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Genotypic Characterization of *Rhizopus* species from Tempeh and *Usar*: Traditional Inoculum of Tempeh in Indonesia

TATI BARUS[1]*, JASON WIRANATA SANJAYA[2], DAVID TANDJUNG[2], ANASTASIA TATIK HARTANTI[3], ADI YULANDI[2], AND VIVITRI DEWI PRASASTY[2]

[1]*Master of Biotechnology,Faculty of Biotechnology, Universitas Katolik Atma Jaya, Jakarta 12930, Indonesia;*
[2]*Department of Biology, Faculty of Biotechnology, Universitas Katolik Atma Jaya, Jakarta 12930, Indonesia;*
[3]*Food Technology, Faculty of Biotechnology, Universitas Katolik Atma Jaya, Jakarta 12930, Indonesia.*

Soybeans tempeh (tempeh) is processed by fermentation using *Rhizopus* spp. Tempeh is an important source of protein in Indonesia. The traditional inoculum in tempeh fermentation locally is known as *Usar,* which is made from the leaves of *Hibiscus tiliaceus*. However, *Rhizopus* information from *Usar* is still limited. Therefore, this study aims to identify and investigate the genetic diversity of *Rhizopus* species from *Usar* and tempeh based on the Internal Transcribed Spacer (ITS) sequences and the Random Amplified Polymorphic DNA (RAPD) markers. Twenty-three *Rhizopus* strains were isolated from *Usar* and ten *Rhizopus* strains were isolated from tempeh. Based on ITS sequences, the isolates were similar to *Rhizopus microsporus* (30 isolates) and *Rhizopus delemar* (3 isolates) with 98-99% similarity. The genetics of *R. microsporus* and *R. delemar* are varied and different from the genetics of *R. microsporus* from tempeh. The growth temperature of *R. microsporus* varies from 33°C to 48°C and *R. delemar* can grow to a maximum at 33°C. This research needs to be continued to obtain information about the role of *Rhizopus* from this study in determining the quality of tempeh.

Key words: diversity, ITS, RAPD, *Rhizopus*, tempeh

Tempe kedelai (tempe) diolah melalui fermentasi menggunakan oleh *Rhizopus* spp. Tempe adalah salah satu sumber protein penting di Indonesia. Inokulum tradisional dalam fermentasi tempe dikenal sebagai *Usar* yang terbuat dari daun *Hibiscus tiliaceus*. Namun, informasi *Rhizopus* dari *Usar* masih terbatas. Oleh karena itu, penelitian ini bertujuan untuk mengidentifikasi dan mengkaji keragaman genetik spesies *Rhizopus* dari *Usar* dan tempe berdasarkan urutan sekuen Internal Transcribed Spacer (ITS) dan penanda Random Amplified Polymorphic DNA (RAPD). Dua puluh tiga strain *Rhizopus* diisolasi dari *Usar* dan sepuluh strain *Rhizopus* diisolasi dari tempe. Berdasarkan sekuens ITS maka semua strain tersebut terdiri atas *Rhizopus microsporus* (30 isolat) dan *Rhizopus delemar* (3 isolat) dengan kemiripan 98-99%. Genetik *R. microsporus* dan *R. delemar* bervariasi dan berbeda dari genetic *R. microsporus* dari tempe. Suhu pertumbuhan *R. microsporus* bervariasi dari 33°C hingga 48°C dan *R. delemar* dapat tumbuh hingga maksimum pada 33°C. Penelitian ini perlu dilanjutkan untuk mendapatkan informasi tentang peran *R. microsporus* dan *R. delemar* dari penelitian ini dalam menentukan kualitas tempe.

Kata kunci: ITS, keragaman, RAPD, *Rhizopus*, tempe

Soybeans tempeh (tempeh) is a traditional fermented food from Indonesia. It has been consumed as main source protein by Indonesian for years. It contains essential compounds such as vitamin B12 (Keuth and Bisping 1994), isoflavon and essential fatty acids. It has also been reported that tempeh have many health benefits such as in preventing free radicals (Esaki *et al.* 1996). Tempeh can prevent diarrhea (Sudigbia 1999) and anemia (Astuti 1999) because of increased iron availability during fermentation. Tempeh can also stimulate the formation of good bacteria populations in the intestines (Stephanie *et al.* 2019).

