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Rhizopus delemar is the proper name for *Rhizopus oryzae* fumaric-malic acid producers

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Abstract: The zygomycete *Rhizopus oryzae* currently is identified by sporangiophore morphology and growth temperature, but heterogeneity of the species has been reported. We examined the suitability of organic acid production as an effective taxonomic character for reclassification of the species. Strains were divided into two groups, LA (lactic acid producer) and FMA (fumaric-malic acid producers) according to organic acid production. These grouping were confirmed as phylogenetically distinct because analyses of rDNA ITS, lactate dehydrogenase B, actin, translation elongation factor-1α and genome-wide AFLP resolved the same two exclusive clusters, corresponding with the organic acid grouping. Reclassification of strains in the FMA group as *R. delemar* was proposed.

Key words: AFLP, lactic acid, phylogeny, *Rhizopus*

INTRODUCTION

Genus *Rhizopus* includes species often used for fermented foods in eastern and southeastern Asia. Therefore the phylogeny, physiology, genetics and biochemistry of those strains were studied, and many researchers reported new species classified by morphological and physiological characters (Hesseltine 1983, Schipper and Stalpers 1984, Schipper 1984). Schipper and Stalpers (1984) and Schipper (1984) revised the classification of the genus mainly by growth temperature, size of sporangia and sporangiophore and branching of rhizoids and classified all species of the genus into three groups, *stolonifer*-group, *R. oryzae* and *microsporus*-group, with the proposed synonymy of many species. After some additions (Yuan and Jong 1984, Ellis 1985, Weitzman et al 1996), *Rhizopus* consists of 13 species. Although there were reports of reclassification based on DNA-DNA hybridization and isozyme analysis (Ellis 1986, Liou et al 2001), Schipper's classification is accepted as the standard of the genus. In the classification by Schipper and Stalpers (1984), *Rhizopus oryzae* was recognized as a species with intermediate morphology and physiological characters. They investigated 47 strains from 21 species and classified all of them as *R. oryzae* because they fundamentally were indistinguishable.

We recently found a clear relationship between rDNA ITS sequence and acid production in *Rhizopus oryzae* (Abe et al 2003). In that work, strains of *R. oryzae* were divided clearly into two types, lactic acid producers and fumaric-malic acid producers from the data of organic acid production and the rDNA ITS sequence. In addition, genus-level analysis of rDNA sequences revealed that the degree of diversity between the two groups was similar to that between species (Abe et al 2006). On the other hand, Saito et al (2004) found a relationship between lactic acid production and the *ldh* genes. *R. oryzae* has two genes for lactate dehydrogenase, *ldhA* and *ldhB*. Fumaric acid producers lacked the *ldhA* gene, which was responsible for lactic acid production in lactic acid producers. In addition, the nucleotide sequences of the *ldhB* gene distinguished the two types. These results indicated the possibility of organic acid production as a physiological category suitable for reclassification of *R. oryzae*, suggesting these two types might be distinct species.

Organic acid production had been studied extensively in *R. oryzae*. Takahashi and Sakaguchi (1925) and Takahashi et al (1926) studied the production of fumaric acid and lactic acid and found that *Rhizopus* spp. could be divided into fumaric acid producers, lactic acid producers and producers of both fumaric and lactic acid, but they did not comment on the taxonomic importance of acid production. Kitahara and Fukui (1949) indicated that there was no clear relationship between acid production and morphological classification. Inui et al (1965) used acid production as the key character to differentiate *R. oryzae*, *R. japonicus* and *R. delemar*. However, the

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TABLE I. *R. oryzae* strains used in this study with accession numbers of sequence data

