HAYATI Journal of Biosciences March 2010
Vol. 17 No. 1, p 21-26
EISSN: 2086-4094

Available online at:
http://journal.ipb.ac.id/index.php/hayati
DOI: 10.4308/hjb.17.1.21

Amplified Fragment Length Polymorphism Diversity of Cultivated White Oyster Mushroom *Pleurotus ostreatus*

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Received November 30, 2009/Accepted February 17, 2010

Amplified fragment length Polymorphism (AFLP) analysis was performed to study the genetic diversity of fifteen isolates of white oyster mushroom (*Pleurotus ostreatus*) originated from different places in Indonesia (Java, Sumatra, Bali, and Kalimantan) and Thailand. The majority of studied isolates originated from commercial mushroom, and some of them were collected from nature. AFLP analysis revealed 202 loci of positive DNA bands. Heterozygosity value showed polymorphisms of the fifteen studied isolates. Correlation and cluster analyses of the isolates showed that isolate from Thailand (BNK isolate), isolate from Bogor (AMD isolate), and isolate from Purwokerto (USX isolate) were distinctly different from the other isolates indicated by positive bands with DNA size greater than 650 bp. Isolate from Kalimantan (BJM isolate) was distinctly different from Java island isolates. Generally there was no correlation of AFLP variability and geographic distribution of commercial strain isolates.

Key words: *Plerotus ostreatus*, biodiversity, AFLP, cluster analysis, breeding

INTRODUCTION

White oyster mushroom (*Pleurotus ostreatus*) is an important edible and cultivated mushroom, and it was the second most important mushroom in the world market, after button mushroom (*Agaricus bisporus*) (Chang 1999). In Indonesia this mushroom was cultivated mushroom by either traditional or modern farming, and the market was very promising. Mushroom cultivation became one alternative of agribusiness in Indonesia. In nature, *P. ostreatus* and other *Pleurotus* sp. live on dead wood as saprobe primary decomposer. Their ability to produce lignin degrading enzymes (Velazquez-Cedeno *et al.* 2004; Widiastuti *et al*. 2008), make them possible to be used as lignocellulosic waste degrading agents.

Despite of economical prospective it is very limited information of their genetics and breeding to support development of mushroom cultivation in Indonesia. Farmers usually used mushroom spawn received from the spawn producers without ability to select quality of the spawn. Thus a study on genetics and breeding of this mushroom is very important for their cultivation. Construction and evaluation of a germplasm or genetic resouces is the first important step of breeding program. The collected materials could be natural or commercial isolates. The purpose of the collection is to build a genetic diversity as foundation of genetic improvement program. White oyster mushroom is propagated somatically, and majority of farmers use spawn produced from the same spawn poducer. This practical culture can lead to a genetic homogenity of cultivated mushroom in a region. In order to build a genetic diversity of the mushroom, genetic materials have to be collected from several locations, and a thourough evaluation have to be performed to analyse the genetic variability.

This paper reported the genetic evaluation of the mushroom analysed by amplified fragment length polymorphism (AFLP) technique (Vos *et al.* 1999). The technique was frequently used to study biodiversity of many organisms such as papaya (Kim *et al*. 2002), sunflower (Al-Chaarani *et al*. 2004), tomato (Park *et al*. 2004), and garlic (Volk *et al.* 2004).

MATERIALS AND METHODS

Genetic Materials. Fifteen white oyster mushroom isolates used in this work were isolated from commercial mushroom or laboratory stocks collected from different places in Indonesia (Java, Sumatera, Kalimantan, and Bali) and Thailand (Table 1). The isolates were kept on slant agar of potato dextrose agar (PDA) media supplemented with 500 mg chloramphenicol.

DNA Extration. DNA was extracted from either mycelia or from fruiting bodies. The mycelia was produced on liquid peptone yeast extract sugar media (Eger 1976). The mycelia was harvested in a sieve after two weeks of incubation and squeezed between filter papers to remove liquid as much as possible. The pre-dried mycelia was peeled off from the filter paper, folded, and immersed into liquid nitrogen.

