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Analysis of the genetic diversity of *Inonotus obliquus* from six countries by sequence-related amplified polymorphism

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Abstract – The genetic diversity within a set of 22 strains of *Inonotus obliquus* from 6 countries was assessed using SRAP marker. The number of polymorphic fragments detected per primer combination ranged from 20 to 52 with an average of 37.74. Twenty three SRAP primer combinations had generated a total of 868 bands of which 859 (98.62%) were polymorphic. The UPMGA cluster analysis revealed that the 22 strains of *Inonotus obliquus* could be divided into 4 groups with the genetic similarity of 0.68. Principal coordinate analysis (PCoA) clusters was consistent with the results of UPGMA. Its results confirmed the results of UPGMA cluster analysis. This is the first report of using SRAP marker as a tool for determining genetic variation among *Inonotus obliquus*.

Clustering analysis / Medicinal fungus / Molecular marker / Principal coordinated analysis

INTRODUCTION

Inonotus obliquus (*Hymenochaetaceae*) is a medicinal fungus inhabiting birches in the cold latitudes of North America, Europe, Japan, Korea and China (Hawksworth *et al.*, 1995). It grows on living trunks of mature birch (*Betula* spp.) trees, the basidiomata emerging from near ground level up to a height of about 3 m. The conks burst through the bark, which remains firmly attached to the trunk, and appear as large black gall-like structures, varying in size (5-20 cm in diam), with a very irregular, cracked and deeply fissured surface (Reid, 1976).

Inonotus obliquus had been used as a traditional remedy to cure various diseases such as cancer, cerebrovascular diseases, diabetes, gastrointestinal diseases since the sixteenth century (Choi *et al.*, 2010; Sun *et al.*, 2008). Triterpenes, polysaccharides, polyphenols and melanin were found in the basidiomata of *I. obliquus*, which are shown to have anticancer and antitumor activities (Handa *et al.*, 2012; Song *et al.*, 2008), anti-inflammatory ability (Van *et al.*, 2009), antioxidant effect (Ma *et al.*, 2012) and hypoglycemic ability (Lu *et al.*, 2010). However, most of the studies about *I. obliquus* were mainly focused on its medicinal aspect.

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The sequence-related amplified polymorphism (SRAP) technique is a simple and efficient marker system, which preferentially targets exonic sequences and regions containing promoters (Li & Quiros, 2001). Previous reports have shown that SRAP marker has been successfully used for a variety of purposes including strain identification, gene mapping construction, gene tagging, genetic diversity assessment and cDNA fingerprinting (Amar *et al.*, 2011; Hao *et al.*, 2008; Youssef *et al.*, 2011). To date, few reports have assessed the genetic diversity of *I. obliquus* using sequence-related amplified polymorphism (SRAP) molecular marker.

The purpose of the present study was to assess the genetic diversity of *I. obliquus* from different geographic origin over the all distribution range on the basis of an analysis of SRAP molecular marker.

MATERIALS AND METHODS

Sampling: Twenty two strains of *I. obliquus* were used in this study. Of these, ten strains originated from Japan, seven originated from different provinces of China, two from USA, one from Russia, one from Finland and one from North Korea. The number, name and source of collection are listed in Table 1.

DNA extraction: Genomic DNA was extracted from mycelium by using cetyltrimethylammonium bromide procedure (Doyle *et al.*, 1987). The quality and concentration of the DNA samples (Table 2) was quantified by NanoDrop 2000. The DNA concentration was adjusted to 50 ng/μL.

SRAP analysis: The SRAP analysis was performed as described by Li and Quiros (2001). A total of 23 SRAP primer combinations from 5 forward primers and 6 reverse primers (Table 3) were screened for polymorphism. SRAP reactions were conducted in a 25 μl volume consisting of 2.5 μl of 10 ×PCR buffer, 1.5 μM $MgCl_2$, 1U of Taq DNA polymerase, 200 μM of dNTPs, 0.3 μM of each primer, and template DNA approximately 50 ng.