CBS No.	Former name[a]	Proposed name	rDNA ITS	*ldhB*	EF-1α	*act-1*
110.17	*R. maydis*	*R. oryzae*	AB181303	AB281556	AB281527	AB281498
112.07[T]	*R. oryzae*	*R. oryzae*	AB097334	AB281557	AB281528	AB281499
120.12	*R. delemar*	*R. delemar*	AB181318	AB281558	AB281529	AB281500
127.08	*R. nodosus*	*R. oryzae*	AB181304	AB281559	AB281530	AB281501
128.08	*R. tritici*	*R. oryzae*	AB181305	AB281560	AB281531	AB281502
257.28	*R. formosaensis*	*R. oryzae*	AB181311	AB281561	AB281532	AB281503
258.28	*R. hangchao*	*R. oryzae*	AB181312	AB281562	AB281533	AB281504
260.28	*R. liquefaciens*	*R. oryzae*	AB181306	AB281563	AB281534	AB281505
264.28	*R. pseudochinensis*	*R. oryzae*	AB181307	AB281564	AB281535	AB281506
266.30	*R. fusiformis*	*R. oryzae*	AB181313	AB281565	AB281536	AB281507
278.38	*R. oryzae*	*R. delemar*	AB097299	AB281566	AB281537	AB281508
279.38	*R. sontii*	*R. delemar*	AB181319	AB281567	AB281538	AB281509
295.31	*R. suinus*	*R. delemar*	AB181320	AB281568	AB281539	AB281510
321.35	*R. kasanensis*	*R. oryzae*	AB181308	AB281569	AB281540	AB281511
330.53	*R. boreas*	*R. oryzae*	AB181314	AB281570	AB281541	AB281512
381.52	*R. arrhizus*	*R. oryzae*	AB181315	AB281571	AB281542	AB281513
385.34	*R. achlamydosporus*	*R. delemar*	AB181321	AB281572	AB281543	AB281514
386.34	*R. bahrnensis*	*R. delemar*	AB181322	AB281573	AB281544	AB281515
387.34	*R. batatas*	*R. oryzae*	AB181309	AB281574	AB281545	AB281516
389.34	*R. chiuniang* var. *isofermentarius*	*R. delemar*	AB181323	AB281575	AB281546	AB281517
390.34	*R. delemar* var. *minimus*	*R. delemar*	AB181324	AB281576	AB281547	AB281518
391.34	*R. javanicus*	*R. delemar*	AB181325	AB281577	AB281548	AB281519
392.95	*R. delemar* var. *multiplicisporus*	*R. delemar*	AB181326	AB281578	AB281549	AB281520
393.34	*R. peka II*	*R. delemar*	AB181327	AB281579	AB281550	AB281521
395.34	*R. semarangensis*	*R. delemar*	AB181316	AB281580	AB281551	AB281522
395.54		*R. delemar*	AB181317	AB281581	AB281552	AB281523
402.51	*R. javanicus* var. *kawasakiensis*	*R. delemar*	AB181328	AB281582	AB281553	AB281524
404.51	*R. shanghaiensis*	*R. delemar*	AB181329	AB281583	AB281554	AB281525
406.51	*R. usamii*	*R. delemar*	AB181330	AB281584	AB281555	AB281526

[a] According to CBS website: http://www.cbs.knaw.nl/.

classification was not accepted and the current taxonomy was based on morphological characters. In this study we propose to use organic acid productivity as the key feature to reclassify *R. oryzae* fumaric-malic acid producers as *R. delemar*, with the molecular phylogeny based on sequence data of the rDNA ITS, *ldhB*, EF-1α and actin as well as genomic AFLP.

MATERIALS AND METHODS

Strains and growth conditions.—*Rhizopus oryzae* strains used in this study are listed (TABLE I). All strains were obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands). For preservation and serial transfer, potato-glucose agar (Difco, Detroit, Michigan) was used. The medium for DNA preparation was malt extract (malt extract [Difco] 20 g/L, polypepton [Nihon Pharmaceutical, Tokyo] 1 g/L, and glucose 20 g/L). Organic acid production was investigated according to Saito et al (2004).

DNA extraction.—Liquid-cultured cells were filtered, air-dried, and lyophilized overnight. The genomic DNA of each strain was extracted from the lyophilized cells according to Sone et al (1997).

Amplification and sequencing of rDNA ITS region, ldhB*, EF-1a and* act1.—rDNA ITS region, *ldhB* and *act1* were amplified and sequenced with primers described respectively in White et al (1990), Saito et al (2004) and Voigt and Wöstemeyer (2001). EF-1α was amplified and sequenced with listed primers (TABLE II). 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), 2.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California) and 100 ng of template DNA of each strain. The reaction conditions were: 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, extension at 72 C for 1 min and a final 5 min of elongation at 72 C in a Model 9700 Thermal Cycler (Applied Biosystems), except that the annealing temperature for actin primers was 50 C. PCR products were purified with Microspin S-300HR (Amersham Biosciences, Piscataway, New Jersey). Sequencing reactions were performed with a BigDye™ Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems) and analyzed by ABI PRISM 3100 Genetic Analyzer or ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence alignments were performed with Clustal X (Thomp-

TABLE II. Primers used for the amplification and sequencing of EF-1α

Primer	Usage[a]	Sequence (5′ to 3′)	Reference
MEF-10	Amp, Seq	GTTGTCATCGGTCACGTCGATTC	This study
MEF-20	Seq	GGATACCACCAAGTGGTCCG	This study
MEF-30	Seq	GTCGAAATGCACCACGAAAC	This study
MEF-50	Seq	GGGTTTCGTGGTGCATTTCG	This study
MEF-60	Seq	CGGACCACTTGGTGGTATCC	This study
MEF-4	Amp, Seq	ATGACACCRACAGCGACGGTTTG	O'Donnell et al (2001)

[a]Amp = PCR amplification; Seq = sequencing.

son et al 1997). Parsimony analysis was performed with PAUP* 4.0b10 (Swofford 2002). Sequence data was deposited in the DDBJ/EMBL/GenBank database. (Accession numbers listed in TABLE I.)

