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The Molecular Phylogeny of the Genus *Rhizopus* Based on rDNA Sequences

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In order to establish the molecular phylogeny of the genus *Rhizopus*, three molecules of the ribosomal RNA-encoding DNA (rDNA), complete 18S, internal transcribed spacer (ITS)1-5.8S-ITS2, and 28S D1/D2 regions of all the species of the genus were sequenced. Phylogenetic trees showed three major clusters corresponding to the three groups in the current morphological taxonomy, *microsporus*-group, *stolonifer*-group, and *R. oryzae*. *R. stolonifer* var. *lyococcos* was clustered independently from the major clusters. *R. schipperae* clustered differently in all trees. Strains of *R. sexualis* had multiple ITS sequences. *A. rouxii* clustered with *R. oryzae*. These results indicate the possibility of molecular identification of species groups using rDNA sequencing. Reclassification of the genus might be appropriate.

Key words: *Rhizopus*; ribosomal RNA-encoding DNA (rDNA); classification; molecular phylogeny

The strains of the genus *Rhizopus* are often used in fermented foods in East and Southeast Asia.[1)] Since long ago, therefore, those strains have been studied variously as to their phylogeny, physiology, genetics, and biochemistry, and many researchers have reported new species, classified by morphological and physiological characteristics.[2)]

Schipper and Stalpers[2)] revised the classification of the genus mainly by growth temperature, size of sporangia and sporangiophore, and branching of rhizoid, and classified all the species of the genus into three groups: the *stolonifer*-group, *R. oryzae*, and the *microsporus*-group, with the re-integration of many species. After the addition of some new species,[3–5)] currently the genus *Rhizopus* consists of 13 species. Although there have been reports of reclassification based on DNA–DNA hybridization and isozyme analysis,[6–8)] this classification is accepted as the standard classification of the genus.

Recently, we developed a new method to screen the lactic acid producers in *Rhizopus oryzae*.[9)] In that work, strains of *R. oryzae* were divided clearly into two types from data on organic acid production and the rDNA ITS sequence, although *R. oryzae* was one species in the classification by Schipper and Stalpers.[2)] In addition, we found that *Amylomyces rouxii* had the same ITS sequence as the lactic acid producer, and that it produced lactic acid. These facts suggest the possibility of reclassifying *R. oryzae* and *A. rouxii*.

In order to consider the reclassification, it is necessary to understand the molecular phylogenetic information of all members of the genus, which should allow us to discuss the relationship between rDNA sequence diversity and the species. Although the current classification of fungi is commonly based upon morphology, molecular techniques, such as DNA sequencing are powerful tools now used in many fungal cases[10)] and in some cases species can be identified using the molecular data,[11)] but the molecular phylogeny of the genus *Rhizopus* has not been studied intensively yet. Voigt *et al.*[12)] and Voigt and Wöstemeister[13)] studied the phylogeny of some species from the genus *Rhizopus*, with many other species from Zygomycete, using genes of rRNA, translation elongation factor EF-1α, and actin. In those studies, only limited numbers of strains were used, and the phylogenetic relationships within the genus *Rhizopus* were not solved well.

In this study, we aimed to establish the molecular phylogeny of the genus *Rhizopus* by rDNA sequencing and to compare it with the current classification of the genus.

Materials and Methods

Strains and growth conditions. The strains used in this study are listed in Table 1. All the strains were obtained from American Type Culture Collection (ATCC, Manassas, VA) and the Centraalbureau voor

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Abbreviations: rDNA, ribosomal RNA-encoding DNA; ITS, internal transcribed spacer; PCR, polymerase chain reaction

