



[doi:10.1016/j.mycres.2006.10.003](https://doi.org/10.1016/j.mycres.2006.10.003) | [How to Cite or Link Using DOI](#)

Copyright © 2006 The British Mycological Society Published by Elsevier Ltd.

[Permissions & Reprints](#)

Polyphasic approach to the taxonomy of the *Rhizopus stolonifer* group

[Guey-Yuh Liou^a](#),  , [Shyue-Ru Chen^a](#), [Yu-Hui Wei^a](#), [Fwu-Ling Lee^a](#), [Hui-Mei Fu^a](#), [Gwo-Fang Yuan^a](#) and [Joost A. Stalpers^b](#)

^aBioresource Collection and Research Center, Food Industry Research and Development Institute, P.O. Box 246, Hsinchu 30062, Taiwan

^bCentraalbureau voor Schimmelcultures, P.O. Box 85167, 3508 AD Utrecht, The Netherlands

Received 8 May 2006;

revised 22 September 2006;

accepted 4 October 2006.

Corresponding Editor: H. Thorsten Lumbsch.

Available online 24 February 2007.

Abstract

Phylogenetic analysis of *Rhizopus* strains based on the D1/D2 region of LSU rDNA sequences yielded a phylogram with four well-supported clades. The *R. microsporus* clade concurs with classification obtained by traditional methods. The *R. oryzae* group was found to include species of the genus *Amylomyces*. The traditional *R. stolonifer* group was divided into two well-supported clades in the phylogram, with one clade comprising *R. stolonifer* var. *stolonifer*, *R. sexualis* var. *sexualis*, and *R. sexualis* var. *americanus* and the other clade comprising taxa with recurved sporangiophores; *R. reflexus*, *R. stolonifer* var. *lyococcus*, and *R. circinans*, identifying recurved sporangiophores as an important taxonomic character. The molecular data supported the recognition of this clade at the species level: *R. lyococcus* (basonym: *Sporotrichum lyococcum*).

Keywords: DNA composition; DNA relatedness; LSU rDNA sequence; *Mucoraceae*; *Rhizopus*

Article Outline

[Introduction](#)

[Materials and methods](#)

[Fungal strains and preparation of genomic DNA](#)

[PCR amplification and direct DNA sequencing of rDNA](#)

[Molecular phylogenetic analysis](#)

[Determination of DNA base composition](#)

[DNA relatedness](#)

[Morphological characteristics](#)

[Results](#)

[Phylogenetic analysis](#)

[DNA composition and DNA relatedness](#)

[Morphological characteristics of *R. stolonifer* and related species](#)

[Taxonomy](#)

[Discussion](#)

[Group concept of *Rhizopus*](#)

[Taxonomy of *Rhizopus sexualis*](#)

[Taxonomy of *Rhizopus* taxa with recurved sporangiophores](#)

[Acknowledgements](#)

[References](#)

Introduction

Members of the fungal genus *Rhizopus* are traditionally used in the preparation of fermented foods in Asia, such as tempeh, peka, and ragi. Recently, the industrial production of enzymes using *Rhizopus* has been investigated ([Kumar et al. 2004](#)). Some species are important post-harvest pathogens that contribute to considerable spoilage of agricultural output ([Vágvölgyi et al. 2004](#)) and contamination of food and animal feed through the production of mycotoxins and other biologically active compounds ([Jennessen et al. 2005](#)). Some *Rhizopus* species are also associated with human disease ([\[Schipper et al., 1996\]](#) and [\[Voigt et al., 1999\]](#)). *Rhizopus* was monographed by [Schipper \(1984\)](#) and [Schipper & Stalpers \(1984\)](#). [Schipper \(1984\)](#) divided the genus into three groups; the *R. stolonifer* group, the *R. oryzae* group, and the *R. microsporus* group, according to the characteristics of the sporangial apparatus and growth temperatures. Thereafter, the characteristics of morphology and growth temperature and the data from mating experiments became the basic criteria for the species definition of *Rhizopus*. Moreover, DNA renaturation for *R. oryzae* ([Ellis 1985](#)) and *R. microsporus* ([Ellis 1986](#)), the proteins of sporangiospores ([Seviour et al. 1985](#)), and antigenic studies for the *R. microsporus* group ([Polonelli et al. 1988](#)) were taken as additional criteria in defining taxa.

In recent years, isozyme analysis and RAPD have been applied in taxonomic studies of *Rhizopus* species. Isozyme analysis has been used to describe the intra- and inter-specific variation ([Liou et al. 2001](#)). RAPD analysis revealed genetic variability in the species *R. stolonifer* ([Vágvölgyi et al. 2004](#)). A specific taxonomic problem was addressed by these techniques; *R. reflexus*, and *R. stolonifer* var. *lyococcus* differ from *R. stolonifer* var. *stolonifer*, suggesting they are separate species. However, the phylogenetic relationships among groups of *Rhizopus* and the criteria in defining species of the *R. stolonifer* group still remain unclear.

Combinations of phenotypic and genotypic characters, such as rDNA sequence, nuDNA base composition, and DNA–DNA reassociation values, have been successfully used in yeast taxonomy. Molecular genetic approaches are now generally used for filamentous fungi too, and are fundamental for modern taxonomy ([\[Binder and Hibbett, 2002\]](#), [\[O'Donnell et al., 2001\]](#), [\[Kurtzman et al., 1986\]](#), [\[Lutzoni et al., 2004\]](#), [\[Moncalvo et al., 2002\]](#), [\[Tamura et al., 1999\]](#) and [\[Voigt et al., 1999\]](#)). The purpose of the present study is to: (1) analyse the phylogenetic relationship of *Rhizopus* species based on D1/D2 region of LSU (28 S) rDNA sequences to

evaluate the validity of the taxonomic classification by morphological characteristics; and (2) use a polyphasic approach to explore species characterisation within the *Rhizopus stolonifer* group.

