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Delimitation of *Rhizopus* varieties based on IGS rDNA sequences

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Rhizopus currently comprises 10 species two of which, *R. arrhizus* and *R. microsporus*, are divided into nine varieties. The molecular phylogenetic relationships among varieties were studied here by using IGS rDNA sequences. All three varieties of *R. arrhizus* were characterized by special short tandem repeat (STR) motifs except for strain CBS 257.28. The phylogram of *R. arrhizus* consisted of four clades. Three clades comprised *R. arrhizus* var. *arrhizus*, *R. arrhizus* var. *delemar* and *R. arrhizus* var. *tonkinensis*, respectively. The fourth clade was composed of CBS 258.28 *R. arrhizus* var. *arrhizus* and CBS 257.28 *R. arrhizus* var. *tonkinensis*. However, the varieties of *R. microsporus* did not correspond with any STR motif or form monophyletic groups. According to this study, the morphological and molecular characters evolve in concert in *R. arrhizus* and at different rate in *R. microsporus*.

Keywords: classification, molecular phylogeny, *Rhizopus arrhizus*, *Rhizopus microsporus*, STR.

Members of *Rhizopus* Ehrenb. are important as agents of food fermentation, agricultural and food spoilage, human mucormycosis, and industrial and medical biotechnology (Schipper *et al.* 1996, Voigt *et al.* 1999, Kumar *et al.* 2004, Vágvölgyi *et al.* 2004, Jennessen *et al.* 2005). They may occur as saprobes on plant debris, soil, and dung, or as air contaminants. *Rhizopus* is characterized by apophysate sporangia, stolons on aerial mycelia, and rhizoids developed from stolons and opposite sporangiophores. According to anamorphic morphology and growth temperature Schipper (1984) and Schipper & Stalpers (1984) divided this genus into two groups (*R. microsporus* Group and *R. stolonifer* Group) and one species (*R. oryzae* Went & Prins. Geerl.), the *R. microsporus* Group including *R. homothallicus* Hesselt. & J. J. Ellis, *R. microsporus* Tiegh. var. *microsporus*, var. *chinensis* (Saito)

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Schipper & Stalpers, var. *oligosporus* (Saito) Schipper & Stalpers, and var. *rhizopodiformis* (Cohn) Schipper & Stalpers, the *R. stolonifer* Group comprising *R. sexualis* (G. Smith) Callen var. *sexualis*, var. *americanus* Hesselt. & J. J. Ellis, *R. stolonifer* (Ehrenb.: Fr.) Vuill. var. *stolonifer*, and var. *lyococcus* (Ehrenb.) Stalpers & Schipper in Stalpers [as *lyococcos*]. The distant relationship between the two varieties of *R. stolonifer*, i.e. var. *lyococcus* and var. *stolonifer*, was observed but treated differently by Abe *et al.* (2006), Liou *et al.* (2007) and Liu *et al.* (2007). Abe *et al.* did not reclassify these two taxa; Liou *et al.* modified the taxonomical scheme of Schipper (1984) with a new combination *R. lyococcus* (Ehrenb.) G. Y. Liou *et al*; however *R. lyococcus* was recognized as a synonym of *R. reflexus* Bainier (Zheng *et al.* 2007). Based on morphology of the sporangial and zygosporic states, maximum growth temperature, mating compatibility, and molecular systematics, a recent monographic study of *Rhizopus* made by Zheng *et al.* (2007) recognized 10 species and nine varieties, but no groups. The ITS rDNA and the orotidine-5'-monophosphate decarboxylase gene (*pyrG*) have successfully supported most of the morphological treatments made by Zheng *et al.* (2007) at the species level, and supported the treatment of nine varieties to be grouped into two species, i.e. *R. arrhizus* [var. *arrhizus*, var. *delemar* (Boidin) J. J. Ellis, and var. *tonkinensis* (Vuill.) R. Y. Zheng & X. Y. Liu] and *R. microsporus* [var. *microsoprus*, var. *azygosporus* (G. F. Yuan & S. C. Jong) R. Y. Zheng, var. *chinensis*, var. *oligosporus*, var. *rhizopodiformis*, and var. *tuberosus* R. Y. Zheng & G. Q. Chen]. But the morphologically well-classified varieties of *R. arrhizus* could not be resolved by using these two genes, nor could those of *R. microsporus* (Liu *et al.* 2007).

Therefore more variable region than the ITS rDNA and *pyrG* gene is needed for discriminating varieties in this genus. The greatest amount of sequence variation within the rDNA exists in the intergenic spacers of ribosomal RNA gene (IGS rDNA) which evolve faster than the ITS rDNA and are suitable for studies at or below the species level (Guidot *et al.* 1999, James *et al.* 2001, Gorokhova *et al.* 2002, Sallares & Brown 2004, Sutar *et al.* 2004, Fukunaga *et al.* 2005). However, owing to the much larger region involved, the IGS rDNA has not been exploited in a routine manner. Nevertheless, the successful use of the IGS rDNA to solve scientific questions under species level (Guidot *et al.* 1999, James *et al.* 2001, Sutar *et al.* 2004, Fukunaga *et al.* 2005) suggests the utility of the marker for investigating the molecular phylogenetic relationships of the three varieties of *Rhizopus arrhizus* and the six varieties of *R. microsporus*. This study specially discusses the molecular phylogenetic relationships among these varieties by analyzing the IGS rDNA sequences.

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Materials and Methods

Fungal cultivation and DNA extraction

Data of *Rhizopus* strains used in this study are listed in Tab. 1. All stock cultures were maintained on potato dextrose agar (PDA). A 50-mL glucose-asparagine-thiamine synthetic medium (Huang & Yu 1988) was inoculated with a one-week old slant culture in a 250-mL Erlenmeyer flask. After incubating for 3–4 d at 25 °C at 150 rpm, mycelia were collected by vacuum filtration, washed three times with sterile distilled water and once with 25 % ethanol, air-dried and then frozen at –20 °C. Total genomic DNA was isolated from mycelia using a CTAB method (Warude *et al*. 2003) with some modifications. Approximately 70 mg of dried mycelia was ground to a fine powder in liquid nitrogen, transferred to a 1.5-mL microfuge tube and then resuspended in 700 µL of CTAB extraction buffer [100 mM Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), 1.5 M NaCl, and 2 % CTAB]. The mixture was shaken vigorously and then incubated at 65 °C for 30 min. After a series of deproteinizations with chloroform-isoamyl alcohol (24:1 in volume), a 500-µL portion of the supernatant was removed to a new 1.5-mL tube, and DNA was precipitated by the addition of 1 mL of cold absolute ethanol. The precipitated DNA was pelleted in a centrifuge, washed with 70 % ethanol, air-dried, dissolved in 50 µL of TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)] and then stored at –20 °C.

