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Genetic diversity of *Rhizopus microsporus* from traditional inoculum of tempeh in Indonesia based on ITS sequences and RAPD marker

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Abstract. *Barus T, Halim R, Hartanti AT, Saputra PK. 2019. Genetic diversity of* Rhizopus microsporus *from traditional inoculum of tempeh in Indonesia based on ITS sequences and RAPD marker. Biodiversitas 20: 847-852.* The main microorganism for tempeh fermentation is *Rhizopus microsporus*. These days, many tempeh producers use commercial inoculum, such as 'Raprima' as resource of *R. microsporus*. As a result, the genetic diversity of *R. microsporus* that had been reported in Indonesia has diminished. Information about genetic diversity is needed as a basis to select *R. microsporus* as tempeh inoculum. This research aims to investigate the genetic diversity of *R. microsporus* from waru leaves based on Internal Transcribed Spacer (ITS) Sequence and Random Amplified Polymorphic DNA (RAPD) markers. A total of 25 *R. microsporus* were isolated from traditional inoculum waru leaves (Inoculum 1) and traditional inoculum other than waru leaves (Inoculum 2). Amplification of ITS sequence was done using universal primer pairs of ITS-4 and ITS-5. Amplification of RAPD markers was done using primers OPC-08, OPC-19, OPQ-6, R-108, OPA-09 and OPJ-20. ITS sequence was not sufficient to compare the similarities among *R. microsporus*. On the other hand, RAPD markers successfully compared the similarities among 25 *R. microsporus*. A total of 25 *R. microsporus* were divided into 9 clusters. *R. microsporus* from Inoculum 1 grouped into Cluster 1, Cluster 3 and Cluster 4-8. Inoculum 2 grouped into Cluster 2 and Cluster 9. *R. microsporus* from tempeh grouped into Cluster 4 and was different from Inoculum 1 and Inoculum 2, except for TB3.

Keywords: Diversity, ITS sequence, RAPD marker, *Rhizopus microsporus*, tempeh

INTRODUCTION

Soybeans tempeh (tempeh) is a popular traditional food in Indonesia that is processed by fermenting soybeans. The most commonly used raw material for making tempeh is yellow seeded soybeans. Fermentation technology in the manufacture of tempeh has been handed down through generations and was changed based on experience. It is predicted to have originated from West Java-Indonesia as mentioned in *Serat Centhini* (1814) (Shurtleff and Aoyagi 2013).

Fermentation of soybeans into tempeh as food yield much superior nutritional value and positive aspects to health. Soybeans as raw form of tempeh contain approximately 40% protein, 35% carbohydrates, 20% fat, and 5% ash (Liu 1997). Fermentation of soybeans into tempeh degrade these macromolecules into smaller units, so that it can easily be digested and utilized by the body (Nout and Kiers 2005). Denter et al. (1998) reported that the concentration of some vitamins, such as riboflavin, B12, niacin, pyridoxine, folic acid, and biotin are higher in tempeh compared within soybeans. Tempeh contains antibacterial compounds that can inhibit pathogenic bacterias (Roubos-van den Hil and Nout 2011) and antioxidants (Klus et al. 1993; Esaki et al. 1996). Furthermore, tempeh also might modulate the composition of gut microbiota toward a healthier gut and stimulate IgA secretion (Soka et al. 2014)).

The main microorganism in fermentation of tempeh is *Rhizopus* spp. Therefore, *Rhizopus* spp. have been known as one of the economically important molds in Indonesia. Currently, diversity of *Rhizopus* in tempeh has been reduced. Please write some sentence as reference of ITS. Thirty-five species (97%) *Rhizopus* species have been isolated from tempeh obtained from 29 locations in Indonesia are *R. microsporus* which is genetically similar based on ITS sequence (Hartanti et al. 2015). This is due to the use of commercialized tempeh inoculum which is composed of particular species of *Rhizopus*, especially *R. microsporus*. Many Strains of *Rhizopus* spp. (Dolatabadi et al. 2014), *R. oryzae*, *R. arrhizus* (Abe et al. 2010), and *R. stolonifer* were previously reported in tempeh (Dwidjoseputro and Frederick 1970; Prihatna and Suwanto 2007). In the past, species of *Rhizopus* spp. for the manufacture of tempeh in Indonesia was highly varied because the tempeh producers use their own traditionally produced inoculums. Now, there are fewer tempeh craftsmen who still use traditional inoculum. *Rhizopus* spp. from the traditional inoculum has not been investigated yet. Identification of *Rhizopus* spp. has been done by comparing physiological and morphological features in the past (Schipper 1984; Schipper and Stalpers 1984). In the past, identification of *Rhizopus* spp. has been done by comparing physiological and morphological features (Schipper 1984; Schipper and Stalpers 1984). At present, many molecular techniques are available, generally based on sequence 18S

