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Full Length Research Paper

Differentiation studies of predominant lactic acid bacteria isolated during *growol* fermentation by using polyphasic taxonomic characterization

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Twelve isolates known as weakly amylolytic lactic acid bacteria were isolated from different time during *growol* fermentation, a cassava based product from Indonesia. Differentiation tests of these strains were performed using molecular and phenotypic characterization. 16S subunit of the ribosomal RNA and phenylalanyl tRNA synthase (*pheS*) gene sequences of all isolates showed similarity with *Lactobacillus plantarum* subsp. *plantarum* NBRC 15891 as a reference strain. But many isolates revealed different pattern of template DNA and carbohydrate fermentation profile which determined employing random amplified polymorphic DNA (RAPD) and API 50 CHL analysis, respectively. This result allowed three groups of isolates belonging to the species L*. *plantarum*, to be distinguished. The comparative methods of identification showed considerable genome variation between lactic acid bacteria isolated from different time of fermentation, thus, becoming useful information for using the isolates as starter culture.

Key words: *Growol*, lactic acid bacteria, polyphasic taxonomic.

INTRODUCTION

Amylolytic lactic acid bacteria (ALAB) play an important role in the preparation of traditional foods fermentation, especially cassava based products. Starch degradation ability of ALAB fermentation causes the change of starch microstructure and induces their amylography and viscosity characteristics (Bertolini et al., 2000; Plata-Oviedo and Camargo, 1998). Even, in sour cassava starch (povilho azedo; Brazil), lactic acid fermentation and sun drying are related to the baking ability of these products (Brabet et al., 1999; Bertolini et al., 2001; Demiate et al., 2000). Many strains of ALAB have been isolated from fermented food products. Strains of *Lactobacillus plantarum* have been isolated from African cassava-based fermented products (Johansson et al., 1995; Nwankwo et al., 1989) and *Lactobacillus manihotivorans* were isolated from cassava starch (Guyot and Morlon Guyot, 2001). ALAB were also present in other traditional fermented food. *L. plantarum* have also been isolated from fermented fish and rice product (Olympia et al., 1995), *Lactobacillus amylovorus* from corn (Nakamura, 1981). Recently, Sanni et al. (2002) described amylolytic strains of *L. plantarum* and *Lactobacillus fermentum* as strains in various Nigerian traditional amylaceous fermented foods. The search for ALAB in fermented amylaceous foods has been justified by the high starch content of the

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raw material.

Growol is an Indonesian traditional food prepared from cassava. Fresh peeled cassava roots are soaked in water for three to five days, followed by pressing to decrease moisture content. Then steamed to make it "ready to eat" *growol*. Microorganisms involved in the fermentation process come from the environment, especially the natural microbiota of water. Substrate composition changes during the fermentation of cassava (Epriliati, 1994). At the beginning, fermentation medium contain sugar that can be utilized by lactic acid bacteria (LAB) as a carbon source. Furthermore, LAB should be able to utilize starch for their growth which allows the change of their characteristics. During the fermentation process, a succession of naturally occurring microorganism results in a population dominated by lactic acid bacteria, that is, a group of *Streptococcus,* C*oryneform,* yeast and a group of *Enterobacteriaceae*; *Bacillus* sp and *Acinetobacter* sp; *Lactobacillus* sp and *Moraxella* sp (Rascana, 1986). Several authors have noted that *Lactobacillus* spp. constitute the majority of lactic acid bacteria of the fermented cassava preparation (Rascana, 1986; Rahayu, 1996). Unfortunately, the identification of these LAB in *growol* is just based on their morphological and physiological characteristics in genus levels and not yet in strain level. Whereas, some important functional properties of lactic acid bacteria should be displayed at strains level (Johansson et al., 1995; Guyot et al., 2003), which requires better knowledge of intra species diversity in relation with potential technological and different applications in food processing, such as the improvement of natural fermentation of amylaceous raw materials.