Materials and methods

Fungal strains and preparation of genomic DNA

Strains used in this study were maintained at the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan, [Table 1](#) lists the names of the strains, the corresponding BCRC numbers, and the original source.

Table 1. Strains used in this study

Species/strain no.	Equivalent strain designation	Source	GenBank accession numbers
<i>Amylomyces rouxii</i> Calmette 1976			
BCRC 32232	ATCC 22962; NRRL 3187		DQ466611
BCRC 32233 ^{NT}	ATCC 42543; CBS 438.76; NRRL 5866	Sweet fermented glutenous rice, Thailand	DQ466612
BCRC 32719		Peka, Taiwan	DQ466616
<i>Rhizopus azygosporus</i> G.F. Yuan & S.C. Jong 1984			
BCRC 31158	ATCC 48108; CBS 357.93	Tempeh, Java, Indonesia	DQ466597
NRRL 28627	CBS 359.92	Liver of premature baby, South Australia	AF113477^a
<i>Rhizopus caespitosus</i> Schipper & Samson 1994			
BCRC 33515	CBS 427.87	Naina Tal, India	DQ466604
<i>Rhizopus circinans</i> Tiegh. 1878			
BCRC 33727	ATCC 52315; NRRL 1475		DQ466608
<i>Rhizopus homothallicus</i> Hesselt. & J.J. Ellis 1962			
BCRC 31146	ATCC 42221; CBS 336.62; NRRL 2538	Soil, Guatemala	DQ466605

Species/strain no.	Equivalent strain designation	Source	GenBank accession numbers
<i>Rhizopus microsporus</i> var. <i>microsporus</i> Tiegh. 1875			
BCRC 31140	ATCC 52813; CBS 699.68	Soil, Ukraine	DQ466601
BCRC 31141	ATCC 52814; CBS 700.68	Forest soil, Republic of Georgia	DQ466602
BCRC 33706	NRRL 2915		DQ466603
NRRL 28775			AF113479^a
<i>Rhizopus microsporus</i> var. <i>chinensis</i> (Saito) Schipper & Stalpers 1984			
BCRC 31148	ATCC 11559; CBS 344.29	USSR	DQ466599
NRRL 28629			AF113478^a
<i>Rhizopus microsporus</i> var. <i>oligosporus</i> (Saito) Schipper & Stalpers 1984			
NRRL 2710		Tempeh, Indonesia	AF157212^b
BCRC 31996	ATCC 22959; CBS 338.62; NRRL 2710		DQ466596
<i>Rhizopus microsporus</i> var. <i>rhizopodiformis</i> (Cohn) Schipper & Stalpers 1984			
BCRC 31994	CBS 343.29	USSR	DQ466598
NRRL 28630			AF113480^a
<i>Rhizopus microsporus</i> var. <i>tuberosus</i> R.Y. Zheng & G.Q. Chen 1998			
BCRC 33955 ^T	NBRC 100014	Chinese Koji, Taishan Country, Guangdong Prov., China	DQ466600
<i>Rhizopus oryzae</i> Went & Prins. Geerl. 1895			
BCRC 31145 ^T	ATCC 56536; CBS 112.07; NRRL 3133	The Netherlands	DQ466613
BCRC 32521	ATCC 6227b; CBS 382.52; NRRL 12939		DQ466617

Species/strain no.	Equivalent strain designation	Source	GenBank accession numbers
BCRC 33497	CBS 120.12	Japan	DQ466615
BCRC 33498	CBS 395.34	Japan	DQ466614
NRRL 28631			AF113481^a
<i>Rhizopus reflexus</i> Bainier 1880			
BCRC 31138	CBS 319.35; ATCC 44468		DQ466609
BCRC 31139	CBS 320.35; ATCC 44469		DQ466610
<i>Rhizopus schipperae</i> Weitzman, McGough, Rinaldi & Della-Latta 1996			
BCRC 33517	ATCC 96514; CBS 138.95	Human lung, Texas, U. S. A.	DQ466606
<i>Rhizopus sexualis</i> var. <i>sexualis</i> (G. Sm.) Callen 1940			
BCRC 32004	CBS 123.64	Decaying fruit of <i>Fragaria</i> sp., UK	DQ466592
BCRC 32005 ^T	CBS 336.39; NRRL 2567	Decaying fruit of <i>Fragaria</i> sp., Cambridge, UK	DQ466590
BCRC 31801	ATCC 36510	Surface of strawberry, England	DQ466591
<i>Rhizopus sexualis</i> var. <i>americanus</i> Hesselt. & J.J. Ellis 1962			
BCRC 32007 ^T	ATCC 56269; CBS 340.62; NRRL 2626	Air, Manhattan, Kansas, U.S.A.	DQ466593
<i>Rhizopus stolonifer</i> (Ehrenb.: Fr.) Vuill. 1902 var. <i>stolonifer</i>			
BCRC 32002	CBS 347.49	Tempeh	DQ466595
BCRC 32003	CBS 263.28	Mushroom spawn, The Netherlands	DQ466594
NRRL 1477			AF113482^a
<i>Rhizopus stolonifer</i> var. <i>lyococcus</i> (Ehrenb.: Fr.) Stalpers & Schipper 1984			

Species/strain no.	Equivalent strain designation	Source	GenBank accession numbers
BCRC 33817	CBS 398.95; JCM 5589		DQ466607
<i>Mucor amphibiorum</i> Schipper (1978)			
NRRL 28633			AF113466^a
<i>Mucor circinelloides</i> f. <i>lusitanicus</i> (Bruderl.) Schipper (1976)			
NRRL 3631			AF113467^a
<i>Mucor indicus</i> Lendn. (1930)			
NRRL 28634			AF113469^a
<i>Mucor hiemalis</i> f. <i>hiemalis</i> Wehmer (1903)			
NRRL 3624		Michigan, USA	AF113468^a
<i>Mucor mucedo</i> Fresen. (1850)			
NRRL 3635			AF113470^a
<i>Mucor racemosus</i> Fresen. (1850)			
NRRL 3640			AF113471^a
<i>Mucor ramosissimus</i> Samouts. (1927)			
NRRL 3042		Nasal lesion of man	AF113472^a
<i>Mucor recurvus</i> var. <i>indicus</i> Baijal & B.S. Mehrotra (1966)			
NRRL 3247			AF157200^b