*Corresponding author: Phone: +62-21-5727615; Email: tati.barus@atmajaya.ac.id

Tempeh is made through the fermentation of soybean, mainly by *Rhizopus* spp. Therefore, *Rhizopus* spp. is known as an economically important mold in Indonesia. Many species of *Rhizopus* spp. such as *R. oligosporus*, *R. oryzae*, *R. arrhizus*, and *R. stolonifer* were previously identified from Indonesian tempeh (Dwidjoseputro and Wolf 1970). In the current taxonomic system, *R. arrhizus* is considered as a synonym of *R. oryzae* (Abe *et al.* 2010), *R. oligosporusas* as synonym of *R. microspores* (Dolatabadi *et al.* 2014), and *R. oryzae* as synonym of *R. delemar* (Abe *et al.* 2007). At present, tempeh producers rarely use *Usar* (traditional inoculum) (Fig 1) because they generally use commercial inoculum. *Usar* is made by growing *Rhizopus* spp. on *Hibiscus tiliaceus* leaves for 24 hours and then dried. After that,

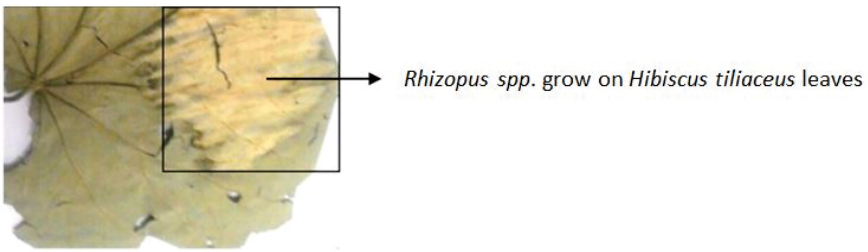

Fig 1 *Usar*: traditional inoculum of tempeh .

it is ready to be used as an inoculum in tempeh fermentation. However, information about *Rhizopus* spp. from Usar was not yet available.

Many molecular techniques are available to study genotypic characterization of *Rhizopus* (Abe *et al.* 2007). To identify of *Rhizopus* spp. often done based on ITS sequences (Iwen *et al.* 2002; Lott *et al.* 1998). Abe *et al.* (2007) reported the classification of three *Rhizopus* species, i.e. *R. microsporus*, *R. stolonifer*, and *R. oryzae*. Therefore, in this study ITS sequences were used to assess the genetic diversity of *Rhizopus* isolates from *Usar* (Fig 1) and tempeh.

Beside ITS sequences, various molecular methods have been developed to get more accurate data on the genetics of an organism. One of them is random amplified polymorphic DNA (RAPD) marker using polymerase chain reaction (PCR). RAPD markers have been successfully used in assessing differentiating fungal genetic diversity, such as the genetic diversity of *Colletotrichum* spp. (Mahmodi *et al.* 2014) and *Rhizopus stolonifera* (Vágvölgyi *et al.* 2004). Therefore, this study aims to identify and investigate the genetic diversity of *Rhizopus* species from *Usar* and tempeh based on the Internal Transcribed Spacer (ITS) sequence and the Random Amplified Polymorphic DNA (RAPD) markers.

MATERIALS AND METHODS

***Rhizopus* Isolation**. *Rhizopus* isolates have been isolated from *Usar* were taken from Yogyakarta, Central Java-Indonesia. *Usar* sampling was carried out from Yogyakarta because to our knowledge that only in this area *Usar* was still used as an inoculum in tempeh production. A total 10 tempeh is collected from Yogyakarta and Solo, Central Java-Indonesia. All these tempeh are produced using commercial inoculums. A total of 23 pieces of *Usar* that grow *Rhizopus* has been cut off and homogenized in sterile 0.85% w/v NaCl by the use of a Stomacherlab-blender 400 (Seward Medical, London, UK) for 1 minute. All isolates were grown on potato dextrose agar (PDA) and incubated at 28°C. All suspected *Rhizopus* isolates were stored at 4°C for further analysis.

