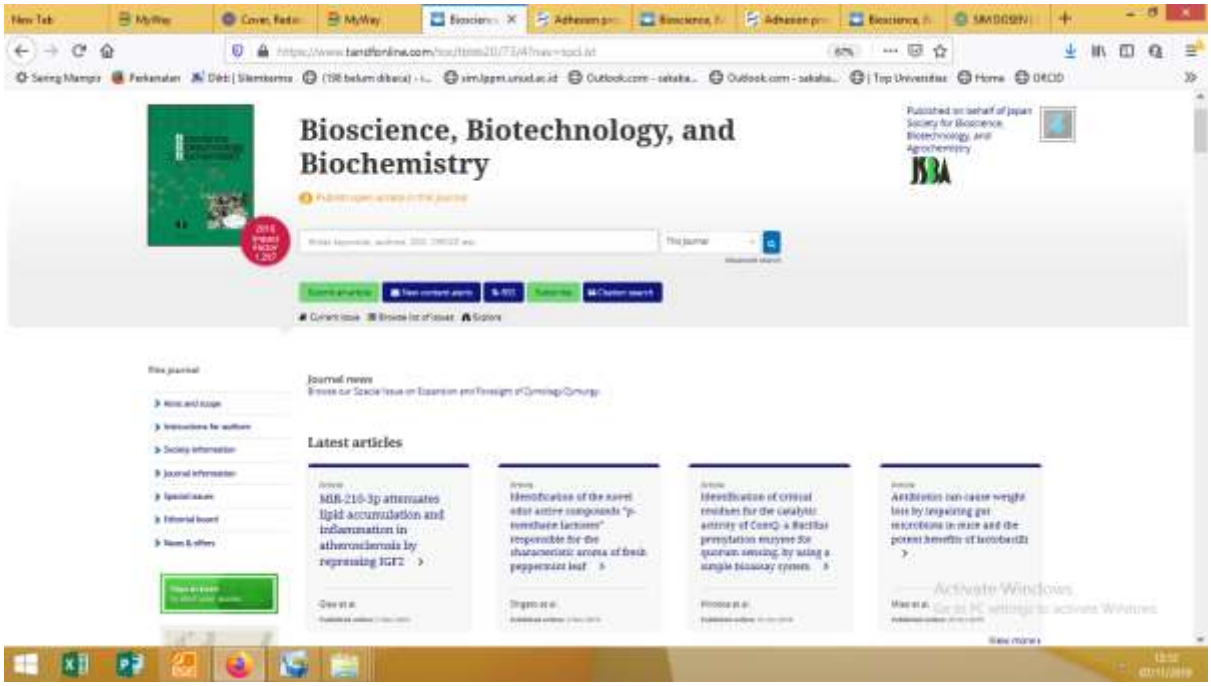
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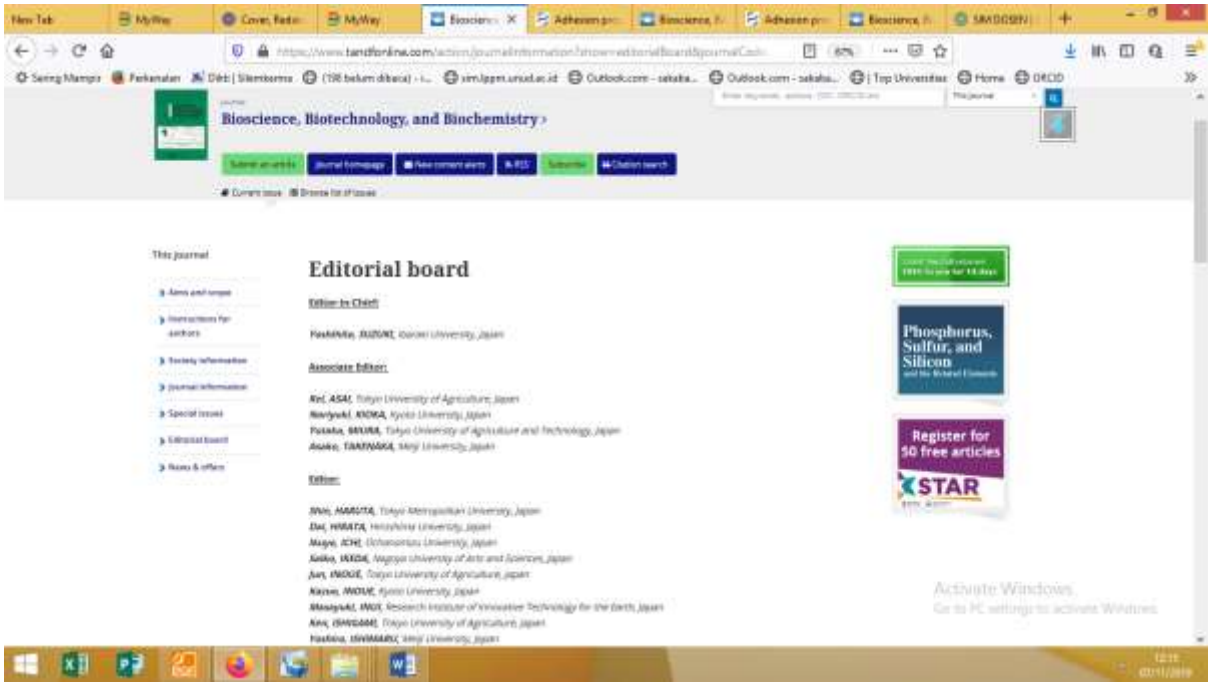
