPA-19	CAAACGTGGG	8	A-05	AGGGGTCTTG
3	A-12	TCGGCGATAG	9	B-11	GTAGACCCGT
4	RA01	AGCGCCATTG	10	A-20	GTTGCGATCC
5	H-07	CTGCATCGTG	11	F-14	TGCTGCAGGT
6	Y-19	TGAGGGTCCC	12	G-15	ACTGGGACTC

RAPD amplification

PCR was performed in a programmable DB80240-33 Thermal Cycler. Reaction mixtures (25 μL) contained ddH_2O, 2.5 μL 10× PCR buffer, 2.0 mmol/L $MgCl_2$, 1.0 mmol/L dNTPs, 10.0 pmol RAPD primer, 40 ng template DNA and 1.0 unit of *Taq* DNA polymerase. All reagents were from the Sino-American Biotechnology Company, Beijing.

PCR conditions were as follows: one cycle of 94 °C for 5 min; 40 cycles of 94 °C for 1 min, 40 °C for 1 min and 72 °C for 1.5 min, and a final extension cycle of 72 °C for 5 min. Reaction products were separated by agarose (1.4% w/v) gel electrophoresis (3–4 h in 0.5 x TBE buffer, 100V), visualized with ethidium bromide and photographed under UV using a GDS-8000 gel documentation system. Amplification using each primer was carried out in triplicate.

Statistical analysis

Binary data based on the presence or absence of fragments and fragment mobility was obtained using LabWorks 4.0 software combined with artificial correction to analyse the bands. Phylogenetic relationships between the eight *I. obliquus* strains were determined by the branch and bound search method from a maximum parsimony tree using the MEGA program.

p-Distances were calculated using the formula: p-distance = 1 – Ssm = (b+c)/(a+b+c+d) and Ssm = (a+d)(a+b+c+d) using MEGA 2.1 software. (Ssm : Simple matching coefficient; a : bands common to two strains; b and c : bands unique to each strain; d : bands absent from both strains). Clustering was performed using the unweighted pair-group method with arithmetic clustering (UPGMA), and a genetic relationship dendrogram was constructed (10).

Results and Discussion

Quality of genomic DNA

DNA bands on agarose gels were distinct and without tailing (*Table I*) indicating high quality and no degradation products. Furthermore, OD_{260}/OD_{280} values were higher than 1.3 confirming that the purity of the DNA samples was of a level suitable for RAPD amplification.

Analysis of RAPD amplification

RAPD marker analysis conducted on the genomic DNA of eight *I. obliquus* strains using 12 10-mer primers revealed a total of 167 stable and reproducible DNA fragments, of which 101 (60.5%) were polymorphic (*Table III, Figure 1*). The number of bands amplified by individual primers ranged from 7 to 21. The high level of polymorphism and band reproducibility achieved demonstrates that RAPD is an effective method for identifying genetic relationships between different strains of *I. obliquus*.

Table III Number of bands amplified using the different primers.

Primer codes	Amplified bands	Polymorphic bands	Common bands	Polymorphic frequency
H-13	11	5	6	45.4%
OPA-19	20	11	9	55.0%
A-12	16	9	7	56.3%
RA01	15	9	6	60.0%
H-07	14	13	1	92.8%
G-15	10	5	5	50.0%
Y-19	13	9	4	69.2%
A-04	15	9	6	60.0%
A-05	7	5	2	71.4%
B-11	21	13	8	61.9%
A-20	13	7	6	53.8%
F-14	12	6	6	50.0%

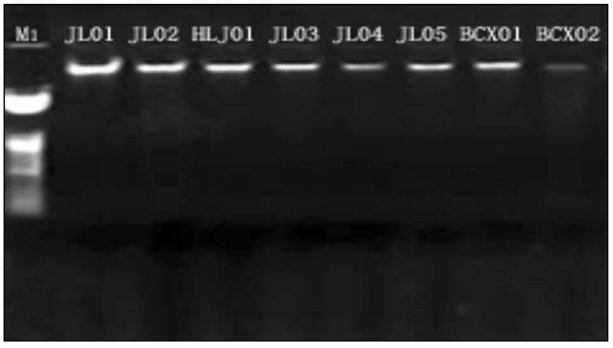

Figure 1 Genomic DNA samples from eight strains of *Inonotus obliquus*.
Lane M1: 23kbp DNA Ladder; Test strains JLO1-BCX02

Clustering analysis

An average genetic similarity coefficient matrix based on RAPD analysis of the eight *I. obliquus* strains is shown in *Table IV*. Genetic distances between strains ranged from 0.031 to 0.523. The low genetic distance value of 0.031 for strains BCX01 and BCX02 indicated a similar hereditary basis, and band patterns obtained for these two strains were actually identical in every case (*Figure 2*). Because of small differences in the activity, however, the possibility of synonymy and variant types having a close genetic relationship could not be excluded. The genetic distance between strains JL02 and JL03 is 0.215, suggesting that there was no significant genetic variation between samples from Yanji and Songjinghe. The sample from Wangqing (JL01) however, was significantly different to that from the Heilongjiang region (HLJ01) indicating considerable genetic variation.

A phenogram constructed using the UPGMA method identified three distinct groups: the first group consisted of strains BCX01 and BCX02, the second included strains JL01, JL02, JL03, JL04 and JL05, and strain HLJ01 formed the third (*Figure 3*).

According to the above RAPD analysis and genetic distance coefficients, genetic differentiation between the eight *I. obliquus* strains is related to geographical distribution and our data is therefore in good agreement with those of Zhao et al. (2). The reason may be that partial strains originated from the same species or variant types of close relationship. Clustering analysis carried out on the amplified fragments generated by the 12 primers also supported this conclusion. These data are in good agreement with results based on peroxide isoenzyme patterns (11) thereby further confirms the credibility of using RAPD as a method of identifying genetic relationships between *I. obliquus* strains.

