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Original research article

Rhizopus Species from Fresh Tempeh Collected from Several Regions in Indonesia

Anastasia Tatik Hartanti,[1,2] Gayuh Rahayu,[1*] Iman Hidayat[3]

[1] *Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Darmaga Campus, Bogor 16680, Indonesia.*
[2] *Department of Biology, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta 12930, Indonesia.*
[3] *Research Center for Biology, Indonesian Institute of Sciences (LIPI), Jalan Raya JakartaeBogor km 46, Cibinong 16911, Indonesia.*

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ABSTRACT

Recent changes in taxonomy of *Rhizopus*, which are now heavily relying on molecular approach, create significant problem in assigning species name to particular *Rhizopus* strains isolated from various sources, including tempeh. The present study aims to determine 36 strains of *Rhizopus* from tempeh originated from 26 locations in Indonesia, using combination of molecular phylogenetic analysis based on internal transcribed spacer ribosomal DNA sequence, physiology, and morphology to species level. The results showed that most of the strains belong to *R. microsporus*–complex, and only one strain belongs to *R. delemar*. Morphological variations within *R. microsporus* were observed, but under current approach they were insufficient for infraspecific delimitation. The current report is an important contribution in validating the identity of *Rhizopus* from fresh tempeh in Indonesia.

1. Introduction

In the last 30 years, taxonomy and identification of species belonging to *Rhizopus* Ehrenb. have been significantly changed from traditional morphological and physiological approaches (Schipper 1984; Schipper and Stalpers 1984; Zheng *et al.* 2007) to molecular phylogeny–based identification method (Abe *et al.* 2006, 2010; Liou *et al.* 2007). The revision of *Rhizopus* published by Schipper (1984) and followed by Schipper and Stalpers (1984) were probably the 1st significant monographs of *Rhizopus* worldwide. These monographs provided the fundamental morphological–based identification of *Rhizopus*, which is still used until recent time (Schipper 1984; Schipper and Stalpers 1984). Three groups, *viz*, *R. microsporus*–group, *R. oryzae* Went & Prins Geerl., and *R. stolonifer*–group, were recognized (Schipper and Stalpers 1984).

In the molecular–based identification of *Rhizopus*, one of the most significant contributions was of Abe *et al.* (2006). By using molecular phylogenetic analysis based on sequence of *Rhizopus* generated from 18S, internal transcribed spacer (ITS), and 28S ribosomal DNA (rDNA) regions, they determined three major clusters, i.e. *R. microsporus*–group, *R. stolonifer*–group, and *R. oryzae* (Abe *et al.* 2006). This result was in concordance with morphological-based identification proposed by Schipper and Stalpers (1984). Similar result was also published based on D2/D2 region of large subunit rDNA (Liou *et al.* 2007). Latest monograph of *Rhizopus* by Zheng *et al.* (2007) recognized 10 species, *viz*, *R. homothallicus* Hesselt & JJ. Ellis, *R. microsporus* Tiegh., *R. stolonifer* (Ehrenb.Fr.) Lindner, *R. sexualis* (G. Smith.) Callen, *R. americanus* (Hesselt & JJ. Ellis) RY. Zheng, GQ. Chen & XY, Liu, *R. arrhizus* A. Fisch, *R. caespitosus* Schipper & Samson, *R. niveus* M. Yamaz, *R. reflexus* Bainier, and *R. schipperae* Weitzman, McGough, Rinaldi & Dell–Latta. This monograph was based on combination of sporangial and zygosporic states morphology, maximum growth temperature, mating compatibility, and molecular systematic. The most important point from Zheng *et al.* (2007) was the proposal of *R. arrhizus* to replace *R. oryzae*, the most popular *Rhizopus* species. However, based on multigene molecular phylogenetic analysis sequences generated from ITS rDNA, actin, and EF–1α regions, Abe *et al.* (2010) recognized only eight species, *viz*, *R. oryzae*, *R. delemar* (Boidin) Wehmer & Hanzawa (basionym: *R. niveus* M. Yamaz), *R. microsporus*, *R. reflexus*, *R. stolonifer*, *R. schippeae*, *R. homothallicus*, and *R. caespitosus*. In that report, *R. stolonifer* was proposed to accomodate *R. sexualis* and *R. americanus*. In the latest molecular phylogenetic analysis combined with morphology, physiology and mating-type analyses, all varieties within

* Corresponding author.
E-mail address: gayuhrahayu@gmail.com (G. Rahayu).
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R. microsporus—group described by Abe *et al.* (2006) and Zheng *et al.* (2007) were reduced into synonymy (Dolatabadi *et al.* 2014).

In Indonesia, *Rhizopus* spp. have been known as one of the economically important mould because of their role as inoculum source for making tempeh, a traditional soybean—based fermented food. However, information regarding diversity of *Rhizopus* of fresh tempeh and its inoculant has been confusing due to limited information, and outdated determination method previously used by Indonesian mycologists. For example, *R. oligosporus* has still commonly been recognized as inoculant of tempeh in Indonesia until now (Dewi and Aziz 2011; Prihatna and Suwanto 2007), although, this name was, a long time ago, transferred as var. *oligosporus* within *R. microsporus*—group by Schipper and Stalpers (1984). In relation to develop a standardization of tempeh quality in Indonesia, it is important to have an accurate name and valid identification of *Rhizopus* species. Therefore, in this study, 36 strains of tempeh inoculant from different regions in Indonesia were identified to provide an accurate and valid information regarding the taxonomy of *Rhizopus* spp. from Indonesian fresh tempeh.