PCR amplification

The entire IGS rDNA was amplified by PCR with the primers (Fig. 1) anchored in the 3' end of the LSU (LR12R: 5'- CTG AAC GCC TCT AAG TCA GAA -3'; Veldman *et al*. 1981) and in the 5' end of SSU rDNA (NR31R: 5'- GGC TTA ATC TTT GAG ACA AG -3'; designed in this study). A serial dilution of genomic DNA was used to optimize the concentration of template for the PCR amplification. The PCR was performed in a 50-µL reaction mixture containing dilute genomic DNA in optimum concentration, 0.25 µM of each primer, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.08 % Nonidet P40, 1.5 mM magnesium chloride ($MgCl_2$), 200 µM each of nucleotide triphosphate (Promega Co., U.S.A.), and two units Taq DNA polymerase (Sangon Ltd., Canada). Mineral oil (40 µL) was overlaid on the reaction mixture. The reaction was performed in a MiniCyclerTM (MJ Research Inc., U.S.A.) for 35 cycles: denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min, with an initial denaturation at 94 °C for 1 min before cycling and final extension at 72 °C for 10 min after cycling. An aliquot (2 µL) of the

Table 1. – *Rhizopus* strains used in this study.

Zheng *et al.* 2007		Strain[a]	Received as[b]	GenBank No. of IGS[c]	
Species	**Variety**			**5' end**	**3' end**
R. arrhizus	var. *arrhizus*	CBS 110.17	*R. oryzae* (*R. maydis* T)	DQ641329	DQ641357
		CBS 112.07	*R. oryzae* T	DQ641326	DQ641354
		CBS 258.28	*R. oryzae* (*R. hangchow* T)	DQ641335	DQ641363
		CBS 328.47	*R. oryzae* (*R. delemar* T)	DQ641331	DQ641359
		IFO 4798	*R. oryzae* (*R. tritici* T)	DQ641334	DQ641362
		JCM 5580	*R. oryzae* (*R. peka* II T)	DQ641327	DQ641355
		JCM 5581	*R. oryzae* (*R. nodosus* T)	DQ641328	DQ641356
		JCM 5584	*R. oryzae* (*R. usamii* T)	DQ641330	DQ641358
		NRRL 1469 (NT)	*R. arrhizus* var. *arrhizus* NT	DQ641333	DQ641361
		NRRL 2710	*R. oligosporus*	DQ990323	DQ990327
		NRRL 5866	*R. arrhizus* var. *rouxii (Amylomyces rouxii* NT)	DQ641332	DQ641360
		R-55		DQ990320	DQ990324
		R-69		DQ990321	DQ990325
		R-612		DQ990322	DQ990326
	var. *delemar*	CBS 279.38	*R. oryzae* (*R. sontii* T)	DQ641353	DQ641381
		CBS 385.34	*R. oryzae* (*R. achlamydosporus* T)	DQ641346	DQ641373
		CBS 386.34	*R. oryzae* (*R. bahrnensis* T)	DQ641341	DQ641369
		CBS 389.34	*R. oryzae* (*R. chiuniang* var. *isofermentarius* T)	DQ641344	DQ641372
		CBS 393.34	*R. oryzae* (*R. peka* I T)	DQ641345	DQ641378
		CBS 402.51	*R. oryzae* (*R. javanicus* var. *kawasakiensis* T)	DQ641347	DQ641374
		HUT 1233	*R. chiuniang* T	DQ641348	DQ641375
		IFO 4735	*R. formosaensis* T	DQ641349	DQ641376
		IFO 5442	*R. javanicus* T	DQ641352	DQ641380
		JCM 5560	*R. oryzae* (*R. delemar* T)	DQ641343	DQ641371
		JCM 5561	*R. oryzae* (*R. formosaensis* var. *chlamydosporus* T)	DQ641350	DQ641377
		NRRL 1472 (T)	*R. arrhizus* var. *delemar* (*R. delemar* T)	DQ641342	DQ641370
		NRRL 2872	*R. oryzae* (*R. delemar* var. *minimus* T)	DQ641351	DQ641379

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R. arrhizus	var. *tonkinensis*	CBS 257.28	*R. oryzae* (*R. formosaensis* T)	DQ641336	DQ641364
		CBS 330.53	*R. oryzae* (*R. boreas* T)	DQ641340	DQ641368
		IFO 5438 (T)	*R. oryzae* (*R. tonkinensis* T)	DQ641338	DQ641366
		JCM 5569	*R. oryzae* (*R. kansho* T)	DQ641339	DQ641367
		JCM 5570	*R. oryzae* (*R. tamari* T)	DQ641337	DQ641365
R. microsporus	var. *azygosporus*	CBS 357.93 (T)	*R. azygosporus* T	DQ641389	EF544120
				DQ641390	
	var. chinensis	CBS 631.82 (T)	*R. microsporus* var. *chinensis* T	DQ641394	EF544121
		R-36		DQ641391	EF544122
	var. *microsporus*	CBS 699.68 (NT)	*R. microsporus* var. *microsporus*	DQ641383	EF544123
		R-39		DQ641382	EF544124
	var. *oligosporus*	AS 3.1161		DQ641387	EF544125
		CBS 337.62 (NT)	*R. microsporus* var. *oligosporus* NT	DQ641386	EF544126
		R-47		DQ641384	EF544127
	var. *rhizopodiformis*	CBS 343.29	*R. microsporus* var. *rhizopodiformis* (*R. pusillus* T)	DQ641388	EF544128
		CBS 536.80 (NT)	*R. microsporus* var. *rhizopodiformis* NT	DQ641392	EF544129
		R-49		DQ641385	EF544130
	var. *tuberosus*	AS 3.1145 (T)		DQ641393	EF544131

[a] Abbreviations: China General Microbiological Culture Collection Center, Beijing, China (AS); Centraalbureau voor Schimmelcultures, Utrecht, Netherlands (CBS); Faculty of Engineering, Hiroshima University, Japan (HUT); Institute for Fermentation, Osaka, Japan (IFO); Japan Collection of Microorganisms, Riken, Japan (JCM); the USDA Agricultural Research Service Collection, Peoria, USA (NRRL); and the Culture Collection of the Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China (with a R- prefix for Rhizopus spp.).

[b] Type and neotype strains are indicated with “T” and “NT”, respectively. The names in parentheses refer to the original names of the strains received.

[c] The two sequences DQ641389 and DQ641390 were amplified and sequenced from strain CBS 357.93 *R. microsporus* var. *azygosporus*.

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PCR amplification product was electrophoresed in a 1.0 % agarose gel, stained with ethidium bromide (EtBr) and visualized under ultraviolet light.

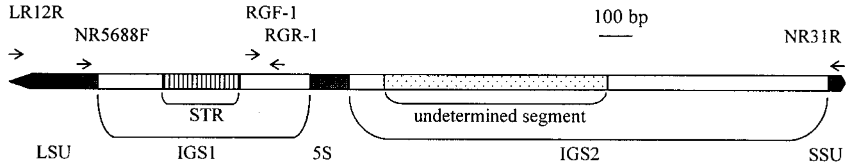

Fig. 1. Locations of regions and primers. STR is short tandem repeats within the IGS1 region. The undetermined segment is about 690 bp long. The arrowheads show the location and direction of the primers. The scale represents 100 bp.