Many studies have shown RAPD technology to be a rapid, precise and sensitive method for detecting and identifying species and strains of edible-medicinal fungi. Although RAPD still has some disadvanta-

Table IV Average genetic similarity coefficient matrix based on RAPD analysis.

Strains	JLO1	JL02	HLJ01	JL03	JL04	JL05	BCX01	BCX02
JLO1	0.000							
JL02	0.354	0.000						
HLJ01	0.523	0.415	0.000					
JL03	0.415	0.215	0.508	0.000				
JL04	0.492	0.385	0.492	0.477	0.000			
JL05	0.492	0.323	0.492	0.415	0.369	0.000		
BCX01	0.508	0.431	0.446	0.400	0.508	0.415	0.000	
BCX02	0.508	0.400	0.477	0.369	0.477	0.415	0.031	0.000

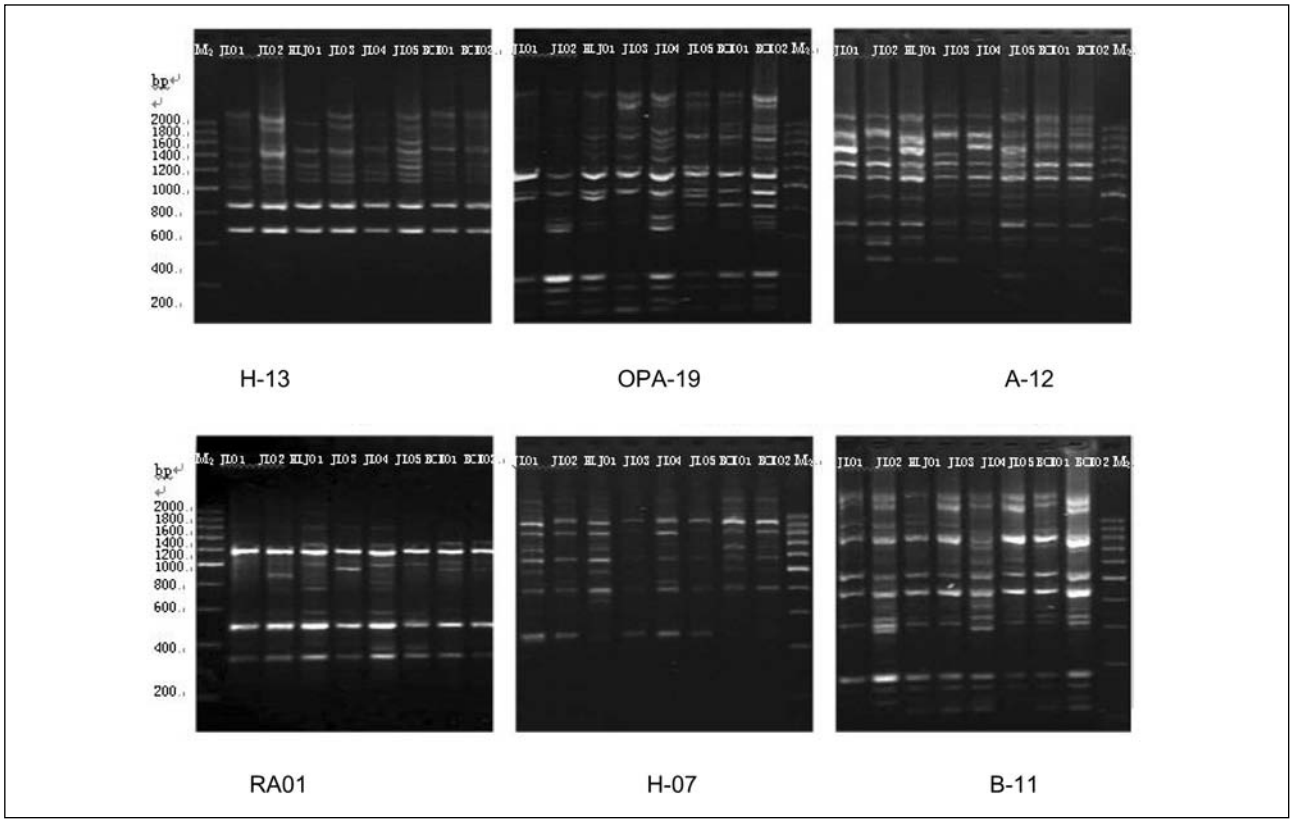

Figure 2 RAPD patterns obtained using primers H-13, OPA-19, A-12, RA01, H-07 and B-11.
Lane M2: 200bp DNA Ladder; Test strains JL01-BCX02

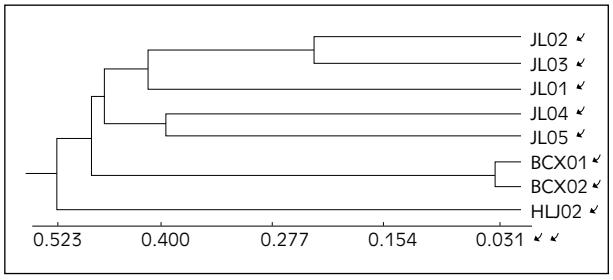

Figure 3 Dendrogram obtained from UPGMA analysis of p-distance values.

ges, such as low reproducibility, poor stability and false positives, experimental procedures associated with RAPD technology are subject to continual development and improvement. Furthermore, the application of RAPD technology to gene localization and the construction of genetic linkage maps will greatly benefit the edible-medicinal fungi industry.

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