A total of 0.1 g mycelia or fruiting bodies from each isolate was immersed into liquid nitrogen, grounded thoroughly on mortar and then transferred into universal bottles containing 3 ml extraction buffer. The bottles were

gently agitated and incubated at 65 °C for 35 minutes. DNA was then extracted using a procedure of Sambrook *et al.* (1989) with an extraction buffer of Reader and Broda (1985). The extracted DNA pellet was resuspended with 50 µl H_2O, and RNase was added and incubated overnight at 37 °C. RNase was inactivated by heating at 70 °C for 10 minutes. The purity and quality of DNA was verified using UV spectrophotometer and electrophoresis.

AFLP Template Preparation. DNA template was employed using restriction enzymes of PstI and MseI AFLP adapters. A total 250-500 ng genomic DNA was digested by 5 U PstI and 5 U MseI restriction enzymes at 37 °C for 3 h in NE Buffer (10x) provided by Biolabs Inc. Then 10 mM ATP was added to the solution. Adapters were prepared by adding top and bottom strands with proportion of 3.4 µl Mtop: 4.17 µl Mbot (50 pMol/µl MSeI adapter), and 0.67 µl Ptop: 0.42 µl Pbot (5 pMol/µl PstI adapter). Ligation was performed by adding 3 U/µl of T4 DNA ligase, then the mixture was diluted up to 10 times using TE buffer (pH 8.0) and stored at -20 °C.

AFLP Fragments Amplification. Amplification process was conducted in two steps. The first step was pre-amplification to produce AFLP template. And the second step was selective amplification to produce specific fragments that will be appeared on electrophoresis gel. The AFLP reaction employed PstI and MseI primers which were produced by Biolegio BV. The AFLP primers for pre-amplification had no selective nucleotides; the sequence of PstI primer (P00) was 5'GACTGCGTACATGCAG3' and MSeI primer (M02) was 5'GATGAGTCCTGAGTAAC3'. The primers for selective amplification had two additional selective nucleotides, i.e. PstI primer (P11) sequence (5'GACTGCGTACATGCAGAA3'), and MSeI primer (M50) sequence (5'GATGAGTCCTGAGTAAACAT3'). The P11 primer was labeled with IRDye 700.

Preamplification using PCR PTC-100TM MJ apparatus was set up for 24 cycles; each cycle consisted of 30 seconds of DNA denaturing (94 °C), 30 seconds of primers annealing (56 °C), and 60 seconds of extension (72 °C). A total of 5 µl RL mixture was added with 0.6 µl (30 ng/µl) Pst I primer (P00), 0.6 µl (30 ng/µl) MSeI primer (M02), 0.4 ìl dNTP 10 mM, 2 ìl PCR buffer 10 time concentration, 0.08 ìl (5 unit/ìl) super Taq and 11.32 ìl ddH_2O. Quality and quantity of preamplification product was verified by using electrophoresis.

Selective amplification had the same procedure as preamplification, except for DNA templates and AFLP primers. The product of preamplification was diluted 5 times, then 5 µl of it was taken as DNA template for selective amplification. The primers used in this step were M50 and P11 primers.

Gel Preparation and Electrophoresis. Electrophoresis of DNA fragments was done using LI-COR 4300 DNA Analyzer apparatus. A plate of 6.5% polyacrylamide gel was made by mixing 23 ml ready to use solution of KB plus (LI COR Inc), 15 µl TEMED and 150 µl of 10% ammonium persulphate. The plate was then fixed on electrophoresis apparatus, and added with TBE buffer (10 X). Pre-electrophoresis was performed for 30 minutes with 20 watt power to increase temperature up to 50 °C. Before loading the DNA samples and DNA standard marker was denaturated at 90 °C for 3 minutes and put on ice.

Data Analysis. LI-COR 4300 DNA Analyzer provided, as output, an image of DNA fragment bands of electrophoretic gel. The bands was then interpreted and scored using Saga Automated AFLP Software; the presence of band was scored as one (1) and the absence was scored as zero (0), and the data were transfered to a SPSS datasheet to be determined of their genetic heterozygosity (Nei 1973), correlation and clustering analysis (Mardia *et al.* 1979).