DNA sequence data

The PCR amplification products were sent to the Invitrogen Corporation (http://www.invitrogen.com.cn) for determination through direct sequencing. In addition to primers LR12R and NR31R, other three primers (Fig. 1) were also designed in this study for sequencing. They are NR5688F (5'- GAG TAG CCT TTG TTG CTA CG -3'), RGF-1 (5'- CGG GAT TAA GGA GCA ACA CCT TAG -3') and RGR-1 (5'- TTC TAG GTG ATG GAC GGC -3'). Sequences were assembled by the program SeqManII (http://www.dnastar.com) and deposited in GenBank under the accession numbers shown in Tab. 1. A total of 46 and 25 IGS rDNA sequences respectively for *Rhizopus arrhizus* and *R. microsporus* were gathered for this study. Two datasets were analyzed: (1) sequences from 23 strains of *R. arrhizus*, (2) sequences from 12 strains of *R. microsporus* except DQ641390 of CBS 357.93. Because the IGS rDNA was hypervariable alignment between different species was not possible. No sequence was suitable as outgroup for either dataset.

Phylogenetic analyses

Multiple alignments were performed using MegAlign (http://www.dnastar.com). All alignments were optimized by eye and manually edited for gap insertions with BioEdit 4.7.1 (Hall 1999) and transferred into NEXUS format files through ClustalX 1.83 (Thompson *et al.* 1997). Phylogeny reconstructions were conducted by using the parsimony analyses implemented in the PAUP* 4.0b10 (Swofford 2002) and using Bayesian inference with the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) techniques implemented by the MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The program TreeView 1.5.0 (Page 1996) was used for creating and manipulating graphical trees.

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Because of the undetermined segment in the IGS2 region (Fig. 1), the partition homogeneity test (PHT; Farris *et al*. 1994) implemented in PAUP* 4.0b10 was used to evaluate the concordance of the sequences flanking both sides of the undetermined segment, by using a heuristic search with 1 000 replicates and 1 000 maxtrees, treating gaps as a new state and excluding uninformative characters.

In parsimony analyses, the short tandem repeat (STR) region was excluded and re-integrated after being coded with a set of 22 and four characters, respectively for *Rhizopus arrhizus* and *R. microsporus* (Tab. 2 and 3), ambiguously aligned regions were removed, and unambiguously aligned gaps were treated as a new state. All characters had equal weight. Parsimony branch support was estimated by performing 1 000 bootstrap analyses (Felsenstein 1985) with heuristic searches. The starting tree was obtained via stepwise addition during which one tree was held at each step. Branches of zero length were collapsed with tree-bisection-reconnection as the branch-swapping algorithm. The MulTree option was in effect and MaxTrees was set to 5 000.

The best-fit models of nucleotide substitution, which were selected by hierarchical likelihood ratio tests (hLRT; Huelsenbeck & Crandall 1997, Posada & Crandall 2001) implemented in MrModelTest 2.2 (Posada & Crandall 1998, Nylander 2004), were used in MrBayes 3.1.2 for inferences of phylogeny. In Bayesian analyses, the short tandem repeat (STR) region was excluded and re-integrated after being coded with a set of 22 and four characters, respectively for *Rhizopus arrhizus* and *R. microsporus* (Tab. 2 and 3), ambiguously aligned regions were removed, and unambiguously aligned insertion and deletion regions (IDR) were also removed but re-integrated after being coded with binary presence / absence characters because the present version of MrBayes cannot treat gaps as a new state. All these binary data were analyzed with the default F81-like model implemented in MrBayes 3.1.2. Two independent Bayesian analyses were run simultaneously each with one cold chain and three heated chains with temperature set to 0.1, each initiated with a random tree and flat prior. For *Rhizopus arrhizus*, one out of every 1 000 trees was sampled for 1 000 000 generations. The first 500 sampled trees from the two runs were discarded before summarizing the information, and the remaining 1 500 sampled trees were used for calculating posterior probabilities which were considered statistically significant when P · 0.95. For *R. microsporus*, one out of every 100 trees was sampled for 500 000 generations. The first 3 000 sampled trees from the two runs were discarded and the remaining 7 000 sampled trees were summarized. The convergence of the 2 runs was diagnosed by the average standard deviation of splits-frequencies

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(less than 0.01) and the potential scale reduction factor (approximate 1; Gelman and Rubin 1992).

The node reliability was assessed by bootstrap proportions (BP) · 70 % and Bayesian posterior probabilities (PP) · 0.95 (Alfaro *et al*. 2003, Douady *et al*. 2003). The tree resolution was measured by tree length (TL), consistency index (CI), homoplasy index (HI), retention index (RI) and rescaled consistency index (RC).

Results

Phylogenetic analyses of *Rhizopus arrhizus* based on IGS rDNA.

PCR products were amplified from all selected strains of *Rhizopus arrhizus* with primers LR12R and NR31R. These PCR products were sequenced on both strands with the following primers: LR12R, NR5688F, RGF-1, RGR-1 and NR31R (Fig. 1). For comparison of the entire IGS rDNA, the sequence of *R. oryzae* (= *R. arrhizus*) ATCC 24794 (GenBank accession number AY847626) was downloaded as a standard. The comparison of ATCC 24794 with 32 sequences determined herein showed that some 690 bp in the IGS2 rDNA could not be sequenced due to poly A or poly T structures. The obtained sequences varied remarkably in length, from 1644 bp for *R. arrhizus* var. *delemar* CBS 279.38 to 1822 bp for *R. arrhizus* var. *arrhizus* CBS 110.17, mainly contributed by the STR in IGS1 rDNA. Owing to the undetermined segments, sequences of 32 strains of *R. arrhizus* were submitted to GenBank under 64 accession numbers (Tab. 1).

The alignment of *Rhizopus arrhizus* sequences resulted in 274 sites within the LSU, 651 sites of IGS1 including the STR region (233 sites), 120 sites of 5S, 794 sites of partial IGS2, and 50 sites of partial SSU rDNA (Fig. 1). The total 1 889 characters contain 1 470 constant characters, eight parsimony-uninformative variable characters and 411 parsimony-informative characters. The nucleotide characters of partial LSU, entire 5S and partial SSU rDNA were all constant. All variable characters were from the entire IGS1 and partial IGS2 rDNA.