ribosomal DNA (rDNA), 28S rDNA and internal transcribed spacer (ITS) (Abe et al. 2006, 2007, 2010). However, ITS sequence is often used because of it is a conserved region in the fungal DNA and has a high mutation rate among species (Abe et al. 2003; Iwen et al. 2002; Lott et al. 1998). To compare the diversity within species, RAPD markers are often used and has been successful in comparing the genetic diversity of molds (Anggriawan 2017; Lusta et al. 2003). *Rhizopus* is a species of mold. Therefore, this study aims to assess the genetic diversity of *Rhizopus* species from traditional inoculums of tempeh. The results will be used as basis for further analysis of the role of *Rhizopus* species in determining the quality of the tempeh.

MATERIALS AND METHODS

Isolation of *Rhizopus* species

Rhizopus species were isolated from traditional inoculum from 18 samples of waru leaves (Inoculum 1), traditional inoculum other than waru leaves (Inoculum 2) and tempeh collected from Yogyakarta-Central Java-Indonesia. Each sample was suspended in sterile 0.85% w/v NaCl by the use of a Stomacher lab-blender 400 (Seward Medical, London, UK) for 1 minute at "normal" speed. Each *Rhizopus* species was grown on potato dextrose agar (PDA) and incubated at 28ºC for 2 days. All obtained *Rhizopus* species were temporarily stored at 4 °C for further analysis.

Growth of *Rhizopus* species at several temperature levels

The growth of each *Rhizopus* species is examined at some temperature variations to know the maximum growth temperature. Therefore, each *Rhizopus* species was grown on PDA and incubated at 33 °C, 42 °C, 45 °C, and 48 °C (Hartanti et al. 2015).

Isolation of 22 *Rhizopus* species genome

A total of 22 *Rhizopus* species were identified based on conserved ribosomal internal transcribed spacer (ITS) region. Mycelium of each *Rhizopus* species grown on PDA has been used as DNA sources for genome isolation using Phytopure™ DNA Extraction Kit (GE Healthcare, UK) according to the manufacturer's protocol. Genome isolation products were checked using 1% electrophoresis agarose gel (Promega, Madison, USA) then stained with ethidium bromide (Sigma-Aldrich, USA). UV transilluminator has been used to visualize DNA genome in gel electrophoresis.

Sequence amplification of ITS

The ITS regions between the small nuclear 18S rDNA and large nuclear 28S rDNA were amplified, including 5.8S rDNA using universal primer pairs of ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al. 1990). Sequence amplification was conducted in GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) with total 50 µL reaction mixture containing 1.5 µL 10 mM dNTPmix; 1 µL DNA template; 2.5 µL each primer (each of ITS-4 and ITS-5); 10 µL 5X KAPA Taq *EXtra Buffer*; 3.5 µL $MgCl_2$ 25mM; 28.5 µL nuclease-free water (NFW); and 0.5 µL (2.5 U/µL) µL KAPA Taq *EXtra HotStart DNA Polymerase*.. The amplification cycle consists of an initial denaturation at 94 °C for 2 minutes followed by 35 cycles of denaturation at 94 °C for 15 seconds, annealing at a temperature of 55 °C for 30 seconds, and extension at 72 °C for 1 minute. Final elongation was set at 72 °C for 5 minutes. Amplified PCR products were checked on agarose gel (1% w/v) (Promega, Madison, USA) then stained with ethidium bromide (Sigma-Aldrich, USA). UV transilluminator was used to visualize the PCR products in gel electrophoresis.

DNA sequencing of ITS region

The PCR products were sequenced in Macrogen Inc., Republic of Korea. The ITS nucleotide sequences for each *Rhizopus* species were aligned with ITS sequence database provided by GenBank (www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was created using Molecular Evolutionary Genetics Analysis 7 (MEGA7). The branch support was analyzed by 1000x bootstrap analysis.