Amplification was carried out with the following PCR program: 5 min of initial denaturing at 94°C, 1 min of denaturing at 94°C, 1 min of annealing at 35°C and 1 min of elongation at 72°C, five cycles of four steps. 35 cycles of 94°C for

Tab.1. The numbers, strain code, and source of collection of 22 *Inonotus obliquus* strains

No.	*Strain code*	*Origin*	*No.*	*Strain code*	*Origin*
1	MAFF420198	Japan	12	JL05	China
2	MAFF420308	Japan	13	JL02	China
3	MAFF420256	Japan	14	CX02	North Korea
4	MAFF420102	Japan	15	Finnish birch	Finland
5	MAFF420239	Japan	16	HLJ01	China
6	MAFF420234	Japan	17	Z2	China
7	MAFF420279	Japan	18	JL03	China
8	NBRC9788	USA	19	MAFF420101	Japan
9	Birch Russia	Russia	20	MAFF410028	Japan
10	JL01	China	21	MAFF420165	Japan
11	JL04	China	22	NBRC8681	USA

1 min, 50°C for 1 min, and 72°C for 1 min; followed by a final extension 10 min at 72°C. The PCR products were mixed with 10 µl of formamide loading buffer (95% formamide, 20 mM EDTA, pH 8.0, Xylene cyanol and Bromophenol blue) analyzed on 8% non-denatured polyacrylamide gels in 1 × TBE buffer running at 200 V constant voltage for 2.5 h and then silver stained according to the reported procedure (Bassam *et al.*, 1991).

Data analysis: The polymorphic bands were identified visually and the distinct fragments were counted. For SRAP analyses, the polymorphic bands were scored as being present (1) or absent (0). Dice's similarity coefficients between strains were calculated by the NTSYSpc 2.10. Cluster analysis was performed using the UPGMA

Tab. 2. The quality and concentration of the DNA samples

No.	*A260*	*A280*	*260/280*	*260/230*	*DNA conc.*
1	0.772	0.360	2.15	2.24	50
2	1.100	0.520	2.11	2.15	50
3	0.627	0.285	2.20	1.62	50
4	0.910	0.443	2.06	2.22	50
5	0.899	0.408	2.20	2.20	50
6	0.614	0.287	2.13	1.71	50
7	3.655	1.758	2.08	2.23	50
8	0.752	0.357	2.10	2.00	50
9	0.705	0.334	2.11	2.23	50
10	0.648	0.319	2.03	2.02	50
11	0.651	0.301	2.16	1.70	50
12	0.849	0.407	2.09	1.92	50
13	1.043	0.550	1.90	1.78	50
14	0.896	0.423	2.12	2.11	50
15	1.240	0.592	2.10	2.32	50
16	0.745	0.753	2.11	2.16	50
17	0.853	0.407	2.09	2.50	50
18	0.548	0.262	2.09	2.07	50
19	0.363	0.159	2.28	1.92	50
20	0.583	0.291	2.00	1.84	50
21	0.543	0.258	2.10	1.64	50
22	1.521	0.809	1.88	1.07	50

Tab. 3. Primer sequences for SRAP analysis

Primer	*Sequence (5'-3')*	*Primer*	*Sequence (5'-3')*
me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT
me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA
me5	TGAGTCCAAACCGGAAG	em5	GACTGCGTACGAATTAAC
		em6	GACTGCGTACGAATTGCA

algorithm, and a dendrogram was produced based on each simple matching matrix. The principal coordinated analysis (PCoA) was performed to show the differentiation in a three-dimensional array of eigenvectors using the DCENTER and EIGEN modules of NTSYSpc 2.10.

RESULTS

Results of SRAP amplification: The 30 pairs of SRAP primers were tested on 2 strains, viz. JL04 and MAFF420198 for effectiveness. Of these primer pairs, 23 pairs produced clear, highly polymorphic, stable bands. Some typical banding profile amplified by Me4Em1 was shown in Fig. 1. The number of polymorphic bands for each primer combination ranged from 20 to 52 with an average of 37.74 (Table 4). 23 primer combinations generated a total of 868 bands, of which 859 (98.62%) were polymorphic.