^{NT}, ex-neotype strain; ^T, ex-type strain; ATCC, American Type Culture Collection, USA; BCRC, Bioresource Collection and Research Centre, Food Industry Research & Development Institute, Hsinchu, Taiwan. CBS, Centraalbureau voor Schimmelcultures; Utrecht, The Netherlands; NBRC, NITE Biological Resource Centre, National Institute of Technology and Evaluation, Japan; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL USA.

^a [Voigt et al. \(1999\)](#).

^b [O'Donnell et al. \(2001\)](#).

Strains were inoculated on malt extract agar (2 % malt extract DIFCO (Foster city, CA, USA), 2 % glucose, 0.1 % peptone, 2 % agar) and incubated at 25 °C for 3–7 d for activation, before being transferred to potato dextrose broth (DIFCO 254920) and then incubated at 25 °C for 3 d to collect mycelia. Additionally, DNA for PCR was extracted using the Chelex method ([Chen 1998](#)).

PCR amplification and direct DNA sequencing of rDNA

The D1/D2 region of LSU rDNA was defined using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5'-GGTCCGTGTTTCAAGACGG) ([O'Donnell 1992](#)). The reaction was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using 30 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min with an initial denaturation at 94 °C for 5 min before cycling and a final extension at 72 °C for 7 min following cycling. PCR products were purified using the QIAquick[®] PCR Purification Kit (QIAGEN[®], Hilden, Germany). Sequencing reactions were performed using the ABI PRISM[™] BigDye[™] Terminator v3.0 Ready Reaction Cycle Sequencing Kit as directed by the manufacturer. Finally, the PCR products were sequenced using the ABI PRISM[™] (Applied Biosystems) 3700 DNA analyser.

Molecular phylogenetic analysis

Sequence ambiguities were identified by comparisons with their opposite strand sequences. The LSU rDNA partial sequences of each strain were aligned using CLUSTAL X 1.83 ([Thompson et al. 1997](#)). The alignment of all sequences was visually assessed and optimised where necessary. Phylogenetic evaluation was accomplished by applying DNADIST and NEIGHBOUR programs from the PHYLIP 3.63 package ([Felsenstein 2004](#)). Phylogenetic trees were rooted with *Mucor recurvus* var. *indicus*. Finally, a BS analysis with 1K replications was performed by applying the SEQBOOT program from the PHYLIP 3.63 package. Trees were viewed using TreeView 1.6.6 ([Page 1996](#)).

Determination of DNA base composition

The isolation and purification of DNA for DNA base composition as expressed in the G + C ratio and DNA relatedness were performed by Genomic DNA Buffer Set and Genomic-tip 500/G (QIAGEN[®]). The purified DNA was hydrolysed to nucleosides as described by [Tamaoka and Komagata \(1984\)](#), and the hydrolysate was analysed by reverse phase HPLC to determine DNA base composition (mol% G + C).

DNA relatedness

DNA–DNA reassociation values expressing the level of DNA relatedness were determined by the photobiotin-labelling microplate method ([\[Lee et al., 1994a\]](#) and [\[Lee et al., 1994b\]](#)). This

method was modified by Kaneko & Banno ([Kaneko & Banno 1991](#)) from that described by Ezaki and colleagues ([\[Ezaki et al., 1988\]](#) and [\[Ezaki et al., 1989\]](#)). DNA from reference strains of *Rhizopus* was labelled with photobiotin to detect DNA relatedness to other DNAs. The main modification involved immobilisation of heat-denatured *Rhizopus* DNA (400 ng) in a microplate well at 28 °C overnight, followed by hybridisation at 39 °C for 24 h. The values represent the averages of two to three determinations.

Morphological characteristics

The established taxonomic system of *Rhizopus* ([\[Schipper, 1984\]](#) and [\[Schipper and Stalpers, 1984\]](#)) was followed for identification of strains. The strains were cultured on Malt extract agar at 25 °C. Slide mounts were prepared and examined under a light microscope (Axiophot, Zeiss, Oberkochen, Germany). Response to temperature was determined by measuring the diameters of colonies on agar plates incubated at temperatures of 25 °C, 30 °C, 33 °C, and 35 °C.

Results

Phylogenetic analysis

The result of the NJ analysis is illustrated in [Fig 1](#). The genus *Rhizopus* appeared monophyletic and falls into four clades with high support using NJ analysis. The species belonging to the *R. microsporus* group form clade A. Clade B comprises *R. oryzae* and *Amylomyces rouxii*. Clade C contains *R. stolonifer* var. *stolonifer*, *R. sexualis* var. *sexualis*, and *R. sexualis* var. *americanus* and the species with recurved sporangiophores, *R. stolonifer* var. *reflexus*, *R. stolonifer* var. *lyococcus*, and *R. circinans*, form clade D.