Fourteen IDR characters were coded with binary presence / absence characters. The STR (233 characters) included six variety-related motifs represented by U, V, W, X, Y and Z (Tab. 2). These motifs have the core sequence TCTG. They are not distributed randomly in all varieties, but particularly in certain varieties. *R. arrhizus* var. *delemar* contained U (TTTGTCTGC) and V (CTTGTCTGC) which was not present in the other two varieties. Hence, one can easily distinguish it from the others according to the presence of U and V. On the other hand, *R. arrhizus* var. *arrhizus* contained W (TTTGTCTG), X (CTTGTCTG) and Z (CCTCTCTG), with the Z motif

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unique to this variety. *R. arrhizus* var. *tonkinensis,* with the exception of strain CBS 257.28, contained W, X and Y (CCTGTCTG), among which motif Y is unique to it. Therefore, in the light of the presence of Y and Z, one can easily differentiate *R. arrhizus* var. *arrhizus* and *R. arrhizus* var. *tonkinensis*. Although both contain W and X, the numbers of W in the two varieties vary considerably. Most members of *R. arrhizus* var. *arrhizus*, except strains CBS 258.28 and R-55, contain more than five Ws, while those of *R. arrhizus* var. *tonkinensis* only one. The two exceptional individuals CBS 257.28 and CBS 258.28 are identical in DNA sequences. They have just one W (like *R. arrhizus* var. *tonkinensis*) and the Z motif (like *R. arrhizus* var. *arrhizus*). In order to capture the features of the STR region for phylogenetic analyses, we coded 22 regions with binary data (Tab. 2).

Results of the partition-homogeneity test (PHT) indicated that the sequences from both sides of the undetermined segment are highly homogeneous (P = 0.998) and could be analyzed as a combined dataset.

The best-fit model selected by hLRT in MrModelTest 2.2 is GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites. The standard deviation of split frequencies was 0.344 at the 1 000th generation and it fell below 0.01 (0.009) after 1 000 000 generations. The potential scale reduction factors are close to 1.0 (0.999–1.014) for all substitution model parameters after summarizing the last 1 500 samples from the two runs.

Phylogenetic trees constructed with the two methods are integrated into Fig. 2. In the IGS rDNA phlogeny, *Rhizopus arrhizus* var. *delemar* formed a monophyletic group supported with 100 % BP and 1.0 PP. *R. arrhizus* var. *arrhizus* was not monophyletic, but most of its members formed a clade supported with 99 % BP and 1.0 PP. *R. arrhizus* var. *tonkinensis* was not monophyletic either, but most of its strains formed a clade with 100 % BP and 1.0 PP support. Strains CBS 258.28 of *R. arrhizus* var. *arrhizus* and CBS 257.28 of *R. arrhizus* var. *tonkinensis* formed a clade with 100 % BP and 1.0 PP support. In the combined phylogeny of *R. arrhizus* four lineages almost consistent with current morphological classification were resolved.

Phylogenetic analyses of *Rhizopus microsporus* based on IGS rDNA.

All of the strains of *Rhizopus microsporus* analyzed by ITS rDNA (Liu *et al.* 2007) were studied by examining the IGS rDNA. Thirteen partial IGS1 sequences were determined from 12 strains with the primer pair NR5688F and RGR-1. While 11 strains possessed single PCR products, two distinct bands strain were amplified for strain CBS 357.93 of *R. microsporus* var. *azygosporus*. The two bands were purified by gel extraction and then sequenced separately.

Table 2. – Motifs and coded characters of the short tandem repeat (STR) sequences of *Rhizopus arrhizus*.

Variety	Strain	STR type[a]	Characters coded from STR motifs[b]																					
			U	V	W	X	Y	Z	UU	UV	VU	VV	WW	WX	WY	WZ	XW	XX	XY	XZ	YX	ZW	ZX	ZZ
var. *arrhizus*	R-69	WXZWZWZWWWWWWXXXZXZXZXZXXZZXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	1
	CBS 112.07	WXZWZWZWZWZWWXWZXXXXXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	1	0	1	0	1	1	0
	JCM 5580	WXZWZWZWZWZWWXWZXXXXXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	1	0	1	0	1	1	0
	JCM 5581	WXZWZWZWZWZWWXWZXXXXXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	1	0	1	0	1	1	0
	CBS 110.17	WXZWZWZWZWZWWXWZXXXXXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	1	0	1	0	1	1	0
	JCM 5584	WXZWZWWWWWWWWXXXZXZXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	0
	CBS 328.47	WXZWZWZWWXWZXXXXXXXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	1	0	1	0	1	1	0
	NRRL 5866	WXZWZWWWWWWXXZXZXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	0
	NRRL 1469	WXZWZWWWWXXXZXZXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	0
	IFO 4798	WXZWZWWWWXXZXZXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	0
	R-612	WXZWZWXWWXXXXZXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	1	0	1	0	1	1	0
	NRRL 2710	WXZWZWWWXXZXZXX	0	0	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	0
	R-55	WXZXZXX	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0
	CBS 258.28	WZZXXXXXXX	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1
var. *tonkinensis*	CBS 257.28	WZZXXXXXXX	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1
	JCM 5570	WYXYXYXYXYXYXYX	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
	IFO 5438	WYXYXYXYXYXYXYX	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
	JCM 5569	WYXYXYXYXYXYX	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
	CBS 330.53	WYXYXYXYXYXYX	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
var. *delemar*	CBS 386.34	UVVUVUUUVUVUUUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	NRRL 1472	UVVUVUUVUVUUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	JCM 5560	UVVUVUUVUVUUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	CBS 389.34	UVVUVUUVUVUUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	CBS 385.34	UVVUVUUVUVUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	CBS 402.51	UVVUVUUVUVUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	HUT 1233	UVVUVUUVUVUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	IFO 4735	UVVUVUUVUVUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	JCM 5561	UVVUVUUVUVUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	CBS 393.34	UVVUVUUVUUUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	NRRL 2872	UVVUVUUVUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	IFO 5442	UVVUVUUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	CBS 279.38	UVVUVUUUUU	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0

[a] Letters U, V W, X, Y and Z are used for the nucleotide sequences corresponding to TTTGTCTGC, CTTGTCTGC, TTTGTCTG, CTTGTCTG, CCTGTCTG and CCTCTCTG, respectively.
[b] Binary data: absent = 0, present = 1.

