Difference in cultivation characteristics and genetic polymorphism between Chinese and Japanese strains of Wolfiporiacocos Ryvarden et Gilbertson (Poria cocos Wolf)

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Original Paper

Difference in cultural characteristics and genetic polymorphism between Chinese and Japanese strains of *Poria cocos* Wolf

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Abstract

Hoelen, a dried sclerotium of *Poria cocos* Wolf (*Polyporaceae*) has been used as a crude drug in both Chinese and Japanese traditional medicines (Kampo). Recently, cultivated Chinese hoelens has accounted for most of the market, while the cultivation of Japanese *Poria cocos* strains has not been successful. Aiming to find out the relationship between the differences in cultivation characteristics and genetic polymorphism, we conducted a field cultivation experiment as well as rot test, and RAPD analysis of *Poria cocos* strains collected from China and Japan, 3 Chinese and 7 Japanese strains. In field cultivation, although there was no marked difference between Chinese and Japanese strains in both mycelium propagation and the rate of sclerotium formation, Chinese strains formed whiter sclerotia with a mean size more than twice that of Japanese strains. Representatives of Chinese and Japanese strains, Yunnan and Kaimondake, respectively, were tested for wood-rotting ability. More wood was utilized and the wood color was darker in trials of the Yunnan strain. Amplifications of total DNA of these 9 fungal strains with 2 primers, PC-6 and PC-11, in RAPD analysis

showed a difference in the amplicon profile between Japanese and Chinese strains, suggesting differences in their genetic background.

Keywords
Hoelen, Poria, locality, cultivation, wood-rotting, RAPD

Introduction

Hoelen, a dried sclerotium of *Poria cocos* Wolf (*Polyporaceae*) has been used as a crude drug in both Chinese and Japanese traditional medicines (Kampo). In Kampo medicine, Hoelen is prescribed in many important formulations, and about 700 tons per year is consumed in the Japanese market, mostly imported from China and some from the Korean Peninsula. Recently, cultivated Chinese Hoelens has accounted for most of the market (1). The Chinese cultivated product is usually whitish, while Japanese and Korean wild products have a reddish appearance (2). As the Guide of Japanese Pharmacopoeia describes, reddish and moist Hoelens are considered good quality (1), suggesting that the Japanese product would be desirable for medicinal use; however, collections of wild Hoelens in Japan have been falling in the last several decades due to a decreased number of experienced collectors and damage to pine woods by pine weevils. The cultivation of Japanese *Poria cocos* strains has not been successful: some failed to produce sclerotia and others only produced sclerotia contaminated with earth and sand, or smaller ones (3) (4) (5) (6). The differences in Japanese and Chinese strains in the color of Hoelen, suitability for cultivation as well as nutrition preference of

mycelia seem to be grounded in their genetic differences. Japanese and Chinese strains have been examined by analyzing the DNA sequence of the nuclear ribosomal 18S rRNA gene, ITS region and 28S rRNA gene; however, there was no difference between these two in the nucleotide sequence of the 18S rRNA gene (7) and the ITS region and 28S rRNA gene (8). Aiming to elucidate the relationship between the difference in cultivation characteristics and genetic polymorphism, we conducted a field cultivation experiment, a rot test, as well as RAPD analysis of *Poria cocos* strains collected from China and Japan.

Materials and Methods

Materials

The 3 Chinese and 7 Japanese *Poria cocos* strains used are listed in Table 1. These were derived from dried or fresh sclerotia, respectively, as follows: sclerotia were bored in the center, and the center parts were removed and pressed on the agar culture medium, described in the following section. Mycelia derived from these sclerotia were

transplanted and propagated on new agar culture medium.

