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rDNA ITS Sequence of *Rhizopus oryzae*: Its Application to Classification and Identification of Lactic Acid Producers

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***Rhizopus oryzae* is an important organism for its production of organic acids such as lactic acid, fumaric acid, *etc*. To date, there were no easy methods to classify strains according to their acid production. The sequences of the ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer (ITS) region of 64 strains of *R. oryzae* were analyzed and found to conserve mutations correspond to acid production. We have devised a way to use these mutations for a novel method to identify lactic-acid-producing *Rhizopus oryzae*, by designing specific polymerase chain reaction (PCR) primers on them. Touch down PCR using these primers amplified the ITS DNA of lactic acid producers specifically. By this method, we could isolate lactic acid producing strains from Indonesian fermented foods.**

Key words: *Rhizopus oryzae*; ribosomal RNA-encoding DNA (rDNA); internal transcribed spacer (ITS); selective polymerase chain reaction (PCR); lactic acid fermentation

The genus *Rhizopus* belongs to Mucorales in Zygomycetes. The characteristic of the genus is rhizoid formation. Some isolates of *R. oryzae* are parasitic on plants or people, but *R. oryzae* includes species that are capable of efficient production of organic acids.[1)] A notable example of its usage as organic acid producer is the production of alcohol beverages and *tempeh* from the ancient times in East Asia and Southeast Asia. Among those organic acids, lactic acid is regarded as an important substrate for biodegradable plastics and food preservatives.

From the points of both lactic acid production and use for food fermentation, *R. oryzae* is actually an important microorganism. *R. oryzae* NRRL395 was shown to ferment ground corn and to produce L(+)-lactic acid in the presence of calcium carbonate.[2)] Recently, Oda *et al*. reported about its new usage as a starter for fermentation of potato pulp, a byproduct of potato starch production in order to use them as feeds for livestock farming in Hokkaido.[3)] In the paper, it was reported that *R. oryzae* strains were clearly divided into 2 groups, namely one that produces lactic acid, and the other produces fumaric acid, even though *R. oryzae* is classified as one species in the current taxonomy.[3)] In order to use *Rhizopus* strains as starters for silage, it is needed to screen the lactic acid producing strains selectively. However, the amount of acid production varies according to the fermentation conditions, thus a simple and clear method for the differentiation is preferred. Here, we tried to find methods to differentiate these 2 groups by molecular genetic information.

Ribosomal RNA-encoding DNA (rDNA) has been used in phylogeny, classification, and identification of fungi, bacteria, and other organisms. In addition to rDNA sequences of large and small subunits, the sequence of the internal transcribed spacer region (ITS) between the large and the small subunits is used for these purposes. The ITS is more diverse than other components of rDNA, so this region is often used to compare between species. Thus, the ITS of *R. oryzae* will be a ideal molecule, but has not been studied well as yet.

In this paper, we analyzed ITS sequences of 65 strains of *R. oryzae* and compared their differences in lactic acid fermentation. Also we established a PCR-based method for the screening of lactic acid producers. The results showed clearly that we could screen strains out for lactic acid production from resources such as fermented foods by the use of our method promptly and successfully.

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

Materials and Methods

Strains and growth conditions. All the strains classified as *R. oryzae* were obtained from the NITE Biological Resource Center (NBRC, Kisarazu, Japan), Agricultural Research Service Culture Collection (NRRL, Illinois, USA), American Type Culture Collection (ATCC, Maryland, USA), and Graduate School of Agriculture, Hokkaido University (AHU, Sapporo, Japan). For the preservation and serial transfer, Potato Glucose Agar (DIFCO, Detroit, USA) was used. The medium for DNA preparation was malt extract medium (malt extract 20 g/l, polypepton 1 g/l, glucose 20 g/l). The type strain of *Amylomyces rouxii* (CBS 438.76) was obtained from Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands). For the identifiaction of *Amylomyces rouxii*, basal synthetic medium (SBM)[4] was used.