This study aims to identify lactic acid bacteria responsible for the *growol* fermentation process, using randomly amplified polymorphic DNA (RAPD) analysis, complemented methods for DNA profiling based on 16S rRNA and *pheS* genes. The results of genotypic characteristics will be compared with phenotypic evaluation of carbohydrates fermentation profiles. The different techniques are combined and used to differentiate the presence of genome similarity clusters in a strain level.

MATERIALS AND METHODS

Microbiological sampling

The samples from the fermentation steps of *growol* were obtained from local producer in Kulonprogo, Yogyakarta and were brought to the laboratory. They were stored at ambient temperatures of 29±1°C and continued for fermentation. Samples for microbiological analysis were taken daily from soaking water and cassava root during five days of fermentation. Twelve isolates detected as lactic acid bacteria, were analyzed for their genotypic and phenotypic characterization in NITE Biological Resource Center, Japan. The identification of isolates were done and compared with lactic acid bacteria type isolates from NBRC cultured collection. The reference isolates were *L. plantarum* subsp. *plantarum* NBRC 15891 and *L. plantarum* subsp *argentoratensis* NBRC 106468.

Morphological and growth ability characterization

The colony of isolates was picked up from MRS-agar plate and their morphological characteristics observed. The colony colors of isolates were determined at the pellet of 24 h isolates. Gram-staining, oxidase and catalase tests (Ehrlich, 1956) were also performed. Gas producing ability of isolates was observed for 72 h incubation in glucose yeast peptone medium.

Physiological characterization

The carbohydrate fermentation profiles of isolates were tested with API 50 CH strips and API CHL medium, and incubated for 48 h with observation every 24 h. (according to the manufacturer's instructions, API system, Bio-Merieux, Japan).

Genotypic characterization

DNA preparation

All isolates were pre-cultivated in MRS broth media. Cells grown for 24 h at 30°C were used for DNA extraction and purification. Cultured cells were then harvested in a 1.5 ml microtube. Resuspended pellet in 180 µl of enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA-2Na, 1.2% Triton X-100, pH 8.0, 20 mg/ml lysozyme), incubated for at least 30 min at 37°C. Adding 25 µl of proteinase K and 200 µl of buffer AL. Mixed by vortexing and incubated at 56°C for 30 min. Purification of bacterial were done by using DNeasy tissue kit (Qiagen no.69504) and procedures of analysis following manufacturer's recommendations. The quantity and purity of the DNA was measured by Nanodrop, ND-100 V 3.31 Spectrophotometer at wavelengths of 260 nm.

16S rRNA gene analysis

A fragment of the 16S rRNA (rDNA) gene was amplified by PCR with conserved primers close to the 3_ and 5_ ends of the gene. The amplified process of DNA products using a TaKaRa Ex Taq Hot Start Version (TAKARA BIO, no. R007A) with Primer 9F; 5'-GAGTTTGATCCTGGCTCAG and Primer 1510R; 5'GGCTACCTTGTTACGA (or 1541R; 5'-AAGGAGGTGATCCAGCC), were designed for *Lactobacillus* specific. Agarose gel electrophoresis was done to examine the purity and amount of 16S rRNA. 1 µl of PCR products were visualized by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA (pH 8)). PCR amplified products were then purified by using a Monofas DNA purification kit I (GL science, no. 5010-21500) to remove primer and free nucleotides following the manufacturer's instruction. The PCR products were directly sequenced (a single strand) by using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, no. 4337454, Foster City, Calif.) by preparing reaction mixture as manufacturer's instruction. Sequencing reaction products were then purify by using an Auto-Seq G-50 (Amersham Biosciences) and analyzed by using an automatic DNA sequence (ABI PRISM 3130 Genetic Analyzer; Applied Biosystems). The closest known relatives were determined by performing database searches at GenBank using the BLAST program.