DNA Extraction. Genomic DNAs of *Rhizopus* isolates were extracted from four days-old mycelia grown on PDA using the Phytopure™ DNA Extraction Kit (GE Healthcare, UK) according to the manufacturer's protocol. DNAs were visualized on 1% electrophoresis agarose gel (Promega, Madison, USA) then stained with ethidium bromide (Sigma-Aldrich, USA).

Amplification of Its Region and Sequencing. Amplification of ITS region was performed using the GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) and the primer pair ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) and ITS5 (5′–GGAAGTAAAAGTCGTAACAAGG–3′) (White et al. 1990). A total 50 µL of reaction mixtures were used, containing 1 µL DNA template, 10 µL 5X KAPA (Sigma-Aldrich, USA), Taq EXtra Buffer, 2.5 µL of each primer, 1.5 µL 10 mM dNTPmix, 3.5 µL MgCl2, 28.5 µL nuclease-free water (NFW), and 0.5 µL KAPA Taq EXtra HotStart DNA Polymerase. PCR conditions were set as follow: 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. PCR products were visualized on 1% agarose gel and stained with ethidium bromide. PCR products were then partially sequenced at Macrogen Inc., Republic of Korea. The DNA sequencing results were compared to the GenBank database using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using MEGA7. The branch support was analyzed by 1000x bootstrap analysis.

Growth of *Rhizopus* Isolates on Various Temperature. All isolates were grown on potato dextrose agar (PDA). To determine the optimal growth temperature, all *Rhizopus* isolates obtained in this study were grown on various temperature, i.e. 33°C,

Table 1 Primers to amplify RAPD markers of *Rhizopus* isolates.

Primer	Sequence	Melting temperature	Sources
OPQ6	GAGCGCCTTG	34°C	Vagvolgyi*et al.* 2004
OPA9	GGGTAACGCC	34°C	Mahmodi*et al.* 2014
OPJ20	AAGCGGCCTC	34°C	Mahmodi*et al.* 2014
R108	GTATTGCCCT	30°C	Vagvolgyi*et al.* 2004
OPA11	CAATCGCCGT	32°C	Mahmodi*et al.* 2014
OPA1	CAGGCCCTTC	34°C	Mahmodi*et al.* 2014

42°C, 45°C, and 48°C.

Amplification of Random Amplification of Polymorphic DNA. Amplifications of RAPD markers were conducted using single six primers (Table 1) through GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA). Amplification of RAPD was carried out with a total of 25 µL of the reaction mixture with the same composition carried out with Barus et al. (2019). Amplifications of RAPD markers were performed with the same condition also with Barus et al. *(2019)*. Annealing conditions were done based on the melting temperature of each primer (Table 1). Products of amplification were separated by electrophoresis in agarose gel (1% w/v). The agarose gel was stained with ethidium bromide and UV transilluminator was used to visualize the PCR products in agarose gel. The 1 kb ladder (Fermentas) was used as weight marker. Clearly resolved each band was manually scored for the presence (1) or absence (0) to make binary data. Dendrogram analysis among all the *Rhizopus* was computed using Roderic D.M. Page software. The unweighted pair group method analysis (UPGMA) was used for clustering and Tree View software was used for interactive visualization of the dendrogram.

RESULTS

A total of twenty-three *Rhizopus* isolates had been isolated from *Usar* (TB23-TB45) and ten *Rhizopus* isolates had been isolated from tempeh (TB46-TB55) (Fig 2). ITS sequences were successfully amplified and each PCR amplification showed DNA fragments with single band at 700 bp (Fig 2). BLASTN results of ITS sequence (± 600 nucleotides) showed that 30 isolates (TB23-TB25, TB27, TB29-TB36, TB38-TB55) were *Rhizopus microporus* with similarity about 98-100%. Only three isolates (TB26,TB28, TB37) were *Rhizopus delemar* with similarity about 98-100%. All the ITS sequences have been submitted to GenBank with accession numbers MF445258 - Mf445290.