Table 1. Strains Used in This Study

Group*	Species	Strain	Accession no.		
			18S	ITS	28S D1/D2
microsporus	*R. schipperae*	ATCC 96514^T	AB250170	AB106340	AB250193
		ATCC 204270	AB250162	AB113015	AB250185
	R. azygosporus	CBS 357.92	AB250159	AB097391	AB250182
		CBS 357.93^T	AB250156	AB097392	AB250179
	R. caespitosus	CBS 427.87	AB250168	AB097387	AB250191
	R. homothallicus	CBS 336.62^T	AB250175	AB097388	AB250198
	R. microsporus var. *chinensis*	CBS 262.28	AB250158	AB097384	AB250181
		CBS 631.82	AB250157	AB097394	AB250180
	R. microsporus var. *microsporus*	CBS 699.68	AB250155	AB097385	AB250178
		CBS 700.68	AB250165	AB097386	AB250188
	R. microsporus var. *oligosporus*	CBS 337.62	AB250177	AB097395	AB250200
		CBS 338.62	AB250166	AB097389	AB250189
	R. microsporus var. *rhizopodiformis*	CBS 343.29	AB250161	AB097390	AB250184
		CBS 536.80	AB250160	AB097393	AB250183
oryzae	*R. oryzae*	CBS 112.07^T	AB250164	AB097334	AB250187
		CBS 278.38	AB250174	AB097299	AB250197
		CBS 404.51	N. D.**	AB181329	N. D.
		CBS 406.51	N. D.	AB181330	N. D.
		CBS 391.34	N. D.	AB181325	N. D.
		CBS 395.34	N. D.	AB181316	N. D.
		CBS 381.52	N. D.	AB181315	N. D.
		CBS 128.08	N. D.	AB181305	N. D.
		CBS 127.08	N. D.	AB181304	N. D.
stolonifer	*R. sexualis* var. *americanus*	CBS 340.62^T	AB250169	AB113010–113012***	AB250192
	R. sexualis var. *sexualis*	CBS 336.39^T	AB250163	AB113016–113021***	AB250186
	R. stolonifer var. *lyococcos*	CBS 319.35	AB250172	AB100449	AB250195
		CBS 320.35	AB250173	AB100450	AB250196
	R. stolonifer var. *stolonifer*	CBS 150.83	AB250176	AB113022	AB250199
		CBS 609.82	AB250167	AB113023	AB25019
Amylomyces (outgroup)	*Amylomyces rouxii*	CBS 438.76^T	AB250171	AB181310	AB250194
	*Mucor miehei*****	ATCC 26282	AF192506	AF198253	AF205941

*The grouping was according to Reference 2.
**N. D., Not determined.
***Multiple sequences of ITS were isolated from each strain.
****According to Reference 18.

Schimmelcultures (CBS, Utrecht, Netherlands). For preservation and serial transfer, potato glucose agar (Difco, Detroit, MI) was used. The medium for DNA preparation was malt extract medium (malt extract [Difco] 20 g/l, polypepton [Nihon Pharmaceutical, Tokyo] 1 g/l, and glucose 20 g/l).

DNA extraction. Each microorganism was grown aerobically by shaking at 27 °C for 3 d using 10 ml medium in a test tube. The fungal cells were filtered, air-dried, and lyophilized overnight. The genomic DNA of each strain was extracted from the lyophilized cells according to the method of Sone *et al.*[14)]

PCR reactions. PCR amplification was performed in 50 µl reaction mixture containing 5 µl of 10 × PCR buffer, 5 µl deoxynucleotide triphosphate (2 mM each), 10 pmole of each primer, 3.5 µl $MgCl_2$ solution (25 mM), and 2.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). The template DNA was 100 ng of each strain for the PCR reaction. The reaction conditions were as follows: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. A final 5 min of chain elongation at 72 °C was carried out after cycling completion in a model 9700 thermal cycler (Applied Biosystems). In the case of *R. sexualis* var. *americanus*, where amplification was not detected under the conditions above, Ampdirect for Semi-Purified DNA (Shimadzu, Kyoto, Japan) was used. The composition of PCR reaction was as follows: 10 µl of Ampdirect, 10 µl of Ampaddition, 4 µl deoxynucleotide triphosphate (2 mM each), 5 pmol of each primer, 1.25 U AmpliTaq DNA polymerase (Applied Biosystems), and template DNA (200 ng) in a total volume of 50 µl. The reaction conditions were as follows: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. A final 5 min of chain elongation at 72 °C was carried out after cycling completion. The PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, visualized with a UV transilluminator, and photographed.

Cloning of heterogeneous PCR products. The PCR products were purified using Microspin S-400HR spin column (Amersham Biosciences, Piscataway, NJ), and used for ligation into pGEM-T Easy (Promega, Madison, WI). The ligation was conducted following the protocol supplied by the manufacturer. The ligation product was precipitated with ethanol and then used for the transformation of *E. coli* JM109. White colonies on an LB-ampicillin-Xgal-IPTG plate were chosen, and the plasmids were extracted from the liquid culture. The molecular weight of the inserted DNA was checked by *Eco* RI digestion, and plasmid with different insert was used for sequencing analysis. Deletion of insert was performed with a Deletion kit for kilo-sequencing (Takara, Ohtsu, Japan) following the manufacturer's instructions.