Culture medium for fungal mycelia cultivation

Culture medium consisted of the following: one liter of medium contained 20 g glucose, 1 g yeast extract, 15 g agar, $CaCl_2 \cdot 2H_2O$ 440 mg, $MgSO_4 \cdot 7H_2O$ 370 mg, $FeSO_4 \cdot 7H_2O$ 27.8 mg, EDTA-Na_2 37.3 mg, *myo*-inositol 100 mg, nicotinic acid 0.5 mg, pyridoxine hydrochloride 0.5 mg, thiamine hydrochloride 0.1 mg, and glycine 2 mg.

Propagation of fungal mycelia on sawdust

Sawdust of American pine 1.52 kg, rice bran 0.44 kg, plaster powder 0.02 kg, glucose 0.02 kg and distilled water were mixed. The mixture was divided into 300 g portions and packed in Biopot BS™ (Mori Industries Co. Ltd), and was sterilized by autoclaving. The mycelia grown on the agar culture were mixed with the sawdust mixture on a clean bench, and incubated in a biotron (model LPH-350SP; Nippon Medical and Chemical Instruments Co. Ltd) at 27 °C and 80% humidity for 2 weeks.

Inoculation of mycelia onto tree logs and field cultivation of inoculated logs

Tree logs (10-15 cm diameter, 30-40 cm length) streaked with 8-10 lines were exposed to the air for 3-4 months for seasoning. Bags filled with propagated mycelia were tied on top of the tree logs. Soil from a spot facing south in the herbal garden of Kanazawa University was mixed with river sand at approximately 50 %. The tree logs inoculated with mycelia were laid under the ground at this spot.

Rot test of fungal strains

Tree logs (3-8 cm diameter) were seasoned for 2 months and cut into approximately 225 cm^3 discs. The discs were dried at 98˚C for 4 hr and weighed, and then soaked in solution containing 1 % glucose and 0.5% yeast extract for 48 hr. The soaked discs were placed in cultivation bags for mushroom culture (1.3 x 380 F, offered by Mori Industries Co. Ltd.) and then sterilized by autoclaving. The tops of the discs in the bags were covered with sawdust filled with mycelia of each fungal strain, and were incubated at 25˚C and 80 % humidity. After incubation for the respective weeks, mycelia, sclerotia and fruiting bodies sprouting up were removed carefully from the discs. The removed

sclerotia were dried at 50˚C for 18 hrs and weighed. The ratio of the sclerotium weight per disc volume was calculated. The discs were dried at 105 ˚C for 18 hrs, cooled in a desiccator, and then weighed. The weight of the remaining wood was calculated as follows: weight of remaining wood (%) = (dried weight after incubation/dried weight at the start) x 100.

Detection of color change of rotted wood.

The discs used for rot test were dried and weighed as in the preceding section. The discs were barked and the wooden parts were powdered. The powder was dried at 105 ˚C for 18 hrs and the color of the powder was assessed with Konica Minolta spectrocolorimeter CM-3500d using software CM-S100w Spectra Magic™ NX Basic.

Extraction of fungal DNA

Total DNA was extracted from 200-400 mg of fresh cultured mycelium material using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol (QIAGEN).

PCR Amplification of ITS2

Polymerase chain reaction (PCR) was performed using 30-100 ng total DNA as the template in 25 μl of a reaction mixture containing 2.5 μl 10×PCR buffer for KOD -Plus-, 0.2 mM of each dNTP, 1.0 μM $MgSO_4$, 0.5 units KOD -Plus- polymerase (Toyobo), and 0.4 mM of each primer. Primers are shown in Table 2. Amplification was carried out under the following conditions: pre-heating at 94˚C for 2 min; 30 cycles of denaturation at 94˚C for 15 s, annealing at 55˚C for 30 s and elongation at 68˚C for 2 min; a final elongation at 68˚C for 5 min. One tenth volume of the PCR products was analyzed by agarose gel electrophoresis and then the remaining part was purified using a QIAquick PCR Purification Kit (Qiagen).

Sequencing Reaction

The purified PCR product was subjected to direct sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 310 sequencer (Applied Biosystem). The DNA sequences were aligned using 'DNASIS' version 3.0 (Hitachi).