The phylogenetic tree based on the ITS sequences showed that the thirty three of *Rhizopus* isolates were divided into two clusters (Fig 3). The first cluster consisted of *R. microsporus* (30 isolates) and the second cluster consisted of *R. delemar* (3 isolates).

All *Rhizopus* spp. isolates were grown at 33°C, 42°C, 45°C, and 48°C. The growth temperature for all isolates *R. microsporus* varied (Table 2). Eight strains of *R. microspores* could grow up to 48°C, thirteen strains could grow up to 45°C, seven strains could grow up to 42°C, and two strains could grow up to 33°C. Conversely, three *R. delemar* isolates could only grow up to 33°C.

Genomic DNAs isolated from 33 *Rhizopus* isolates were subjected to obtain RAPD-PCR markers using six primers (Table 1), but only 9 out of 33 *Rhizopus* isolates produced distinc and reproducible band RAPD marker using these primers. The dendogram (Fig 4) describes the genetic similarity *R. microsporus* and *R. delemar* was successfully created. UPGMA dendrogram based on RAPD – PCR separated the *R. microsporus* and *R. Delemar* in two main clusters. Among all *R microporus*, the smallest genetic similarity (GS) (35%) was found between TB34 and TB35 and the largest GS (63%) was found between TB32 and TB33. *R. microporus* from tempeh (TB49) is most similar to TB32 with GC 54% and most different with TB34 with GC 38%. *R. delemar* TB26 and *R. delemar* TB37 have genetic similarity 64%.

DISCUSSION

A study by Bressa *et al.* (2017) showed that lifestyle enhanced health-promoting bacteria. A previous

Table 2 Growth on various temperatures of thirty-three *Rhizopus* isolates isolated from*Usar* and tempeh.

Source	Isolate code	Species	Growth temperature (°C)			
			33	42	45	48
Usar	TB24	*R. microsporus*	✔	✔	✔	✔
Usar	TB25	*R. microsporus*	✔	✔	✔	✔
Usar	TB31	*R. microsporus*	✔	✔	✔	✔
Usar	TB32	*R. microsporus*	✔	✔	✔	✔
Usar	TB33	*R. microsporus*	✔	✔	✔	✔
Usar	TB39	*R. microsporus*	✔	✔	✔	✔
tempeh	TB48	*R. microsporus*	✔	✔	✔	✔
tempeh	TB54	*R. microsporus*	✔	✔	✔	✔
Usar	TB23	*R. microsporus*	✔	✔	✔	-
Usar	TB27	*R. microsporus*	✔	✔	✔	-
Usar	TB30	*R. microsporus*	✔	✔	✔	-
Usar	TB35	*R. microsporus*	✔	✔	✔	-
Usar	TB36	*R. microsporus*	✔	✔	✔	-
Usar	TB38	*R. microsporus*	✔	✔	✔	-
Usar	TB40	*R. microsporus*	✔	✔	✔	-
Usar	TB43	*R. microsporus*	✔	✔	✔	-
Usar	TB45	*R. microsporus*	✔	✔	✔	-
tempeh	TB46	*R. microsporus*	✔	✔	✔	-
tempeh	TB52	*R. microsporus*	✔	✔	✔	-
tempeh	TB53	*R. microsporus*	✔	✔	✔	-
tempeh	TB49	*R. microsporus*	✔	✔	✔	-
Usar	TB29	*R. microsporus*	✔	✔	-	-
Usar	TB34	*R. microsporus*	✔	✔	-	-
Usar	TB41	*R. microsporus*	✔	✔	-	-
Usar	TB42	*R. microsporus*	✔	✔	-	-
Usar	TB44	*R. microsporus*	✔	✔	-	-
tempeh	TB47	*R. microsporus*	✔	✔	-	-
tempeh	TB51	*R. microsporus*	✔	✔	-	-
tempeh	TB50	*R. microsporus*	✔	-	-	-
tempeh	TB55	*R. microsporus*	✔	-	-	-
Usar	TB26	*R. delemar*	✔	-	-	-
Usar	TB28	*R. delemar*	✔	-	-	-
Usar	TB37	*R. delemar*	✔	-	-	-