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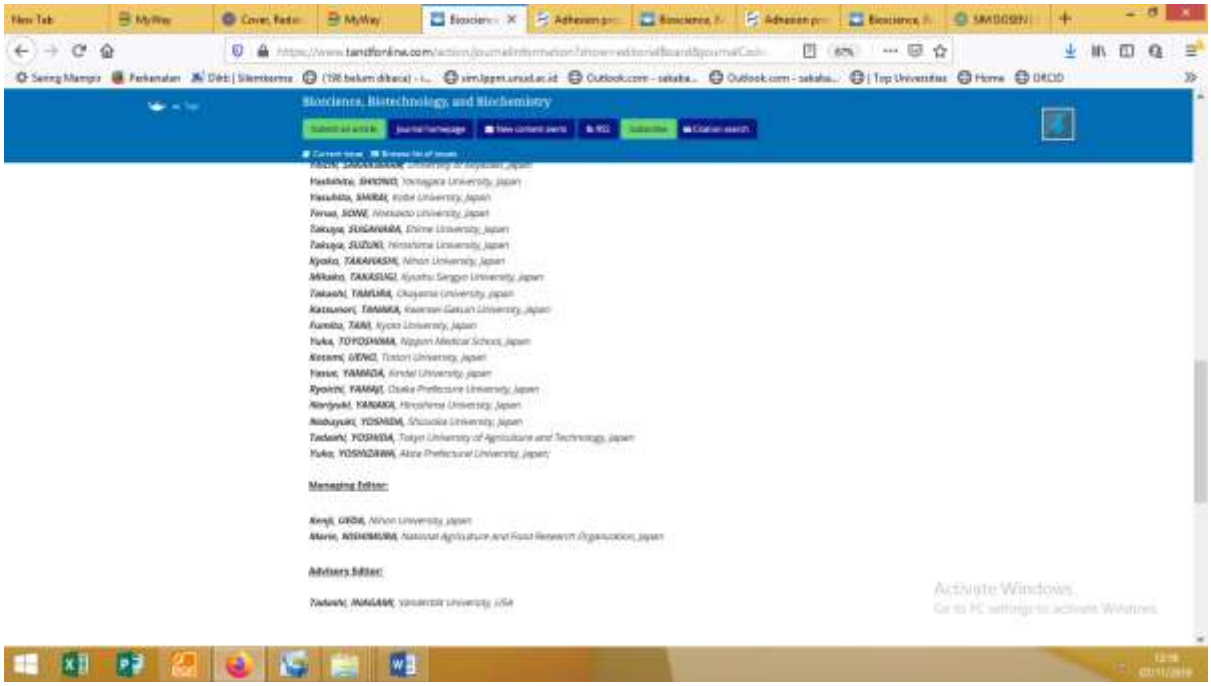