Organic acid analysis. The cultivation medium and condition for organic acid analysis were selected and analyzed using high-pressure liquid chromatography according to the method of Oda *et al.*[5]

DNA extraction. The microorganisms were grown aerobically with shaking at 27°C for 3 d using 10 ml of 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 by the method of Sone *et al.*[6] The *tape* and *tempeh* are Indonesian traditional foods, and *ragi tape* and *ragi tempeh*, are dry starters for these foods. We used one *tape* (home-made with traditionally prepared glutinous rice and *ragi tape*), three kinds of *tempeh* (one was Muchlar, industrially made, and the other two purchased in the Market in Yogyakarta, Indonesia), one *ragi tape* (Na Kok Liong, Solo, Indonesia), and one *ragi tempeh* (Raprima, Bandung, Indonesia). Each food sample of 0.1 g in a 1.5-ml microcentrifuge tube was suspended in 1.0 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and incubated for 15 min at 37°C in the presence of lysozyme and *N*-acetylmuramidase at the final concentration of 2.5 mg/ml and 30 mg/ml, respectively. After centrifugation (12,000 × *g*, 3 min), the supernatant was discarded and the precipitates were used for DNA extraction with an UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc. CA, USA).

PCR reactions. The ITS region amplification was done with the primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′).[7] The PCR amplifications were done in 50 μl containing 5 μl of 10 × PCR buffer, 5 μl deoxynucleotide triphosphate (2 mM each), 1 pM each primer, 3.5 μl $MgCl_2$ solution (15 mM) and 2.5 U AmpliTaq DNA polymerase (PE Applied Biosystems Inc., Foster City, USA). 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 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and a final 5 min of chain elongation at 72°C was done after the cycling completion in a model 9700 thermal cycler (PE Applied Biosystems Inc., Foster City, USA). Two primers were designed based on the base comparison of the ITS region sequences. The corresponding positions of each primer were named based on the *R. oryzae* ITS region sequence (Fig. 1). The Primer sets, which are supposed to produce fragments of about 550 bp, were tested for specific PCR amplification. The PCR amplifications were done in the above PCR reaction composition. The template DNA was 0.2 ng of each strain for the specificity test. The PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, made visible with a UV transiluminator, and photographed.

Sequence analysis. The PCR products were purified by a Microspin column S-300 (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The ITS region sequencing was done using a BigDye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., Foster City, USA) and analyzed by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). Phylogenetic relationships were estimated using the Clustal W,[8] PHYLIP (Distributed by Dr. J. Felsenstein, Department of Genetics, University of Washington, Seattle, USA) and TREEVIEW (Distributed by Dr. R. D. M. Page, Institute of Biomedical and Life Sciences, University of Glasgow, Scotland, UK) softwares. The sequence data obtained in this study was deposited in the DDBJ/EMBL/GenBank database, under accession Nos. AB097271–AB097298, AB097300–AB097333, and AB097383.

Isolation of lactic acid-forming fungi from food samples. One g of the sample was diluted in 9 ml of saline solution (0.85% NaCl) and serial diluents were plated on potato dextrose agar (PDA, Merck, Germany) and incubated at 27°C for 24–48 h. The filamentous fungal colonies showing a different morphology were isolated from plates and purified using the same medium and maintained in PDA slants at 5°C.

Table 1. Strains Used in This Study and Their Classification by Acid Production and ITS Sequence