RAPD analysis

Amplification of 16S rRNA gene with RAPD Analysis was done using Ready To Go™ RAPD analysis beads (Amersham Biosciences) as manufacturer's recommendation. Three pairs of primers were used for this analysis, that is, RAPD analysis primer 1 – (5'-d(GGTGCGGGAA)-3'), RAPD analysis primer 4 – (5'-d(AAGAGCCCGT)-3') and RAPD analysis primer 6 – (5'-d(CCCGTCAGCA)-3'). PCR condition of RAPD analysis were 95°C, 5 min, 45 cycles consisting 95°C, 1 min, 36°C, 1 min 72°C, 2 min and final elongation at 72°C, 5 min. Then, PCR fragments were separated on 2% agarose gel using 1 × TAE buffer. RAPD patterns were visualized by UV transillumination after staining in ethidium bromide.

pheS genes analysis

The amplification of *pheS* gene was done using a TaKaRa Ex Taq hot start version (TAKARA BIO, no. R007A) following manufacturer's instruction. The primers used a forward primer *pheS*-21-F CAYCCNGCHCGYGAYATGC and reverse primer *pheS*-23-R GGRTGRACCATVCCNGCHCC (which were used for *Lactobacillus* specific). Agarose gel electrophoresis was done to examine the purity and amount of amplified PCR products. Then PCR products were purified using a Monofas DNA purification kit I (GL science, no. 5010-21500) to remove primers and free nucleotides. Sequencing reaction of purified PCR products were done by a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, no. 4337454), using the same primers of *pheS* amplification. PCR condition started at 96°C for 3 min, 30 cycles consisting of 96°C for 15 s, 35°C for 1 s, 60°C for 4 min and final elongation at 60°C for 3 min. Purification of sequencing reaction products was done using AutoSeq G-50 (Amersham Biosciences, GE Heathcare, no. 27-5340-01), then was analyzed by using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems). All these analysis were done as manufacturer's recommendation.

Nucleotides sequence accession number

Nucleotide sequences determined in this study were deposited in the GenBank database under the accession numbers AB603677 to AB603688 for 16S rRNA gene and AB603689 to AB603700 for *pheS* gene.

Phylogenetic analyses

The sequences data were sent to online international DNA database for homology search by basic local algorithm search tools (BLAST). Sequences were aligned using CLUSTALX (Thompson et al., 1994) and were adjusted manually. The neighbor-joining (NJ) method (Saitou and Nei, 1987) was used to construct all phylogenetic trees. The distance matrix for the aligned sequences was calculated using the two-parameter method of Kimura (1980). The robustness for individual branches was estimated by bootstrapping with 1000 resampling.

RESULTS

Morphological characteristics

All isolates isolated from different time of cassava fermentation were Gram-positive, catalase-negative, short rods in shape and white to yellowish colony color. They had uniform turbidity and sediment formation on liquid medium and were not producing gas from glucose (Table 1).

Physiological characteristics of isolates by API 50 CHL

Physiological characterization of isolates was preformed using API 50 CHL with *L. plantarum* subsp. *plantarum* NBRC 15891 and *L. plantarum* subsp. *argentoratensis* 104648 as reference strain (Table 2). Isolates AA12 exhibited assimilation pattern which had 100% similarity with *L. plantarum* subsp. *plantarum* NBRC 15891, whereas, the distinction of other isolates was based on their ability to use L-rhamnose as a carbon source. It could also be noticed that, the ability to use particular carbon sources is isolates-specific, that is, isolates AA7, UA6 and UB9 could not consume D-melezitose, UA3 could use methyl-αD- glucopyranose and D-tagatose, UB8 used D-tagatose and AA11 could use inulin as a carbon source for producing organic acid.

Phylogenetic analyses based on the 16S-rRNA genes, *pheS* genes and RAPD analyses

The comparative analyses of 16S rRNA and *pheS* gene sequences showed the same results of isolates belonging species. The phylogenetic tree of the 16S rRNA genes of isolates grouped AA7, UA6, UB8, UB9, AA12, AB1, AA10, AA5, AA2, AB1, AA11 and UA3 had similarity with *Lactobacillus pentosus* and *Lactobacillus plantarum* subsp. *plantarum* (Figure 1). But *pheS* genes analyses identified that all isolates were *L. plantarum* subsp. *plantarum* (Figure 2).