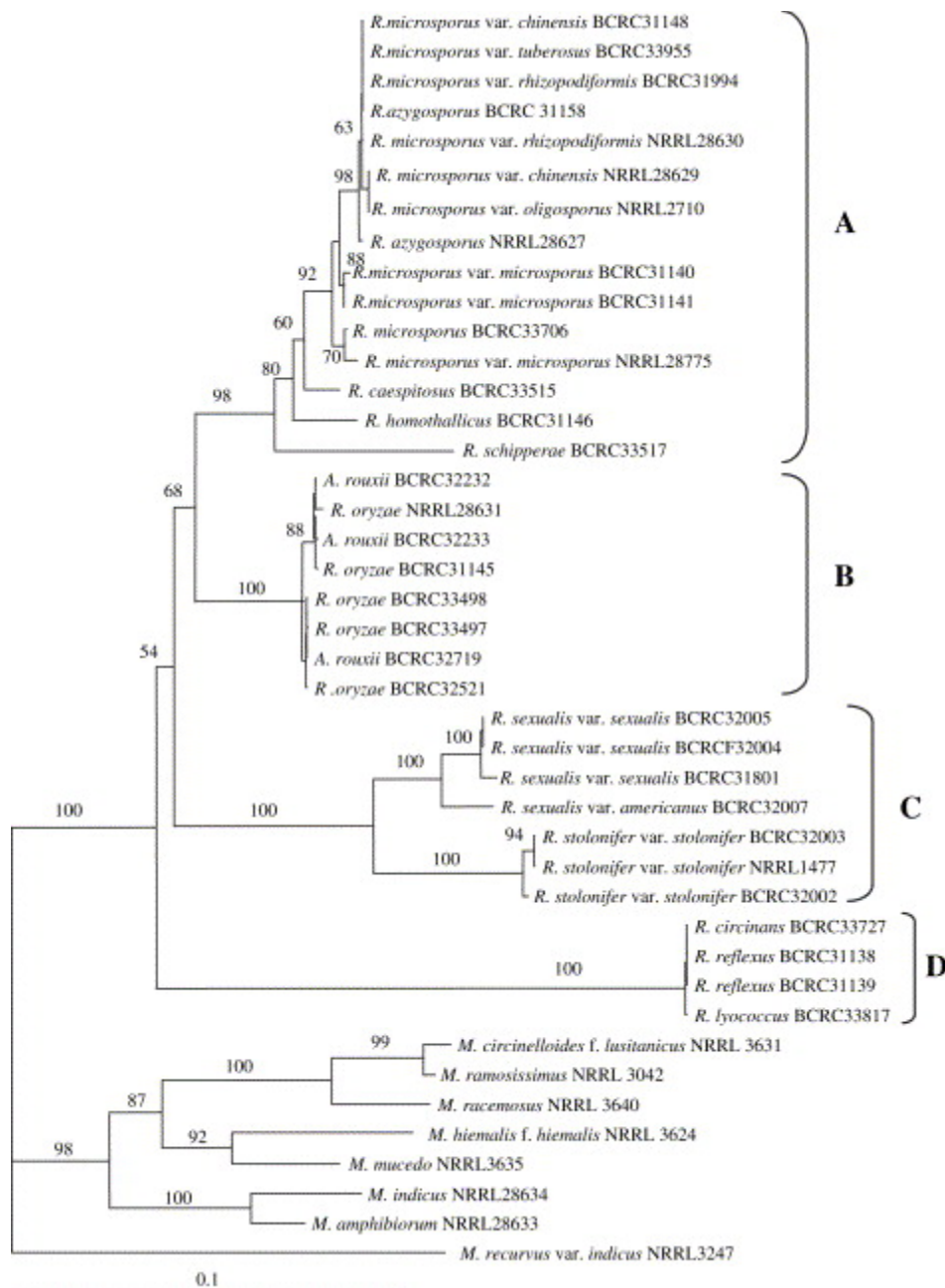


Fig 1. NJ tree of *Rhizopus* strains inferred from 622 nucleotides from LSU rDNA D1 and D2 region. Topology was rooted with *Mucor recurvus* var. *indicus* NRRL3247. Numbers given on branches indicate the confidence level from 1K replicates BS sampling.

DNA composition and DNA relatedness

[Table 2](#) lists the nuDNA base compositions and the result of DNA relatedness between type strains of *R. sexualis* var. *sexualis* and *R. sexualis* var. *americanus*. The G + C contents of the

two varieties of *R. sexualis* were 30.8 and 34.4 mol%. The degree of the reassociation between the DNAs from the type strains of *R. sexualis* var. *sexualis* and *R. sexualis* var. *americanus* was 76 %.

Table 2. DNA base composition and nuclear DNA relatedness between *Rhizopus sexualis* var. *sexualis* and *R. sexualis* var. *americanus*

Species/strain no.	G + C (mol %)	Percent relative binding of DNA from	
		BCRC 32005 ^T	BCRC 32007 ^T
<i>R. sexualis</i> var. <i>sexualis</i>			
BCRC 32005 ^T	30.8	100 %	
<i>R. sexualis</i> var. <i>americanus</i>			
BCRC 32007 ^T	34.4	76 %	100 %

[Table 3](#) lists the nuDNA base compositions and the result of DNA relatedness among strains of *R. stolonifer* and *Rhizopus* with recurved sporangiophores; *R. reflexus*, *R. stolonifer* var. *lyococcus* and *R. circinans*. *R. stolonifer* var. *stolonifer* strains BCRC 32002 and BCRC 32003 demonstrated 35.9 and 36.3 % of DNA base composition. For *R. stolonifer* with recurved sporangiophores, DNA base composition ranged from 34.8 to 37.6 %. The degree of relative binding between the two strains of *R. stolonifer* var. *stolonifer* was 96 %. The degree of relative binding between the strains of *Rhizopus* with recurved sporangiophores ranged from 93 to 100 %. However, the DNA relatedness values between strains of *R. stolonifer* var. *stolonifer* and *Rhizopus* with recurved sporangiophores ranged from 21 to 32 %, clearly indicating a more remote relationship.