2. Materials and methods

2.1. Isolates

Thirty six *Rhizopus* strains used in this study were isolated from tempeh collected from 29 locations in Indonesia (Table 1). One additional *Rhizopus* strain isolated from pear fruit was included in the analysis (IPBCC 13.1138). All strains were grown on potato dextrose agar (PDA) and incubated at room temperature. Cultures obtained in this study were deposited at Bogor Agricultural University Culture Collection (IPBCC).

2.2. DNA isolation, polymerase chain reaction (PCR) amplification, and sequencing

Four days' old mycelia grown on PDA was scrapped and used as DNA sources. DNA was extracted using Phytopure™ DNA Extraction Kit (GE Healthcare, UK) according to the manufacturer's protocol. Amplification of ITS rDNA region was performed by PCR using primer pair of ITS5 (5′—GGAAGTAAAAGTCGTAACAAGG—3′) and ITS4 (5′—TCCTCCGCTTATTGATATGC—3′) (White *et al.* 1990). All PCR amplifications were performed in a total 25 μL of reaction mixture, containing ±100 ng of DNA template, 0.25 μM of each primer, PCR buffer 1×, dNTPmix 0.2 mM, $MgCl_2$ 1.75 mM, and 1 unit of Taq DNA polymerase. The reaction condition was set as follows: 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 run in 1% agarose gel by electrophoresis at 100 V for 30 minutes, and soaked in ethidium bromide for 15 minutes. Gel was then visualized using Gel Doc™ XR + system (Bio-Rad, Germany). PCR products were sent to First BASE (Malaysia) for sequencing.

2.3. Phylogenetic analysis

Nucleotide sequence obtained from the respective primer, ITS5 and ITS4, was assembled in Chromas Pro 1.41 (Technelysium Pty Ltd., Australia). The sequences were aligned with sequences retrieved from DNA databases (DDBJ and NCBI) using multiple

Table 1. Name, code, locality, and GenBank accession number of strains used in this study

Species	Strain code	Location of sample collected	IPBCC accession number	GenBank accesion number (ITS)
R. delemar	ATH53	Palu, Central Sulawesi	IPBCC 13.1126	KF710007
R. delemar	ATHpr	Bogor, West Java	IPBCC 13.1138	KF710008
R. microsporus	ATH1	Bogor, West Java	IPBCC 13.1102	AB894622
R. microsporus	ATH9	Cilacap, Central Java	IPBCC 13.1103	AB894623
R. microsporus	ATH10	Bekasi, West Java	IPBCC 13.1104	KF709996
R. microsporus	ATH11	Sukabumi, West Java	IPBCC 13.1105	KF710000
R. microsporus	ATH13	Surabaya, East Java	IPBCC 13.1106	KF710004
R. microsporus	ATH14	Makasar, South Sulawesi	IPBCC 13.1107	KF710005
R. microsporus	ATH15	Bukit Tinggi, North Sumatra	IPBCC 13.1108	KF709999
R. microsporus	ATH23	Denpasar, Bali	IPBCC 13.1109	KF710006
R. microsporus	ATH24	Malang, East Java	IPBCC 13.1110	KF709978
R. microsporus	ATH25	Samarinda, East Kalimantan	IPBCC 13.1111	KF710001
R. microsporus	ATH26	Bogor, West Java	IPBCC 13.1112	KF709986
R. microsporus	ATH27	Surabaya, East Java	IPBCC 13.1113	KF709985
R. microsporus	ATH29	Medan, North Sumatra	IPBCC 13.1114	AB894624
R. microsporus	ATH31	Mataram, Nusa Tenggara	IPBCC 13.1115	KF709984
R. microsporus	ATH32	Pontianak, West Kalimantan	IPBCC 13.1116	KF709992
R. microsporus	ATH33	Raja Ampat, West Papua	IPBCC 13.1117	KF709997
R. microsporus	ATH35	Brebes, Central Java	IPBCC 13.1118	KF709983
R. microsporus	ATH38	Yogyakarta	IPBCC 13.1119	KF709995
R. microsporus	ATH40	Yogyakarta	IPBCC 13.1120	AB894625
R. microsporus	ATH41	Yogyakarta	IPBCC 13.1121	KF709987
R. microspores	ATH43	Lampung	IPBCC 13.1122	KF709988
R. microsporus	ATH47	Kebumen, Central Java	IPBCC 13.1123	KF710002
R. microsporus	ATH48	Magelang, Central Java	IPBCC 13.1124	KF709982
R. microsporus	ATH50	Kutoarjo, Central Java	IPBCC 13.1125	KF710003
R. microsporus	ATH54	Medan, North Sumatra	IPBCC 13.1127	KF709979
R. microsporus	ATH55	Labuhan Batu, North Sumatra	IPBCC 13.1128	AB894626
R. microsporus	ATH58	Kendari, Southest Sulawesi	IPBCC 13.1129	KF709990
R. microsporus	ATH59	Bogor, West Java	IPBCC 13.1130	KF709991
R. microsporus	ATH60	Cilacap, Central Java	IPBCC 13.1131	KF709998
R. microsporus	ATH61	Mataram, Nusa Tenggara Barat	IPBCC 13.1132	KF709989
R. microsporus	ATH63	Mataram, Nusa Tenggara Barat	IPBCC 13.1133	AB894627
R. microsporus	ATH64	Manokwari, West Papua	IPBCC 13.1134	KF709980
R. microsporus	ATH65	Nabire, West Papua	IPBCC 13.1135	KF709981
R. microsporus	ATH66	Pontianak, West Kalimantan	IPBCC 13.1136	KF709993
R. microsporus	ATH67	Jambi, Sumatra	IPBCC 13.1137	KF709994