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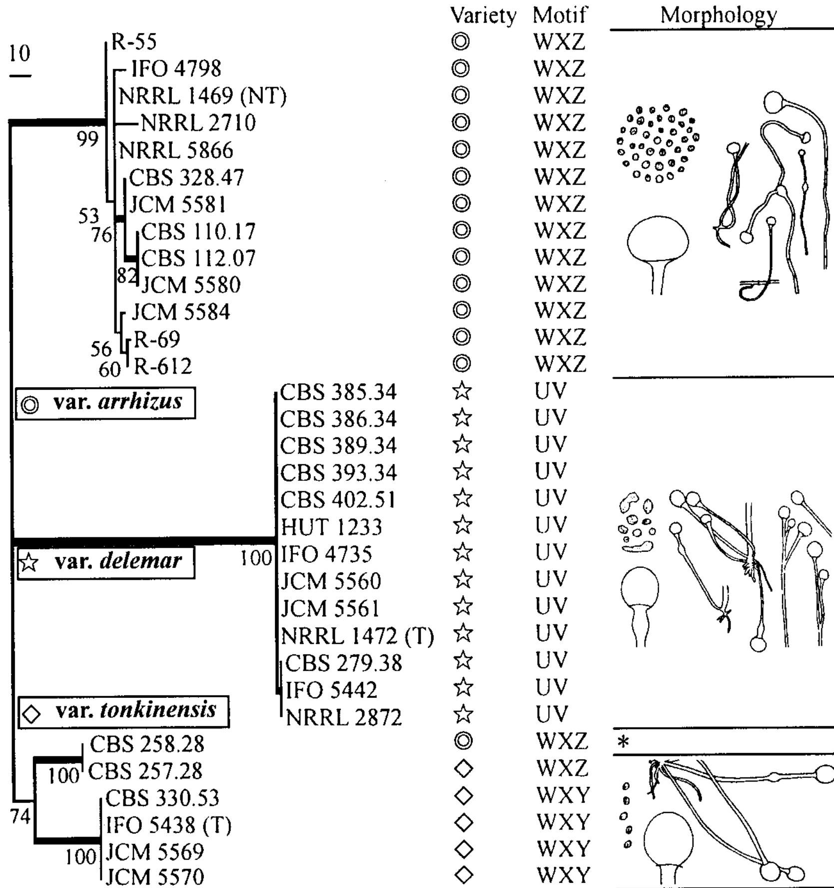

Fig. 2. Bootstrap 50% majority-rule consensus tree constructed with unweighted parsimony analyses in PAUP* 4.0b10 illustrating molecular phylogeny of 32 strains of *Rhizopus arrhizus* based on IGS rDNA. Tree length = 291.16, Consistency index (CI) = 0.8894, Homoplasy index (HI) = 0.1106, Retention index (RI) = 0.9865, Rescaled consistency index (RC) = 0.8774. Bootstrap values above 50 % are indicated at internodes. The bar at the upper left indicates 10 steps. Thickened branches indicate more than 0.95 posterior probabilities obtained from Bayesian analyses. Motifs are described in Tab. 2. The drawings represent the typical morphology of sporangiospores, collumellae, swellings and sporangiophores (Tab. 4). The marker "*" refers to the morphology of CBS 258.27 which is the same as other *R. arrhizus* var. *arrhizus*.

The sequencing was carried out on both strands with the same primers as used for PCR. Twelve sequences from the IGS2 region were obtained from the same set of strains. The PCR primers for this region were RGF-1 and NR31R. The sequencing was performed with primer NR31R. All 25 sequences from the 12 strains were deposited in GenBank under the accession numbers shown in Tab. 1. The

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comparison of these sequences with the entire IGS rDNA of *R. microsporus* var. *oligosporus* ATCC 48011 (Genbank accession number AY847625) showed that a 1187 bp segment in the IGS2 rDNA could not be determined due to poly A or poly T structures. The obtained sequences varied remarkably in length, from 1364 bp for *R. microsporus* var. *rhizopodiformis* R-49 to 1432 bp for *R. microsporus* var. *chinensis* CBS 631.82, mainly contributed by the STR in IGS1 rDNA. Owing to the undetermined segments, sequences of 12 strains of *R. microsporus* were submitted to GenBank under 25 accession numbers (Tab. 1).

Results of the partition-homogeneity test (PHT) indicated that the sequences from the partial IGS1 and partial IGS2 rDNA were highly homogeneous (P = 1.0) and could be analyzed as a combined dataset, when the IGS1 type 1 sequence (DQ641389; Tab. 1) of *Rhizopus microsporus* var. *azygosporus* CBS 357.93 was used. The PHT did not support the combinability if the other type of the IGS1 rDNA sequence of CBS 357.93 (DQ641390; Tab. 1) was used (P = 0.001). So the phylogenetic analyses of *R. microsporus* were carried out based on combined data of the partial IGS1 and IGS2 rDNA without DQ641390.

The alignment of 12 IGS rDNA sequences of *Rhizopus micrsoprus* resulted in 1447 sites in total, including 55 LSU sites, 516 partial IGS1 sites, 826 partial IGS2 sites, and 50 SSU sites. The alignment consists of 1 050 constant, 17 variable and parsimony-uninformative, and 380 parsimony-informative characters. All of the variable characters were contributed by the partial IGS1 and partial IGS2 rDNA region.

The unambiguously aligned insertion and deletion regions (IDR), including 15 sites, were coded with binary characters. The short tandem repeat (STR) region spans 107 sites. Four types of STR containing different kinds and numbers of motifs, with a core sequence CCA, are shown in Tab. 3. They were PQQQQQQ (type I), PQPQQQQ (type II), PQQQ (type III) and PQQ (type IV). The letters P and Q represent motif sequences CACCACCATT and CACCAAGACCATT, respectively. Five sequences did not have short tandem repeats at the same locus. They were able to be treated as the fifth STR type. According to these motifs, a set of four binary characters was designed to represent the molecular information for phylogeny inference (Tab. 3).

The best-fit model selected by hLRT in MrModelTest 2.2 is HKY model with gamma-distributed rate variation across sites. The standard deviation of split frequencies was 0.082 at the 1000th generation and fell below 0.01 (0.009) after 500 000 generations. The potential scale reduction factors were close to 1.0 (1.0–1.029) for all

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Table 3. – Characters coded for the short tandem repeat (STR) sequences of *Rhizopus microsporus*.

Type:motifs[a]	Strain[b]	Variety	Coded characters[c]			
			P	**Q**	**PQ**	**QP**
I: PQQQQQQQ	CBS 631.82	var. *chinensis*	1	1	1	0
II: PQPQQQQ	CBS 337.62	var. *oligosporus*	1	1	1	1
III: PQQQ	AS 3.1145	var. *tuberosus*	1	1	1	0
	AS 3.1161	var. *oligosporus*	1	1	1	0
	CBS 343.29	var. *rhizopodiformis*	1	1	1	0
	CBS 536.80	var. *rhizopodiformis*	1	1	1	0
	CBS 357.93 (DQ641389)	var. *azygosporus*	1	1	1	0
IV: PQQ	R-49	var. *rhizopodiformis*	1	1	1	0
V: no motif	R-39	var. *microsporus*	0	0	0	0
	CBS 357.93 (DQ641390)	var. *azygosporus*	0	0	0	0
	R-47	var. *oligosporus*	0	0	0	0
	CBS 699.68	var. *microsporus*	0	0	0	0
	R-36	var. *chinensis*	0	0	0	0

[a] Motifs P and Q are designated for the sequences of CACCACCATT and CACCAAGACCATT, respectively.
[b] Two sequences, DQ641389 and DQ641390, were amplified and sequenced from strain CBS 357.93 *R. microsporus* var. *azygosporus*.
[c] Binary data: absent = 0, present = 1.