Table 3. DNA base composition and nuclear DNA relatedness among strains of *Rhizopus stolonifer* and related species

Species/ strain no.	G+C (mol %)	Percent relative binding of DNA from					
		BCRC 32002	BCRC 32003	BCRC 31138	BCRC 31139	BCRC 33817	BCRC 33727
R. stolonifera var. stolonifera							
BCRC 32002	35.9	100 %					
BCRC 32003	36.3	96 %	100 %				
R. reflexus							
BCRC 31138	36.7	30 %	32 %	100 %			
BCRC 31139	34.8	22 %	21 %	97 %	100 %		
R. stolonifera var. lyococcus							
BCRC 33817	36.9	28 %	26 %	100 %	97 %	100 %	
R. circinans							
BCRC 33727	37.6	27 %	24 %	94 %	93 %	97 %	100 %

Morphological characteristics of *R. stolonifera* and related species

Rhizopus reflexus, *R. stolonifera* var. *lyococcus* and *R. circinans* display similar morphological characteristics and temperature responses as *Rhizopus stolonifera* var. *stolonifera*. However, these taxa differ from *R. stolonifera* var. *stolonifera* by producing recurved sporangiophores and smaller sporangiospores ([Table 4](#)).

Table 4. Morphological characteristics of *Rhizopus stolonifera* and related species

	<i>Rhizopus stolonifera</i> var. <i>stolonifera</i>	<i>Rhizopus reflexus</i>	<i>Rhizopus lyococcus</i>	<i>Rhizopus circinans</i>
Morphology of sporangiophores	Erect	Both recurved and erect	Both recurved and erect	Both recurved and erect
Length of sporangiophores	Up to 3181 μm	Up to 3000 μm	Up to 2550 μm	Up to 2000 μm
Sporangia	120–280 μm	80–250 μm	130–230 μm	80–290 μm
Columellae	100–170 \times 95–150 μm	90–200 \times 60–190 μm	85–200 \times 85–175 μm	90–190 \times 80–170 μm
Sporangiospores	6–11.5 \times 5.5–10 μm	5.5–8 \times 4.5–8 μm	4.5–8 \times 4.5–7 μm	5–8.5 \times 4.5–6.5 μm
Maximum growth temp.	33 $^{\circ}\text{C}$	35 $^{\circ}\text{C}$	35 $^{\circ}\text{C}$	35 $^{\circ}\text{C}$

	<i>Rhizopus stolonifer</i> var. <i>stolonifer</i>	<i>Rhizopus reflexus</i>	<i>Rhizopus lyococcus</i>	<i>Rhizopus circinans</i>
Specimens examined	BCRC 32002; BCRC 32003	BCRC 31138; BCRC 31139	BCRC 33817	BCRC 33727

Taxonomy

Rhizopus lyococcus (Ehrenb.: Fr.) G.Y. Liou, F.L. Lee, G.F. Yuan & Stalpers, **comb. nov.**

Mycobank no.: [MB510307](https://www.mycobank.org/MB510307)

Basionym: *Sporotrichum lyococcum* Ehrenb. [as ‘*lyococcus*’], *Sylvae mycol. Berol.* 22 (1818).

Synonyms: *Collarium lyococcum* (Ehrenb.: Fr. Fr., *Syst. Mycol* 3(2): 442 (1832).

Rhizopus stolonifer var. *lyococcus* (Ehrenb.: Fr.) Stalpers & Schipper [as *lyococcus*], *Stud. Mycol.* 24: 68 (1984).

Rhizopus circinans Tiegh., *Annl. Sci. Nat., Bot., sér. 6* 4: 369 (1878).

Mucor circinans (Tiegh.) Mig., *Kryptogamenflora von Deutschland, Deutsch-Oesterreich und der Schweiz* 3(1): 198 (1910).

Rhizopus reflexus Bainier, *Bull. Soc. Bot. Fr.* 27: 226 (1880).

Mucor reflexus (Bainier) Mig., *Kryptogamenflora von Deutschland, Deutsch-Oesterreich und der Schweiz* 3(1): 197 (1910).

Key to the *Rhizopus* groups (modified from [Schipper 1984](#))

1 Sporangiohores partly recurved **R. lyococcus**

Sporangiophores erect 2

2(1) Sporangiohores mostly not exceeding 0.8 mm (rarely up to 1 mm) in height; rhizoids simple; sporangia up to 100 µm in diam; maximum growth temperature over 45 °C **R. microsporus group**

R. microsporus group

Sporangiophores often more than 1 mm in height; rhizoids with secondary branching; sporangia often over 100 µm in diam; no growth at 45 °C 3

3(2) Sporangia commonly up to 275 µm; no growth at 36 °C **R. stolonifer group**

Sporangia not exceeding 240 µm; growth at 36 °C **R. oryzae**

Key to the taxa of *Rhizopus stolonifer* group (modified from [Schipper 1984](#))

1 Zygosporangia absent in single-sporangium isolates ***R. stolonifer***

Zygosporangia present in single-sporangium isolates 2

2(1) Sporangia more than 150 µm in max. diam ***R. sexualis* var. *sexualis***

Sporangia less than 100 µm in max. diam ***R. sexualis* var. *americanus***

Discussion

Group concept of *Rhizopus*

Classification and identification of *Rhizopus* species has hitherto largely been based on morphology. [Schipper \(1984\)](#) divided the genus into three groups: the *R. stolonifer* group, the *R. oryzae* group, and the *R. microsporus* group according to characteristics of the sporangial apparatus and growth temperatures. In this study, for the first time, sequence data have been used to assess the definition of the genus *Rhizopus*. In contrast to the extreme variation of the ITS rDNA (G.Y.L., unpubl.), the D1/D2 region of LSU rDNA has proved to be valuable in the phylogeny of the genus *Rhizopus*. The phylogram based on LSU rDNA partial sequences shows that the genus *Rhizopus* is comprised of four clades.