Similarity coefficients analysis: The similarity matrix of the 22 strains used in SRAP analysis is listed in Table 5. It showed that the range of similarity vary between 0.600 and 0.985 with an average of 0.771. The lowest similarity was showed between MAFF420239 and MAFF420165 genotypes (0.600) whereas the highest similarity was between JL02 and Z2 strains (0.985).

Cluster analysis: A dendrogram based on Dice's similarity coefficients was depicted in Fig. 2. In this dendrogram, 22 *Inonotus obliquus* strains were clustered into four major clusters at Dice's similarity coefficients of 0.68. The first cluster included

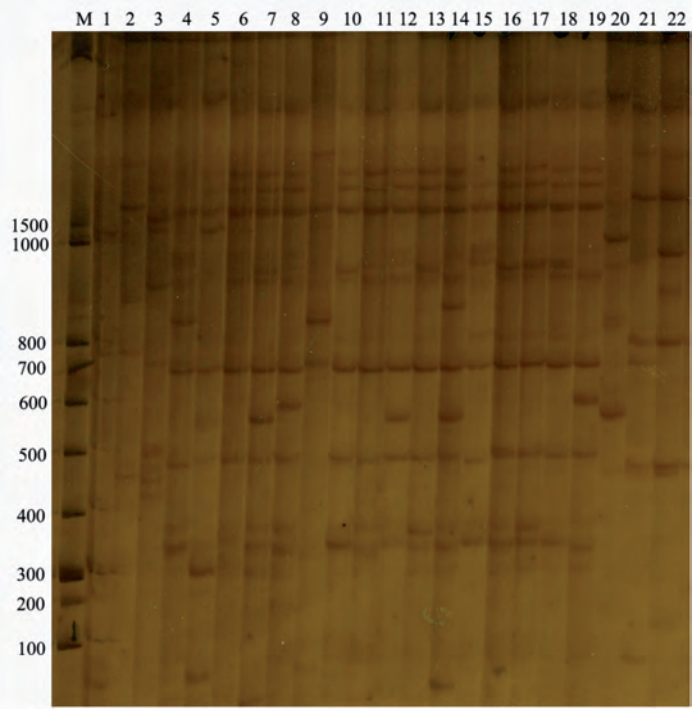

Fig. 1. A typical example of SRAP profiles of the investigated *Inonotus obliquus*, obtained from Me4Em1. M indicates DNA marker ladder. For the strains number, see Table 1.

Tab. 4. Amplified result of SRAP analysis for the 22 *Inonotus obliquus* strains

Primer combination	*Total bands*	*Polymorphic bands*	*% of polymorphic bands*
Me1Em2	33	33	100
Me1Em3	28	28	100
Me1Em4	45	45	100
Me1Em6	52	52	100
Me2Em3	27	27	100
Me2Em4	33	33	100
Me2Em6	46	46	100
Me3Em1	22	19	88.36
Me3Em2	37	37	100
Me3Em3	20	18	90
Me3Em4	27	27	100
Me3Em5	35	35	100
Me4Em1	32	32	100
Me4Em2	43	43	100
Me4Em3	39	39	100
Me4Em4	50	50	100
Me4Em6	38	38	100
Me5Em1	30	30	100
Me5Em2	45	45	100
Me5Em3	50	49	98
Me5Em4	51	51	100
Me5Em5	35	32	91.86
Me5Em6	50	50	100
Average	37.74	37.34	98.62
Total	868	859	–