AFLP analysis.—Amplified fragment length polymorphism (AFLP) analysis was performed with an AFLP Microbial Fingerprinting Kit (Applied Biosystems) according to the manufacturer's protocol. AFLP samples were prepared with 10 combinations of selective primers; *Eco*R I + A and *Mse* I + T, *Eco*R I + C and *Mse* I + A, *Eco*R I + C and *Mse* I + C, *Eco*R I + C and *Mse* I + G, *Eco*R I + C and *Mse* I + T, *Eco* R I + G and *Mse* I + C, *Eco* R I + G and *Mse* I + G, *Eco*R I + G and *Mse* I + T, *Eco*R I + T and *Mse* I + C, and *Eco*R I + T and *Mse* I + T. Amplified samples were analyzed with an ABI PRISM 3100 genetic analyzer and Genescan software (Applied Biosystems). Electrophoresis results were analyzed and converted to binary data with Genotyper software (Applied Biosystems). The binary data were converted with Windist (Yap and Nelson 1996) to a distance matrix, and the data were subjected to clustering using Neighbor software of PHYLIP (Felsenstein 2004). Bootstrap analysis was performed with Winboot (Yap and Nelson 1996) software with 1000 iterations. DNA sequence alignments, AFLP matrix and trees were deposited in TreeBase (study accession number = S1869).

RESULTS

R. oryzae strains were chosen from the strains used in the study by Schipper and Stalpers (1984), which is the current classification of the genus. This enabled us to compare directly the results of our study with the morphological classification.

Production of organic acids and ethanol of all *R. oryzae* strains was determined as listed (TABLE III). Thirteen strains produced mainly lactic acid and other strains produced fumaric acid and malic acid. The amounts of malic acid and ethanol produced in the latter group were higher than those of the former. From these data strains were divided into two groups, LA (lactic acid producer) and FMA (fumaric acid and malic acid producer) (TABLE III).

rDNA ITS sequences of all strains were classified into four types (TABLE IV). These sequences were subjected to parsimony analysis (FIG. 1). They grouped into two clusters. Members of each cluster corresponded to that of each organic acid production group (TABLE III).

Further cluster analyses were performed with the DNA sequences of lactate dehydrogenase B (*ldhB*), actin (*act1*) and translation elongation factor-1α (EF-1α) (FIG. 2). Larger diversity of sequence was detected in these three phylogenetic trees than in the rDNA ITS tree. Two large clusters corresponding to the LA and FMA clusters in rDNA ITS tree were resolved with high bootstrap values, except the tree based on *ldhB*. In the *ldhB* tree CBS391.34 was located outside the major clusters, but the bootstrap value for the FMA group including CBS391.34 was the highest, at 100, indicating a strong relationship among CBS391.34 and other FMA group strains.

Two subclusters were detected in each of the large clusters in the EF-1α tree. These subclusters corresponded to the groups of the ITS sequence A, B, C and D. In the other two trees at least one subcluster consisted of strains with a different ITS sequence. In the *ldhB* tree all strains of the FMA group except CBS 391.34 formed a cluster. In the *act1* tree as well, three strains with ITS sequence B clustered with the ITS sequence A strains (FIG. 2).

AFLP analysis was performed to infer phylogenetic relationships at the whole genome level. A total of 301 polymorphic markers, selected from bands amplified with 10 pairs of selective primers, were used for the analysis. The neighbor joining tree based on AFLP data is shown (FIG. 3). Two large clusters corresponding to the LA group and FMA group were resolved with high bootstrap values. Subclusters A, B, C and D also were resolved.