reported that gut microbiota in obese subjects and/or with Type-2 Diabetes were different from lean and non-diabetic subjects (Patterson *et al.* 2016). To get beneficial gut microbiota population, probiotics consumption and dietary fibers are strongly recommended. Holscher (2017) reported that low fiber intake is associated with increased chronic diseases, such as obesity, cardiovascular disease, type 2 diabetes, and colon cancer. Tempeh, a popular fermented food in Indonesia, is one source of fiber-rich food.

The main microorganism in fermentation of tempeh is *Rhizopus* spp. At present, many molecular techniques are available for identification of *Rhizopus* spp. However, internal transcribed spacer (ITS) is often used (Abe *et al.* 2003. Based on ITS sequence showed that 30 isolates (TB23-TB25, TB27, TB29-TB36, TB38-TB55) were *Rhizopus microporus* with similarity about 98-100% and three isolates (TB26, TB28, TB37) were *Rhizopus delemar* with similarity about 98-100%.The ITS regions have become an important molecular target for fungal taxonomy and identification (Iwen *et al.* 2002). Due to greater sequence variations, the ITS domains are more suitable for species identification (Iwen *et al.* 2002; Lott *et al.* 1998). Therefore ITS sequences are widely used to identify and assess fungal genetic diversity.

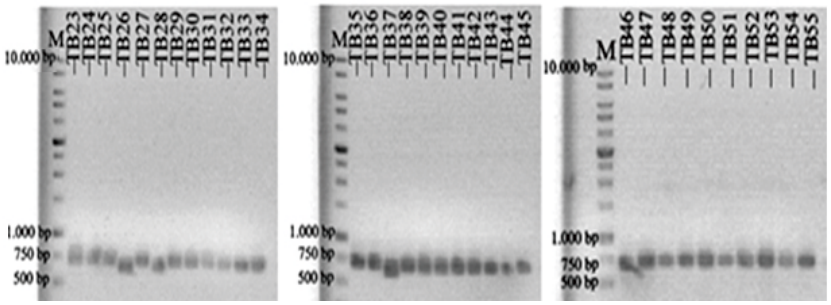

Fig 2 Results of PCR amplification sequences of internal transcribed spacer (ITS) sequence of *Rhizopus* isolates. M: Marker 1-kb lambda ladder. TB23-TB45: *Rhizopus* isolates from *Usar*. TB46-TB55: *Rhizopus* isolates from tempeh.