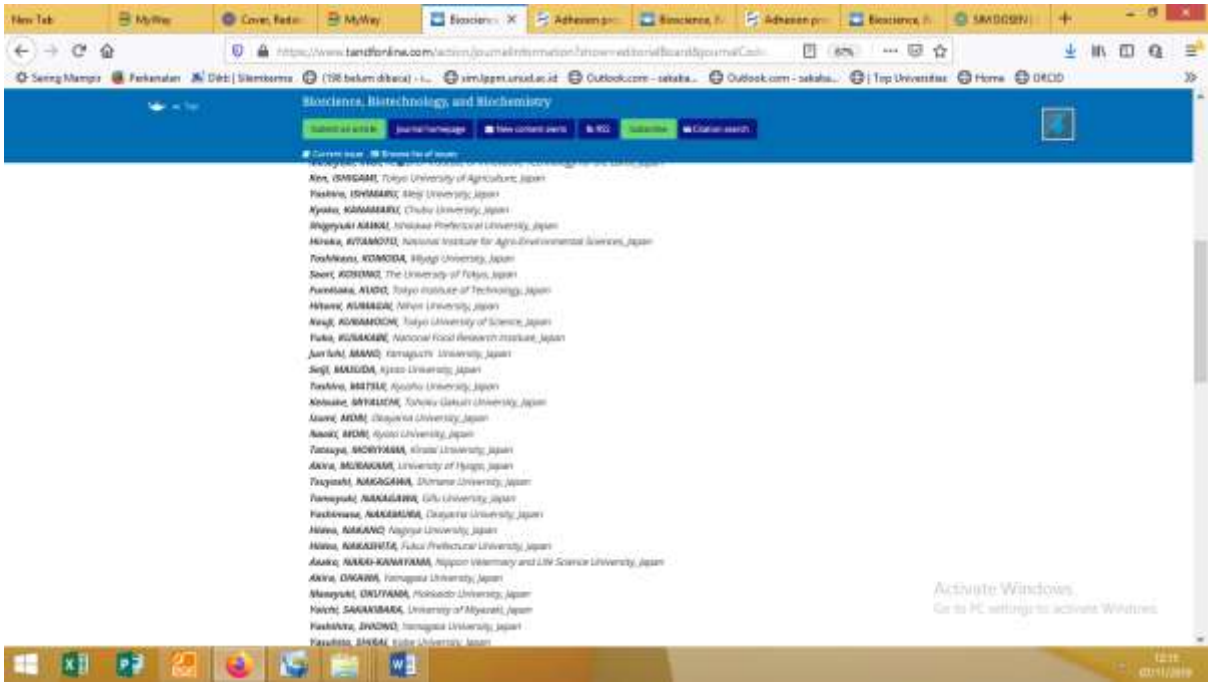

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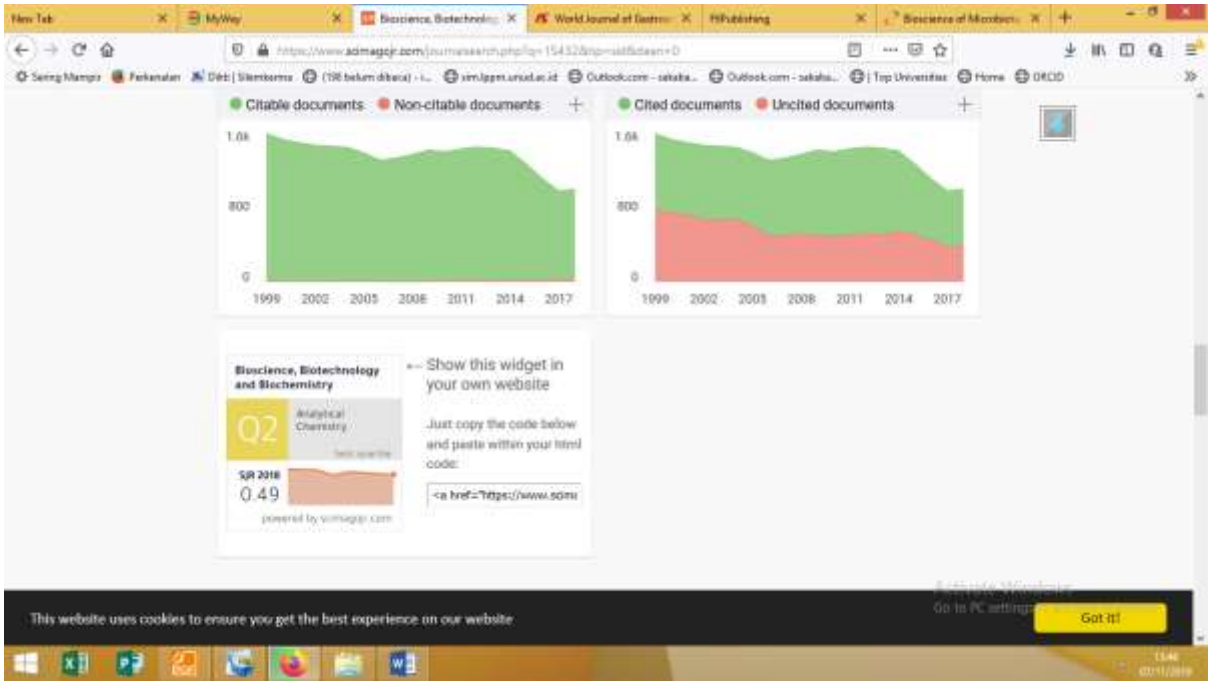