DISCUSSION

The aim of this study was to evaluate organic acid production as the key to reclassify *R. oryzae*. Organic acid production of *R. oryzae* was reported in recent studies to divide the species into two groups, lactic acid producers and fumaric-malic acid producers, but strains used in those studies did not contain authentic

TABLE III. Production of organic acids and ethanol by the *Rhizopus oryzae* strain used

Group	CBS No.	Lactic acid (mg mL^{-1})	Fumaric acid (mg mL^{-1})	Malic acid (mg mL^{-1})	Ethanol (mg mL^{-1})	ITS type
LA	110.17	43.2	0.0	<0.1	4.7	A
LA	112.07^{T}	42.9	0.0	<0.1	3.6	A
LA	127.08	42.9	0.0	<0.1	4.6	A
LA	128.08	42.9	0.0	0.4	3.7	A
LA	257.28	39.5	0.0	0.3	5.6	B
LA	258.28	40.2	0.0	<0.1	3.6	B
LA	260.28	41.9	0.0	<0.1	5.2	A
LA	264.28	45.4	0.0	0.5	5.9	A
LA	266.30	40.8	0.0	<0.1	2.9	B
LA	321.35	43.4	0.0	0.3	4.5	A
LA	330.53	34.6	0.0	0.8	7.1	B
LA	381.52	16.4	0.0	1.9	13.4	B
LA	387.34	33.9	0.0	0.6	5.9	A
FMA	120.12	0.0	0.8	11.7	13.1	D
FMA	278.38	0.0	3.6	10.9	14.1	D
FMA	279.38	0.0	1.3	8.5	15.0	D
FMA	295.31	0.0	0.7	9.0	14.7	D
FMA	385.34	0.0	1.1	3.2	17.0	D
FMA	386.34	0.0	0.9	6.2	15.7	D
FMA	389.34	0.0	1.3	9.0	14.6	D
FMA	390.34	0.0	1.1	10.4	13.6	D
FMA	391.34	0.0	0.5	5.7	15.4	D
FMA	392.95	0.0	1.0	11.2	11.7	D
FMA	393.34	0.0	0.8	8.7	16.1	D
FMA	395.34	0.0	0.4	9.6	17.0	C
FMA	395.54	0.0	<0.1	3.2	18.3	C
FMA	402.51	0.0	4.3	12.0	12.6	D
FMA	404.51	0.0	0.8	11.1	14.4	D
FMA	406.51	0.0	0.6	6.1	17.7	D

type strains so they could not make detailed taxonomic delimitations (Abe et al 2003, Oda et al 2003, Saito et al 2004, Abe et al 2006). In this study we used the strains on which the current classification is based to evaluate species limits.

These strains were classified into two groups, LA (lactic acid producers) and FMA (fumaric-malic acid producers) (TABLE III) as shown by Oda et al (2003) and Saito et al (2004) with a different set of strains. FMA strains did not produce lactic acid at all. Saito et al (2004) found that the lack of lactic acid was due to the absence of the *ldhA* gene, which is responsible for its production. On the other hand LA strains did not produce fumaric acid at all, but this does not indicate the lack or mutation of the gene responsible for fumaric acid production because fumaric acid is produced as an intermediate of the TCA cycle (Saito et al 2004).

The lactic acid production of the LA strains and ethanol production of FMA strains was about twice as much as that of strains used by Saito et al (2004). This could be due to a difference in culture conditions. We tried to cultivate them under the same conditions, but there might have been less aeration in our case because both metabolites are produced from pyruvate anaerobically. Consequently the differentiation of strains based on organic acid production became clearer than in the study of Saito et al (2004).

Organic acid production once was used as a key taxonomic character within *Rhizopus* by Inui et al (1965), but this was not accepted in other works, probably because these physiological characteristics are easily affected by environmental factors, such as media and aeration. In this study it was confirmed strongly that the grouping of *R. oryzae* strains into LA and FMA was phylogenetically robust from the sequence of the four independent genes, rDNA-ITS, *ldhB*, *act1*, and translation elongation factor-1α as well as the genomewide AFLP analysis. Bootstrap values of the clusters for LA groups and FMA groups were more than 70%. This also strengthens the opinion by Saito et al (2004) that the loss of lactic acid production in FMA strains was not due to a mutation of LA strains.

TABLE IV. Four types of rDNA ITS sequence found in *R. oryzae* strains

Sequence type	Position in D type sequence					
	52	54	174	414	517	559
A	—	—	T	G	T	T
B	—	—	C	G	T	T
C	—	—	C	A	C	C
D	C	T	C	A	C	C

In each organic acid production group subclusters corresponding to the ITS sequence type were detected. However all four subclusters corresponding to four rDNA ITS types were found only from the trees of EF-1α and AFLP. This might indicate the occurrence of recombination between strains of the two ITS types within each organic acid producers in the past. The limit of species accordingly can be made at the organic acid groupings if we apply genealogical concordance phylogenetic species recognition (GCPSR, Taylor et al 2000) in this situation.