RAPD analysis of fungal DNA

PCR for RAPD analysis was performed using 5 ng total DNA as the template in 25 µl of a reaction mixture containing 2.5 µl 10×PCR buffer for Takara Taq, 0.1 mM of each dNTP, 2 mM $MgCl_2$, 1 unit Taq polymerase (Takara), and 0.4 mM of each primer listed in Table 2. Amplification was carried out under the following conditions: pre-heating at 94˚C for 2 min; 45 cycles of denaturation at 94˚C for 1 min, annealing at 45˚C for 1 min and elongation at 72˚C for 2 min; final elongation at 72˚C for 10 min. Ten microliters of PCR reaction mixture was analyzed by agarose gel electrophoresis operated under 50 V in 0.8 x TAE buffer for 70 min.

Cloning of an amplified band of RAPD using PC-11

An amplified band with 2250 bps (f) in RAPD analysis was isolated from the gel and cleaned up using a kit (Wizard SV Gel and PCR Clean Up System; Promega). The band was re-amplified using PC-11 HD primer (ATA AAA GCT TTG CTC TGC CCC) under the following conditions: pre-heating at 94˚C for 2 min; 30 cycles of denaturation at

94˚C for 30 sec, annealing at 45˚C for 30 sec and elongation at 72˚C for 2.5 min; final elongation at 72˚C for 10 min. The reaction mixture was isolated by agarose gel electrophoresis, and the band was isolated from the gel and cleaned up. The purified band was digested with restriction enzymes, Hind III and EcoRI. The reaction mixture was heated at 70˚C for 15 min. The digested PCR product was ligated with the pBluescript SK(-) digested with the same enzymes using a ligation kit (Ligation High; Toyobo). The ligation mixture was applied to competent cells (DH a; Toypbo), and colonies with ampicillin resistance were selected.

Results

Field cultivation of fungal strains

Tree logs inoculated with mycelia of either Chinese or Japanese fungal strains were buried in the herbal garden of Kanazawa University from April to November 2009. Table 3 summarizes the propagation of mycelia and formation of sclerotia of the fungal strains. Both of 2 trials of Hakui and Zhejiang strains failed to propagate mycelia, as did

one of these of Matsukawa, Shibusi and Yunnan strains. There was no marked difference between Chinese and Japanese strains in both mycelium propagation and the rate of sclerotium formation. On the other hand, Chinese strains formed whiter sclerotia than Japanese strains. Moreover, one sclerotium of Japanese Ikeda strain contained earth and sand (Fig. 1). The mean size of Chinese sclerotia was more than twice that of Japanese sclerotia.

Rot test of fungal strains

Representatives of Chinese and Japanese strains, Yunnan and Kaimondake, respectively, were rot-tested using 1035 cm^3-incubation bags for mushroom cultivation. For cultivation of Hoelen in a bottle, Kubo reported that the optimal ratio of bottle/wood disk volume was 2300 cm^3/500 cm^3 (9) (10). Based on Kubo's data, we used wood disks of about 225 cm^3. The mycelia of these strains grew well on the surface of the wood disks and covered them completely in 10 weeks (Fig. 2). The mycelia of the Yunnan strain were whitish and sclerotia formed (Fig. 2-a) in every trial. On the other hand, the mycelia of the Japanese strain were brownish and fruiting bodies were formed in some

trials, but no sclerotium was formed (Fig. 2-b). After removing these mycelia, sclerotia or fruiting bodies, the weight of wood disks were measured. As Fig. 3-b shows, more wood was consumed in the trials of Yunnan strains. After being dried and powdered, the color of the wood was assessed. The wood in trials of the Yunnan strain was darker when evaluated by the change in wood color detected by colorimeter (Fig. 3-a).