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Hideki KITO, Ayumi ABE, I-Nengah SUJAYA, Yuji ODA, Kozo ASANO & Teruo SONE

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Molecular Characterization of the Relationships among *Amylomyces rouxii*, *Rhizopus oryzae*, and *Rhizopus delemar*

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Twenty-one strains of *Amylomyces rouxii* isolated from starters of Asian fermented foods were divided into two groups, lactic acid (LA) and fumaric and malic acid (FMA) producers, by organic acid productivity in liquid culture. Phylogenetic analysis based on the *ldhB* gene, ribosomal RNA encoding DNA (rDNA) internal transcribed spacer (ITS) sequence, and genome-wide amplified fragment length polymorphism (AFLP) revealed that *A. rouxii* was grouped into two major clusters as to organic acid accumulation, corresponding to *Rhizopus oryzae* and *Rhizopus delemar*. These observations suggest that the species *A. rouxii* is composed of two distinct types, derived from *R. oryzae* or *R. delemar* *via* domestication in the starters.

Key words: *Amylomyces rouxii*; *Rhizopus*; internal transcribed spacer; *ldh*; amplified fragment length polymorphism

Mucoromycotina, including *Rhizopus*, *Amylomyces*, and *Mucor*, are indispensable microorganisms for the production of fermented foods in East Asia.[1] *Rhizopus oligosporus* is the principal species used to make Indonesian *tempeh*.[2] During fermentation, this fungus binds dehulled and cooked soybeans into a solid cake covered with a dense cottony mycelium and improves the nutritive quality of the soybeans.[3] Sweet desserts and alcoholic beverages are traditionally produced from rice, cassava, and sorghum in many countries of Southeast Asia, China, and the Indian subcontinent.[4] The fermentation starters for these products contain Mucoromycotina fungi, yeasts, and bacteria in rice flour. Among them, the fungus *Amylomyces rouxii* (Calmette) plays a definite role in saccharifying starch in the crops as raw materials.[5]

Taxonomic study of *A. rouxii* had been done by Ellis *et al.*, fundamentally based on morphological aspects such as the production of enormous numbers of chlamydospores produced in the aerial and substrate mycelium, and the lack of rhizoids, stolons, and black-pigmented sporangia, compared with the relative species, *Rhizopus oryzae*.[6] In addition, some differences have been found in the utilization of carbon sources between *A. rouxii* and *R. oryzae*. Ellis[7] reported similarity of *A. rouxii* and *R. oryzae* revealed by DNA-DNA complementation, and proposed to re-classify *A. rouxii* as *R. arrhizus* var. *rouxii*. The similarity was also proved by the sequence of rDNA genes, as the type strain of *A. rouxii* has been shown to be closely related to strains of *R. oryzae* that accumulate lactic acid.[8]

On the other hand, the classification of *R. oryzae* was reconsidered based on the organic acid production, combined with molecular data.[9] Lactic acid producers and fumaric and malic acid producers were divided into two distinct species, *R. oryzae* and *R. delemar*, supported by gene genealogy of *ldhB*, *act1*, rDNA ITS, translation elongation factor-1α, and AFLP (amplified fragment length polymorphism).[10] The *ldhB* gene has been used in the phylogenetic analyses of *R. oryzae* because DNA sequences of the gene are polymorphic among the strains of those species. *act1*, rDNA ITS, and translation elongation factor-1α are DNA markers often used in the phylogenetic study of fungi. This reclassification indicated that the phylogenetic relationships among *A. rouxii*, *R. oryzae*, and *R. delemar* should be re-evaluated according to organic acid production and other molecular data, because the possibility of the existence of *A. rouxii* strains of fumaric-malic acid producers could not be excluded, although all the specimens used in the phylogenetic study were lactic acid producers.

In the present study, we collected 21 strains of *A. rouxii* and analyzed their organic acid production, and the DNA sequences of the lactate dehydrogenase (*ldh*) genes and internal transcribed spacer (ITS) region of 18S–28S rRNA genes to clarify their relationships to *R. oryzae* and *R. delemar*.