Sequence analysis. The PCR products were purified by Microspin S-300HR (Amersham Biosciences). Sequencing reaction was performed using a BigDye™ Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems) and analyzed with an ABI PRISM 3100 Genetic Analyzer or an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Phylogenetic relationships were estimated using the Clustal X Package.[15] The sequence data obtained in this study were deposited in the DDBJ/EMBL/GenBank database under the accession numbers listed in Table 1.

Results

PCR amplification and sequencing

From all strains except *Mucor miehei*, genomic DNAs were prepared, and the 18S, ITS, and 28S D1/D2 regions were amplified and sequenced with the primers previously described,[16,17] except for ITS of *Rhizopus stolonifer* and *R. sexualis*. For *R. stolonifer* var. *lyococcos* strains, four additional primers, listed in Table 2, were designed to sequence the full length of ITS. In *R. stolonifer* var. *stolonifer* strains, sequencing reactions were inhibited, probably by many secondary structures, and hence we analyzed them by sequencing the deletion mutants, after the PCR products were cloned in pGEM-T easy vector. In the case of *R. sexualis* the situation was more complex, because multiple bands were amplified from each single strain. From *R. sexualis* var. *sexualis*, multiple bands of similar-length fragments were detected. Each fragment in the mixture was separated by cloning into plasmid vector. Sequencing of some independent clones revealed that there were six types of sequences (SS1, 2, 4, 5, 9, and 11). From *R. sexualis* var. *americanus*, multiple bands of different lengths were detected. Cloning strategy, the same as in *R. sexualis* var. *sexualis*, was applied. Sequence analysis revealed that the strain had three independent ITS sequences (SA1, 2, and 3).

Table 2. Oligonucleotide Primers Designed for This Study

Primer	Sequence (5′ to 3′)
Reflexus-F1	CTATAAACATTAGCCTTGAAATTCAGT
Reflexus-F2	ACAGGTTAGCTTTAGCTTGCCTTT
Reflexus-R1	TTTAGGCAGGTTTCCCCAA
Reflexus-R2	GCAAGTGTGCTCTAGGGAAG

Molecular phylogeny

Phylogenic trees were made with 18S, 28S, and ITS rDNA data, those of *Mucor miehei* ATCC 26282[18] serving as outgroups (Fig. 1). The topologies of phylogenetic trees were almost conserved except for clusters of *R. schipperae* and *R. stolonifer* var. *lyococcos*. Bootstrapping analysis indicated three major clusters, A, B, and C. Cluster A consisted of *R. oryzae* and *A. rouxii*, cluster B of *R. microsporus*, *R. azygosporus*, *R. homothallicus* and *R. caespitosus*, and cluster C of *R. stolonifer* var. *stolonifer* and *R. sexualis*. These clusters corresponded to the morphological grouping *oryzae*, the *microsporus*-group, and the *stolonifer*-group respectively. The cluster of *R. schipperae* was located within cluster A in the 18S rDNA tree, but in the 28S tree, it was located just outside cluster A, co-clustered with relatively high bootstrap value. In the ITS tree, *R. schipperae* was located outside clusters A and B, and co-clustered with them with high bootstrap value. *R. stolonifer* var. *lyococcos* clustered independently in all trees. The cluster of *R. stolonifer* var. *lyococcos* in the ITS tree was related to cluster C, but a low bootstrap value indicated the relationship was not robust.

In cluster A, no strains of *R. azygosporus* were separable from *R. microsporus* var. *rhizopodiformis* in any phylogenetic tree, *i.e.*, all the sequences were shared. *R. microsporus* var. *oligosporus* also shared the 18S and ITS sequences with the strains above, but was slightly different in the 28S D1/D2 region sequence. Two strains of *R. microsporus* var. *microsporus* shared all the sequences and clustered separately from the other *R. microsporus* strains. In all trees, *R. microsporus* var. *chinensis* CBS 631.82 was clustered with *R. azygosporus*, *R. microsporus* var. *oligosporus*, and *R. microsporus* var. *rhizopodiformis*, but another strain of the same species, CBS 262.28, was clustered with *R. microsporus* var. *microsporus*. *R. caespitosus* and *R. homothallicus* were clustered independently with the subcluster consisting of *R. microsporus* and *R. azygosporus*.