ITS 2 sequence and RAPD analyses of fungal DNA

The ITS 2 sequences of the fungal strains were analyzed to confirm their species identity. Every strain had an ITS 2 sequence identical to NCBI accession number EF397597, obtained in our previous study (8). The same total DNA extracts analyzed for the ITS 2 sequence were used for RAPD analysis. Seventeen primers for RAPD analysis were designed in reference to DNA polymorphism studies on various mushrooms (11) (12) (13) (14) (15). Amplification with 2 primers, PC-6 and PC-11, showed a difference in the amplicon profile between Japanese and Chinese strains, as Fig. 4 shows: the amplified bands (b) and (e) in the profile of PC-6 as well as band (f) in that of PC-11 appeared only in the amplification of Chinese fungal strains; however, the

appearance of amplified bands (b) and (e) was unstable.

Cloning of amplified band (f)

An amplified band with 2250 bps (f) of RAPD using PC-11 was isolated for further analysis. The band was reacted with restriction enzymes, BamHI, EcoRI and HindIII, and only digestion with EcoRI gave 2 distinctive digested bands. The RAPD reaction with a primer which had the same sequence of PC-11 and Hind III tag gave same RAPD profile as that of PC-11 including a product of the same length as the amplified band (f). The product was subjected to digestion with Hind III and EcoRI, and ligated with a plasmid for cloning. Four clones were isolated and analyzed for their DNA sequences. Clones 13 and 47 had the same insert of 1140 bps, whereas clone 1 and clone 11 had inserts of 700 bps and 1400 bps, respectively, without sequences homologous to clone 13/47. The sequences of the clones, 1, 11, 13, 47, were submitted to GenBank: JF960946, JF960947, JF960948, JF960949, respectively. The longest ORF within these sequences, which was found in clone 13/47, encoded a 103 amino acid sequence. The nature of the amino acid sequence has not yet been verified.

Discussion

Using various Japanese *Poria cocos* strains for field cultivation, we found that sclerotia, with an internal color of pale brown or red, were formed from most strains; however, one was contaminated with earth and sand, as in previous results of the cultivation of Japanese strains (3) (4) (5) (6). Compared with the results with Japanese strains, 2 Chinese strains formed whiter, heavier, and larger sclerotia under the same cultivation conditions, which could be explained the difference in rotting ability. This was partly demonstrated by a rot test of the representatives of Chinese and Japanese strains. *Poria cocos* was classified as a brown wood-decaying fungus (9) (10) (16) (17), which decomposes cellulose and hemicellulose but not lignin (18). As a result, wood rotted by the fungus becomes brownish because of the remaining oxidized lignin; therefore, the color change of inoculated wood reflects the extent of wood decay. The relationship between color change and wood decay was clearly confirmed by the rot test in our study, and was found useful to screen the rotting ability of fungal strains. The genomic difference between Chinese and Japanese *Poria cocos* strains had been

surmised but there was no evidence (7) (8). Our RAPD result here showed the genomic polymorphism of Chinese and Japanese strains, suggesting their difference in genetic background. One of the amplified bands in RAPD analysis was cloned and its sequence was revealed. The identification of the amplified sequence is under investigation.

References

1. Editorial committee of guidebook to Japanese Pharmacopoeia 15th (eds.), Guidebook to Japanese Pharmacopoeia 15th pp, D-582-D585, Hirokawa Shoten, Tokyo, 2006.

2. Anjiki N (1996) Master's Thesis submitted to the Faculty of Pharmaceutical Sciences of Kanazawa University

3. Sakata T (2002) Attempt to produce sclerotium from cultured Wolfiporia cocos. Proc. Hiroshima Prefectural Center for Forestry Techniques 34: 33-39

4. Tominaga Y (1986) Studies on the life history of Tuckahoe, Poria cocos Wolf. Proc. Hiroshima Agricultural Junior College 8: 115-141

5. Tominaga Y (1987) Study on Culture of Tuckahoe I. Germination of fruit body and formation of sclerotium in log culture and wood chip/ sawdust bag culture. Proc. Hiroshima Agricultural Junior College. 8: 451-463