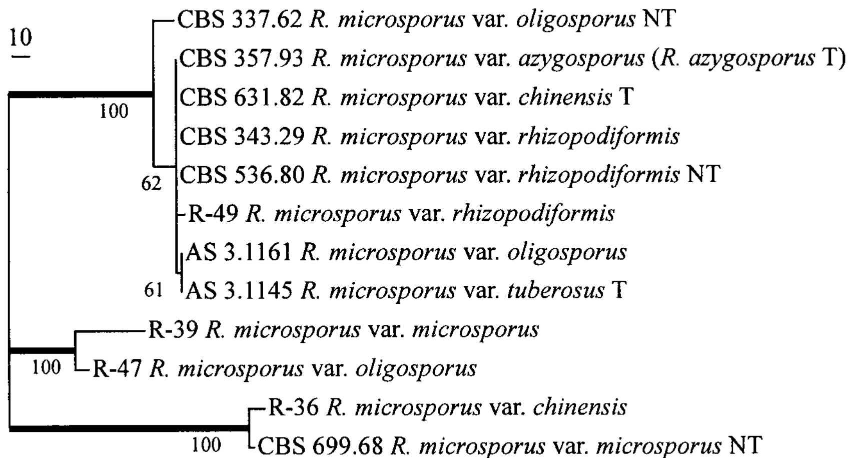

Fig. 3. Bootstrap 50% majority-rule consensus tree constructed with unweighted parsimony analyses in PAUP* 4.0b10 illustrating molecular phylogeny of 12 strains of *Rhizopus microsporus* based on IGS rDNA. Tree length = 351.91, Consistency index (CI) = 0.9518, Homoplasy index (HI) = 0.0482, Retention index (RI) = 0.9596, Rescaled consistency index (RC) = 0.9133. Bootstrap values above 50 % are provided at internodes. The bar at the upper left indicates 10 steps. Thickened branches indicate more than 0.95 posterior probabilities obtained from Bayesian analyses.

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substitution model parameters after summarizing the last 7 000 samples from the two runs.

Phylogenetic trees constructed with the two methods are integrated into Fig. 3. In the IGS rDNA phlogeny, *Rhizopus microsporus* var. *chinensis* R-36 and *R. microsporus* var. *microsporus* CBS 699.68 (Neotype) form a clade. *R. microsporus* var. *microsporus* R-39 and *R. microsporus* var. *oligosporus* R-47 formed a separate clade, and a third clade consists of the others including *R. microsporus* var. *azygosporus* (= *R. azygosporus*, Type) CBS 357.93, R. *microsporus* var. *chinensis* (= *R. chinensis*, Type) CBS 631.82, AS 3.1161 and *R. microsporus* var. *oligosporus* CBS 337.62 (Neotype), *R. microsporus* var. *rhizopodiformis* CBS 343.29, CBS 536.80 (Neotype) and R-49, and R. *microsporus* var. *tuberosus* AS 3.1145 (Type). These three lineages of *R. microsporus* were supported with 100 % BP and 1.0 PP; however, they are incompatible with the current morphological classification.

Discussion

Molecularly identical CBS 257.28 and CBS 258.28 are morphologically distinct

The major differences among the three varieties of *Rhizopus arrhizus* based on a monographic study (Zheng *et al.* 2007) are summarized in Tab. 4 and illustrated in Fig. 2. The major characteristics for discriminating these varieties involve the position of sporangiophore swellings, length of sporangiophores, shape and base of columellae, and shape and size of sporangiospores. In this study, an important molecular characteristic for distinguishing these varieties is the STR motif (Tab. 2 and Fig. 2). The delimitation of *R. arrhizus* varieties based on IGS rDNA sequences support the morphological treatments made by us except for strains CBS 257.28 and CBS 258.28. IGS rDNA of these two strains were identical and contained the Z motif, a special motif related to *R. arrhizus* var. *arrhizus*. If we consider variety-related motif only, these strains would be classified as *R. arrhizus* var. *arrhizus*. However the phylogram integrating more characters appears to be more informative. These two strains form an independent clade separated from all other *Rhizopus* species examined, with 100 % BP and 1.0 PP support (Fig. 2). This lineage is discordant with the morphological classification (Zheng *et al.* 2007). CBS 257.28 was received as the type culture of *R. formosaensis* Nakazawa, one of the synonyms of *R. arrhizus* var. *delemar*, but morphologically appeared to be *R. arrhizus* var. *tonkinensis* CBS 258.28 was received as *R. oryzae*, the type of *R. hangchow* M. Yamaz, but morphologically belongs to *R. arrhizus* var. *arrhizus*. This

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inconsistency between the molecular phylogeny and morphological classification requires further study. Here, we treat them as a distinct variety based on morphological differences.

Table 4. – Major characteristics of three varieties of *Rhizopus arrhizus*

Varieties	Swellings	Sporangiophores	Columellae		Sporangiospores	
	Position on sporangiophores	Length	Shape[a]	Base	Shape	Size (μm in length)
var. *arrhizus*	middle	< 1000 μm	subglobose	wide	regular	(4-)5-8(-9)
var. *delemar*	upper-half	< 1000 μm	ovoid	wide	irregular	(4-)5.5-14.5(-23)
var. *tonkinensis*	middle	> 1000 μm	ovoid	narrow	regular	(4-)5-7.5(-10)

[a] Only the most common shape is listed.

Correction of NRRL 2710 from *Rhizopus microsporus* var. *oligosporus* to *R. arrhizus* var. *arrhizus*

Strain NRRL 2710 was classified as *Rhizopus microsporus* var. *oligosporus* (= *R. oligosporus*) by Schipper & Stalpers (1984). Zheng *et al.* (2007) observed its intermediate morphology between *R. arrhizus* var. *arrhizus* and *R. arrhizus* var. *tonkinensis*. Most of the sporangiophores of NRRL 2710 arise from the aerial mycelium or stolons are not opposite rhizoids and seldom in groups, rhizoids are usually poorly developed or absent and hence more similar to *R. arrhizus* var. *arrhizus*; but the colour of the sporangiophores are darker and not yellowish brown, many collumellae are ovoid or oblong-ovoid and seldom hemiglobose or roundish conical, and thus more similar to *R. arrhizus* var. *tonkinensis*. This study finds that it possesses the STR Z motif, a variety-specific characteristic of *R. arrhizus* var. *arrhizus*, and is nested in the *R. arrhizus* var. *arrhizus* clade in the partial IGS rDNA gene tree. Therefore, strain NRRL 2710 is classified as *R. arrhizus* var. *arrhizus* based on morphological and molecular evidence (Fig. 2).