sequence alignment based on fast Fourier transform (Katoh *et al.* 2002). *Phycomyces blakesleeanus* strain, the Centraalbureau voor Schimmelcultures (CBS) 284.35 (JN206308), was assigned as outgroup. GenBank accession number, strain code, and taxon name used in this study are given in Table 1. Phylogenetic analysis was conducted using the maximum likelihood (ML) method implemented in MEGA 5.05. Model of T92 + G + I (Tamura 3−parameter and Gamma distributed with invariant sites) was selected as the best−fit substitution model for the current analysis. Strength of the internal branches of the phylogenetic trees was tested with bootstrap (BS) analysis (Felsenstein 1985) using 1000 replications. Other parameters used in the ML analysis were selected according to the default standard in MEGA 5.05 software. BS values of 50% or higher were shown. Tree generated from ML analysis was edited in TreeGraph version 2 (Stöver and Müller 2010).

2.4. Morphology and physiology examination

To support species determination or to evaluate the possibilities of adopting infraspecific classification of Zheng *et al.* (2007) for those 35 strains in *R. microsporus*, morphological and physiological characteristics were then observed. Morphological characteristics, such as sporangiophore (length and colour), columellae (shape), sporangiospore (shape, size, and colour), and rhizoid−type were examined according to Zheng *et al.* (2007) by using light microscope Olympus™BX53 (Olympus, Japan). Measurements of sporangiophore length and sporangiospore size were made in 30 replications ($n = 30$). In physiological characterization, the ability of *Rhizopus* spp. to grow at 33 °C, 42 °C, 46 °C, 48 °C, and 51 °C was examined.

3. Results

3.1. Phylogenetic analysis

Based on the ITS tree generated from ML analysis, *Rhizopus* spp. from Indonesian tempeh were placed into *R. delemar*−clade (one strain) and *R. microsporus*−clade (35 strains) with 60% and 99% BS, respectively (Figure 1). The *R. delemar*−clade, of which containing only one *Rhizopus* strain from Indonesian tempeh, was sister to *R. oryzae*−clade with 100% BS. The strain that was obtained from pear was also in *R. delemar*−clade. The *R. microsporus*−clade was divided into two monophyletic subclades. The 1st subclade consisted of reference strains of *R. microsporus* var. *tuberosus*, *R. microsporus* var. *rhizopodiformis*, *R. microsporus* var. *oligosporus*, *R. microsporus* var. *chinensis*, and *R. microsporus* var. *azygosporus*, and all *R. microsporus* strains collected in this study. The 2nd subclade contained two sequences of reference strain of *R. microsporus* var. *microsporus* only, and none of the strains that were currently studied belongs to this subclade. The phylogenetic tree (Figure 1) clearly showed that the resolution of sequence from ITS region was not sufficient in determining *Rhizopus* species into variety level, particularly in *R. microsporus*−complex of the non-*R. microsporus* var. *microsporus*. No distinct monophyletic clade was formed within this subclade.

3.2. Morphological and physiological characters

Identification of *Rhizopus* spp. from tempeh based on morphology and physiology characteristics supported the results from the phylogenetic analyses. The *Rhizopus* strain ATH53 from tempeh was determined as *R. delemar* based on their long sporangiophores (up to 800 μm), strongly striated and pointed ends of sporangiospores, and the maximum growth temperature up to 42 °C. Further, the remaining strains that were placed within *R. microsporus*−complex had distinct characters comparing to those of *R. delemar*. The *R. microsporus* has shorter sporangiophores (38.8−267 μm long), faintly striated and not pointed ends sporangiospores, and higher maximum growth temperature (up to 48 °C). Nevertheless, these strains highly varied in their shape of columellae and sporangiospores, in the presence of azygosporangia, and in their maximum temperature growth. These characterizations supported the ITS phylogeny at species level, all the species found in this study are redescribed below.