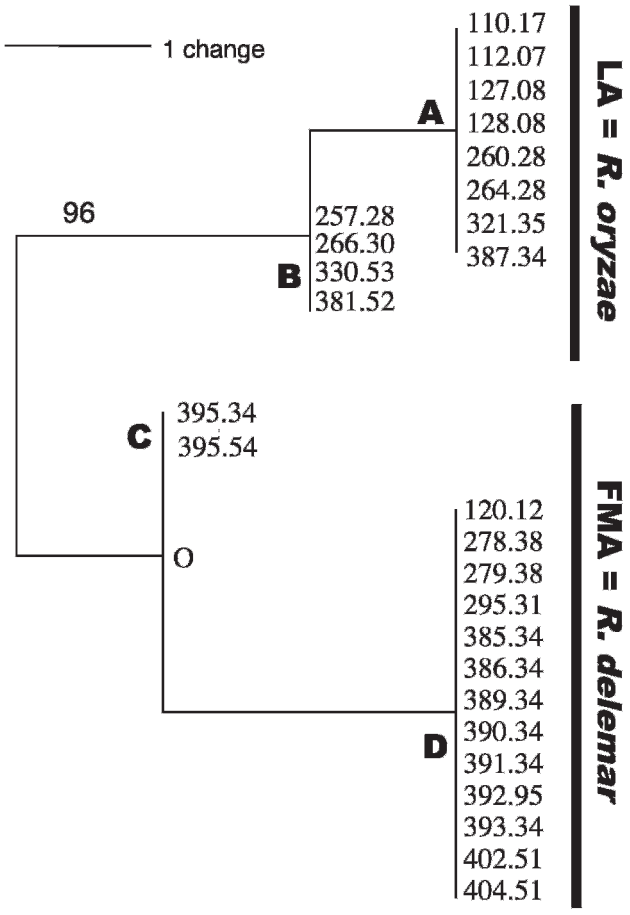

FIG. 1. Single most parsimonious tree based on rDNA - ITS sequence of *R. oryzae* and *R. delemar*. Taxa are indicated with CBS culture numbers. Gaps were treated as fifth base. Tree is written by treating the data of C and D type sequences as outgroup cluster, indicated by the letter O. Tree parameters: length = 6, CI = 1.0. The bootstrap value is for the A and B cluster of sequences. Sequence types are indicated with A, B, C and D. Organic acid production groups are shown at right.

On the other hand, considering that the occurrence of A + B clade in only the *act1* tree might be due to ancestral polymorphism (Takahata and Nei 1985) or homoplasy rather than ongoing gene flow, two cryptic species corresponding to groups A and B can be recognized. However, the fact that we could not detect any morphological or physiological polymorphism between groups A and B prevented us from placing the species limit at this level.

Ellis (1985) proposed to reclassify *R. oryzae*, *R. delemar* and its varieties, *R. chungkuoensis* var. *isofermentarius*, *R. javanicus* var. *kawasakiensis*, *R. arrhizus* and its varieties, and *A. rouxii* into three varieties of *R. arrhizus*, (i.e. var. *arrhizus*, var. *delemar*, and var. *rouxii*) based on DNA-DNA hybridization data. Compared with our AFLP data, his proposed varieties, *R. arrhizus* var. *arrhizus* and var. *delemar*, correspond respectively to LA group and FMA group in our study, although the strains in his study were not the same as those used in ours. His basis for the proposal of ''variety'' instead of ''species'' were small morphological difference of sporangiospore and intermediate DNA complementarity (65%) between two varieties of *R. arrhizus*, in comparison with the higher rate (>90%) within the same variety and the lower rate (18%) among species *R. arrhizus* and *R. microsporus*. Shipper and Stalpers (1984) proposed three groups of *Rhizopus* species, the *stolonifer*-group, *microsporus*-group and *oryzae*. The significance of this grouping recently was confirmed by Frye and Reinhardt (1993) and Abe et al (2006), using respectively G + C content and rDNA ITS sequence. The lower DNA complementarity between *R. arrhizus* and *R. microsporus* corresponds with the genetic distance between the species. Further the intermediate complementarity between the two varieties of *R. arrhizus* is enough for them to be divided into separate species.

Another important issue for species recognition is mating. Schipper and Stalpers (1984) reported that zygospores were obtained in some crosses between *R.*

oryzae strains, CBS346.36 × 112.07; 127.08; 110.17; 148.22; 257.28; 264.28; 266.30; 329.47; 382.52; and 285.55. In this study strains except 346.36, 148.22, 329.47, 382.52, 285.55 were shown to be the LA type, and no strains identified as the FMA type in this study were included in the list of fertile strains. This suggests that all fertile crosses observed by Schipper and Stalpers (1984) were between LA type strains, and no crosses between LA and FMA have been observed yet. This supports the idea that LA and FMA strains represent two species.