RESULTS

AFLP Loci and Polymorphisms. AFLP analysis of fifteen white oyster mushroom isolates has revealed 202 loci differentiated by size of DNA bands consisting of 42 loci with DNA fragment size greater than 650 bp, 60 loci in the range of 350-650 bp, and 100 loci in the range of 50-350 bp (Figure 1, Table1). The pragment size greater than 650 bp was possessed by four isolates i.e. BNK, USX, AMD and BJM isolates. The DNA fragment size of 350-650 bp was possessed by all isolates, however it was still dominated by the four isolates mentioned previously. The last DNA fragment of 50-350 bp was appeared almost similar on all isolates. Total mean value of heterozygosity was 0.31, signifying the genetic polymorphisms of the fifteen isolates.

Correlation and Cluster Analyses. Correlation analysis generated several correlating functions of the fifteen isolates and 202 AFLP loci. Those correlations could be presented by similar projection coordinates of the isolates and loci on a same correlation plane spanned by correlation axis (Figure 2). The correlation plane was divided into two correlation axis and four quadrants namely as northwest, northeast, southeast, and southwest. AMD, BNK, and USX isolates were separated from the other thirteen isolates, the BNK isolate was on northwest quadrant, and AMD and USX isolates were on southwest quadrant. On these quadrants, they were projected mostly the loci with DNA bands greater than 450 bp for the 1st up to 80th loci. The other isolates were mostly concentrated on northeast quadrant which were corresponded to the loci with DNA size of 50-450 bp.

On clustering dendogram of fifteen isolates (Figure 3) it was seen that BNK isolate was separated from the others with 25% dissimilarity, AMD isolate was separated with 17% dissimilarity, USX isolate was separated with 10% dissimilarity, and BJM isolate was separated with 6% dissimilarity. The rest of eleven isolates consisted of the isolates in northeast quadrant (Figure 2) were gathered in one group.

To see better diversity among the twelve isolates instead of BNK, AMD, and USX isolates, it was performed the second correlation and clustering analyses for those twelve isolates and 130 loci possessed by the isolates (Figure 4 & 5). BJM isolate from Kalimantan was took a