No.	Strain	Potato pulp ferment.[a]	ITS sequence	Organic acid (mg/ml)[b]		Liquid ferment.[c]	No.	Strain	Potato pulp ferment.[a]	ITS sequence	Organic acid (mg/ml)[b]		Liquid ferment.[c]
				Lactic	Fumaric						Lactic	Fumaric	
1	NBRC4697	D	II	1.8	8.4	B	36	NBRC5319	A	I	N.D.	N.D.	—
2	NBRC4698	D	II	2.3	5.8	B	37	NBRC5378	A	I	N.D.	N.D.	—
3	NBRC4705	A	I	N.D.	N.D.	—	38	NBRC5379	A	I	N.D.	N.D.	—
4	NBRC4706	A	I	N.D.	N.D.	—	39	NBRC5380	D	I	31.7	0.3	A
5	NBRC4707	A	I	N.D.	N.D.	—	40	NBRC5384	A	I	N.D.	N.D.	—
6	NBRC4716	C	I	36.3	0.0	A	41	NBRC5406	D	II	0.9	3.7	B
7	NBRC4726	B	II	N.D.	N.D.	—	42	NBRC5413	D	I	31.6	0.4	A
8	NBRC4730	D	II	2.3	9.5	B	43	NBRC5414	D	I	36.0	0.4	A
9	NBRC4732	C	II	1.9	6.4	B	44	NBRC5418	D	I	37.8	0.0	A
10	NBRC4734	B	II	N.D.	N.D.	—	45	NBRC5438	A	I	N.D.	N.D.	—
11	NBRC4735	B	II	N.D.	N.D.	—	46	NBRC5440	A	I	N.D.	N.D.	—
12	NBRC4736	B	II	N.D.	N.D.	—	47	NBRC5441	B	II	N.D.	N.D.	—
13	NBRC4744	A	I	N.D.	N.D.	—	48	NBRC5442	B	II	N.D.	N.D.	—
14	NBRC4746	D	II	2.2	5.9	B	49	NBRC5780	A	I	N.D.	N.D.	—
15	NBRC4747	B	II	N.D.	N.D.	—	50	NBRC5781	C	N	3.2	7.2	B
16	NBRC4749	B	II	N.D.	N.D.	—	51	NBRC6154	C	N	3.0	10.3	B
17	NBRC4754	B	II	N.D.	N.D.	—	52	NBRC6155	A	I	N.D.	N.D.	—
18	NBRC4756	D	II	1.3	6.4	B	53	NBRC6300	A	N	N.D.	N.D.	—
19	NBRC4757	B	II	N.D.	N.D.	—	54	NBRC9364	A	I	N.D.	N.D.	—
20	NBRC4758	D	I	35.0	0.0	A	55	NBRC30795	C	N	8.0	1.3	A
21	NBRC4766	A	I	N.D.	N.D.	—	56	NBRC31005	A	I	N.D.	N.D.	—
22	NBRC4770	B	II	N.D.	N.D.	—	57	NRRL395	D	I	34.9	0.0	A
23	NBRC4771	D	II	2.3	8.4	B	58	AHU6532	D	I	35.2	0.0	A
24	NBRC4772	C	N	2.5	0.0	A	59	AHU6533	D	I	35.1	0.0	A
25	NBRC4773	D	II	1.9	7.4	B	60	AHU6535	D	I	43.4	0.0	A
26	NBRC4775	D	II	2.0	9.1	B	61	AHU6536	D	I	33.2	0.0	A
27	NBRC4776	D	II	0.0	7.4	B	62	AHU6586	D	I	21.7	0.5	A
28	NBRC4780	A	I	N.D.	N.D.	—	63	AHU6587	D	I	34.0	0.0	A
29	NBRC4783	A	I	N.D.	N.D.	—	64	AHU6596	D	I	35.5	0.0	A
30	NBRC4785	D	I	43.9	0.0	A	65	ATCC48005	D	II	1.3	9.6	B
31	NBRC4798	D	I	35.8	0.0	A	66	ATCC48006	D	II	1.3	9.8	B
32	NBRC4801	B	II	N.D.	N.D.	—	67	ATCC48007	D	II	1.0	9.8	B
33	NBRC4804	A	I	N.D.	N.D.	—	68	ATCC48008	D	II	2.1	9.1	B
34	NBRC4809	A	I	N.D.	N.D.	—	69	ATCC48009	D	II	1.2	8.4	B
35	NBRC5318	D	I	23.6	0.4	A							

[a] According to Oda *et al.*,[3] strains were classified into 4 groups: A (Lactic acid producer), B (Fumaric acid producer), C (Acid productivity was low), and D (Not used in the study).
[b] Strains classified as C or D in Potato pulp fermentation were used for liquid fermentation. N.D. = Not determined because the strain was already classified in Potato pulp fermentation.
[c] A = Lacic acid producer, B = Fumaric acid producer.

Results

PCR amplification of ITS region and sequences of PCR products

We used the 69 *R. oryzae* strains listed in Table 1. These strains were divided into 4 groups following the results of potato-pulp fermentation[3] as follows; lactic acid producing group (A), fumaric acid producing group (B), a little organic acid producing group (C), and unknown (D).