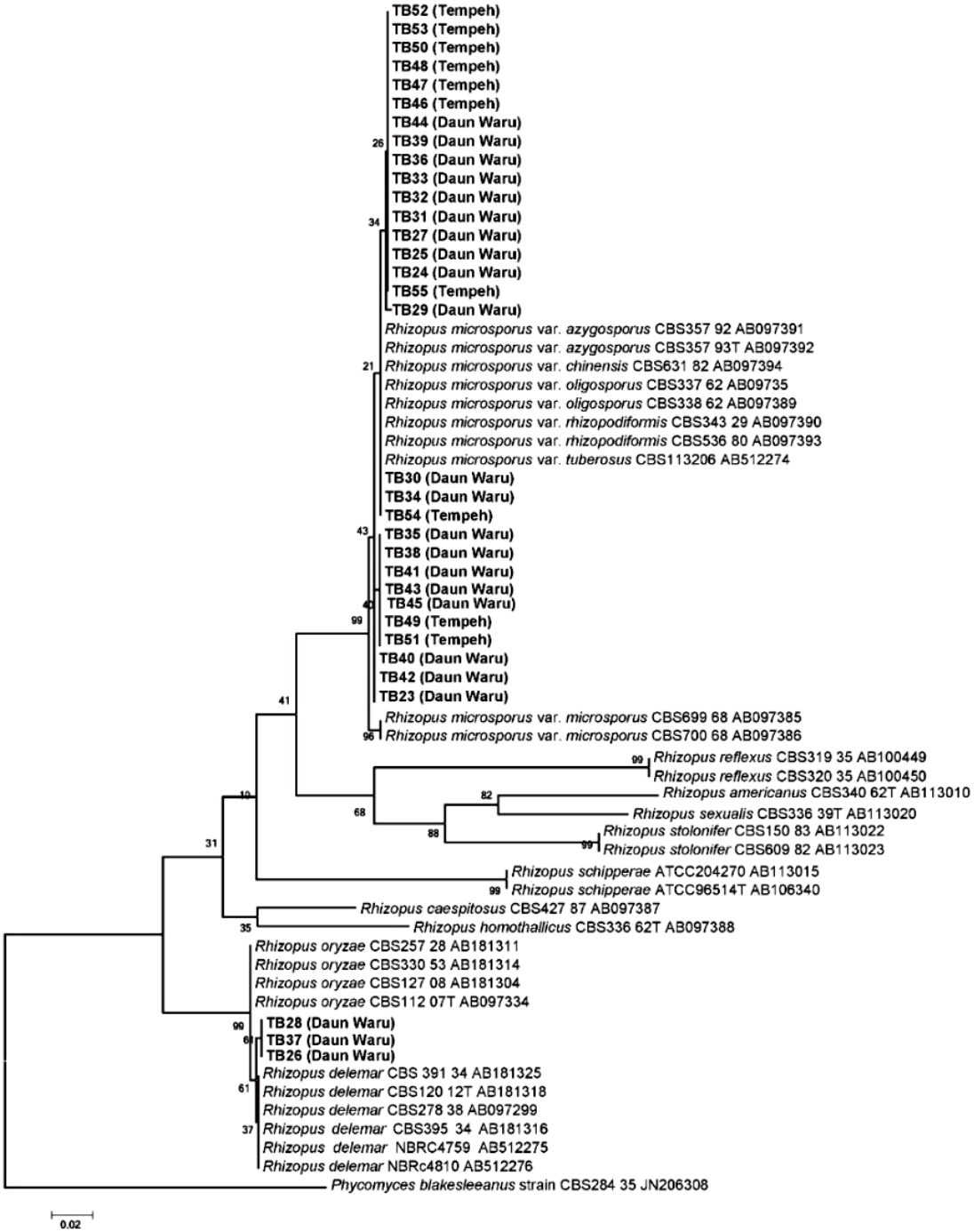

Fig 3 Phylogenetic tree generated from the internal transcribed spacer (ITS) sequences of 33 isolates of *Rhizopus* spesies isolated from *Usar* and tempeh.

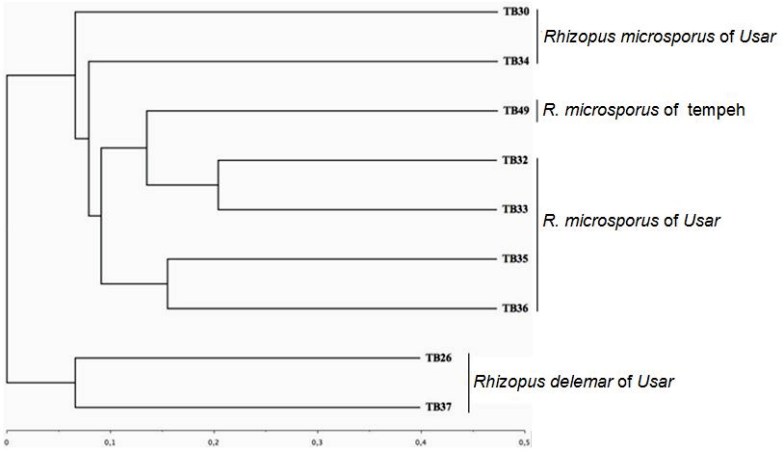

Fig 4 UPGMA dendrogram of *Rhizopus* strains isolated from *Usar* and tempeh based on RAPD – PCR.