3.2.1. *Rhizopus delemar* (Boidin) Wehmer & Hanzawa

Colonies on PDA at 1st are white, becoming grey to blackish grey when mature, covering the plate [9 cm in diameter (diam.)] about 3−4 days at room temperature. Stolons well-developed, subhyaline to grayish brown, septate or not septate. Rhizoids sometimes absent, or finger−like or branched when present, unequal in length, grayish brown, paler at the tip. Sporangiophores arising from stolon and opposite rhizoids, sometimes arising directly from mycelia and without rhizoids, solitary or 2−3 in groups (Figure 2A), simple, straight to slightly curved, 403.2−812.0 μm long, 7.4−12.1 μm wide, light brown to dark brown, aseptate, smooth to verruculose, sometimes forked or trifurcate at the apex and swollen at the middle (Figure 2B). Apophyses conspicuous. Sporangia globose to subglobose (55−185 μm diam.), yellowish to dark brown, without collar (Figure 2C). Columellae ovoid (41.1−85.0 μm × 37.6−85.7 μm), smooth, light brown. Sporangiospores ovoid, sometimes subglobose, smooth, with distinct striation (4.8−9.5 μm diam.), subhyaline, becoming dark grey in mass (Figure 2D). Chlamydospores not seen and zygosporic state not found.

Material examined: Indonesia, Central Sulawesi: Palu, from tempeh, 28 August 2012, AT Hartanti, ATH53 (IPBCC 13.1126); West Java province: Bogor, from pear (*Pyrus* sp.), 28 August 2012, AT Hartanti, ATHpr (IPBCC 13.1138).

3.2.2. *Rhizopus microsporus*−complex

Colonies on PDA at 1st are white, becoming brownish, brownish grey, grey to blackish when mature, covering the plate (9 cm diam.) about 3−4 days at room temperature. Stolons well-developed, subhyaline to light brown or grayish brown, septate or not septate, sometimes swollen at the point where rhizoids are formed. Rhizoids mostly simple or sometimes branches, unequal in length or variable in length (very short to comparatively long), grayish brown, paler at the tip. Sporangiophores arising from stolon and opposite rhizoids, or directly from aerial hyphae and not opposite rhizoids, solitary or 2−3 in groups, simple, straight to slightly curved, rarely forked at the apical part (38.8−267.0 μm long, 3.9−17.0 μm wide), light brown, paler at the apex, usually aseptate, and smooth. Apophyses conspicuous or shallow. Sporangia globose to depressed globose (24−110 μm diam.), yellowish, dark brown when mature, with a small conspicuous collar or without collar. Sporangia rapidly deliquescent. Columellae variable in shape, mostly roundish to depressed globose, occasionally pyriform to oblong−ovoid (23.8−64.1 μm long × 17.6−43.8 μm wide), smooth, subhyaline to light brown. Sporangiospores vary or uniform in size and shape, ovoid to subglobose (2.7−6.4 μm long × 3.3−4.6 μm wide), smooth, without or slightly striated, subhyaline, becoming grayish brown or dark grey in mass. Chlamydospores solitary or often in short chains, globose to irregular (12.0−66.5 μm × 4.0−18.9 μm). Azygosporangia occasionaly found and zygosporic state not found.

Material examined: Indonesia, from tempeh, AT Hartanti. North Sumatra province: Medan, 4 September, 2012, ATH54 (IPBCC 13.1127) and 23 May 2012, ATH29 (IPBCC 13.1114); Labuhan Batu, 28 August 2012, ATH55 (IPBCC 13.1128). West Sumatra province: Bukit Tinggi, 17 February 2012, ATH15 (IPBCC 13.1108). Jambi province: Jambi, 26 December 2012, ATH67 (IPBCC 13.1137). Lampung: Lampung, 7 July 2012, ATH43 (IPBCC 13.1122); West Java province: Bogor, 2 February 2012, ATH1 (IPBCC 13.1102); 5 May