Using the genomic DNA extracted from these strains, the ITS region of rDNA was amplified with the primers ITS4 and ITS5. The amplification resulted in four different patterns. From each of 64 strains out of 69 strains, a single band of approximately 650-bp was amplified. Among the other 5 strains, 2 strains resulted in the amplification of several bands, a single 750-bp fragment was amplified in 1 strain, and from 2 strains was detected a 900-bp fragment. These 5 strains belonged to group C.

The DNA sequences of the amplified 650-bp fragments were analyzed. Among these DNA sequences, a major difference that could divide the sequences into two groups was detected (Fig. 1). According to these differences, the sequences were divided into 2 major groups, I and II, with a Type I sequence and a Type II sequence, respectively. In the Type I sequence, the 53rd and 54th bases were lost, and the 416th (A) was changed to G, the 519th (C) was replaced by T, and the 561st (C) was replaced by T, compared with the Type II sequence. The group I contains other minor base substitutions, resulting in the formation of three subgroups, groups I-1, I-2,

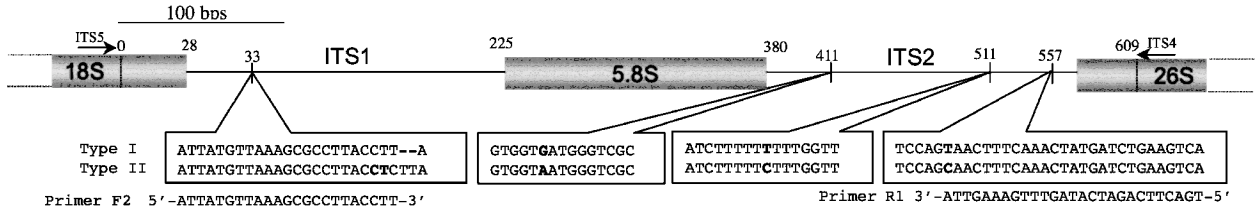

Fig. 1. The Schematic Illustration of *R. oryzae* rDNA-ITS Sequence.
A total map of the region is represented by the bar. Sites of the primers ITS5 and ITS4 are indicated with arrows. The three subunits (partial 18S, 5.8S, 28S) of the rDNA are indicated with boxes. The mutation sites are presented by the lower boxes, with the sequences of the Type I (upper) and Type II (lower). Mutations were indicated with bold letters. The positions indicated in the bar correspond to the 5′ site of each region, of the Type II sequence. The sequence of selective primers F2 and R1 are presented. Please note that the R1 sequence is indicated in the 3′ to 5′ direction.

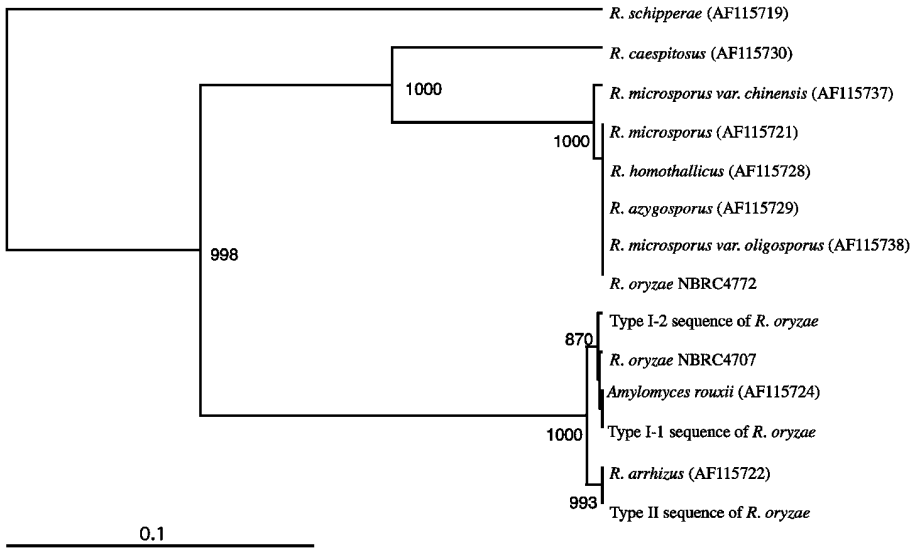

Fig. 2. The UPGMA-Phylogenetic Tree of rDNA-ITS Sequence of Genus *Rhizopus*.
Bootstrap percentages over % from 1000 replicates are shown on each branch. The numbers in parentheses indicate their Database accession numbers.