Materials and Methods

Organisms. The strain numbers of *Amylomyces rouxii* and their origins are as follows: CBS 438.76^T, LP1, LP2, LP3, and LP4 from *look-pang*; ATCC 48370, SDM1, SDM18, SDM23, SDM26, SDM28, and SDM34 from *ragi tapé*; CBS 111757, BM3, BM4, BM7, BM8, BM11, BM13, BM15, and BM16 from *banh-men*. *Look-pang*,[11] *ragi tapé*,[12,13] and *banh-men*[11] are starters used in the production of fermented foods and alcoholic beverages in Thailand, Indonesia, and

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Abbreviations: rDNA, ribosomal RNA encoding DNA; ITS, internal transcribed spacer; AFLP, amplified fragment length polymorphism; LA, lactic acid; FMA, fumaric and malic acid; PDA, potato dextrose agar

Table 1. Organic Acid Production, Ethanol Production and Accession Numbers of DNA Sequences of the Fungal Strains Used in This Study

Species	Strain	Origin	Amount (mg ml^{-1})				Accession number	
			LA	FA	MA	EtOH	*ldhB*	ITS
R. oryzae	CBS112.07^T		43.2[a]	0.0[a]	<0.1[a]	3.6[a]	AB281557	AB097334
	CBS257.28		39.5[a]	0.0[a]	0.3[a]	5.6[a]	AB281561	AB181311
	CBS330.53		34.6[a]	0.0[a]	0.8[a]	7.1[a]	AB281570	AB181314
R. delemar	CBS120.12^T		0.0[a]	0.8[a]	11.7[a]	13.1[a]	AB281558	AB181318
	CBS395.34		0.0[a]	0.8[a]	8.7[a]	16.1[a]	AB281580	AB181316
A. rouxii	CBS438.76^T	*Look-pang*	33.6	0.0	0.2	5.4	AB440606	AB181310
	LP1	*Look-pang*	31.9	0.0	0.5	4.4	AB440607	AB440598
	LP2	*Look-pang*	30.0	0.1	0.6	4.6	AB440608	AB440599
	LP3	*Look-pang*	30.9	0.1	0.5	4.3	AB440609	AB440600
	LP4	*Look-pang*	34.7	0.0	0.2	5.9	AB440610	AB440601
	ATCC48370	*Ragi tapé*	18.0	0.3	1.5	10.0	AB440611	AB181332
	SDM1	*Ragi tapé*	11.1	0.3	0.8	11.7	AB440612	AB181333
	SDM18	*Ragi tapé*	5.3	0.1	0.2	13.6	AB440613	AB181334
	SDM23	*Ragi tapé*	6.8	0.2	0.4	14.3	AB440614	AB181335
	SDM26	*Ragi tapé*	4.5	0.1	0.5	10.3	AB440615	AB181336
	SDM28	*Ragi tapé*	9.1	0.3	0.7	12.1	AB440616	AB181337
	SDM34	*Ragi tapé*	8.5	0.1	0.7	15.1	AB440617	AB181338
	CBS111757	*Banh-men*	0.0	0.2	2.6	11.8	AB440618	AB181347
	BM3	*Banh-men*	0.0	0.2	1.3	4.9	AB440619	AB181342
	BM4	*Banh-men*	0.0	0.2	1.6	6.7	AB440620	AB440602
	BM7	*Banh-men*	0.0	0.3	2.8	10.0	AB440621	AB181343
	BM8	*Banh-men*	0.0	0.4	2.2	7.5	AB440622	AB181344
	BM11	*Banh-men*	0.0	0.5	2.8	9.0	AB440623	AB440603
	BM13	*Banh-men*	0.0	0.2	1.6	6.1	AB440624	AB181345
	BM15	*Banh-men*	0.0	0.3	2.2	8.4	AB440625	AB440604
	BM16	*Banh-men*	0.0	0.3	2.2	8.2	AB440626	AB440605

[a]According to Abe *et al.*[9]

Vietnam, respectively (Table 1). SDM strains were previously isolated by Abe *et al.*[14] LP and BM strains were isolated for the present experiments as follows: Each starter was suspended in 0.85 M NaCl with a series of dilutions, followed by plating out on potato dextrose agar (PDA, Difco, Detroit, MI). After overnight incubation at 25 °C, tips of fungal hyphae were picked up microscopically and moved to a new PDA plate as a single isolate because *A. rouxii* does not produce sporangiospores. After confirmation of abortive sporulation and rDNA ITS sequence similarity to *R. oryzae*, cultivation in a BSM (basal synthetic medium)[6] containing sucrose, maltose, or glycerol as a carbon source confirmed whether they were *A. rouxii* or not.[14] *Rhizopus oryzae* CBS112.07^T, 257.28, and 330.53, *R. delemar* CBS120.12^T, and 395.34 were used as reference strains.