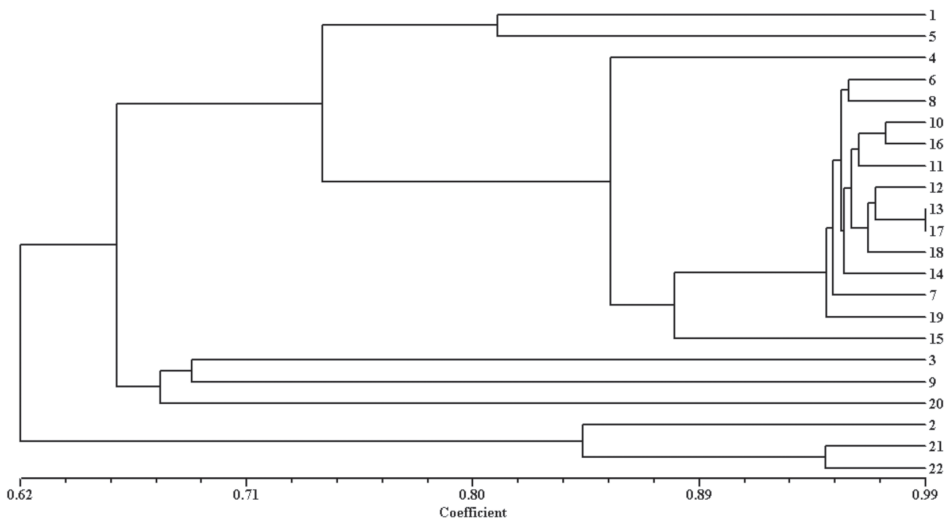

Fig. 2. Dendrogram of 22 *Inonotus obliquus*, derived from the UPGMA cluster analysis.

Tab. 5. Average genetic similarity coefficient matrix based on SRAP of 22 *Inonotus obliquus* strains

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*
1																						
2	0.690																					
3	0.743	0.657																				
4	0.714	0.623	0.674																			
5	0.812	0.608	0.668	0.773																		
6	0.699	0.618	0.659	0.858	0.802																	
7	0.688	0.601	0.650	0.856	0.786	0.947																
8	0.692	0.611	0.659	0.856	0.783	0.954	0.949															
9	0.722	0.627	0.689	0.665	0.666	0.668	0.659	0.666														
10	0.698	0.614	0.662	0.866	0.789	0.960	0.955	0.960	0.665													
11	0.692	0.608	0.654	0.858	0.788	0.952	0.952	0.949	0.668	0.962												
12	0.691	0.612	0.658	0.859	0.796	0.948	0.946	0.955	0.676	0.961	0.960											
13	0.691	0.607	0.662	0.855	0.794	0.953	0.946	0.957	0.667	0.963	0.957	0.968										
14	0.691	0.609	0.656	0.859	0.782	0.941	0.948	0.950	0.662	0.954	0.953	0.961	0.949									
15	0.710	0.626	0.676	0.876	0.766	0.886	0.879	0.884	0.672	0.896	0.881	0.887	0.882	0.880								
16	0.690	0.604	0.650	0.854	0.783	0.947	0.947	0.945	0.661	0.969	0.954	0.950	0.950	0.948	0.888							
17	0.695	0.611	0.664	0.851	0.795	0.947	0.942	0.949	0.664	0.957	0.952	0.962	0.985	0.944	0.881	0.942						
18	0.700	0.623	0.672	0.857	0.798	0.948	0.946	0.957	0.672	0.961	0.960	0.963	0.965	0.956	0.885	0.946	0.957					
19	0.691	0.621	0.669	0.848	0.787	0.941	0.934	0.944	0.674	0.945	0.944	0.956	0.956	0.940	0.876	0.939	0.953	0.945				
20	0.695	0.645	0.684	0.649	0.634	0.634	0.624	0.634	0.668	0.635	0.629	0.639	0.628	0.637	0.642	0.627	0.638	0.639	0.639			
21	0.682	0.844	0.639	0.613	0.600	0.614	0.603	0.612	0.621	0.613	0.609	0.604	0.604	0.608	0.622	0.605	0.612	0.620	0.618	0.639		
22	0.682	0.849	0.644	0.613	0.603	0.616	0.603	0.612	0.632	0.615	0.609	0.608	0.608	0.611	0.627	0.607	0.614	0.624	0.618	0.628	0.945	