and NBRC4707. Interestingly, this division of groups I and II was completely consistent with the division of fermentation group A and B respectively, in 32 strains whose acid production profiles were already identified in potato pulp fermentation (Table 1). The other strains the fermentation groups of which were not identified clearly (groups C and D) had one of the Type I or II sequences (Table 1). Then these strains were cultivated in liquid medium for 3 days in order to clarify their ability of acid production. The organic acid production was completely consistent with the DNA sequence grouping (Table 1).

The phylogenic tree was made with these data and some known sequences on the database (Fig. 2). Four clusters appeared. Two of them consisted of one of each sequence, *R. schipperae* and *R. caespitosus*. For the other two clusters, one cluster consisted of *R. microsporus* var. *chinensis*, *R. microsporus*, *R. homothallicus*, *R. azygosporus*, *R. microsporus* var. *oligosporus* and *R. oryzae* NBRC4772. The other cluster consisted of all of *R. oryzae* except NBRC4772, *R. arrhizus*, and *Amylomyces rouxii*. The group I-1 had same sequence for *A. rouxii*. The strain NBRC4772 strain had same sequence of *R. microsporus*, *R. homothallicus*, *R. azygosporus*, and *R. microsporus* var. *oligosporus*.

Design of selective primers and study of reaction condition

The final goal of this paper was to establish a PCR-based method to screen lactic acid producers. Thus primers were designed to amplify the lactic acid production group A (Type I sequence) specifically. The primers F2 (5′-ATTATGTTAAAGCGCCTTACCTT-3′) and R1 (5′-TGACTTCAGATCATAGTTTGAAAGTTA-3′) which contain bases specific to the Type I sequence at each 3′ end and will amplify 553-bp DNA were finally adopted for the selective PCR. The PCR conditions were examined mainly by changing the annealing temperature, by using *R. oryzae* NBRC4783 and NBRC4735 for the control templates for the Type I and II, respectively. Non-

specific bands were still visible in the amplification from group II DNA with annealing at 61°C, but almost not detected at 63°C. Based on the results of this combination with the touch down PCR,[9] where the annealing temperature is lowered in each PCR cycle, finally clearly resulted in specific amplification from Type I (Fig. 3). The specific PCR conditions were established as follows: Initial denaturation at 94°C for 2 min, 8 cycles of denaturation at 94°C for 15 sec, annealing at 69°C (the temperature was lowered 1°C for each cycle) for 30 sec, and extension at 72°C for 1 min, and 20 cycles of denaturation at 94°C for 15 sec, annealing at 61°C for 30 sec, and extension at 72°C for 1 min, and a final 5 min of chain elongation at 72°C. These conditions allowed us to detect the amplification of Type I from 1 ng of template DNA, but there was no amplification of the Type II sequence from 100 ng of template (Fig. 3).

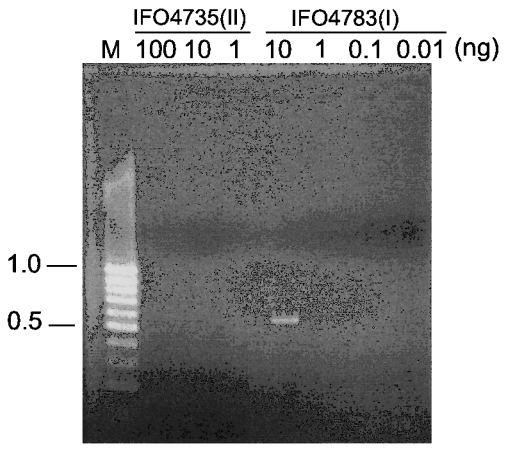

Fig. 3. Touch Down PCR Products Separated on Ethidium Bromide Stained 1.5% Agarose Gel.