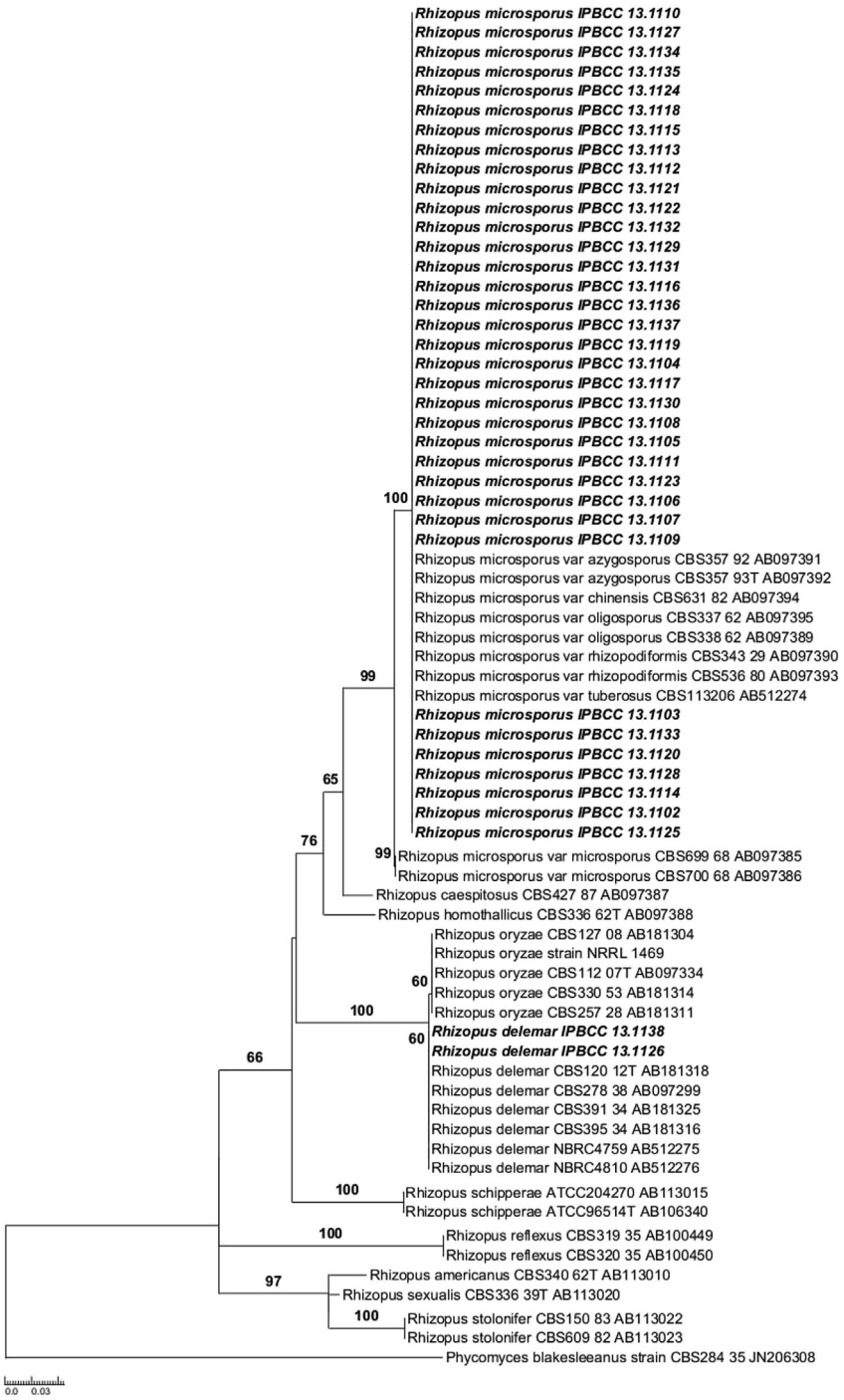

Figure 1. A maximum likelihood (ML) tree based on sequence data of internal transcribed spacer region for 37 strains of *Rhizopus* from Indonesia, 31 *Rhizopus* from GenBank, and single outgroup taxon. Bootstrap values (>50%) for ML analysis are given above nodes.

2012, ATH26 (IPBCC13.1112); 17 February 2012, ATH59 (IPBCC 13.1130); Bekasi, 10 February 2012, ATH10 (IPBCC 13.1104); Sukabumi, 13 February 2012, ATH11 (IPBCC 13.1105); Central Java province: Brebes, 7 July 2012, ATH35 (IPBCC 13.1118); Kebumen, 12 July 2012, ATH47 (IPBCC13.1123); Magelang, 12 July 2012, ATH48 (IPBCC 13.1124); Kutoarjo, 12 July 2012, ATH50 (IPBCC 13.1125); Cilacap, 8 February 2012, ATH9 (IPBCC 13.1103); 1 September 2012, ATH60 (IPBCC13.1131). Special region of Yogyakarta: Yogyakarta, 7 July 2012, ATH41 (IPBCC 13.1121), 11 July 2012, ATH40 (IPBCC13.1120) and 17 July 2012, ATH38 (IPBCC 13.1119). East Java province: Surabaya, 13 February 2012, ATH13 (IPBCC 13.1106); 21 May 2012, ATH27 (IPBCC 13.1113); Malang, 23 April 2012, ATH24 (IPBCC 13.1110). South Sulawesi province: Makassar, 17 February 2012, ATH14 (IPBCC 13.1107); Southeast Sulawesi province: Kendari, 28 August 2012, ATH58 (IPBCC 13.1129). Bali province: Denpasar, 21 February 2012, ATH23 (IPBCC 13.1109). East Kalimantan province: Samarinda, 1 May 2012, ATH25 (IPBCC 13.1111). West Kalimantan province: Pontianak, 9 June 2012, ATH32 (IPBCC13.1116), and 20 September 2012, ATH66 (IPBCC 13.1136). Nusa Tenggara Barat province: Mataram, 25 May 2012, ATH31