Molecular characterization using RAPD marker

The genetic diversity of 32 *Rhizopus* species was analyzed using RAPD marker in this study. *R. microsporus* TB1-TB2 were isolated from tempeh. *R. microsporus* TB3-TB12, TB14-TB15, TB16-TB22 were isolated from Inoculum 1. *R. microsporus* TB69-TB82 were isolated from Inoculum 2 and identified based on ITS sequences by Jodi (2017). Amplification of RAPD marker of each *Rhizopus* species was performed in GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) using six primers (Table 1). RAPD amplification was conducted with total 25 µL reaction mixture containing 1µL DNA *template* (150 ng/µL); 12.5 µL *GoTaq green* (*Thermoscientific*); 5 µL each primer (100 µg) and 6.5 µL NFW. PCR amplification was performed as follows: initial denaturation at 93°C for 1 minute; followed by 45 cycles of denaturation at 93°C for 1 minute; annealing at 30°C or 34°C (Table 1) for 1 minute and extension at 72°C for 2 minutes. Final elongation was set at 72°C for 5 minutes.

Each PCR product was separated by electrophoresis at a constant voltage of 60 V for 50 minutes in 1x TAE (Tris Acetate EDTA) buffer and resolved on agarose gel (1% w/v) stained with ethidium bromide. The gel was visualized in a UV-transilluminator and photographed in a Gel Doc system (Vilber™). The 1 kb ladders (Fermentas) was used as known molecular weight marker. The bands of RAPD marker were scored as 1 or 0 on the basis of presence and absence of bands to make binary data. Phylogenetic tree was constructed using the Free Tree program (Pavlicek et al. 1999) and was *clustered* by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. Dendrogram was visualized using Tree View X (Roderic D.M. Page) software.

Table 1: Primers used to amplify RAPD marker of *Rhizopus* species

Primer	Sequence	Meltin Temp.	Sources
OPC-08	5'-TGGACCGGTG-3'	34°	Vagvolgyi et al. (2004)
OPC-19	5'-GTTGCCAGCC-3'	34°	Vagvolgyi et al. (2004)
OPQ-06	5'-GAGCGCCTTG-3'	34°	Vagvolgyi et al. (2004)
R-108	5'-GTATTGCCCT-3'	30°	Vagvolgyi et al. (2004)
OPA-09	5'-GGGTAACGCC-3'	34°	Mahmodi et al. (2014)
OPJ-20	5'-AAGCGGCCTC-3'	34°	Mahmodi et al. (2014)

RESULTS AND DISCUSSION

Identification of *Rhizopus* species based on ITS sequence

A total of twenty *Rhizopus* species from tempeh and Inoculum 1 were isolated and identified. To obtain the genome of all *Rhizopus* species, the Phytopure™ DNA Extraction Kit was used. ITS sequences were successfully amplified. Each PCR amplification of ITS sequences showed DNA fragments with single band at 700 bp. The representative PCR amplification sequences of ITS of *Rhizopus* species are shown in Figure 3. BLASTN results of the ITS sequence (± 650 nucleotides) showed similarity with *Rhizopus microsporus* in the range of 99%-100% with E-value 0. The ITS sequences of each *R. microsporus* was deposited in NCBI GenBank with accession numbers listed in Table 2.

Diversity of *Rhizopus* species based on ITS sequence

The phylogenetic tree based on ITS sequences was successfully created (Figure 2). However, the ITS sequence cannot show variation among species of *R. microsporus*. Phylogenetic tree grouped all of *R. microsporus* into one big cluster. Thus, ITS sequences cannot distinguish genetic diversity of *R. microsporus* up to the variation level.

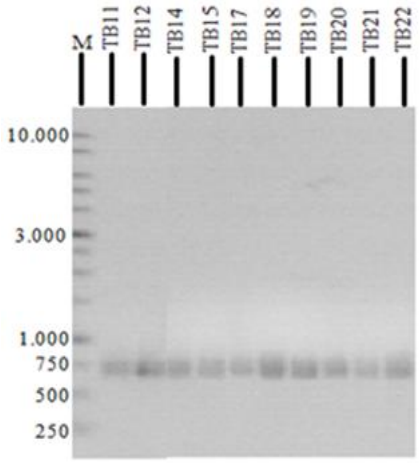

Figure 1: Results of PCR amplification sequences of internal transcribed spacer (ITS) as representative. M: Marker 1-kb ladder. TB11-TB12, TB14-B15, TB17-TB22: *Rhizopus* species were isolated from Inoculum 1.