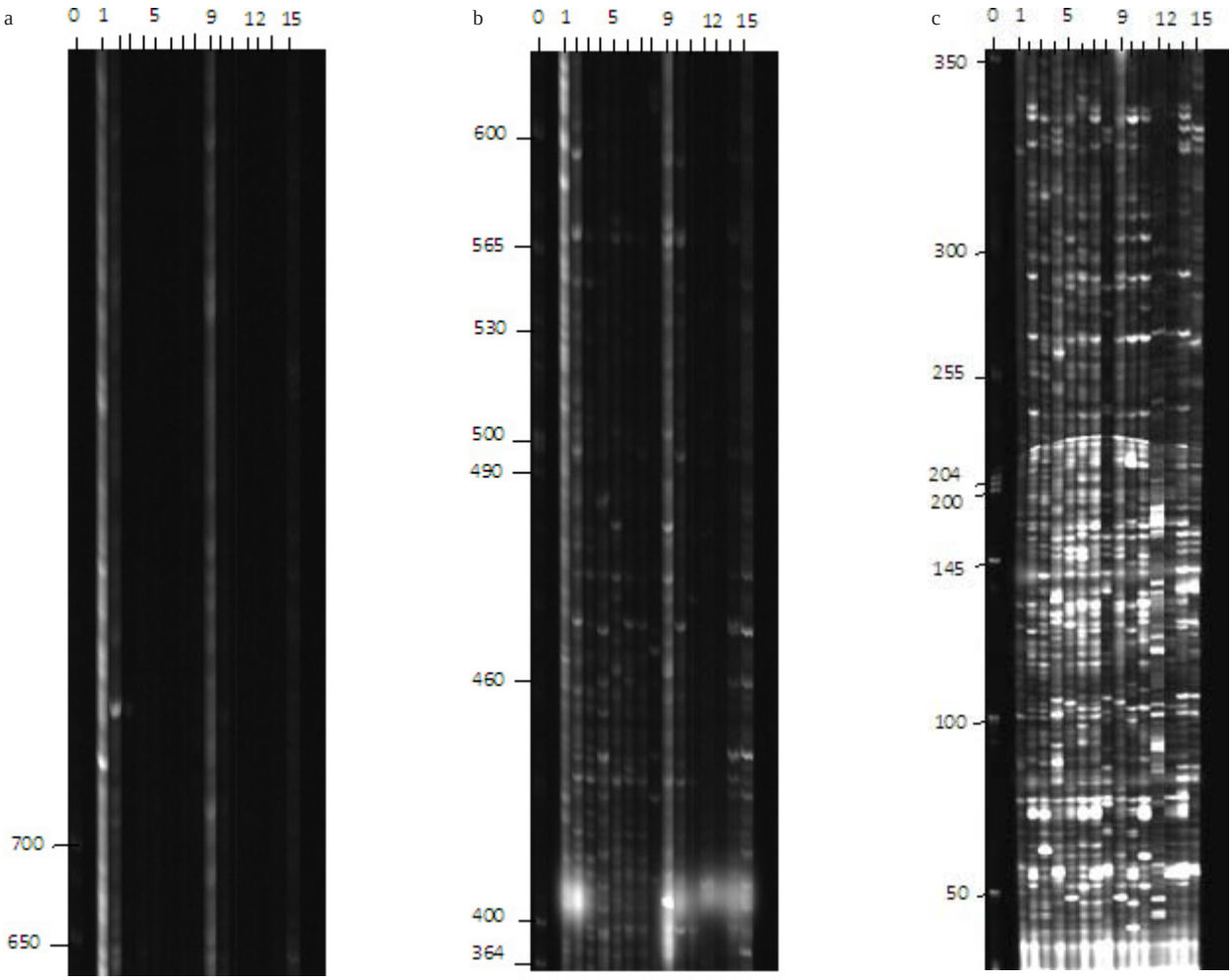

Figure 1. AFLP gel electrophoresis of three groups based on DNA sizes (a) >= 650 bp, (b) 350 bp-650 bp, (c) 50 bp – 350 bp. The number indicated labels of (0) DNA molecular weight standard, (1) BNK,(2) USX, (3) CBB, (4) PGN, (5) TBN, (6) CLM, (7) WRM, (8) LIP, (9) AMD, (10) KRT, (11) BBR, (12) MTR, (13) CSR, (14) USO, and (15) BJM isolates.

Table 1. Number of AFLP loci and the origins of fifteen isolates of white oyster mushroom (*Pleurotus ostreatus*)

Isolates	Origin	DNA fragments size and number of positive loci			Total
		>= 650 bp	350-650 bp	50-350 bp	
Total existing loci		42	59	101	202
BNK	Supermarket, Bangkok	40	41	10	91
USX	UNSOED Purwokerto	6	35	52	93
CBB	Traditional market Cibeber, Cianjur	0	18	77	95
PGN	Spawn grower Pagentongan, Bogor	0	12	73	85
TBN	Supermarket, Tabanan, Bali	0	9	75	84
CLM	Farmer Cileumeuh, Cilacap	0	5	69	74
WRM	Farmer Wiramastra Banjarnegara	0	2	70	72
LIP	LIPI, Bogor	0	6	50	56
AMD	Farmer Bogor	27	40	53	120
KRT	Farmer Kerataon, Magetan	0	9	60	69
BBR	Farmer Bibrik, Madiun	0	3	55	58
MTR	Supermarket Metro-Lampung	0	2	44	46
CSR	Spawn grower Cisarua, Sukabumi	0	4	48	52
USO	UNSOED, Perwokerto	0	8	53	61
BJM	Traditional market, Banjarmasin.	2	23	61	86
Heterozygosity		0.21 ± 0.029	0.30 ± 0.012	0.35 ± 0.010	0.31 ± 0.010