In the past, it was reported that various *Rhizopus* species were used to make tempeh (Dwidjoseputro and Wolf 1970). In this study it was shown that only *R. microspores* were found in tempeh samples. This finding is similar to the report by Hartanti *et al.* (2015), where tempeh collected from 28 locations throughout Indonesia only contained *R. microspores*. This is caused by the use of commercial inoculums that only contain the *R. microsporus*.

Surprisingly, in this study found three isolates of *R. delemar* from Usar. Based on the RAPD marker (Fig 4), TB26 and TB27 are in the same cluster, but they are different types represented by different RAPD markers. Information on *R. delemar* in tempeh is still limited. Therefore, the role of *R. delemar* in determining the quality of tempeh needs to be further investigated. The species of *Rhizopus* may have an important contribution to the variety of tempeh flavor and nutritional value. The different species of *Rhizopus* have different metabolic activities. Moreover, it has been reported that *R. delemar* produced fumaric acid and malic acid (Abe *et al*. 2007).

Figure 3 showed that ITS sequences were not sufficent to distinguish *R. microsporus*up to the variety level. This can be seen from the phylogenetic tree which *R. microsporus* var. *azygosporus*, *R. microsporus* var. *chinensis*, *R. microsporus* var. *oligosporus*, *R. microsporus* var. *rhizopodiformis*, and *R. microsporus* var. *tuberosus* were all grouped as one cluster (Cluster 1). This indicated that the ITS sequences were not sufficent to distinguish *R. microsporus*up to the variety level. This report is in line with Hartanti *et al.* (2015) which ITS sequences were not sufficent to distinguish *R. microsporus* spesies from tempeh up to the variety.

Rhizopus is the main microorganism in making tempeh. Information about the growth of *Rhizopus* isolates on various temperature is important as a basis for selecting isolates to be used as inoculums in tempeh fermentation. The growth temperature for all isolates *R. microsporus strains* varied (Table 2). This was found that *Rhizopus microsporus* (TB32) can grow up to 48°C and *Rhizopus microsporus* (TB55) can grow up to 32°C (Table 2). Barus *et al.* (2019) reported that *Rhizopus microsporus* (TB32) produced tempeh with higher antioxidant activity compared to *Rhizopus microsporus* (Tb55).

Figure 4 showed that RAPD markers can show genetic variation in nine *Rhizopus*. Previously it has been reported that RAPD marker can be used as an important technique to investigate for the genetic variations of fungal (Dwivedi *et al.* 2018). These reported are in line with our result, where RAPD marker can also distinguish the genetic variations of nine *R. microsporus* and two *R. delemar* well.

Genetic of all *R. microsporus* isolate from *Usar* (TB26, TB30, TB32-TB37) were different from the genetic *R. microsporus* from tempeh (TB49). Furthermore, the genetics of *R. microsporus* and *R. delemar* derived from *Usar* also varied. RAPD markers of 24 isolates (TB 23-TB25, TB27-TB29, TB31, TB38, TB39-TB48, TB50-TB55) have not been successfully amplified using several primers (Table 1) even though they have been repeated several times. This might be accessible using another primer.

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REFERENCES

Abe A, Asano K, Sone T. 2010. A molecular phylogeny-based taxonomy of the genus *Rhizopus*. Biosci Biotechnol Biochem 74(7): 1325-1331. doi: 10.1271/bbb.90718.

Abe A, Oda Y, Asano K, Sone T. 2007. *Rhizopus delemar* is the proper name for *Rhizopus oryzae* fumaric-malic acid producers. Mycologia 99(5): 714-722. doi: 10.3852/mycologia.99.5.714.

Astuti M. 1999. Iron availability of tempe and uses in iron deficiency anemia. The Complete Handbook of Tempe: The Unique Fermented Soyfood of Indonesia 41-45.

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 Journal of Biological Diversity 20(3): 847-852. doi: 10.13057/biodiv/d200331.