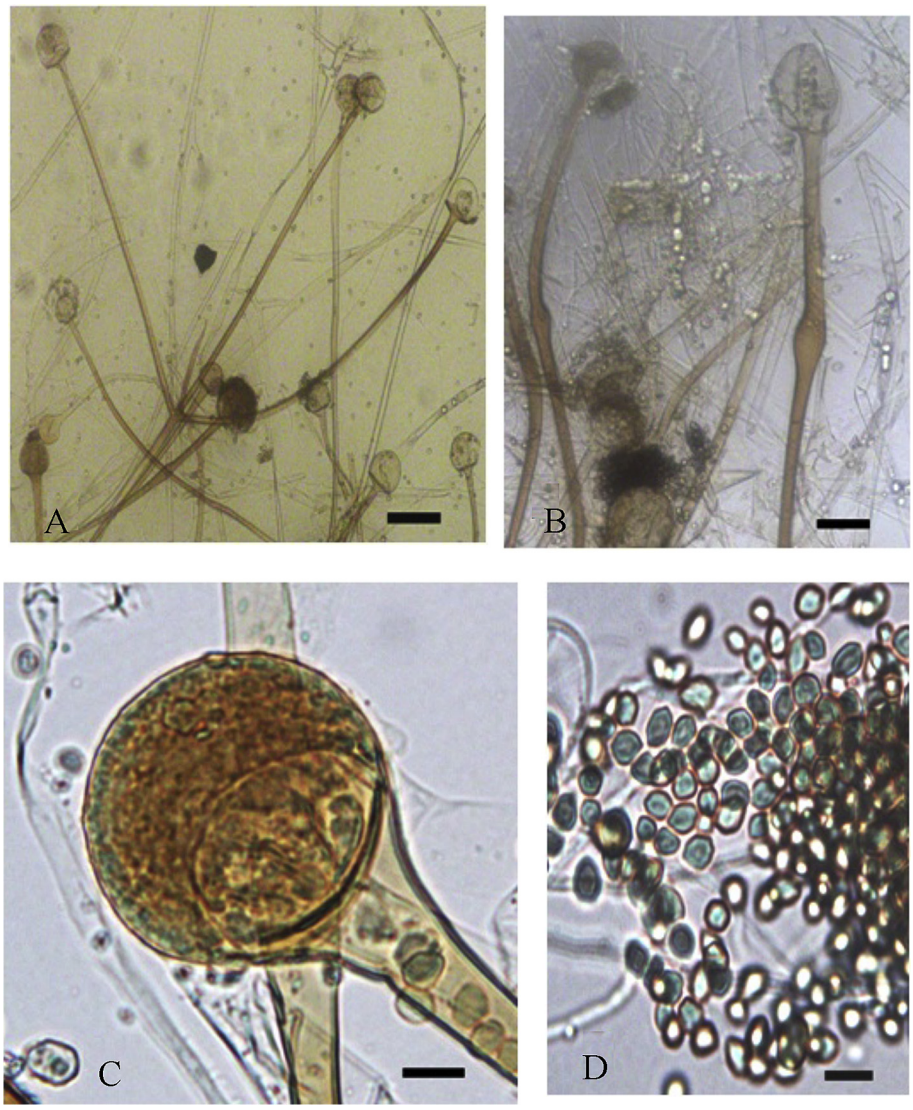

Figure 2. *R. delemar* strain from tempeh in Indonesia. (A) Sporangiophores (three, in groups) arising from mycelia with ovoid-oblong columellae and distinct apophyses (arrow). (B) Swollen sporangiophores. (C) Globose sporangium. (D) Irregular shape of sporangiospores. Bars in (A) = 100 μm, (B) = 20 μm, (C) = 10 μm, and (D) = 10 μm.

(IPBCC 13.1115), 4 September 2012, ATH61 (IPBCC 13.1132) and 4 September 2012, ATH63 (IPBCC 13.1133). West Papua province: Raja Ampat, 27 May 2012, ATH33 (IPBCC 13.1117); Manokwari, 4 September 2012, ATH64 (IPBCC 13.1134); Nabire, 4 September 2012, ATH65 (IPBCC 13.1135).

The description above accomodates all variations observed in *R. microsporus* isolated from fresh tempeh. Most strains (32 strains) collected had typical colony colour (brownish grey when mature) on PDA, simple rhizoids (Figure 3A), roundish to depressed globose columellae (Figure 3B), variable size and shape of sporangiospores (Figure 3C), neither azygospore nor zygospore present, and maximum growth temperature up to 46 °C. These characters are in accordance with that of *R. microsporus* var. *oligosporus* sensu Zheng *et al.* (2007).

The characteristics of strain ATH40 and ATH59 conformed with *R. microsporus* var. *rhizopodiformis* sensu Zheng *et al.* (2007). Their colonies on PDA become grey to blackish grey with well-developed and very abundant rhizoids (Figure 3D), typical pyriform columellae when mature (Figure 3E), and uniform in size and shape (Figure 3F) and without striation sporangiospores. The chlamydospores are solitary or often in short chains (Figure 3G). No azygosporangia and zygosporangia were found. The maximum growth temperature was up to 48 °C.

Strain ATH24 clearly resembled *R. microsporus* var. *azygosporus* sensu Zheng *et al.* (2007). This strain has ovoid to oblong columellae (Figure 3H) and produced many globose to subglobose, hyaline, crenulate azygospore (Figure 3I), with a single light brown suspensor that was mostly constricted at the base. The zygospore was not found. The colony of this strain on PDA was grey to blackish grey when mature. The maximum growth temperature was 48 °C. The columellae are variable in shape from pyriform to oblong–ovoid, and the sporangiospores are uniform in size and shape and faintly striated.