Combination of *Rhizopus arrhizus* var. *rouxii* in *R. arrhizus* var. *arrhizus*

Ellis (1985) recognized variety *Rhizopus arrhizus* var. *rouxii* (Calmette) J. J. Ellis. The main reason is that its sporangia and sporangiospores are extremely degenerate. Yet, Zheng *et al.* (2007) recombined *R. arrhizus* var. *rouxii* into *R. arrhizus* var. *arrhizus* based on the interpretation that the degeneration is a result of long-term use for fermentation. Molecular proof for this treatment come from the STR Z motif which is shared by *R. arrhizus* var. *arrhizus* CBS 110.17, CBS 112.17, CBS 258.28, CBS 328.47, IFO 4798, JCM

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5580, JCM 5581, JCM 5584, NRRL 1469 and NRRL 2710 and *R. arrhizus* var. *rouxii* NRRL 5866, R-55, R-69 and R-612. In addition to these, RAPD analyses also support the recombination of *R. arrhizus* var. *rouxii* to *R. arrhizus* var. *arrhizus* (data not shown). Except those used in this study, other strains of *R. arrhizus* var. *rouxii* included in the RAPD analysis were CBS 266.30, R-209 and R-237. These strains possess the same pattern as members of *R. arrhizus* var. *arrhizus*. In fact, Ellis (1985) found high DNA complementary between *R. arrhizus* var. *arrhizus* and *R. arrhizus* var. *rouxii*.

Reduction of *Rhizopus tonkinensis* to *R. arrhizus* var. *tonkinensis*

Rhizopus arrhizus var. *tonkinensis*, which was originally classified as *R. tonkinensis* Vuill., is a new combination proposed by Zheng *et al*. (2007) according to morphological characteristics shared with *R. arrhizus* var. *arrhizus* and *R. arrhizus* var. *delemar*: rhizoids usually branching when present; sporangiophores often reaching 1.5 mm in length; sporangiospores with visible striations; zygosporangia brown, reaching 140 but not exceeding 180 μm diam.; suspensors equal or unequal; and maximum growth temperature reaching 42 °C. ITS rDNA and *pyrG* genes indicated *R. arrhizus* var. *tonkinensis* was a member of *R. arrhizus* but they could not distinguish it from the other two varieties (Liu *et al*. 2007). In this study, except for CBS 257.28, every member of *R. arrhizus* var. *tonkinensis* was separated from *R. arrhizus* var. *arrhizus* and *R. arrhizus* var. *delemar*, based on the special STR Y motif and they formed a highly supported clade in the molecular phylogeny (Fig. 2). These results support the new combination.

Reduction of *Rhizopus azygosporus* to *R. microsporus* var. *azygosporus*

CBS 357.93 of *Rhizopus microsporus* var. *azygosporus*, which was originally classified as *R. azygosporus* G. F. Yuan & S. C. Jong, was transferred to *R. microsporus* and treated as *R. microsporus* var. *azygosporus* comb. nov. by Zheng *et al*. (2007) based on the shared morphology with other varieties of *R. microsporus*: sporangiophores mostly arising from stolons and determinate in length; zygosporangia/azygosporangia and zygospores when formed not exceeding 100 μm diam.; outer wall of zygospores crenulate and never verrucose; the two suspensors neither equal in shape nor size; and sexual reproduction heterothallic. The partial IGS, entire ITS rDNA and partial *pyrG* gene support this recombination. The ITS rDNA and partial *pyrG* gene indicate it is a member of *R. microsporus* rather

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than a separate species (Liu *et al*. 2007). Its two different IGS rDNA variants (DQ641389, 515 bp; Q641390, 349 bp) differentiate it from all other varieties which possess just a single variant. Although definite conclusions cannot be drawn owing to insufficient evidences, especially those from sexual compatibility test, the phenomenon of occurrence of these two distinct variants of IGS1 rDNA in *R. microsporus* var. *azygosporus* CBS 357.93 can possibly be interpreted as transposition of rDNA during hybridization based on the finding that one type sequence (DQ641389) is the same as *R. microsporus* var. *oligosporus* AS 3.1161 and the other (DQ641390) is the same as *R. microsporus* var. *oligosporus* R-47. Previously, it was reported that the IGS rDNA sequences of *Setaria italica* (L.) P. Beauv. (plant; Fukunaga *et al*. 2005), *Vicia sativa* L. (plant; Macas *et al*. 2003), *Anopheles sinensis* Wiedemann (animal; Whang *et al*. 2002), *Daphnia pulex* Leydig (animal; Gorokhova *et al*. 2002) and *Schizophyllum commune* Fr. (Fungi, Basidiomycota; James *et al*. 2001), as well as the ITS rDNA sequences of the *Larix potaninii* Batalin (plant; Wei *et al*. 2003), *Orconectes* spp. (animal; Harris and Crandall 2000), *Trichaptum abietinum* (Dicks.) Ryvarden (Fungi, Basidiomycota; Kauserud & Schumacher 2003), possessed several variants of different length within an individual due to different numbers of STRs. This paper is the first report of rDNA variants in Zygomycota, Fungi.

Inconsistence of molecule and morphology in *Rhizopus microsporus*

The STR in *Rhizopus microsporus* lacked a unique motif for any variety which was resolved by morphology (Zheng *et al*. 2007). But different strains of some varieties contain different types of STR (Tab. 3), e.g. *R. microsporus* var. *oligosporus* type II (CBS 337.62), III (AS 3.1161) and V (R-47). These strains can be identified easily by morphology and physiology. This means that the traditional characteristics used in identifying varieties of *R. microsporus* are more stable than by the types of STR within the IGS rDNA. In comparison, the STR of *R. arrhizus* provides taxonomic information not only at the strain level but also at the variety level (Tab. 2). It is possible that the morphology and molecule evolve in concert in *R. arrhizus* and that the morphological characters evolve faster than IGS rDNA in *R. microsporus*. Morphologically *R. microsporus* has formed six stable and obviously differentiable varieties (Zheng *et al*. 2007) but phylogenetically only three lineages were developed (Fig. 3), also that these morphological varieties are not consistent with the phylogenetic lineages. In this study, the STR within the IGS rDNA was a useful taxonomic criterion for *R. arrhizus* but not for *R. microsporus*. Therefore we suggest that one should be careful to deduce a useful criterion in related taxa without experimental confirmation.

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Difference of *Rhizopus arrhizus* and *R. microsporus*

Besides the different meaning at variety and strain level, the STR motifs of the two species are completely different. The six motifs of *Rhizopus arrhizus* share a core sequence TCTG, while the two motifs of *R. microsporus* contain another distinct core sequence CCA. These two core sequences display no correlation; in other word the core sequences are species-specific.

Based on the result of the present study, the morphological and molecular characters of *R. arrhizus* evolve in concert and the varieties of *R. arrhizus* can be classified morphologically and molecularly, but the morphology and molecule develop at different rate in *R. microsporus*.

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References

Abe A., Oda Y., Asano K., Sone T. (2006) The molecular phylogeny of the genus *Rhizopus* based on rDNA sequences. *Bioscience, Biotechnology, and Biochemistry* **70**: 2387–2393.

Alfaro M. E., Zoller S., Lutzoni F. (2003) Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution* **20**: 255–266.

Douady C. J., Delsuc F., Boucher Y., Doolittle W. F., Douzery E. J. P. (2003) Comparison of Bayesian and Maximum Likelihood Bootstrap Measures of Phylogenetic Reliability. *Molecular Biology and Evolution* **20**: 248–254.