Table 2: Isolate code, References species (GenBank), and GenBank accession numbers ITS sequences of *Rhizopus* species from tempeh and Inoculum 1.

Isolate code	References species (GenBank)	GenBank accession numbers
TB1 (tempeh)	*R. microsporus* var. *oligosporus* strain ATCC 22959 (KU729104.1)	MF445236
TB2 (tempeh)	*R. microsporus* var. *oligosporus* strain ATCC 22959 (KU729104.1)	MF445237
TB3 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445238
TB4 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445239
TB5 waru leaves	*R. microsporus* strain SHLSYD (KP340799.1)	MF445240
TB6 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445241
TB7 waru leaves	*R. microsporus* var. *oligosporus* strain ATCC 22959 (KU729104.1)	MF445242
TB8 waru leaves	*R. microsporus* var. *oligosporus* strain ATCC 22959 (KU729104.1)	MF445243
TB9 waru leaves	*R. microsporus* voucher KN2 (KU253769.1)	MF445244
TB10 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445245
TB11 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445246
TB12 waru leaves	*R. microsporus* strain SHLSYD (KP340799.1)	MF445247
TB14 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445249
TB15 waru leaves	*R. microsporus* strain SHLSYD (KP340799.1)	MF445250
TB16 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445252
TB17 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445252
TB18 waru leaves	*R. microsporus* strain SHLSYD (KP340799.1)	MF445253
TB19 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445254
TB20 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445255
TB21 waru leaves	*R. microsporus* strain QTYC54 (KM103343.1)	MF445256
TB22 waru leaves	*R. microsporus* strain SHLSYD (KP340799.1)	MF445257

Rhizopus microsporus growth in some temperature variations

Each *Rhizopus* species was incubated to grow at 33 °C, 42 °C, 45 °C, and 48 °C. The growth temperature of *Rhizopus* species varies. The results were shown that all *Rhizopus* species can grow up to 42 °C. There are eleven of *Rhizopus* species can grow up to 45°C, but only seven (TB4, TB6, TB8, TB9, TB19, TB20, TB22) can grow up to 48°C.

Diversity of *R. microsporus* based on RAPD analysis

Dendrogram is shown as a representative of similarity among thirty-two isolates of *Rhizopus* species appear to form 9 clusters (Figure 3). Some of this *R. microsporus* have diverse genetic and some of them are not diverse, which can be seen by the position of the same isolates in the same cluster (Figure 3). Rhizopus from Inoculum 1 (Cluster 1, Cluster 3, Cluster 4-Cluster 8) seemed different than *Rhizopus* from Inoculum 2 (Cluster 2, Cluster 9). *Rhizopus* from tempeh (TB1 and TB2) was different from *Rhizopus* from Inoculum 1 and Inoculum 2, with the exception of TB3 which exist in the same group as TB 1 and TB2.

Table 3. RAPD marker amplification result using six different primers

Primer	Formed bands	Polymorphic bands	Polymorphic bands (%)
OPC-08	48	20	
OPC-19	82	12	
OPQ-06	58	35	
R-108	50	11	
OPA-09	89	2	
OPJ-20	126	0	
Total	453	80	17%