4. Discussion

The majority of *R. microsporus*–complex members are widely recognized as having a close association with soybean–based fermented foods, such as tempeh (Indonesia), koji (Japan and China) (Zheng *et al.* 2007). Tempeh, a soybean–based traditional fermented food, has been consumed as a main source of protein by Indonesians for years. All specimens associated with fresh tempeh samples from

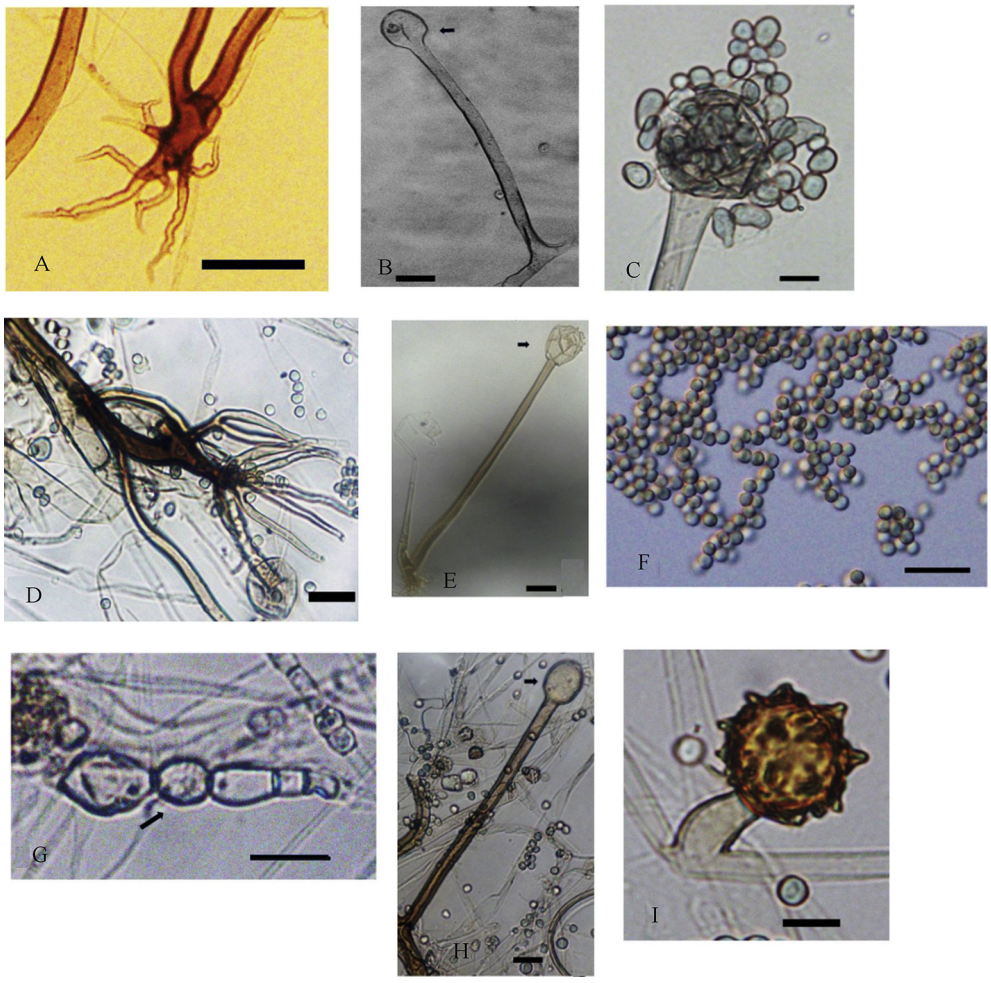

Figure 3. *R. microsporus* isolated from tempeh in Indonesia. (A) Simple rhizoid. (B) General characteristics of sporangiophore arising from stolon with depressed globose columellae (arrow). (C) Various shapes of sporangiospores. (D) Well-developed rhizoids. (E) Sporangiophore arising from stolon with pyriform shape columellae. (F) Uniform shape and size of sporangiospores. (G) Chlamydospores in chain. (H) Sporangiophore arising from stolon with ovoid-oblong columellae (arrow). (I) Azygospore with a single suspensor. Bars in (A) and (E) = 50 μm; (B), (C), and (I) = 10 μm; and (D) and (F–H) = 20 μm.

Indonesia in this study belonging to *R. delemar* and *R. microsporus*. Indeed, several members of *Rhizopus*, such as *R. oligosporus*, *R. oryzae*, *R. arrhizus*, and *R. stolonifer* were previously reported in Indonesia from inoculants of tempeh (ragi) and from fresh tempeh (Dewi and Aziz 2011; Dwidjoseputro and Frederick 1970; Prihatna and Suwanto 2007; Saono *et al.* 1974). In the current systematic of *Rhizopus*, *R. arrhizus* is treated as a synonym of *R. oryzae* as proposed by Abe *et al.* (2010) and *R. oligosporus* is treated as a synonym of *R. microsporus* as proposed by Dolatabadi *et al.* (2014). Therefore, *R. oryzae* and *R. stolonifer* are the remaining species that could not be found in this study. This finding indicates that commercialization of inoculant of tempeh (ragi) by using certain *Rhizopus* species in Indonesia possibly threatens the genetic diversity of *Rhizopus* associated with this traditionally soybean–based fermented foods. However, to verify whether *R. stolonifer* and *R. oryzae* have lost from tempeh in Indonesia, it is necessary to examine more samples in many regions of Indonesia. Re-examination of isolates published by Prihatna and Suwanto (2007) along with Dewi and Aziz (2011) is also important in the further study.