6. Tabata M, Hiraoka N (1994) Studies on the formation of fruit body and sclerotium in Poria cocos Wolf native to Japan. Natural Medicines 48: 18-27

7. Kozuka A (1999) Master's Thesis submitted to the Faculty of Pharmaceutical

Sciences of Kanazawa University

8. Atsumi T, Kakiuchi N, Mikage M (2007) The DNA sequencing analysis of ITS and 28S rRNA of *Poria* cocos. Biol Pharm Bull 30: 1472-1476

9. Kubo T (2002) Studies on indoor cultivation of Poria cocos strains and their cultivation characteristics. Doctor's thesis submitted to the Graduate School of Natural Science and Technology, Kanazawa University

10. Kubo T, Terabayashi S, Takeda S, Sasaki H, Aburada M, Miyamoto K (2006) Indoor cultivation and cultural characteristics of Wolfiporia cocos sclerotia using mushroom culture bottles. Biol Pharm Bull 29: 1191-6

11. Zervakis GI, Venturella G, Papadopoulou K (2001) Genetic polymorphism and taxonomic infrastructure of the *Pleurotus eryngii* species-complex as determined by RAPD analysis, isozyme profiles and ecomorphological characters, Microbiology 147: 3183-3194

12. Nakajima T, Miki T, Miyazaki K (2003) Identification of *Phellinus linteus* (Berk. et Curt.) Aosh. calibers by DNA analysis, Kyushu J For Res No.56

13. Obatake Y, Matsumoto T, Mimura K, Fukumasa-Nakai Y (2002) Genetic

relationships in the natural population of *Pholiota nameko* japan based on DNA polymorphism. *Mycoscience* 43: 463-469

14. Khush RS, Becker E, Wach M (1992) DNA amplification polymorphisms of the cultivated mushroom Agaricus bisporus. Appl Environ Microbiol 58: 2971-7.

15. Yan P-S, Lou X-C, Zhou Q (2004) RAPD molecular differentiation of the cultivated strains of the jelly *mushrooms, Auricularia auricula* and *A. polytricha*. World Journal of Microbiology & Biotechnology 20:795-799

16. Kobayashi I (1993) Studies on a Chinese Medicine, Fu-ling. Doctor's thesis submitted to the Graduate School of Pharmaceutical Sciences, Kanazawa University

17. Kiba K (1994) Studies on a Chinese Medicine Fu-ling. Studies on the growth characteristics of Poria cocos strains. Doctor's thesis submitted to the Graduate School of Pharmaceutical Sciences, Kanazawa University

18. Furukawa H (1992) Science of Mushrooms, Kyoritsu Publishers, Tokyo

Legends for Figures

Fig 1. Examples of sclerotia formed by cultivation of Chinese (Hubei, upper) and Japanese (Ikeda, lower) strains.

Fig 2. The growth of mycelia of Yunnan (a) and Kaimondake (b) strains on the surface of the wood disks in 10 weeks.

Fig 3. Result of rot test evaluated by (a) the change in wood color, and (b) remaining weight of wood. Each 4 trials with Yunnan (diamond) and with Kaimondake (square) were conducted for 5, 10, 14 and 18 weeks. Data express the mean values of these 4 trials with error bars.

Fig 4. RAPD analysis using primers PC-6 (a) and PC-11 (b). Lane 1: DNA molecular weight marker, Lane 2: Zhejiang, Lane 3: Hubei, Lane 4: Yunnan, Lane 5: Shiojiri, Lane 6: Ikeda, Lane 7: Hakui, Lane 8: Shibushi, Lane 9: Kaimondake, Lane 10: Matsukawa strains.