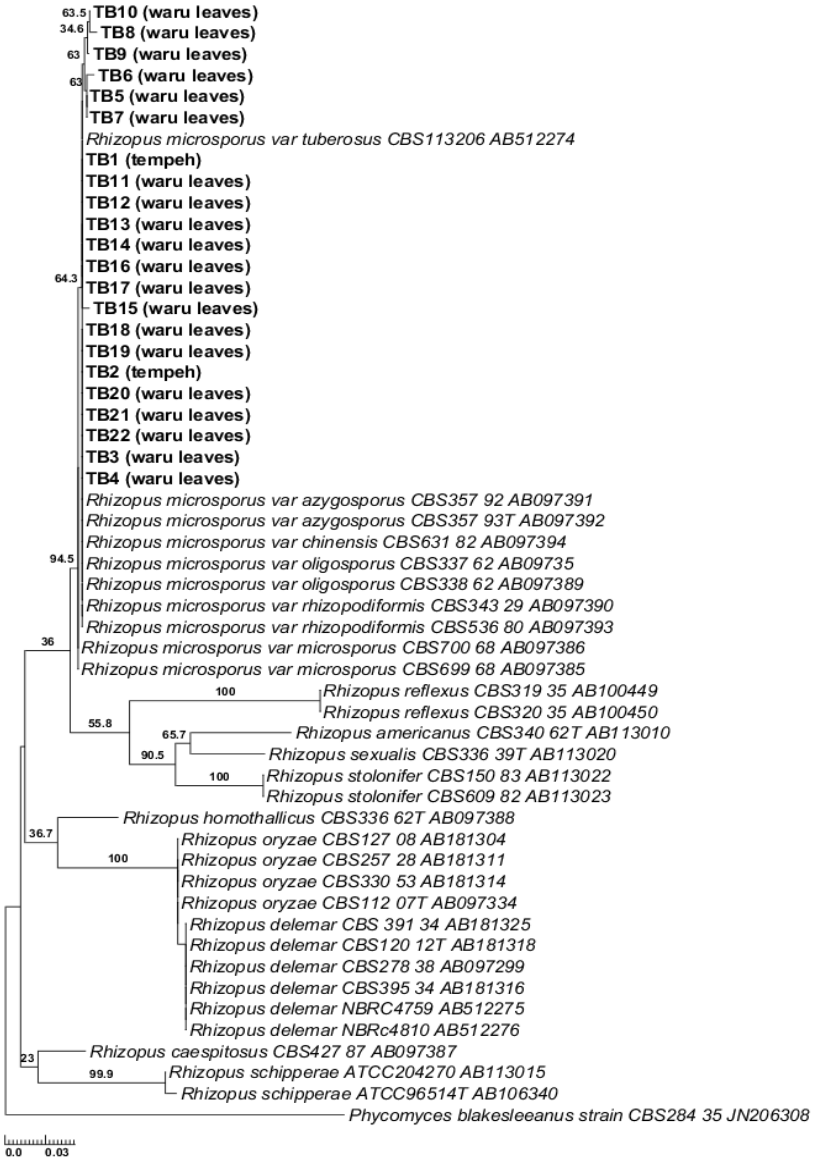

Figure 2: The phylogenetic tree of *Rhizopus* species from tempeh and inoculum I based on ITS sequences

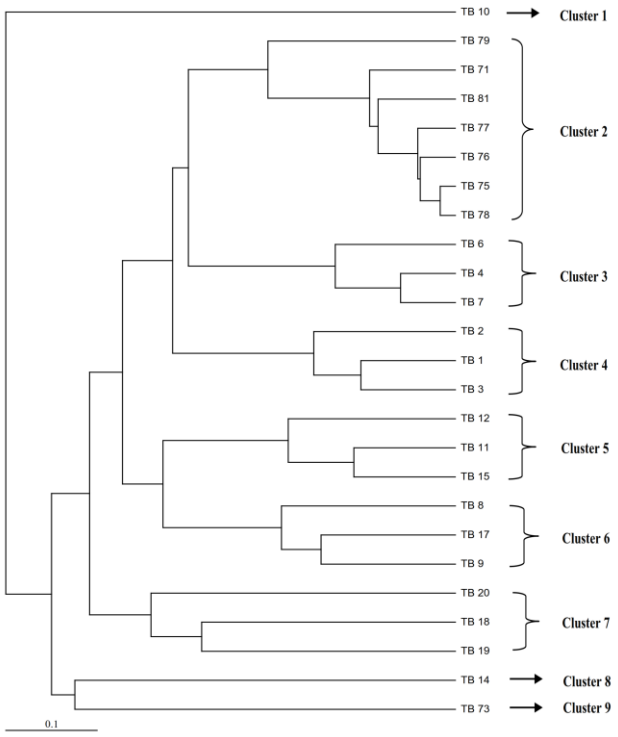

Figure 3. The phylogenetic tree of *Rhizopus* species constructed based on RAPD images. TB1-TB2 *Rhizopus* species from tempeh. TB3-TB12, TB14-TB15, TB16-TB22 *Rhizopus* species from Inoculum 1. TB 69-TB82 *Rhizopus* species Inoculum 2

Discussion

Tempeh is the original fermented food from Indonesia although it has been produced and consumed in various countries in the world. The quality of tempeh is determined by the technology, quality of soybeans used, and the microorganisms involved during the fermentation process. *Rhizopus* species is the main microorganism in making tempeh by fermentation process. Tempeh cannot be produced without involving *Rhizopus* spp. Since long ago, those species have been known as one of the economically important molds in Indonesia.