M is a DNA size marker (Stable 100-bp DNA Ladder, SIGMA Genosis, Japan). Sizes are given in kb. Genomic DNA of NBRC4783 and NBRC4735 were used as controls for the Type I sequence and the Type II sequence, respectively. The number above each lane indicates the amount of template DNA in ng, used in each 50-μl reaction.

PCR amplification was done for all extracted DNA samples. By using of established PCR conditions and a designed primer set, the samples of lactic acid producing *R. oryzae* were amplified correctly (Fig. 4).

PCR amplification of natural samples

Using the selective PCR technique established above, the DNAs from Indonesian fermented food were tested for the identification of lactic-acid-producing *R. oryzae* strains. A clear and expected length band was amplified from the DNA samples of *tape* and *ragi tape*, but no amplification was detected from *tempeh* and *ragi tempeh* (Fig. 5). This result was consistent with the fact that ATCC strains used in this study, which were originally isolated from *tempeh*, belonged to fermentation group B, *i.e.* they had the group II sequence.

Based on this data, one fungal strain named SDM-23 was isolated from *ragi tape*. The ITS sequence of this strain was completely the same as that of Type I-1. However, the isolate was quite different from that of *R. oryzae* in morphology, such as poor sporangia formation and abundant chlamydospore formation. These characteristics indicated to us that this strain, SDM-23, is similar to *Amylomyces rouxii*.

The growth on basal synthetic medium (SBM) with various carbon sources was tested between *R. oryzae* AHU6532, *A. rouxii* CBS 438.76 (type culture), and the strain SDM-23. SDM-23 and *A. rouxii* grew vigorously on SBM containing sucrose or maltose but poorly on SBM-glycerol, whereas *R. oryzae* grew vigorously on glycerol but poorly on sucrose or maltose. Since these characteristics were used in the identification of the genus,[4] the strain was identified as *Amylomyces rouxii* SDM-23.

In order to test if SDM-23 produces lactic acid or not, a liquid culture was done. The strain produced 25 mg/ml of lactic acid in the ninth day of the cultivation.

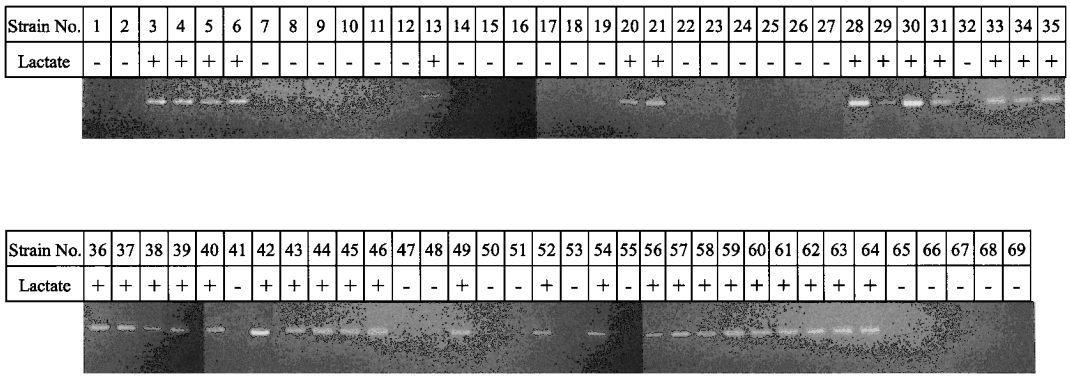

Fig. 4. Touch Down PCR Products for All Strains Separated on Ethidium Bromide Stained 1.5% Agarose Gel.

Strain numbers correspond to the strain numbers listed in Table 1. Lactate production is represented by + (produce) or − (no production).