Ellis J. J. (1985) Species and varieties in the *Rhizopus arrhizus – Rhizopus oryzae* group as indicated by their DNA complementarity. *Mycologia* **77**: 243–247.

Farris J. S., Kallersjo M., Kluge A. G., Bult C. (1994) Testing significance of incongruence. *Cladistics* **10**: 315–319.

Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783–791.

Fukunaga K., Ichitani K., Taura S., Sato M., Kawase M. (2005) Ribosomal DNA intergenic spacer sequence in foxtail millet, *Setaria italica* (L.) P. Beauv. and

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its characterization and application to typing of foxtail millet landraces. *Hereditas* **142**: 38–44.

Gelman A., Rubin D. B. (1992) Inference from iterative simulation using multiple sequences. *Statistical Science* **7**: 457–472.

Gorokhova E., Dowling T. E., Weider L. J., Crease T. J., Elser J. J. (2002) Functional and ecological significance of rDNA intergenic spacer variation in a clonal organism under divergent selection for production rate. *Proceedings of the Royal Society B – Biological Sciences* **269**: 2373–2379.

Guidot A., Lumini E., Debaud J. C., Marmeisse R. (1999) The nuclear ribosomal DNA intergenic spacer as a target sequence to study intraspecific diversity of the ectomycorrhizal basidiomycete *Hebeloma cylindrosporum* directly on *Pinus* root systems. *Applied and Environmental Microbiology* **65**: 903–909.

Hall T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Harris D. J., Crandall K. A. (2000) Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and short tandem studies. *Molecular Biology and Evolution* **17**: 284–291.

Huang H., Yu M.-q. (1988) Identification of *Rhizopus* by electrophoresis of proteins and esterase. *Mycosystema* **1**: 61–71.

Huelsenbeck J. P., Crandall K. A. 1997. Phylogeny estimation and hypothesis testing using maximum likelihood. *Annual Review of Ecology and Systematics* **28**: 437-466.

Huelsenbeck J. P., Ronquist F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.

James T. Y., Moncalvo J.-M., Li S., Vilgalys R. (2001) Polymorphism at the ribosomal DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. *Genetics* **157**: 149–161.

Jennessen J., Nielsen K. F., Houbraken J., Lyhne E. K., Schnürer J., Frisvad J. C., Samson R. A. (2005) Secondary metabolite and mycotoxin production by the *Rhziopus microsporus* group. *Journal of Agricultural and Food chemistry* **53**: 1833–1840.

Kauserud H., Schumacher T. (2003) Ribosomal DNA variation, recombination and inheritance in the basidiomycete *Trichaptum abietinum*: implications for reticulate evolution. *Heredity* **91**: 163–172.

Kumar S., Sharma N. S., Saharan M. R., Singh R. (2004) Biochemical changes during ripening of cheddar cheese prepared by milk clotting enzyme from *Rhizopus oryzae*. *Journal of Food Science and Technology* **41**: 279–283.

Liou G.-y., Chen S.-r., Wei Y.-h., Lee F.-l., Fu H.-m., Yuan G.-f., Stalpers J. A. (2007) Polyphasic approach to the taxonomy of the *Rhizopus stolonifer* group. *Mycological Research* **111**: 196–203.

Liu X.-y., Huang H., Zheng R.-y. (2007) Molecular phylogenetic relationships within *Rhizopus* based on combined analyses of ITS rDNA and *pyrG* gene sequences. *Sydowia* **59**: 235–253.

Macas J., Navrátilová A., Mészáros T. (2003) Sequence subfamilies of satellite repeats related to rDNA intergenic spacer are differentially amplified on *Vicia sativa* chromosomes. *Chromosoma* **112**: 152–158.

Nylander J. A. A. (2004) *MrModeltest v2. Program distributed by the author.* Evolutionary Biology Centre, Uppsala University.

Page R. D. M. (1996) TreeView: An application to display phylogenetic trees on personal computers. *Computational and Applied Biosciences* **12**: 357–358.

Posada D., Crandall K. A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

Posada D., Crandall K. A. (2001) Selecting the best-fit model of nucleotide substitution. *Systematic Biology* **50**: 580–601.

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Ronquist F., Huelsenbeck J. P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Sallares R., Brown T. A. (2004) Phylogenetic analysis of complete 5' external transcribed spacers of the 18S ribosomal RNA genes of diploid *Aegilops* and related species (Triticeae, Polaceae). *Genetic Resources and Crop Evolution* **51**: 701–712.

Schipper M. A. A. (1984) A revision of the genus *Rhizopus* I. The *Rhizopus stolonifer*-group and *Rhizopus oryzae*. *Studies in Mycology* **25**: 1–19.

Schipper M. A. A., Stalpers J. A. (1984) A revision of the genus *Rhizopus* II. The *Rhizopus microsporus*-group. *Studies in Mycology* **25**: 20–34.

Schipper M. A. A., Maslen M. M., Hogg G. G., Chow C. W., Samson R. A. (1996) Human infection by *Rhizopus azygosporus* and the occurrence of azygospore in Zygomycetes. *Journal of Medical and Veterinary Mycology* **34**: 199–203.

Sutar R., David J. K., Ganesan K., Ghosh A. K., Singhi S., Chakrabarti A., Bachhawat A. K. (2004) Comparison of ITS and IGS1 regions for strain typing of clinical and non-clinical isolates of *Pichia anomala*. *Journal of Medical Microbiology* **53**: 119–123.

Swofford, D. L. (2002). *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts, U.S.A.

Thompson J. D., Gilbson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.

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**: 181–188.

Veldman G. M., Klootwijk J., de Regt V. C. H. F., Rudi R. J. (1981) The primary and secondary structure of yeast 26S rRNA. *Nucleic Acids Research* **9**: 6935–6952.

Voigt K., Cigelnik E., O'Donnell K. (1999) Phylogeny and PCR identification of clinical important zygomycetes based on nuclear ribosomal-DNA sequence data. *Journal of Clinical Microbiology* **37**: 3957–3964.

Warude D., Chavan P., Joshi K., Patwardhan B. (2003) DNA isolation from fresh and dry plant samples with highly acidic tissue extracts. *Plant Molecular Biology Reporter* **21**: 467a–467f.

Wei X.-x., Wang, X.-q., Hong D.-Y. (2003) Marked Intragenomic Heterogeneity and Geographical Differentiation of nrDNA ITS in *Larix potaninii* (Pinaceae). *Journal of Molecular Evolution* **57**: 623–635.

Whang I.-j., Jung J., Park J.-k., Min G.-s., Kim W. (2002) Intragenomic length variation of the ribosomal DNA intergenic spacer in a malaria vector, *Anopheles sinensis*. *Molecules and Cells* **14**: 158–162.

Zheng R.-y., Chen G.-q., Huang H., Liu, X.-y. (2007) A monograph of *Rhizopus*. *Sydowia* **59**: 273–372.

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