Barus et al. (2008) reported that tempeh is produced in uncontrolled fermentation conditions. Tempeh fermentation is still using conventional methods with uncontrolled condition. It has been reported that the taste of tempeh may vary due to the different types of microorganisms during fermentation. Particularly in the diversity between *Rhizopus* species and variations in the *Rhizopus* species. Previously reported that *R. oligosporus*, *R. oryzae*, *R. arrhizus*, and *R. stolonifer* were found in tempeh fermentation (Dwidjoseputro and Frederick 1970; Prihatna and Suwanto 2007). Hartanti et al. (2015) reported that tempeh was taken from 28 locations spread in Indonesia only contains *R. microsporus.* It has been reported that *R oligosporus* is synonym of *R. microsporus* (Dolatabadi et al. 2014). However, *Rhizopus* species isolated from traditional inoculum of tempeh has not been reported. Thus, *Rhizopus* species from traditional inoculums waru leaves needs to be preserved and further investigated in determining the quality of tempeh. The results obtained in this study will be further used as a base for further analysis of *R.microsporus* in determining the quality of the tempeh.

Genome of each species of *R.microsporus* was isolated. One of the difficulties in studying fungi molecularly is in the process of genome extraction. In this study, extraction using Phytopure™ DNA Extraction Kit (GE Healthcare, UK) according to the manufacturer's protocol found no difficulty and the obtained genome quality was good. Based on the BLASTN results of ITS sequence, *Rhizopus* TB1-TB12, TB14-TB15, TB16-TB22 were isolated from Inoculum 1 in this study were identified as *R. microsporus* by Saputra (2017). *Rhizopus* TB 69-TB82 were isolated from traditional inoculum not waru leaves (Inoculum 2) were identified also as *R. microsporus*. Iwen, et al. (2002) reported that ITS sequences have become important molecular targets for identification of fungal. The ITS domains are more suitable for species identification than the 18S region (small subunit), the 5.8S region, and the 28S region (large subunit) due to greater sequence variation (Iwen, et al. 2002; Lott et al. 1998). However, ITS sequence was not sufficient to show genetic diversity of *R. microsporus* into variation level. This can be seen from the phylogenetic tree (Figure 2) as an example. There are *R. microsporus* var. oligosporus, *R. microsporus* var. azygosporus, *R. microsporus* var. chinensis, and *R. microsporus* var. *microsporus* are still remained on one cluster.

The amplification of RAPD marker (Figure 3) with six primers produced diversity in DNA patterns of RAPD marker with size ranging from 70 bp to 4000 bp. The phylogenetic tree as a representation of the genetic diversity of 25 *Rhizopus* species showed that thirty-two isolates of *Rhizopus* species appear to form 9 clusters (Figure 3). Some *R. microsporus* have diverse genetic and some of them are not diverse, which the position of the same isolates have been shown in the same cluster. It has been reported that RAPD -PCR technique can be used as an important tool for the genetic differentiation of fungal such as *Fusarium* species (Bonde et al. 2013; Haghighi and Shahdoust 2014). In this study, RAPD marker can also distinguish the genetic diversity of *Rhizopus* species which cannot be distinguished by the ITS sequences (Figure 2). There was no relation between genetic diversity and growth temperature of *R. microsporus*. Genetic *R. microsporus* of tempeh generally looks different with *R. microsporus* of Inoculum 1 and Inoculum 2.

The selection of primer used in PCR-RAPD marker is necessary. Some primers can produce many variations of the RAPD marker but some also are unsuccessful in generating RAPD marker variations. In this study, the highest variations in RAPD marker resulted from primers OPQ-06 (43 polymorphic) and OPC-08 (20 polymorphic), OPC-19 (12 polymorphic) patterns, respectively, while lowest variations resulted from primer of OPA-09 (2 polymorphic).

One of the important sources of protein for Indonesians is tempeh. The survey results indicate that each person on

average consumes tempeh three times a week. Therefore, the results of this investigation are important as a basis for the selection of *R. microsporus* for further investigation of its role in determining the quality of tempeh.

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