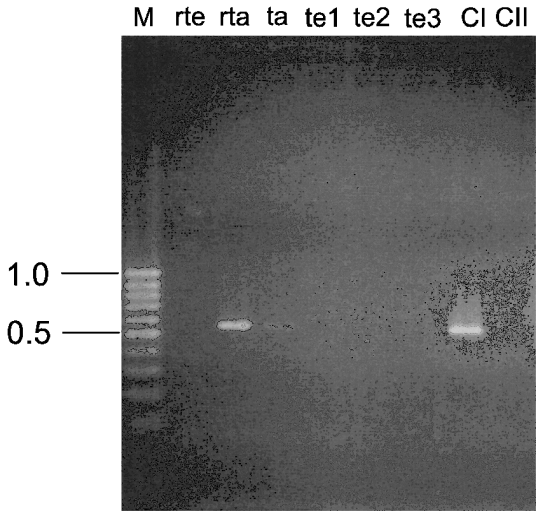

Fig. 5. Touch Down PCR Products from Fermentation Food Samples.

M is DNA size marker. Other lanes contain amplification products from food samples; rte = *ragi tempeh*, rta = *ragi tape*, ta = *tape*, te1 = *tempeh*1 (Muchlar), te2 = *tempeh*2 (Market 1), te3 = *tempeh*3 (Market 2), CI = Type I positive control (NBRC4783), and CII = Type II Negative control (NBRC4735).

Discussion

We examined the sequences of the rDNA ITS region and found that the sequences was applicable to differentiate *R. oryzae* with respect to their organic acid production. Strains belonging to *R. oryzae* were divided into 4 groups by fermentation patterns for potato pulp and 3 groups by sequence data. In a comparison of these data, all strains of lactic acid producing group A had the type I sequence and all strains of fumaric acid producing group B had the type II sequence. The ability of organic acid production of these strains was further confirmed to coincide with the sequence by liquid culture of strains belonged to fermentation groups C and D on potato pulp. Obviously, the organic acid production was not directly related to rRNA, but the existence of a clear relationship between rDNA ITS sequence and organic acid production indicates the importance of the ability for organic acid production as a systematic criterion, and the effectiveness of ITS sequencing for a simpler method of systematics.

It can be concluded that the PCR by the primers designed from ITS sequence is very effective for the isolation of lactic-acid-producing *R. oryzae*. In particular, it was shown by the experiment using Indonesian traditional fermented foods; the *tempeh* strain (*R. oligosporus*) had low activity of lactic acid production and the *tape* strain had a high one. The *tempeh* was made of soybeans, *Ragi tempeh* (*R. oligosporus* starter) and a little addition of lactic acid. The lactic acid fermentation ability of *R. oligosporus* might be lost during the history of their usage in *tempeh* production, where lactic acid was added externally.

It was expected that the lactic-acid-producing *R. oryzae* could be isolated from fermented food samples, but *Amylomyces rouxii*, instead of *R. oryzae* was isolated in this experiment. However, this is not contradictory to our finding, *i.e.*, in the phylogenic tree (Fig. 2), the ITS sequence of *A. rouxii* was completely consistent with the group I (I-1) sequence. Historically, the *A. rouxii* had been included in the genus of *Rhizopus* as *R. chlamydosporus*, but later it was concluded that the two genera were distinct in both the pattern of carbon sources use and morphological characteristics.[9] This suggests that *A. rouxii* was diverged from lactic acid-producing *R. oryzae*, after the separation from fumaric acid producers. This is also followed by the report by Ellis,[10] where DNA-DNA hybridization between *A. rouxii* and *R. oryzae* resulted in very high similarity. The fact that *A. rouxii* is often used in alcoholic fermentation in China may suggest the participation of human usage in its divergence.

From the fact that *A. rouxii* and *R. oryzae* share the rDNA ITS sequence and ability of lactic acid production, it is suggested that the re-classification of the genus *Rhizopus* and *Amylomyces* may be necessary. However, in this report, we did not consider characteristics other than organic acid production and the ITS sequence. Schipper *et al.*[11] had re-classified the genus *Rhizopus* using morphological and physiological characteristics, into three main groups, the microsporus group, the stolonifer group, and *R. oryzae*. From this point of view, *Rhizopus oryzae* in current taxonomy could be a population in which some variations of morphological or physiological traits are involved. Thus, more detailed investigations including molecular and other characteristics are necessary for the completion of the re-classification of the genus.

The *Amylomyces* strain isolated in this study pointed out the possibility of its use as a starter for potato-pulp fermentation of silage or foods, because it was isolated from a fermented food, *i.e.* safe material for humans and animals.

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