Clade A of the phylogram comprises *R. azygosporus*, *R. caespitosus*, *R. homothallicus*, *R. microsporus*, and *R. schipperae*. The sporangial apparatus and growth temperatures of these species accord with diagnostic characters of the *R. microsporus* group ([Schipper 1984](#)). The composition of the *R. microsporus* group defined by morphological characteristics was relatively well supported by phylogenetic analysis based on LSU rDNA partial sequences. In addition, many species of the *R. microsporus* group have been shown by mating tests to be distinct varieties ([Schipper & Stalpers 1984](#)): results which have also been supported by DNA complementarity studies ([Ellis 1986](#)). The phylogram places *R. azygosporus* within the *R. microsporus* clade, indicating that the taxonomic status of *R. azygosporus* needs further investigation.

[Schipper \(1984\)](#) suggested that all strains of the *R. oryzae* group are synonymous with *R. oryzae*. Subsequently, [Ellis \(1985\)](#) proposed two taxa in the *R. oryzae* group, namely, *R. arrhizus* var. *arrhizus*, and *R. arrhizus* var. *delemar*. *Amylomyces rouxii* was reduced to *R. arrhizus* var. *rouxii* based on their DNA relatedness. Clade B of the phylogram ([Fig 1](#)) included *R. oryzae* and *A. rouxii*. Strains of *R. oryzae* can be divided into two groups. These results support the taxonomic status of *R. oryzae* and *A. rouxii* described by [Ellis \(1985\)](#). Also, *A. rouxii*, which falls into two groups in the phylogram, may be polyphyletic. Because *A. rouxii* is used as a starter culture of oriental fermented food and has not been isolated from nature, it might be a domesticated form of *R. oryzae* that has arisen through adaptation to the ragi or peka environment. The situation is probably comparable with that of *R. microsporus* var. *oligosporus*, which is used for tempeh production. For resolving the taxonomic concept in the *R. oryzae* and *A. rouxii* complex, more isolates and studies have to be included.

Clade C comprises *R. stolonifer* and *R. sexualis*, and contains two subclades. One includes strains of *R. stolonifer* var. *stolonifer*, the other contains strains of *R. sexualis* var. *sexualis* and *R. sexualis* var. *americanus* with high support values. *R. sexualis* is a homothallic species; *R. sexualis* var. *americanus* has smaller sporangia, causing its recognition as a distinct variety.

Clade D in the phylogram is well supported, and comprises the taxa with recurved sporangiophores. Most of these strains are currently classified as varieties of *R. stolonifer* by mating experiments ([Schipper 1984](#)). However, both isozyme analysis ([Liou et al. 2001](#)) and RAPD studies ([Vágvölgyi et al. 2004](#)) revealed that *R. stolonifer* var. *stolonifer* and *R. reflexus* might better be considered a separate species. The phylogram based on partial LSU rDNA supports the thesis that *Rhizopus* strains with recurved sporangiophores are relatively remote from the other *Rhizopus* species.

In conclusion, based on the phylogenetic analysis inferred from the D1/D2 region of LSU rDNA sequences, the genus *Rhizopus* contains four clades, the *R. lycococcus* group, the *R. stolonifer* group, the *R. oryzae* group, and the *R. microsporus* group. The presence of recurved sporangiophores is a diagnostic morphological character in *Rhizopus*. The revised group concept from [Schipper \(1984\)](#) is listed in [Table 5](#).

Table 5. Revision of diagnostic characters of *Rhizopus* groups from [Schipper \(1984\)](#)

	<i>Rhizopus lycococcus</i>	<i>Rhizopus stolonifer</i> group	<i>Rhizopus oryzae</i>	<i>Rhizopus microsporus</i> group
Rhizoid	Complex	Complex	Medium	Simple
Morphology of sporangiophores	Both erect and recurved	Erect	Erect	Erect
Length of sporangiophores	Up to 1–3 mm	Up to 1–3(4) mm	Up to 1–2.5 mm	Up to 0.5 mm, rarely up to 1 mm
Sporangia	Max. diam 300 µm	Max. diam 300 µm	Max. diam 160–240 µm	Max. diam 100 µm
Maximum growth temp.	35 °C	33 °C	45 °C	Over 45 °C

Taxonomy of *Rhizopus sexualis*

Rhizopus sexualis var. *americanus* differs from *R. sexualis* var. *sexualis* by having smaller sporangiophores, sporangia, columellae, and larger zygospores ([Schipper 1984](#)). The G + C content of *R. sexualis* var. *sexualis* is 3.6 % lower than that of *R. sexualis* var. *americanus* ([Table 2](#)). Also, the phylogram based on the D1/D2 region of LSU rDNA sequences showed divergence between *R. sexualis* var. *sexualis* and *R. sexualis* var. *americanus* ([Fig 1](#)). However, the level of DNA relatedness between *R. sexualis* var. *sexualis* and *R. sexualis* var. *americanus* was over 70 %. The lower limit of DNA relatedness indicating species delimitation among the yeasts is

less than 70 %. [Kurtzman et al. \(1986\)](#) suggested that findings from the yeast studies are relevant for estimates of biological relatedness among filamentous fungi. DNA relatedness data complement and support the concept of *R. sexualis* var. *americanus* as a separate variety on a morphological basis.

Taxonomy of *Rhizopus* taxa with recurved sporangiophores

Rhizopus stolonifer var. *stolonifer* and *R. stolonifer* var. *lyococcus* differ mainly in the presence of recurved sporangiophores and the size of sporangiospores ([Schipper 1984](#)). Ex-type strains are not available for these taxa. Isolates confirmed by [Schipper \(1984\)](#) were used for DNA relatedness. The DNA relatedness demonstrated that *Rhizopus* with recurved sporangiophores, with a G + C content very close to that of *R. stolonifer* var. *stolonifer*, represents a distinct species ([Table 3](#)). These results are supported by LSU rDNA partial sequences ([Fig 1](#)). Therefore, the recurved sporangiophore is considered an important morphological character for species definition in *Rhizopus*.