Cultivation and organic acid analysis. Fungal cells were grown on potato dextrose agar prepared horizontally in a standing test tube (12 × 90 mm). The mycelium was taken from the surface of the agar and inoculated in 50 ml of a medium containing 50 g/l glucose, 6.7 g/l yeast nitrogen base without amino acids (Difco), 5.0 g/l casamino acids (Difco), and 25 g/l calcium carbonate.[15] After cultivation for 7 d at 25 °C with shaking (90 rpm), the mycelium was separated from the culture fluid by centrifugation. Organic acids in the culture fluid were determined by high-performance liquid chromatography.[15]

DNA analysis. Genomic DNA was isolated as described previously.[14] DNA fragments of *ldhA* and *ldhB* were amplified by a method described elsewhere,[16] with some modification. For detection of *ldh* loci, a partial fragment of the *ldh* genes was amplified using f*Eco* (5′-GAATTCGCARAGCTGGATAAAA-3′) and r1 (5′-ATGATWTR-TTATTTGTAAATT-3′). In cloning of the *ldh* genes, primers f1 (5′-TTTTCTTTWCWATATAATTC-3′) and r1 were used. DNA fragments were cloned into pUC19 plasmid and transformed into *E. coli* JM109, following isolation with QIAquick spin (Qiagen, Hilden, Germany) and sequencing with an ABI PRISM 310 Genetic analyzer sequencer (Applied Biosystems, Foster City, CA). Ten clones of each *ldh* served for the definition of sequence. PCR amplification of the ITS region was conducted as Abe *et al.*[14] and sequenced directly. Sequence alignments were performed using the Clustal X Package.[17] Parsimony analysis was performed using PAUP* 4.0b10.[18] The nucleotide sequences have been assigned the EMBL/GenBank/DDBJ accession numbers listed in Table 1. AFLP analysis was performed according to the method described by Abe *et al.*[9] Polymorphic markers (n = 199) were selected from DNA bands amplified with 10 pairs of selective primers. The existence or lack of a particular DNA band (marker) was scored as 1 or 0, respectively, and combined binary data were used in the distance calculation.

Results

Organic acid production was examined for 21 strains of *Amylomyces rouxii* (Table 1). Nine strains isolated from *banh-men* were shown to produce fumaric acid and malic acid with similarity to *R. delemar*, although the productivity was relatively low. On the other hand, strains from *look-pang* and *ragi tapé* produced lactic acid as *R. oryzae*. The productivity of the strains from *look-pang* was higher than those from *ragi tapé*. Diagnostic amplification of *ldh* loci from *A. rouxii* strains and *R. oryzae* CBS strains was conducted using f*Eco* primer and r1 primer designated for *R. oryzae* NBRC strains.[16] Fifteen lactic acid producing strains presented two fragments of 394 bp and 434 bp amplified from *ldhA* and *ldhB*, respectively, whereas fumaric acid strains bore a single fragment of 434 bp derived from *ldhB* by PCR amplification, as indicated by Saito *et al.* (data not shown).

The phylogenetic relationships of *A. rouxii*, *R. oryzae*, and *R. delemar* were investigated using the DNA sequences of the *ldhB* gene, rDNA ITS, and AFLP. All the *A. rouxii* strains isolated from *banh-men* harbored the same DNA sequence of *ldhB*, and clustered inside the *R. delemar* cluster in the most parsimonious tree of *ldhB* (Fig. 1). On the other hand, strains isolated from *look-pang* and *ragi tapé* were strongly related to

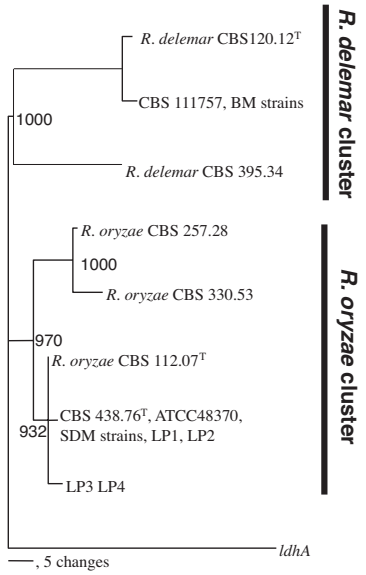

Fig. 1. One of Most Parsimonious Trees for the *ldhB* Genes of *Amylomyces rouxii*, *Rhizopus oryzae*, and *R. delemar* Strains.