Table 1 Strains used for this study

Sample	Locality	Date	Status
	Chinese samples		
Yunnan	Yunnann	2006 May	Cultivated
Hubei	Hubei	2007 October	Cultivated
Zhejiang	Zhejiang	1994 June	Cultivated
	Japanese samples		
Ikeda	Ikeda, Nagano Pref.	2008 April	Wild
Shiojiri,	Shiojiri, Nagano Pref.	2008 April	Wild
Matsukawa	Matsukawa, Nagano Pref.	2008 April	Wild
Hakui	Hakui, Ishikawa Pref	2008 December	Wild
Kaimondake	Kaimondake, Kagoshima Pref.	2009 February	Wild
Shibushi	Shibushi, Kagoshima Pref.	2009 February	Wild
Miyazaki	Hyuga, Miyazaki Pref.	2009 February	Wild

Table 2 Sequence of primers

a Primers used for PCR and Sequencing

Primer name	Sequence
Poria 5.8SF	5'-GAAGAACGCAGCGAAATGCG-3'
Poria ITS2 nes.R	5'-GGTAGTCCTGCCTGATCTGA-3'
Poria ITS2 200F	5'-GTTGAACGGGAACCCTAGAA-3'
Poria ITS2 300F	5'-ACCTCGATGTGAGGAGTTTG-3'
Poria ITS2 400R	5'-GTCGAGATCTTTTATTTTCCC-3'
Poria 28S cent.R	5'-CGATCGATTTGCACGTCAGA-3'
Poria 28S 100R	5'-TCTTCACTCGCAGTTACTAG-3'

b Primers used for RAPD

Primer name	Sequence
PC-1	5'-TGCCGAGCTG-3'
PC-2	5'-AGTCAGCCAC-3'
PC-3	5'-AATCGGGCTG-3'
PC-4	5'-GAAACGGGTG-3'
PC-5	5'-GTTTCGCTCC-3'
PC-6	5'-TGATCCCTGG-3'
PC-7	5'-CTGCTGGGAC-3'
PC-8	5'-TCCGCTCTGG-3'
PC-9	5'-CCACAGCAGT-3'
PC-10	5'-TGCGCCCTTC-3'
PC-11	5'-TGCTCTGCCC-3'
PC-12	5'-GTAGACCCGT-3'
PC-13	5'-CCTTGACGCA-3'
PC-14	5'-AGGGAACGAG-3'
PC-15	5'-CAGGCCCTTC-3'
PC-16	5'-GTGACGTAGG-3'
PC-17	5'-GAGTCCGCAA-3'

Table 3 Results of field cultivation

Origin of mycelium	Mycelium formation	Sclerotium formation	Number of sclerotia	Mean size of sclerotia attached to one log (cm)	Mean size of sclerotia (cm)	Standard deviation
Hakui A					3.3	1.1
B						
Ikeda A	●	●	1	3.9		
B	●	●	1	5.0		
Shiojiri A	●	●	3	3.1		
B	●					
Matsukawa A	●	●	1	4.0		
B						
Shibusi A						
B	●	●	4	3.4		
Kaimonndake A	●	●	1	4.4		
B	●	●	2	1.8		
Miyazaki A	●	●	1	2.0		
B	●	●	1	2.2		
Zhejiang A					7.7	2.2
B						
Hubei A	●	●	2	8.4		
B	●	●	1	9.5		
Yunnan A						
B	●	●	2	5.3		