The levels of DNA relatedness among strains of *R. stolonifer* var. *lyococcus*, *R. reflexus* and *R. circinans* ranged from 93 to 100 %, supporting the combination of these taxa into a single species. Because *R. lyococcus* has priority over the other names ([\[Schipper, 1984\]](#) and [\[Stalpers, 1984\]](#)), *R. reflexus* and *R. circinans* are considered synonyms of *R. lyococcus*.

In conclusion, molecular data obtained from analysis of LSU rDNA partial sequences and DNA relatedness are valuable for establishing phylogenetic relationships and defining the species in the genus *Rhizopus*. The phylogenetic analysis evaluates the taxonomic scheme, which relies on morphological characteristics, and revises the group concepts. Moreover, the DNA relatedness data support both the morphological characteristics and the phylogram based on LSU rDNA partial sequences in the taxonomy of the *R. stolonifer* group. This investigation indicates that *R. lyococcus* is a distinct species and can be morphologically and genetically distinguished from *R. stolonifer* and other *Rhizopus* species. Also, the present study, and other publications ([\[Ellis, 1985\]](#) and [\[Ellis, 1986\]](#)), confirmed that DNA relatedness is a valid tool in evaluating the species concept in *Rhizopus*.

Acknowledgements

This work was supported by a grant (Contract No. 91-EC-17-A-17-R7-0525) from the Ministry of Economic Affairs, Taiwan. Li-Ling Liaw and Ing-Er Hwang, FIRDI, are appreciated for their assistance with DNA sequencing.

References

[Binder and Hibbett, 2002](#) M. Binder and D.S. Hibbett, Higher-level phylogenetic relationships of homobasidiomycetes (mushroom-forming fungi) inferred from four rDNA regions, *Molecular Phylogenetics and Evolution* **22** (2002), pp. 76–90. [Abstract](#) |  [PDF \(260 K\)](#) | [View Record in Scopus](#) | [Cited By in Scopus \(76\)](#)

[Chen, 1998](#) C.J. Chen, Morphological and molecular studies in the genus *Tremella*, *Bibliotheca Mycologica* **174** (1998), pp. 1–225.

[Ellis, 1985](#) J.J. Ellis, Species and varieties in the *Rhizopus arrhizus*–*Rhizopus oryzae* group as indicated by their DNA complementarity, *Mycologia* **77** (1985), pp. 243–247. [Full Text via CrossRef](#)

[Ellis, 1986](#) J.J. Ellis, Species and varieties in the *Rhizopus microsporus* group as indicated by their DNA complementarity, *Mycologia* **78** (1986), pp. 508–510. [Full Text via CrossRef](#)

[Ezaki et al., 1988](#) T. Ezaki, Y. Hashimoto, N. Takeuchi, H. Yamamoto, S.-L. Liu, H. Miura, K. Matsui and E. Yabuuchi, Simple genetic method to identify viridans group streptococci by colorimetric dot hybridization and fluorometric hybridization in microdilution wells, *Journal of Clinical Microbiology* **26** (1988), pp. 1708–1713. [View Record in Scopus](#) | [Cited By in Scopus \(94\)](#)

[Ezaki et al., 1989](#) T. Ezaki, Y. Hashimoto and E. Yabuuchi, Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains, *International Journal of Systematic Bacteriology* **39** (1989), pp. 224–229. [Full Text via CrossRef](#)

[Felsenstein, 2004](#) J. Felsenstein, PHYLIP (Phylogenetic Inference Package). Version 3.6 Distributed by the author, Department of Genome Sciences and Department of Biology, University of Washington, Seattle, WA (2004).

[Jennessen et al., 2005](#) J. Jennessen, K.F. Nielsen, J. Houbraken, E.K. Lyhne, J. Schnürer, J.C. Frisvad and R.A. Samson, Secondary metabolite and mycotoxin production by the *Rhizopus microsporus* group, *Journal of Agricultural and Food Chemistry* **53** (2005), pp. 1833–1840. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(23\)](#)

[Kaneko and Banno, 1991](#) Y. Kaneko and I. Banno, Reexamination of *Saccharomyces bayanus* strains by DNA–DNA hybridization and electrophoretic karyotyping, *IFO Research Communication* **15** (1991), pp. 30–41.

[Kumar et al., 2004](#) S. Kumar, N.S. Sharma, M.R. Saharan and R. Singh, Biochemical changes during ripening of cheddar cheese prepared by milk clotting enzyme from *Rhizopus oryzae*, *Journal of Food Science and Technology* **41** (2004), pp. 279–283. [View Record in Scopus](#) | [Cited By in Scopus \(2\)](#)

[Kurtzman et al., 1986](#) C.P. Kurtzman, M.J. Smiley, C.J. Robnett and D.T. Wicklow, DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group, *Mycologia* **78** (1986), pp. 955–959. [Full Text via CrossRef](#)


[Lee et al., 1994a](#) C.F. Lee, F.L. Lee and W.H. Hsu, Synonymy of *Candida methylica* with *Candida boidinii* and of *Candida methanolophaga* with *Candida succiphila*, *International*

Journal of Systematic Bacteriology **44** (1994), pp. 839–841. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(9\)](#)

[Lee et al., 1994b](#) C.F. Lee, F.L. Lee, W.H. Hsu and H.J. Phaff, *Arthroascus fermentans*, a new yeast species isolated from soil in Taiwan, *International Journal of Systematic Bacteriology* **44** (1994), pp. 303–307. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(9\)](#)

[Liou et al., 2001](#) G.Y. Liou, C.C. Chen, G.F. Yuan and C.Y. Chien, A taxonomic study of the genus *Rhizopus* by isozyme patterns, *Nova Hedwigia* **72** (2001), pp. 231–239. [View Record in Scopus](#) | [Cited By in Scopus \(7\)](#)