In conclusion, we propose to separate FMA group strains into separate species. To our knowledge *Rhizopus delemar* (Boidin)Wehmer and Hanzawa reported in Hanzawa (1912) is the first name given to the strains of the FMA group, thus it is the name we propose for this species. *R. oryzae* should remain the name of LA group strains because the current type culture of *R. oryzae* is included in this group. The key feature for the separation is organic acid production, but one can identify *R. delemar* by sequencing of rDNA-ITS region based on the different nucleotides (TABLE IV). The description for *R. oryzae* by Schipper (1984) should remain as that for the common feature of *R. oryzae* and *R. delemar*, and thus these species will form the third group in the genus *Rhizopus*, the *oryzae-delemar* group.

It was impossible for us to assign the new species name for *R. tonkinensis*, *R. japonicus*, *R. norwegicus*, *R. chunkuoensis* and *R. thermosus* among the species listed in Shipper (1984) due to the unavailability of type culture for those species. However, we were able to assign the new species name to these, with the exception of *R. norwegicus*, using the rDNA ITS sequence data of the culture stocked in our laboratory, some of which were provided by the original identifiers, *R. tonkinensis* AHU6559 and AHU6560 (GenBank accession Nos. AB097338 and AB097339), *R. japonicus* AHU6524 and AHU6525 (GenBank accession Nos. AB097346 and AB097347), *R. chunkuoensis* AHU6513 (GenBank accession Nos. AB097365) and *R. thermosus* AHU6557 andAHU6558 (GenBank accession Nos. AB097357 and AB097358).

We have no clear explanation for the factors that led to the speciation of these two groups. Many strains of *R. oryzae* including some strains used in this study were isolated from fermented foods or their starters. One guess is that human activity, such as the selection of appropriate strains for fermentation foods, might have played a role.

DESCRIPTION OF SPECIES

Rhizopus delemar (Boidin) Wehmer & Hanzawa, Mykol. Zentralbl. 1:77. 1912 emend. A Abe, Y Oda, K Asano and T Sone

≡*Mucor delemar* Boidin,
= *R. sontii*
= *R. suinus*
= *R. aclhlamydosporus*
= *R. bahrensis*
= *R. chiuniang* var. *isofermentarius*
= *R. delemar* var. *minimus*
= *R. javanicus*
= *R. peka*
= *R. semarangensis*
= *R. javanicus* var. *kawasakiensis*
= *R. usamii*
= *R. shanghaiensis*
= *R. delemar* var. *multiplicisporus*
= *R. chunkuoensis*

Sporangiophores up to 1500 μm long, with local swellings, brown, single or aggregated in small groups. Sporangia grayish black, powdery, up to 200 μm diam. Columellae ellipsoidal on a truncate base, mouse-gray or brownish. Sporangiospores angular, subglobose to ellipsoidal, with ridges on the surface, up to 12 μm long. No growth at 45 C, growth at 40 C. Does not produce lactic acid from a carbon source in liquid rotary culture.

Cultures examined: JAPAN, isolated by J. Hanzawa CBS 120.12 = former type culture of *R. delemar*; FRANCE, isolated by H. Boulard CBS 278.38; INDIA, CBS279.38 = former type culture of *R. sontii*; GERMANY, isolated from pig CBS 295.31 = former type culture of *R. suinus*; JAPAN, CBS 385.34 = former type culture of *R. achlamydosporus*; CBS 386.34 = former type culture of *R. bahrnensis*; CBS 389.34 = former type culture of *R. chiuniang* var. *isofermentarius*; CBS 390.34 = former type culture of *R. delemar* var. *minimus*; CBS 391.34 = former type culture of *R. javanicus*, CBS 393.34 = former type culture of *R. peka*; CBS 395.34 = former type culture of *R. semarangensis*; CBS 402.51 = former type culture of *R. javanicus* var. *kawasakiensis*; CBS 406.51 = former type culture of *R. usamii*; CHINA, CBS 404.51 = former type culture of *R. shanghaiensis*; UNITED STATES. GEOGIA: Chamblee, isolated from man, Mucor mycosis of diabetic patient, by B. West CBS 395.54; CBS 392.95 = former type culture of *R. delemar* var. *multiplicisporus*.