The majority of tempeh samples contained *R. microsporus*. One of the fresh tempeh sample originated from Palu (Central Sulawesi) associated with *R. delemar*. Surprisingly, this study did not find any *R. delemar* on fresh tempeh from Java island as *R. delemar* (as *R. oryzae*) were previously reported by Indonesian researchers (Dwidjoseputro and Frederick 1970) on tempeh from different localities in Java, such as Jakarta (Special City District) and Surabaya, Malang (East Java province) and by Zheng *et al.* (2007) such as CBS 385.34, Institute for Fermentation Osaka (IFO) IFO4770, HUT 1220 and 1223. Abe *et al.* (2007) recognised *R. delemar* as *R. oryzae* that produce fumaric–malic acid. The *R. delemar* (written as *R. oryzae*) and *R. microsporus* (written as *R. oligosporus*) were commonly used as tempeh's inoculant at that time (Dwidjoseputro and Frederick 1970). Our survey, indicated that tempeh producers in Java and Sumatera islands use "Ragi Raprima®", an industrial made and commercialized tempeh inoculant. The "Ragi Raprima®" is produced in Bandung, West Java by using *R. microsporus* var. *oligosporus* (synonym *R. microsporus*) as inoculum. These might relate to the fact that no fresh tempeh from Java associated with *R. delemar*.

Based on the morphology and physiology characteristics, *R. microsporus* strains from this study resemble several varieties described by Zheng *et al.* (2007). These include *R. microsporus* var. *oligosporus*, var. *rhizopodiformis* and var. *azygosporus*. The ex–type cultures of *R. microsporus* var. *oligosporus* (CBS 337.62) and *R. microsporus* var. *azygosporus* (CBS 357.93) are indeed originated

from tempeh in Indonesia (Schipper 1984; Schipper and Stalpers 1984; Zheng *et al.* 2007). Another variety, *R. microsporus* var. *rhizopodiformis* (CBS 388.34) was firstly reported from Indonesia as an inoculant of ragi (Zheng *et al.* 2007).

The remaining sequences within the *R. microsporus* clade (var. *chinensis*, var. *microsporus*, and var. *tuberosus*) have never been reported to be found in Indonesia. *Rhizopus microsporus* var. *chinensis* and *R. microsporus* var. *tuberosus* have been reported from Chinese Koji (China) (Zheng *et al.* 2007). While *R. microsporus* var. *microsporus* has commonly been known as fungal pathogen on human that frequently causing fatal infectious diseases called mucormycosis (West *et al.* 1995). The *R. microsporus* var. *microsporus* is a common soil-borne fungus, and also often isolated from manure (Zheng *et al.* 2007). Prihatna & Suwanto (2007) stated that the physiological characters of *R. oligosporus*, now recognized as *R. microsporus* sensu Dolatabadi *et al.* (2014), did not relate to their DNA fingerprinting phenotype. Furthermore, Dolatabadi *et al.* (2014) found that infraspecific classification was not supported by phylogenetic analysis. They stated that it is inappropriate to divide *R. microsporus* into infraspecific taxa on the basis of the concept of Genealogical Concordance Phylogenetic Species Recognition (*ITS* gene, *actin* gene, and *EF 1-α* gene), the physiological properties including growth temperature, spore morphology, mating-type tests, generated MALDI-ToF profiles, and ecological grouping. As this study adopted Dolatabadi *et al.* (2014) concept of *R. microsporus*, only two species of *Rhizopus*, namely *R. delemar* and *R. microsporus* were recognised to associate with fresh tempeh from Indonesia.

The impact of heavy commercialization of tempeh inoculant using particular species of *Rhizopus* has shifted the diversity of *Rhizopus* species associated with tempeh. Reduction of genetic diversity of *Rhizopus* in tempeh is predicted to be affecting the quality of tempeh. It is probably due to different species of *Rhizopus* provide different valuable metabolites for human health. For example, *R. oligosporus* produces phytase that degrades phytate and consequently increases the availability of several minerals such as iron, magnesium, and zinc which are strongly bound to phytate. *Rhizopus oligosporus* is also known for producing ergosterol (provitamin D2) and some vitamins (Feng *et al.* 2007). The effect of different species of *Rhizopus* on the nutritional quality of tempeh has not widely been studied. However, utilization of *R. stolonifer* and *R. oryzae* as tempeh inoculants, at least, change the texture, aroma and the colour of tempeh (Omosebi and Otunola 2013).

In addition, several ex−type cultures of *Rhizopus* species from Indonesia have neither been preserved nor available in microbial culture collection institutions in Indonesia, but these ex−type cultures are available in other countries culture collection, such as the CBS, Netherland, IFO, Japan (Schipper 1984; Schipper and Stalpers 1984; Zheng *et al.* 2007). Above all, conservation of Indonesian economically important microbial genetic resources is a serious issue. Further inventory of *Rhizopus* from tempeh in many other areas in Indonesia is therefore urgently needed to save the diversity of tempeh associated *Rhizopus* resources.

Conflict of interest

The authors declare no conflict of interest. All experiments in this study comply with the current law of the country where they were performed.

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