A. rouxii strains are indicated by strain numbers. Bootstrap values were calculated from 1,000 trees. The *ldhA* sequence of *R. oryzae* NRRL 395 (accession no. AF226154, Skory 2000) was used as an outgroup. Tree parameters: length, 108; consistency index (CI), 0.9722. Two major clusters are indicated by bars to the right of the tree.

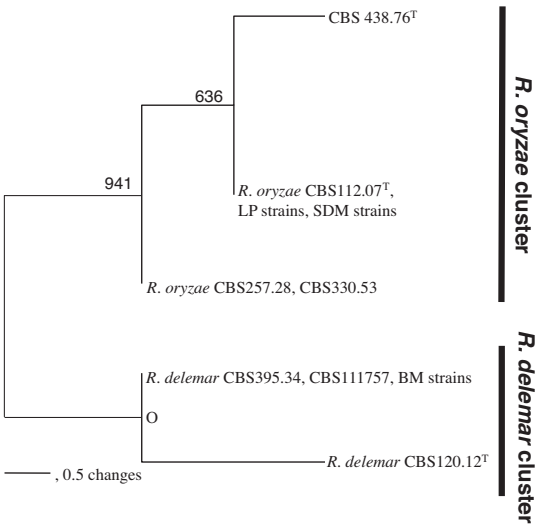

Fig. 2. Single Most-Parsimonious Tree Based on rDNA-ITS Sequence of *A. rouxii*, *R. oryzae*, and *R. delemar*.

A. rouxii strains are indicated by strain numbers. Gaps were treated as fifth base. The tree was written by treating the cluster of *R. delemar* as an outgroup, indicated by the letter O. Parameters of the trees: length, 7; CI, 1.0. Bootstrap values were calculated from 1,000 trees. Two major clusters are indicated by bars to the right of the tree.

R. oryzae CBS112.07^{T}, although two strains, LP3 and LP4, from *look-pang* had slightly varied sequences. The cluster including *A. rouxii* strains from *look-pang* and *ragi tapé* was found to be part of a large robust cluster of *R. oryzae*.

The rDNA ITS parsimonious tree (Fig. 2) indicates similar phylogenetic relationship to Fig. 1, although the variation in the DNA sequence is rather small. All the *A. rouxii* strains from *look-pang* and *ragi tape*, except CBS438.76^{T}, shared a sequence identical to that of

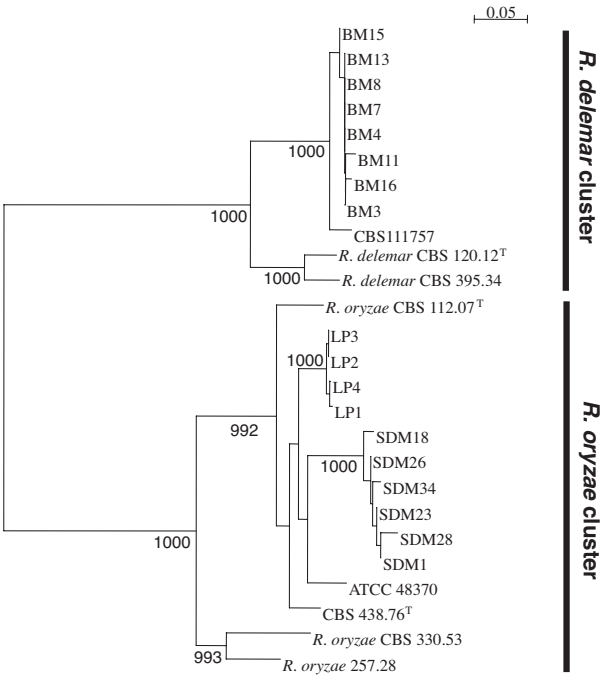

Fig. 3. A Phylogenetic Tree Constructed for AFLP Fingerprints of the *Amylomyces rouxii*, *Rhizopus oryzae*, and *R. delemar* Strains by the Neighbor-Joining Method.

A. rouxii strains are indicated by strain numbers. Bootstrap values were calculated from 1,000 trees. Two major clusters are indicated by bars to the right of the tree.

R. oryzae CBS112.07^{T}. The *Banh-men* strains of *A. rouxii* shared a rDNA sequence with *R. delemar* CBS395.34.

Phylogenetic relationships were examined at the whole genome level using AFLP (Fig. 3), with 199 polymorphic markers. A neighbor-joining tree revealed two robust clusters including *A. rouxii* strains, with more variations than DNA sequence analyses. *A. rouxii* isolated from *banh-men* formed a single robust cluster, related with another cluster of *R. delemar*. The LP and SDM *A. rouxii* strains isolated in this study formed distinct clusters with high bootstrap robustness, showing relationships with other *A. rouxii* strains, ATCC48370, CBS438.76, and *R. oryzae* CBS112.07^{T}. The cluster including the *A. rouxii* strains isolated from *ragi tapé* and *look-pang* was found to be a part of the large cluster of *R. oryzae*, as indicated by the other phylogenetic trees.