Rhizopus oryzae Went. & Prinsen Geerl., Verh. K. Akad. Wet., Sect. 2, 4:16. 1895 emend. A Abe, Y Oda, K Asano and T Sone

= ? *R. arrhizus*
= *R. maydis*
= *R. nodosus*
= *R. tritici*
= *R. formosaenensis*
= *R. hangchao*
= *R. liquefaciens*
= *R. pseudochinensis*
= *R. fusiformis*
= *R. kasanensis*
= *R. boreas*

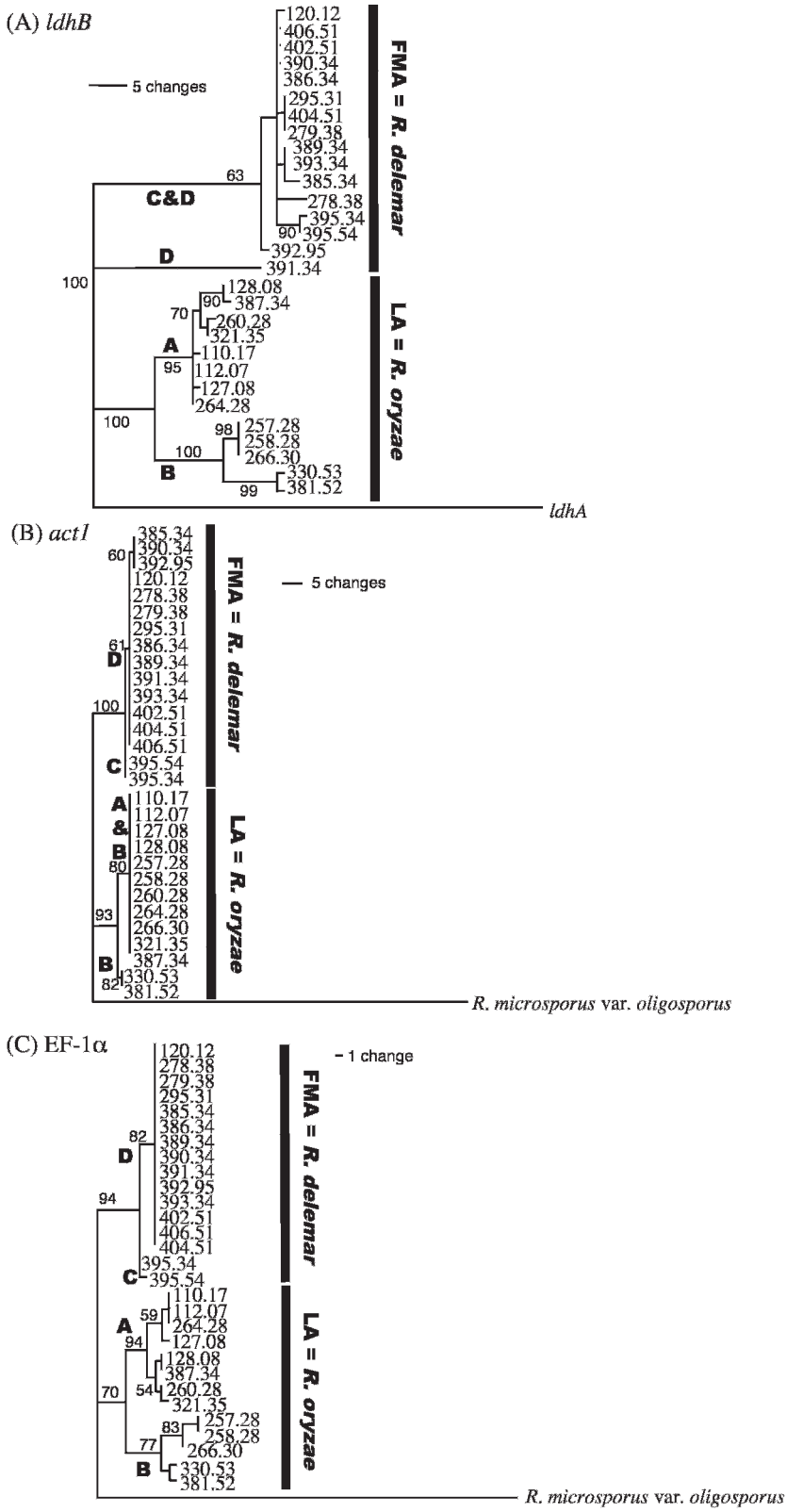

FIG. 2. Parsimonious trees based on DNA sequences of *ldhB, act1* and EF-1α of *R. oryzae* and *R. delemar*. Taxa are indicated with CBS culture numbers. Bootstrap values are shown as percentage calculated from 500 iterations. rDNA-ITS sequence types are indicated by A, B, C or D. Organic acid production groups are shown at the right of taxa. A. One of 64 most parsimonious trees based on *ldhB* sequence. *ldhA* sequence of *R. oryzae* NRRL 395 (accession No. AF226154, Skory 2000) was used as