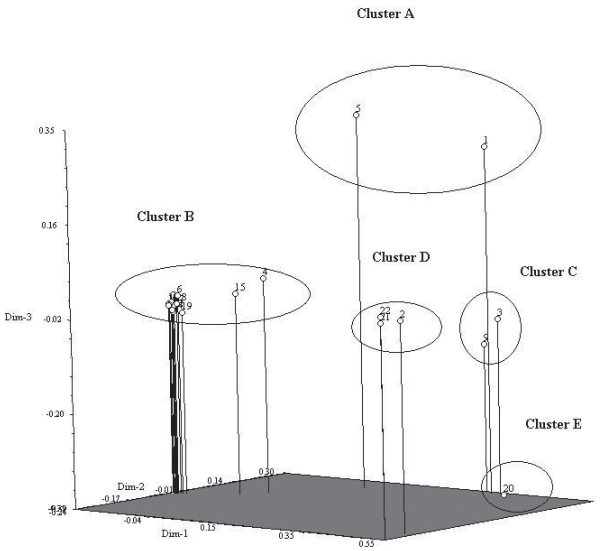

Fig. 3. Dimensional plot from the principal coordinated analysis of the SRAP markers associated with *Inonotus obliquus*. The serial numbers of the strains are shown in Table 1.

16 strains (MAFF420239, MAFF420198, JL04, JL05, JL03, MAFF420101, JL02, Z2, MAFF420234, NBRC9788, JL01, HLJ01, CX02, MAFF420279, Finnish birch and MAFF420102). Seven strains originating from China are included in the first cluster, indicating a close affinities. Birch Russia and MAFF420256 occurred together in the second cluster. MAFF410028 form the third cluster. The fourth cluster included MAFF420308, MAFF420165 and NBRC8681.

Principal coordinated analysis (PCoA): Principal coordinated analysis (PCoA) was applied to the raw data from the SRAP "1" and "0" matrix, and the contribution rates of the first three principal coordinates were 39.52%, 13.65%, and 7.78%, respectively. The analysis resulted in partitioning the strains into 5 distinct groups (Fig. 3). PCoA cluster A included the strains MAFF420239 and MAFF420198. PCoA cluster B included the strains JL04, JL05, JL03, MAFF420101, JL02, Z2, MAFF420234, NBRC9788, JL01, HLJ01, CX02, MAFF420279, Finnish birch and MAFF420102. PCoA cluster C included the strains Birch Russia and MAFF420256, PCoA cluster D included the strains MAFF420308, MAFF420165 and NBRC8681. The cluster E included the strains MAFF410028. The clustering using PCoA is consistent with the clustering resulting from the UPGMA analysis.

DISCUSSION

Many previous studies have indicated that using SRAP allow to quantify the genetic dissimilarity by using polymorphism in amplified fragment across the genome. Recently, Hao *et al.* (2008) revealed genetic diversity between *Paeonia* cultivars with up to 80.35% of polymorphism across the SRAP amplicons. Youssef *et al.* (2011) observed 87.59% polymorphism in Musa accessions. Yanchun *et al.* (2011) used SRAP for the estimation of genetic similarities among 51 tested

Monascus strains. A total of 183 scorable bands were produced, of which 173 were polymorphic (94.5%), and the polymorphism ranged from a maximum of 100% to a minimum of 87%.

This study is the first attempt to use SRAP as a basic molecular marker for genetic variation among the medicinal fungus I. *obliquus*. The number of polymorphic bands for each primer combination ranged from 20 to 52. The 23 primer combinations generated a total of 868 bands, of which 859 (98.62%) were polymorphic. The similarity matrix of 22 strains used in SRAP analysis showed that the range of similarity varied between 0.600 and 0.985. This indicates that all the 22 strains belonging to the same species. The differences among them belonged to intraspecies diversity.

According to the previous published studies, PCoA often demonstrated more accuracy graphical results than UPGMA (Zhang *et al.*, 2009; Yang *et al.*, 2011). In this study, the PCoA divided 22 *Inonotus obliquus* into five main clusters. The differences between two analyses was MAFF420239 and MAFF420198 have been placed into another cluster. The other four clusters were consistent with the results of UPGMA. As a whole, PCoA results confirmed the results of the UPGMA cluster analysis (Fig. 3).

In conclusion, this is the first report of using SRAP marker as a tool for determining genetic variation among *I. obliquus* and it had provided enough information about the genetic relationships between *I. obliquus* strains come from different countries and different places. The genetic diversity shown by SRAP markers in this study can also be applied to the revealing of an internal structure of the species. This provides a methodical basis and guidance for further screening, purification and identification in the future.

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