In cluster B, strains of *R. oryzae* and *A. rouxii* were included. In the cluster, there were two distinct types of all three molecules, and these formed two subclusters, BI and BII. Subcluster BI corresponded to a fumaric acid type sequence, and the subcluster BII corresponded to a lactic acid type sequence determined by Abe *et al.*[9] In order to clarify the bipolarity of the ITS sequence, ITS sequences of additional six strains of *R. oryzae* were obtained and re-clustered. Each strain was co-clustered into one of the clusters of BI or BII. *A. rouxii* CBS 438.76^T co-clustered in subcluster BII.

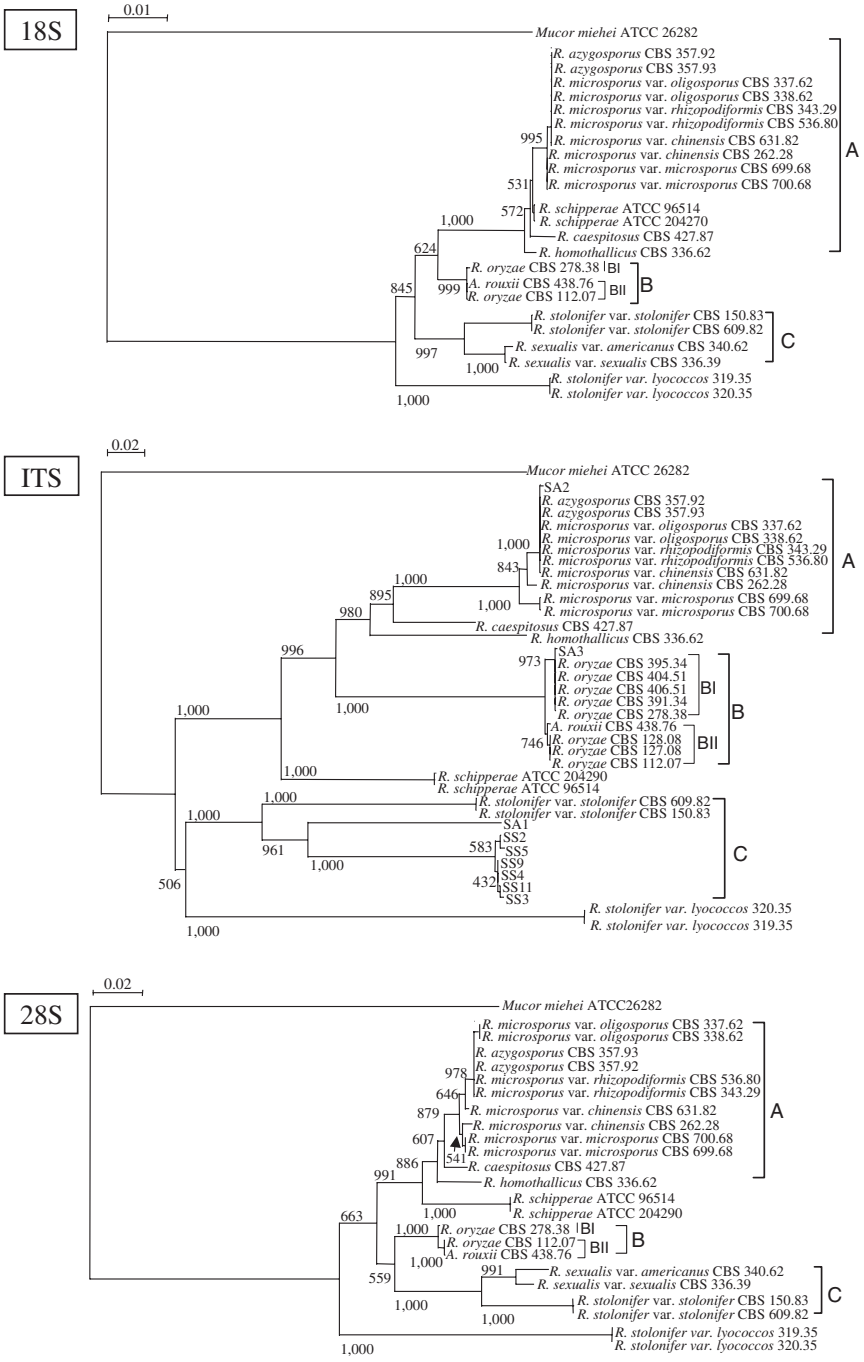

Fig. 1. Phylogenetic Tree of Genus *Rhizopus* Based on rDNA Sequences.