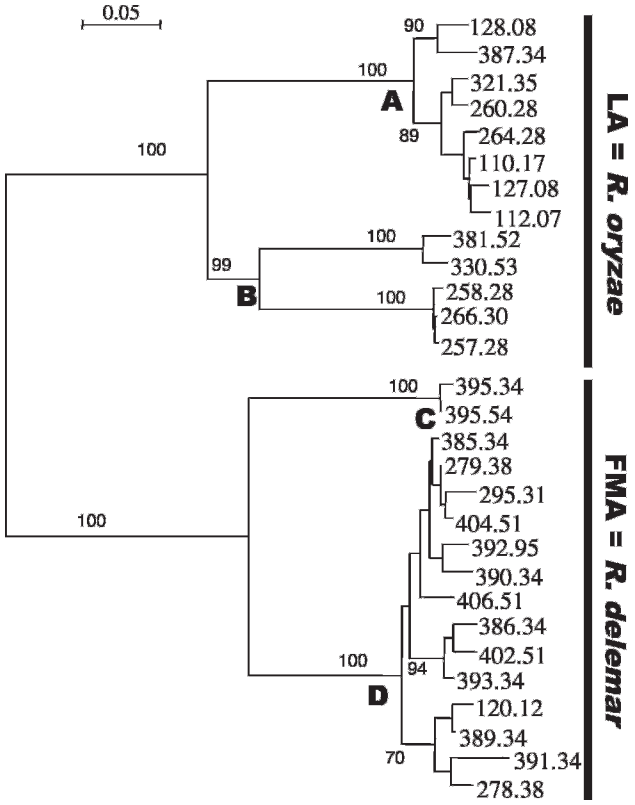

FIG. 3. Neighbor joining tree based on AFLP data for *R. oryzae* and *R. delemar*. Taxa are indicated with CBS culture numbers. Bootstrap values are shown in percentage calculated from 1000 iterations. rDNA-ITS sequence types are indicated by A, B, C or D. Organic acid production groups are shown at right of taxa.

= *R. batatas*
= *R. tonkinensis*
= *R. japonicus*
= *R. thermosus*

Sporangiophores on stolons up to 1500 µm long, with local swellings, brown, single or aggregated in small groups. Sporangia grayish black, powdery, up to 200 µm diam. Columellae ellipsoidal on a truncate base, mouse-gray or brownish. Sporangiospores angular, subglobose to ellipsoidal, with ridges on the surface, up to 10 µm diam. No growth at 45 C, growth at 40 C. Produces lactic acid from a carbon source in liquid rotary culture.

Cultures examined: NETHERLANDS, isolated by F.A.F.C. Went CBS 112.07 = former type culture of *R. oryzae*; SWITZERLAND, isolated by A. Lendner CBS110.17 = former type culture of *R. maydis*; CBS 127.08 = former authentic culture of *R. nodosus*; CHINA, isolated by K. Saito CBS128.08 = former authentic culture of *R. tritici*; TAIWAN, isolated by R. Nakazawa CBS 257.28 = former type culture of *R. formosaensis*; CHINA, isolated by M. Yamazaki CBS 258.28 = former type culture of *R. hangchao*; CHINA, isolated by M. Yamazaki CBS 260.28 = former type culture of *R. liquefaciens*; CHINA, isolated by M. Yamazaki CBS 264.28 = former type culture of *R. pseudochinensis*; UNITED STATES, isolated by B.B. Kanouse CBS 266.30 = former type culture of *R. fusiformis*; CBS 321.35 former *R. kasanensis*; JAPAN, CBS 330.53 = former type culture of *R. boreas*; UNITED STATES, isolated by H.C. Murray CBS 381.52; JAPAN, isolated by R. Nakazawa CBS 387.34 = former type culture of *R. batatas*.

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outgroup. Tree parameters: length = 138, CI = 0.899. B. Single most parsimonious tree based on *act1* sequence. Sequence of *R. microsporus* var. *oligosporus* CBS 338.62 (accession No. AJ287197, Voigt et al 2001) was used as outgroup. Tree parameters: length = 112, CI = 0.982. C. One of eight most parsimonious trees based on EF-1α sequence. Sequence of *R. microsporus* var. *oligosporus* CBS 338.62 (accession No. AF157288, O'Donnell et al 2001) was used as outgroup. Tree parameters: length = 96, CI = 0.854.

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