Discussion

Amylomyces is a monotypic genus, composed of the single species, *A. rouxii*.[6] To date, the species had been investigated as to morphology and physiology, or genetically, but no investigation showed clear evidence of heterogeneity.[6,7] This study first revealed the existence of two distinct types of *A. rouxii* that differ in organic acid productivity and molecular genetic aspects. CBS438.76, ATCC48370, the LP strains, and the SDM strains from *look-pang* and *ragi tapé* produced lactic acid, whereas the CBS111757 and BM strains from *banh-men* accumulated fumaric and malic acid. These two groups of strains were clearly divided into two distinct clusters in the three molecular phylogenetic

trees. These two groups, lactic acid producers and fumaric acid producers, showed strong relationships with *R. oryzae* and *R. delemar*, respectively, which are also discriminated from each other by organic acid production.[9)]

The similarity of *A. rouxii* to *R. oryzae* has been reported by many researchers. Hesseltine[5)] pointed out the possibility that *A. rouxii* is a mutant of *R. oryzae*. Later findings by Ellis[7)] strengthened this possibility by showing 95% hybridization similarity of the DNA between *A. rouxii* and *R. oryzae*. Our results again showed high similarity between the *R. oryzae* group (*R. oryzae* and *R. delemar*)[9)] and *A. rouxii* by the sequence of *ldhB* and rDNA ITS, and AFLP genome fingerprinting. However, based on our results, the hypothesis should be revised as follows: the mutation responsible for the distinct morphology and sugar utilization of *Amylomyces rouxii* occurred independently in both lineages of *R. oryzae* and *R. delemar*.

Saito *et al.*[16)] and Abe *et al.*[9)] indicated the importance of lactate dehydrogenase (*ldh*) genes for the specification of *R. delemar* and *R. oryzae*. Two *ldh* genes, *ldhA* and *ldhB*, are possessed by the *R. oryzae* genome, whereas *R. delemar* has only *ldhB*. *ldhA* is considered to be responsible for lactic acid production, and presumed to be deleted in the genome of *R. delemar*. The lack of the *ldhA* gene in the *A. rouxii* isolated from *banh-men* indicates that *A. rouxii* fumaric-malic acid producers and *R. delemar* derived from a common ancestral species which lacks the *ldhA* gene. If *A. rouxii* is a mutant of *R. oryzae* or *R. delemar* as Hesseltine[5)] asserted, what mutations occurred in *R. oryzae* and *R. delemar*? The clear difference between *A. rouxii* and the *R. oryzae* group is morphology. *A. rouxii* hardly produces mature sporangia or sporangiospores, but produces abundant chlamydospores. No genes responsible for sporangiospore formation have been isolated from the *R. oryzae* group. Another distinct characteristic of *A. rouxii* is the sugar utilization. *A. rouxii* can utilize sucrose but not glycerol. *R. oryzae* can utilize glycerol but not sucrose.[6)] This difference is conserved not only in the case of lactic acid producers but also in the case of the fumaric-malic acid producers in this study. This indicates that the genes responsible for sugar assimilation also altered in *A. rouxii*. The ongoing *R. oryzae* genome project (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/MultiHome.html) should provide useful information and opportunities to explore detailed analysis of genetic differentiation between *A. rouxii* and the *R. oryzae* group.

Recently, Zheng *et al.*[19)] proposed to reclassify *A. rouxii* as *R. arrhizus* var. *arrhizus* (*R. oryzae* in our study), based on morphology and molecular data of rDNA ITS. They explained that the distinct morphology of the *A. rouxii* is due to degeneration, by adaptation to fermentative environmental conditions through generations. Our study also showed similar adaptation occurred in *Rhizopus delemar* (*R. arrhizus* var. *delemar* in their study). From this, *A. rouxii* should be reclassified as *R. oryzae* or *R. delemar*, based on organic acid production as proposed in a previous study on *R. oryzae*.[9)] However, the usefulness of the name "*Amylomyces rouxii*" to indicate the sporangiospore-less mutants of *R. oryzae* and *R. delemar* should remain as points for consideration from practical viewpoints, particularly in the fermentation industries.

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