●: Mycelium or sclerotium was formed

Size of sclerotium: (major axis＋minor axis)/2

Fig 1

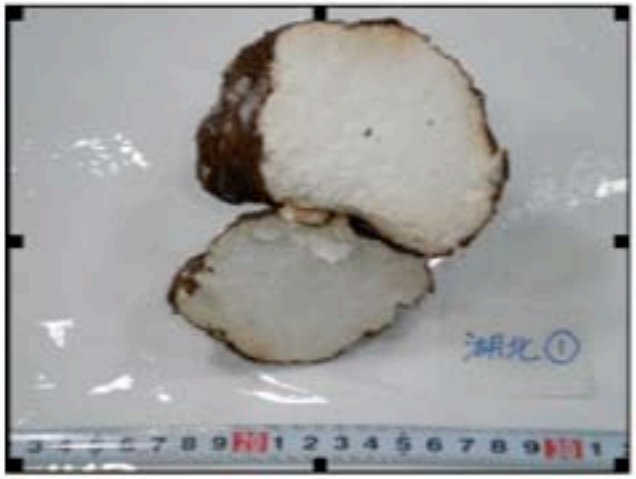


Fig 2

a

b

Fig 3

a

b

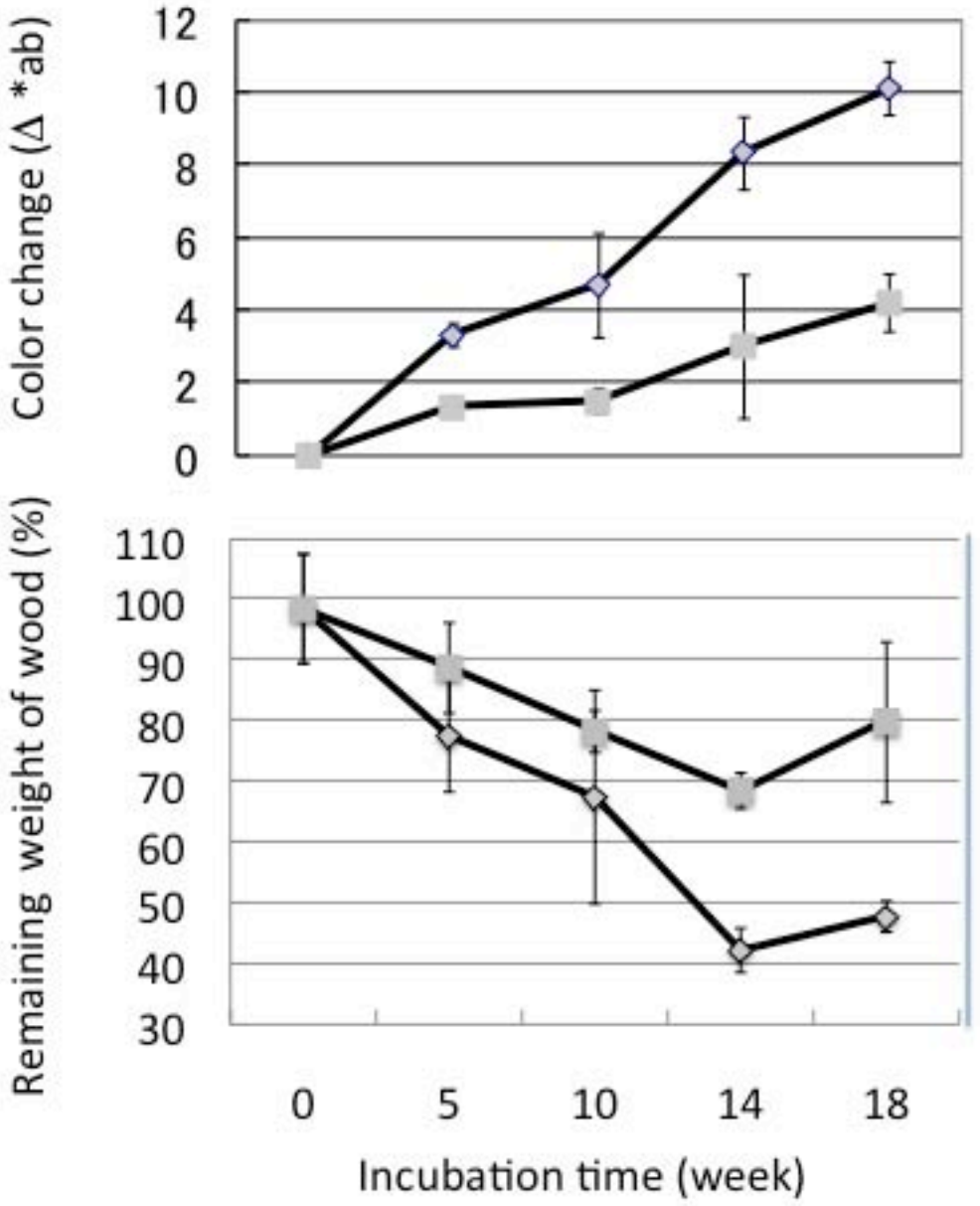
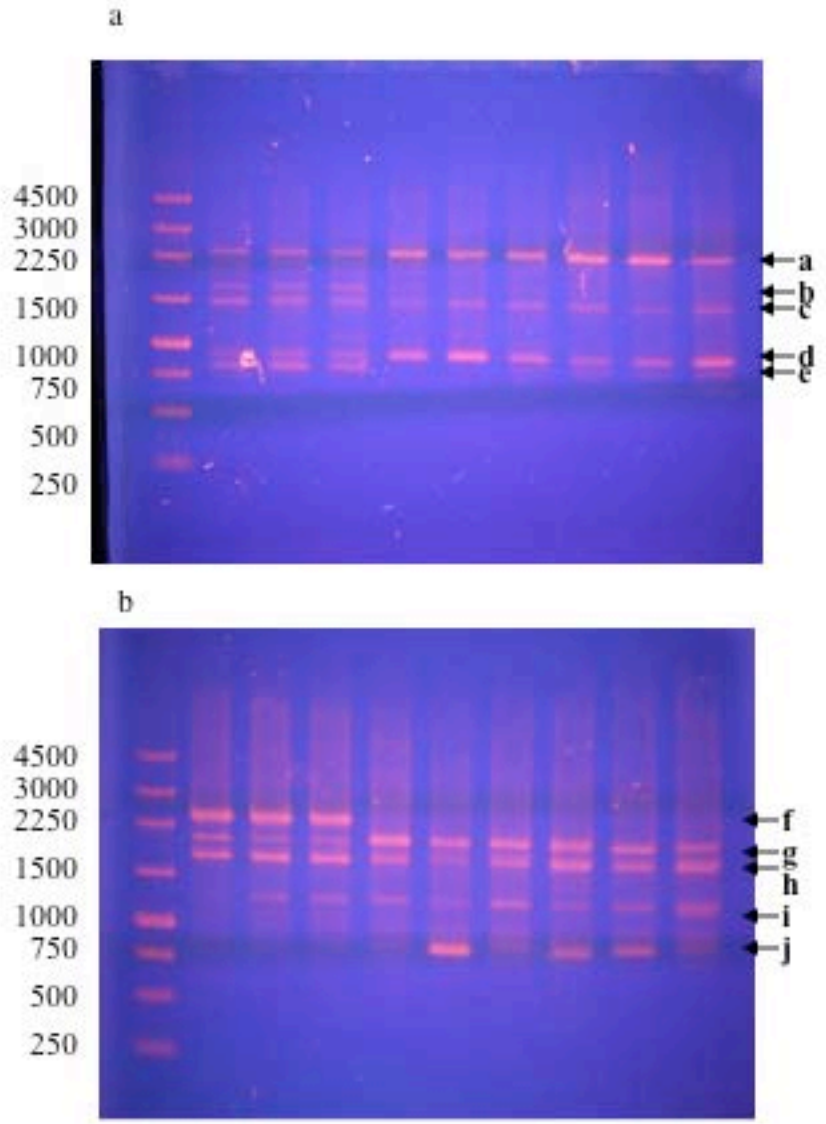

Color change (Δ *ab)
12
10
8
6
4
2
0
Remaining weight of wood (%)
110
100
90
80
70
60
50
40
30
0
5
10
14
18
Incubation time (week)

Fig 4


a
4500
3000
2250
1500
1000
750
500
250
a
b
c
d
e
b
4500
3000
2250
1500
1000
750
500
250
f
g
h
i
j