position on southeast quadrant separated from the other isolates which majority came from Java Island. The BJM isolate corresponded to the loci with 350-450 bp band size of the 70th to 102nd loci. Two other isolates, CBB and PGN isolates, took places on positive coordinate of dimension one, while the rests of isolates occupied on negative part of dimension one. On the dimension two, BBR, MTR, and USO isolates was in southwest quadrant separated from LIP, BBR, WRM, CLM, KRT, and TBN isolates which were in northwest quadrant.

On dendogram, it was seen that BJM isolate was separated from others with 25% dissimilarity, CBB and PGN isolates were separated with 11 and 10% dissimilarity respectively, KRT, and LIP isolates had 6% dissimilarity (Figure 5). The seven isolates were separated into three groups i.e. firstly the group of MTR and CSR isolates, secondly the group of CLM, WRM, BBR, and TBN isolates, and thirdly the group of USO isolate.

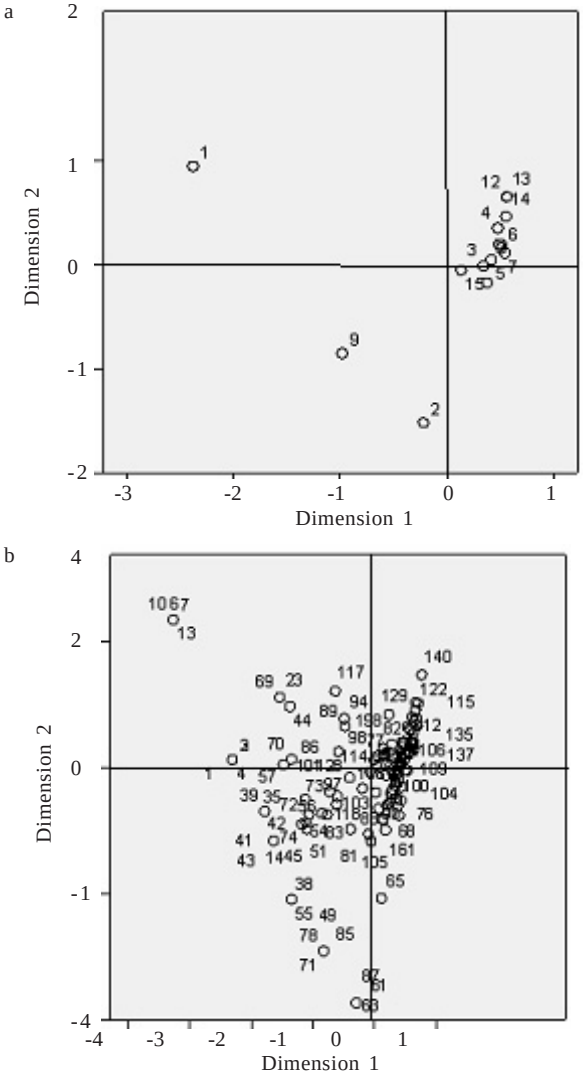

Figure 2. Projection of (a) fifteen isolates and (b) 202 loci on correlation plane. The number indicated labels of (1) BNK, (2) USX,(3) CBB,(4) PGN, (5) TBN, (6) CLM, (7) WRM, (8) LIP, (9) AMD, (10) KRT, (11) BBR, (12) MTR, (13) CSR, (14) USO, and (15) BJM isolates.