Three large clusters A, B, and C, are indicated. Two subclusters of cluster B are marked BI and BII. SA1, 2, 3, and 4 are three independent ITS sequences from *R. sexualis* var. *americanus* CBS 340.62, and SS2, 3, 4, 5, 9, and 11 are six independent sequences from *R. sexualis* var. *sexualis* CBS 336.39. *Mucor miehei* sequences were used as an outgroup. Bootstrap values out of 1,000 iterations are indicated on clades.

R. sexualis strains have shown a variety of ITS sequences in each single strain. In the case of *R. sexualis* var. *americanus*, three distinct types of sequences were isolated. SA1 clustered with *R. sexualis* var. *sexualis*, corresponding with trees of the 18S rDNA and 28S D1/D2 regions. The SA2 sequence was similar to *R. microsporus* var. *oligosporus*/*R. microsporus* var. *rhizopodiformis*. The SA3 sequence was co-clustered with the fumaric acid type of *R. oryzae*. From the ITS sequences of *R. sexualis* var. *sexualis*, a cluster of related but slightly different sequences was formed and co-clustered with the sequence SA1 of *R. sexualis* var. *americanus*. These six different sequences were classified into two groups: *viz.*, the short type and the long type (Fig. 2). A remarkable difference was the insertion of a 22-bp AT-rich sequence in the long-type sequence. In addition, some base substitutions were detected among them.

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                          76   81  266 448                          471  493    579 638     655  709
SS- 3(AB113018)     1 ...C....T...T...ATATA----------------------...-...TTT-...-...CTCTT...GG...A...817
SS- 2(AB113017)     1 ...T....-...T...ATATA----------------------...-...TTTT...-...TCCTC...G-...A...816
SS- 5(AB113020)     1 ...C....T...T...ATATA----------------------...-...---T...-...TCCTC...--...-...812
SS- 4(AB113019)     1 ...T....-...A...ATATATTTTTTTTTTAAAAAAAATATA...A...TTTT...G...CTCTT...GG...-...840
SS- 9(AB113021)     1 ...T....-...A...ATATATTTTTTTTTTAAAAAAAATATA...A...TTTT...-...CTCTT...GG...-...839
SS-11(AB113016)     1 ...T....-...T...ATATATTTTTTTTTTAAAAAAAATATA...A...TTTT...-...CTCTT...GG...-...839
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Fig. 2. Alignment of Six Independent ITS Sequences of *R. sexualis* var. *sexualis*.
Only the parts containing base substitutions or insertions are presented. The number on the top indicates the base number in the SS-3 sequence.

Discussion

To establish the molecular phylogeny of the genus *Rhizopus* and compare it with the current classification, sequences of rDNA 18S, ITS, and 28S D1/D2 of all species of the genus were analyzed. The results indicated that the molecular phylogeny was similar to the morphological grouping, except for *R. schipperae* and *R. stolonifer* var. *lyococcos*. We could identify three major clusters, A, B and C, corresponding to the *microsporus*-group, *R. oryzae*, and the *stolonifer*-group respectively. The high bootstrapping values of these three major clusters indicated the robustness of the above grouping. This relationship might be utilized in the molecular identification of species of the genus *Rhizopus*.

R. schipperae was located in a position different from but related to *R. oryzae* and the *microsporus*-group among the three phylogenetic trees. *R. schipperae* is a species newly identified after the publication of the current taxonomy.[5] The species belonged to the *microsporus*-group morphologically, but showed characteristics different from that of the group, and was similar to *A. rouxii*. The results presented in this study indicate that the relationship of *R. schipperae* to the species of the *microsporus*-group was not strong enough to be in the same group, and thus should be treated as a species independent from any group.

R. stolonifer var. *lyococcos* was also located in a distinct position in all trees. The sequence similarity between the two varieties of *R. stolonifer* was not high. In the trees of 18S and 28S D1/D2, they were not clustered with each other. Morphologically, the species was similar to *R. stolonifer* var. *stolonifer*, but distinct in the form of sporangiophores.[2] The result of rDNA sequence might indicate the possibility of reclassification of these two into two distinct species, but the fact that these two varieties produce the sexual stage[2] strongly supports the current taxonomy, which treat them as two varieties of the same species.