Bressa C, Bailén-Andrino M, Pérez-Santiago J, González-Soltero R, Pérez M, Montalvo-Lominchar MG, Maté-Muñoz JL, Domínguez R, Moreno D, Larrosa M. 2017. Differences in gut microbiota profile between women with active lifestyle and sedentary women. PLoS One 12(2): 1-20. doi: 10.1371/journal.pone.0171352.

Dolatabadi S, Walther G, Van Den Ende AG, De Hoog G. 2014. Diversity and delimitation of *Rhizopus microsporus*. Fungal Diversity 64(1): 145-163. doi 10.1007/s13225-013-0229-6.

Dwidjoseputro D, Wolf FT. 1970. Microbiological studies of Indonesian fermented food stuffs. Mycopathologia et Mycologia Applicata 41(3-4): 211-222. doi: 10.1007/BF02051099.

Dwivedi S, Singh S, Chauhan UK, Tiwari MK. 2018. Inter and intraspecific genetic diversity (RAPD) among three most frequent species of macrofungi (Ganoderma lucidum, Leucoagricus sp. and Lentinus sp.) of Tropical forest of Central India. Journal of Genetic Engineering and Biotechnology 16(1):133-141. doi:10.1016/j.jgeb.2017.11.008.

Esaki H, Onozaki H, Kawakishi S, Osawa T. 1996. New antioxidant isolated from tempeh. Journal of Agricultural and Food Chemistry 44(3): 696-700. doi: 10.1021/jf950454t.

Hartanti AT, Rahayu G, Hidayat I. 2015. *Rhizopus* species from fresh tempeh collected from several regions in Indonesia. Hayati Journal of Biosciences 22(3):136-142. doi: 10.4308/hjb.22.3.136.

Holscher HD. 2017. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut microbes 8(2):172-184. doi: 10.1080/19490976.2017.1290756.

Iwen PC, Hinrichs SH, Rupp ME. 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Medical mycol. 40(1): 87-109. doi: 10.1080/mmy.40.1.87.109.

Keuth S, Bisping B. 1994. Vitamin B12 production by *Citrobacter freundii* or *Klebsiella pneumoniae* during tempeh fermentation and proof of enterotoxin absence by PCR. Appl Environ Microbiol. 60(5): 1495-1499. doi: 10.1128/AEM.60.5.1495-1499.1994.

Lott TJ, Burns BM, Zancope-Oliveira R, Elie CM, Reiss E. 1998. Sequence analysis of the internal transcribed spacer 2 (ITS2) from yeast species within the genus Candida. Current microbiology 36(2): 63-69. doi: 10.1007/s002849900280.

Mahmodi F, Kadir J, Puteh A, Pourdad S, Nasehi A, Soleimani N. 2014. Genetic diversity and differentiation of *Colletotrichum* spp. isolates associated with Leguminosae using multigene loci, RAPD and ISSR. The Plant Pathology Journal 30(1): 10-24. doi:10.5423/PPJ.OA.05.2013.0054.

Patterson E, Ryan PM, Cryan JF, Dinan TG, Ross RP, Fitzgerald GF, Stanton C. 2016. Gut microbiota, obesity and diabetes. Postgraduate Medical Journal 92(1087):286-300. doi: 10.1136/postgradmedj-2015-133285.

Stephanie, Kartawidjajaputra F, Silo W, Yogiara, Suwanto, A. 2018. Tempeh consumption enhanced beneficial bacteria in the human gut. Food Res. 3(1):57-63. doi: 10.26656/fr.2017.3(1).230.

Sudigbia I. 1999. Tempe in the management of infant diarrhea in Indonesia. The Complete Handbook of Tempeh: the Unique Fermented Soyfood of Indonesia 23-40.

Vágvölgyi C, Heinrich H, Ács K, Papp T. 2004. Genetic variability in the species *Rhizopus stolonifer*, assessed by random amplified polymorphic DNA analysis. Antonie van Leeuwenhoek 86(2): 181-188. doi: 10.1023/B:ANTO.0000036123.48215.fc.