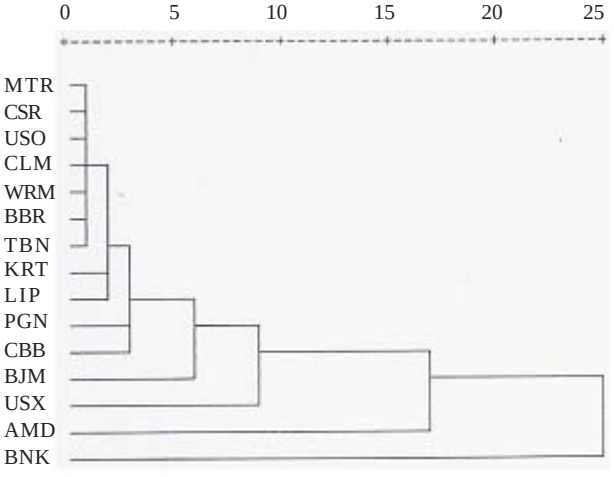

Figure 3. Dendogram of clustering analysis of fifteen isolates of white oyster mushrooms.

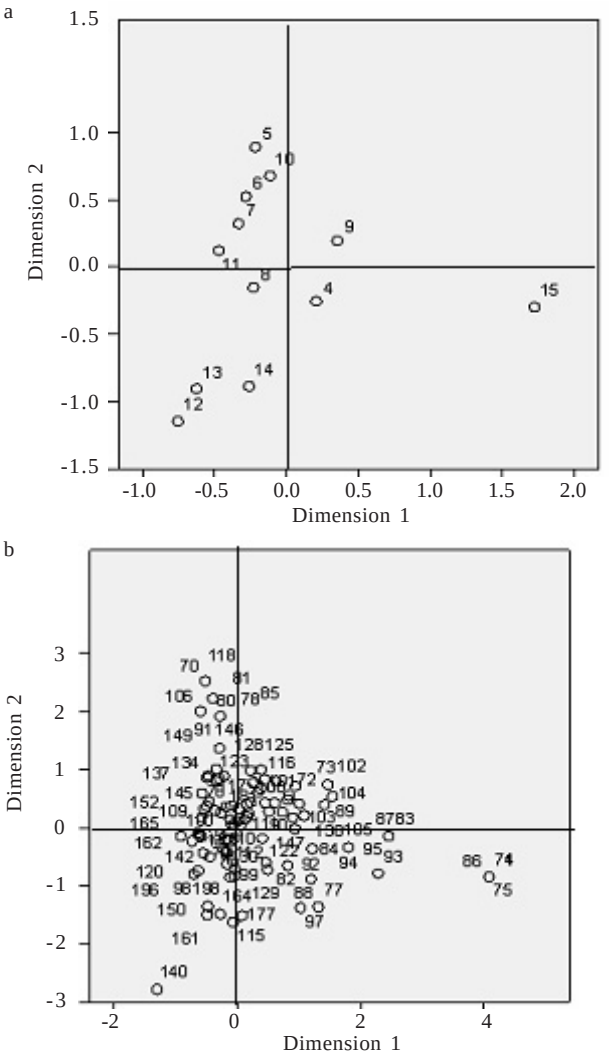

Figure 4. Projection of (a) tweleve isolates and (b) 130 AFLP loci on correlation plane. The number indicated labels of (4) PGN, (5) TBN, (6) CLM, (7) WRM, (8) LIP, (9) CBB, (10) KRT, (11) BBR, (12) MTR, (13) CSR, (14) USO, and (15) BJM isolates.

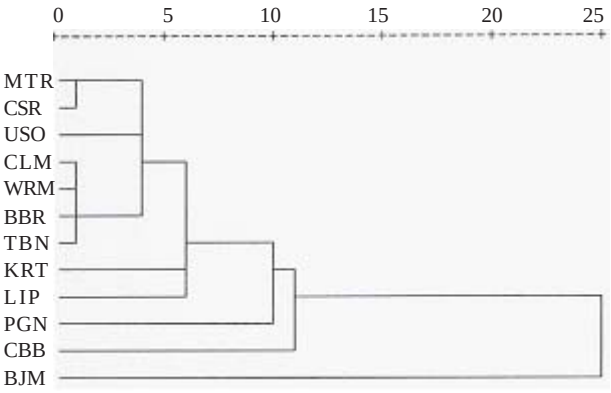

Figure 5. Dendogram of clustering analysis of tweleve isolates of white oyster mushrooms.