[Lutzoni et al., 2004](#) F. Lutzoni, F. Kauff, C.J. Cox, D. McLaughlin, G. Celio, B. Dentinger, M. Padamsee, D. Hibbett, T.Y. James, E. Baloch, M. Grube, V. Reeb, V. Hofstetter, C. Schoch, A. Arnold, J. Miadlikowska, J. Spatafora, D. Johnson, S. Hambleton, M. Crockett, R. Shoemaker, G.-H. Sung, R. Lücking, T. Lumbsch, K. O'Donnell, M. Binder, P. Diederich, D. Ertz, C. Gueidan, K. Hansen, R.C. Harris, K. Hosaka, Y.-W. Lim, B. Matheny, H. Nishida, D. Pfister, J. Rogers, A. Rossman, I. Schmitt, H. Sipman, J. Stone, J. Sugiyama, R. Yahr and R. Vilgalys, Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits, *American Journal of Botany* **91** (2004), pp. 1446–1480. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(184\)](#)

[Moncalvo et al., 2002](#) J.-M. Moncalvo, R. Vilgalys, S.A. Redhead, J.E. Johnson, T.Y. James, M.C. Aime, V. Hofstetter, S.J.W. Verduin, E. Larsson, T.J. Baroni, R.G. Thorn, S. Jacobsson, H. Cléménçon and O.K. Miller Jr., One hundred and seventeen clades of euagarics, *Molecular Phylogenetics and Evolution* **23** (2002), pp. 357–400. [Article](#) |  [PDF \(1024 K\)](#) | [View Record in Scopus](#) | [Cited By in Scopus \(217\)](#)

[O'Donnell, 1992](#) K. O'Donnell, Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*), *Current Genetics* **22** (1992), pp. 213–220. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(186\)](#)

[O'Donnell et al., 2001](#) K. O'Donnell, F.M. Lutzoni, T.J. Ward and G.L. Benny, Evolutionary relationships among mucoralean fungi (*Zygomycota*): evidence for family polyphyly on a large scale, *Mycologia* **93** (2001), pp. 286–297. [Full Text via CrossRef](#)


[Page, 1996](#) R.D.M. Page, TREEVIEW: an application to display phylogenetic trees on personal computers, *Computer Applications in the Biosciences* **12** (1996), pp. 357–358. [Full Text via CrossRef](#)

[Polonelli et al., 1988](#) L. Polonelli, G. Dettori, G. Morace, R. Rosa, M. Castagnola and M.A.A. Schipper, Antigenic studies on *Rhizopus microsporus*, *Rh. rhizopodiformis*, progeny and intermediates (*Rh. chinensis*), *Antonie van Leeuwenhoek* **54** (1988), pp. 5–18.


[Schipper, 1984](#) M.A.A. Schipper, A revision of the genus *Rhizopus*. 1. The *Rh. Stolonifer* group and *Rh. oryzae*, *Studies in Mycology* **25** (1984), pp. 1–19.

[Schipper and Stalpers, 1984](#) M.A.A. Schipper and J.A. Stalpers, A revision of the genus *Rhizopus*. 2. The *Rh. Microsporus* group, *Studies in Mycology* **25** (1984), pp. 20–34.

[Schipper et al., 1996](#) M.A.A. Schipper, M.M. Maslen, G.G. Hogg, C.W. Chow and R.A. Samson, Human infection by *Rhizopus azygosporus* and the occurrence of azygospores in Zygomycetes, *Journal of Medical and Veterinary Mycology* **34** (1996), pp. 199–203. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(14\)](#)

[Seviour et al., 1985](#) R.J. Seviour, L.M. Pethica and J.A. Soddell, Electrophoretic patterns of sporangiospore proteins of *Rhizopus* isolates as taxonomic characters, *Transactions of the British Mycological Society* **84** (1985), pp. 701–708. [Abstract](#) |  [PDF \(532 K\)](#)

[Stalpers, 1984](#) J.A. Stalpers, A revision of the genus *Sporotrichum*, *Studies in Mycology* **24** (1984), p. 68.

[Tamaoka and Komagata, 1984](#) J. Tamaoka and K. Komagata, Determination of DNA base composition by reversed-phase high-performance liquid chromatography, *FEMS Microbiology Letters* **25** (1984), pp. 125–128. [Abstract](#) |  [PDF \(263 K\)](#) | [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(1083\)](#)

[Tamura et al., 1999](#) M. Tamura, M. Hamamoto, C.F. Cañete-Gibas, J. Sugiyama and T. Nakase, Genetic relatedness among species in *Aspergillus* section *Clavati* as measured by electrophoretic comparison of enzymes, DNA base composition, and DNA–DNA hybridization, *Journal of General and Applied Microbiology* **45** (1999), pp. 77–83. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(5\)](#)

[Thompson et al., 1997](#) J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acids Research* **25** (1997), pp. 4876–4882. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(17710\)](#)

[Vágvölgyi et al., 2004](#) C. Vágvölgyi, H. Heinrich, K. Ács and T. Papp, Genetic variability in the species *Rhizopus stolonifer*, assessed by random amplified polymorphic DNA analysis, *Antonie van Leeuwenhoek* **86** (2004), pp. 181–188. [Full Text via CrossRef](#) | [View Record in Scopus](#) | [Cited By in Scopus \(11\)](#)

[Voigt et al., 1999](#) K. Voigt, E. Cigelnik and K. O'Donnell, Phylogeny and PCR identification of clinically important zygomycetes based on nuclear ribosomal-DNA sequence data, *Journal of Clinical Microbiology* **37** (1999), pp. 3957–3964. [View Record in Scopus](#) | [Cited By in Scopus \(85\)](#)

 Corresponding author.

[Mycological Research](#)

Volume 111, Issue 2, February 2007, Pages 196-203