Another interesting fact is that each of the two varieties of *R. sexualis* had multiple distinct ITS sequences in a single strain. One of the explanations of this phenomenon is the occurrence of heterokaryosis. This conclusion is also supported by the fact that *R. sexualis* is homothallic, whereas most species in the genus *Rhizopus* are heterothallic. In the genus *Neurospora*, a similar relationship was found in *Neurospora tetrasperma*, a heterokaryon of two different mating types, known to be pseudohomothallic,[19] but the homogeneity of the 18S and 28S D1/D2 region sequences among *R. sexulalis* species indicates the possibility of recombination or base substitution as alternate causes of ITS heterogeneity. Ueda and Mitaka studied the heterogeneity of the 18S rDNA sequence, and proposed that direct sequencing of PCR product contributed to distinguish the main sequence.[20] In the present study, we also tried to sequence the PCR product, but it was impossible to determine the main sequence because of the mixed peak in the electrophoresis. This indicates that the proportion of each sequence in the total copy of rDNA was not distinct, and that the mutation in a small portion of the ITS sequence was not the cause of the heterogeneity, but the mechanism of such integration of different ITS sequences remains to be studied.

For the two major clusters A and B, which correspond to the *microsporus*-group and *R. oryzae* respectively, the relationship between the molecular phylogeny and morphological taxonomy was rather complicated. *R. azygosporus* shared completely the same sequence with *R. microsporus* var. *rhizopodiformis* in all the molecules investigated, although these are separated at the species level in the current taxonomy. Azygospore formation is the distinct phenotype of *R. azygosporus* for classification at the species level.[3] The result of molecular phylogeny indicates that this distinct phenotype might be due to mutations in the gene(s) related to azygospore formation, occurring at a relatively late time in the phylogenetic process. Liou *et al.*[8] also reported a similarity between the *R. azygosporus* and *R. microsporus* strains, and indicated the possibility of diploidy or aneuploidy. On these points, *R. azygosporus* might better be reclassified as a variety of *R. microsporus*.

On the other hand, the two strains of *R. microsporus* var. *chinensis* did not share a single molecule tested, indicating that these two strains are distinct in phylogeny. Schipper and Stalpers[2] pointed out the difference in sporangiospore ornamentation between these two strains. Thus strain CBS 262.28 can be reidentified as *R. microsporus* var. *microsporus*.

A similar problem exists in the single species of *R. oryzae*. In this species, two distinct groups of strains were detected, corresponding to two ITS sequence types,

I and II, in our previous report.[9] These two distinct sequence types coincided with physiological characteristics such as organic acid production and lipid composition of plasma membrane.[21] Moreover, Saito *et al.*[22] analyzed the structure of *ldh* (lactate dehydrogenase) genes from both ITS types and found that they are distinct from each other. Type II strains, *viz.*, fumaric acid producers, lacked the *ldhA* gene, which is responsible for lactic acid production in type I strains, *viz.*, lactic acid producers. In addition, the nucleotide sequences of the *ldhB* genes were also distinct as between the two types. These data indicate that these two types of *R. oryzae* might be distinct at the species level.

A. rouxii CBS 438.76^T shares one of the sequences (type I) of *R. oryzae* in all molecules investigated. The taxonomic key of *A. rouxii* is the formation of abortive sporangiophores and an abundance of chlamydospores.[23] The sequence similarity between *A. rouxii* and *R. oryzae* indicates the recent diversification of these two genera. Voigt and Wöstemeister[13] reported that *A. rouxii* and *R. oryzae* were distinct at the 18S rDNA sequence, but the strains used by them were distinct from the ones used in this paper. The 18S rDNA sequences deposited by them were compared with those we determined in this study. *R. oryzae* strain NRRL 28631, used in their paper, was found to be type I in our results, and their *A. rouxii* NRRL3139 sequence was the same as type II in our results. In addition, Schwarz *et al.*[24] reported in a recent paper that the homology of the ITS sequence of *Mucor rouxii* (= *A. rouxii*) and *R. oryzae* was only 56.5%. This suggests that *A. rouxii* is a species that contains several genetically distinct populations. More intensive study using a large collection of strains is necessary to determine the relationship between *R. oryzae* and *A. rouxii*.

These facts indicate that re-examination of the classification of genus *Rhizopus* and *Amylomyces* might be appropriate. The three major groupings, the *microsporus*-group, the *stolonifer*-group, and *R. oryzae*, remain as the core criteria, with the addition of *R. schipperae* and *R. stolonifer* var. *lyococcos* as distinct species. On the other hand, the construction of each group should be reconsidered. In the *microsporus* group, a combination of several varieties might be necessary, whereas for *R. oryzae*, reclassification into two species and consideration of the *oryzae*-group might be applicable. For completion of the re-examination of the genus, however, more precise study, including morphological and physiological characterization, and also a molecular approach such as AFLPs are necessary. The genomic sequence of *R. oryzae*[25] might be a good tool for such studies.

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