DISCUSSION

AFLP of the fifteen isolates had revealed 202 loci of positive DNA bands, and the average value of heterozygosity of those loci was 0.31, which was signified the polymorphisms of the isolates. This information indicated that strategy to collect genetic materials from many locations resulted a collection of polymorphic genetic resources.

Correlation and clustering analyses based on 202 AFLP loci showed that BNK, AMD, and USX isolates were distinctly different from the other isolates. The BNK isolate originated from Thailand had 40 loci from a total of 42 loci greater than 650 bp. The loci were not possessed by the majority of Indonesian isolates. In the contrary, BNK isolate had only 10 loci from a total of 100 loci with DNA bands size of 50-350 bp which was possessed by the majority of Indonesian isolates.

AMD isolate received from a farmer in Bogor had a high degree of dissimilarity to the other Indonesian isolates. This isolate was originally from IPB (Bogor Agricultural University) strain (Triyatno, personal communication); and this strain was possibly the isolate which was originally introduced from Thailand (Sudirman, personal communication). Similarly to BNK isolate, this isolate had a number of loci with DNA bands greater than 650 bp, that was not possessed by other Indonesian isolates. This result showed genetic differences between Thailand origin mushroom isolates (BNK and AMD isolates) and Indonesian mushroom isolates. Other laboratory experiments showed that AMD and BNK isolates had a high similarity in quantitative traits, and they were different from Indonesian isolates. AMD and BNK isolates had higher biomass production than that of Indonesian isolates (unpublished data).

USX isolate had the third highest differences from the majority of studied isolates. The USX and USO isolates are the strains isolated from Indonesian indigenous mushrooms. The AFLP analysis showed the differences between those two isolates. the USX isolate had different fruiting bodies texture than that of USO isolate (Mumpuni 2007, personal communication). It was suggested that USX isolate belonged to *Pleurotus* sp., but it was not the species of *P. osteratus*. This AFLP data supported that opinion, however to confirm this indication it is needed an interfertility test of USX isolate and a *P ostreatus* strain.

Correlation and clustering analyses of the isolates instead of BNK, AMD, and USX isolates appeared that BMJ isolate was very distinct from the other isolates (Figure 4). The BJM isolate was originally from indigenous Kalimantan mushroom collected from nature and sold in a traditional market. Two other isolates isolated from nature were USO and PGN isolates. Those isolates were originally from University of General Sudirman (UNSOED) Purwokerto and Research Center for Forest Product, Bogor, Indonesia respectively. The BJM, OSU, and PGN isolates were shown distinctly different on either correlation plane or clustering dendogram (Figure 4 & 5). The dissimilarity between those three indigenous isolates can be interpreted as the diversity of three regional origins.

The other isolates were originaly from commercial strains. Separation or regrouping of those isolates did not correspond to the geographical distribution. For instance MTR and TBN isolates from cultivated mushrooms collected from Metro Lampung, and Tabanan Bali respectively, were not distinctly separated from Java isolates, i.e BJM isolate (Figure 5). This fact could be caused by inter region exchange of mushroom spawn, because many mushroom cultivators outside of Java Island looked for mushroom spawn in Java (Parlindungan 2003).

In summary, the effort to collect genetic materials from different locations resulted a high diversity of polymorphic genetic resources. This result is very important as basic data for future breeding program. And interfertility experiments can be performed between genetically different isolates. AMD and BNK isolates that were very different from the other isolates can be used as genetic sources to improve Indonesian indigenous strains of white oyster mushroom.

ACKNOWLEDGEMENT

This research was supported by Hibah Bersaing research grant of Directorate General of Higher Education, Department of Education, Indonesia. The author thanks to Bogor Agricultural University and Directorate General of Higher Education for the awarded grant. We also thank to Aris Mumpuni from University of General Soedirman, Indonesia for providing several mushroom isolates used in this research, and Lisdar I. Sudirman for scientific discussion. We also thank to Elvavina, Sri Maria Ulfa, and Puspiari for DNA preparation and LI-CORR Analyzer operation, and Yulianto for laboratory supports.

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