The genetic relationships among isolates on the basis of shared amplification products were estimated more than 50, 75 and 55% using primer 1, 4 and 6, respectively (Figures 3 to 5). All dendrograms clearly grouped *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis* into a different cluster, A and B, respectively, and placed the same template DNA of *L. plantarum* subsp. *plantarum* (NBRCa) in the same group as evidence the accuracy of this method. The dendrogram obtained using primer 4 showed a single lineage pattern (Figure 4). There was indication of less genetic variation among isolates compare with dendro-gram using primer 1 and 6. Interestingly, the three pattern of these DNA fingerprinting could place AA2, AA5, AA10 and AB13 in the same cluster, which proved that those for isolates were closely related among each others. RAPD profile also differentiated the low similarity of isolate UA3 with the others (Figures 3 and 5).

Table 1. General characteristics of isolates.

Isolate	Cell shape	Colony color (*)	Gram coloration	Catalase activity	Gas producing	Development on liquid medium	Source and time of isolation**
L. plantarum subsp. *plantarum* NBRC 15891	Rod	White-yellowish	+	-	-	Sediment formation	-
L. plantarum subsp. *argentoratensis* NBRC 106468	Rod	White	+	-	-	Sediment formation	-
AA10	Rod	Yellowish	+	-	-	Sediment formation	Water,120 h
AA7	Rod	White	+	-	-	Sediment formation	Water, 24 h
AA11	Rod	Yellowish	+	-	-	Sediment formation	Water, 120 h
UA6	Rod	White-yellowish	+	-	-	Sediment formation	Cassava root, 24 h
UB9	Rod	Yellowish	+	-	-	Sediment formation	Cassava root, 0 h
UB8	Rod	Yellowish	+	-	-	Sediment formation	Cassava root, 96 h
AB13	Rod	White	+	-	-	Sediment formation	Water, 48 h
AA12	Rod	Yellowish	+	-	-	Sediment formation	Water, 48 h
AA2	Rod	Yellowish	+	-	-	Sediment formation	Water, 0 h
UA3	Rod	White-yellowish	+	-	-	Sediment formation	Cassava root, 96 h
AA5	Rod	Yellowish	+	-	-	Sediment formation	Water, 72 h
AB1	Rod	White-yellowish	+	-	-	Sediment formation	Water, 72 h

*White yellowish means the white color dominantly than yellow color; **refer to the time of fermentation. Key: + = positive reaction; - = negative reaction.

Table 2. Carbohydrate fermentation profiles of lactic acid bacteria isolated from *growol* fermentation using API CHL identification systems.

Carbohydrate*	106468	15891	AA10	AA7	UA6	UB9	UB8	AB13	AA12	AA2	AB1	AA11	AA5	UA3
D arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L arabinose	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L rhamnose	-	-	+	+	w	w	w	+	+	+	+	+	w	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl-αd-mannopyranoside	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-αd-glucopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Inulin	-	-	-	-	-	-	-	-	-	+	-	+	-	-
D melezitose	-	+	+	-	-	-	-	+	+	+	+	+	+	+
D raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D turanose	-	+	-	+	+	+	+	-	+	-	+	+	+	+
D tagatose	-	-	-	-	-	-	+	-	-	-	-	-	-	+
L arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2 keto gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 keto gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*24 results is taking from 49 of carbohydrate types which fermented by isolates. Key: + = positive reaction; - = negative reaction; w = weak reaction.

DISCUSSION

There is no differences on morphological characters between isolates isolated at different time of fermentation and also when compared with type strain isolates, except on colony color. Isolates which were isolated in the same time of fermentation but from different batch had different colony color (Table 1). The specific color of isolate's colonies can be influenced by the age of cell and source of the isolates, although, they belong to the same species. The ability of isolates in fermenting carbohydrates by using API 50 CH also showed slightly different patterns (Table 2). Biochemical profiles on carbohydrate usage were influenced by the condition and composition of medium in their isolate, although they were from the same origin, it was also noticed that, the ability to use particular carbon sources is isolates-specific. The isolates from the beginning of fermentation when the substrate is contained a lot of sugar, will utilize these sugars easily. But after more than two days the sugar will be exhausted, therefore, LAB should be able to utilize starch as a carbon

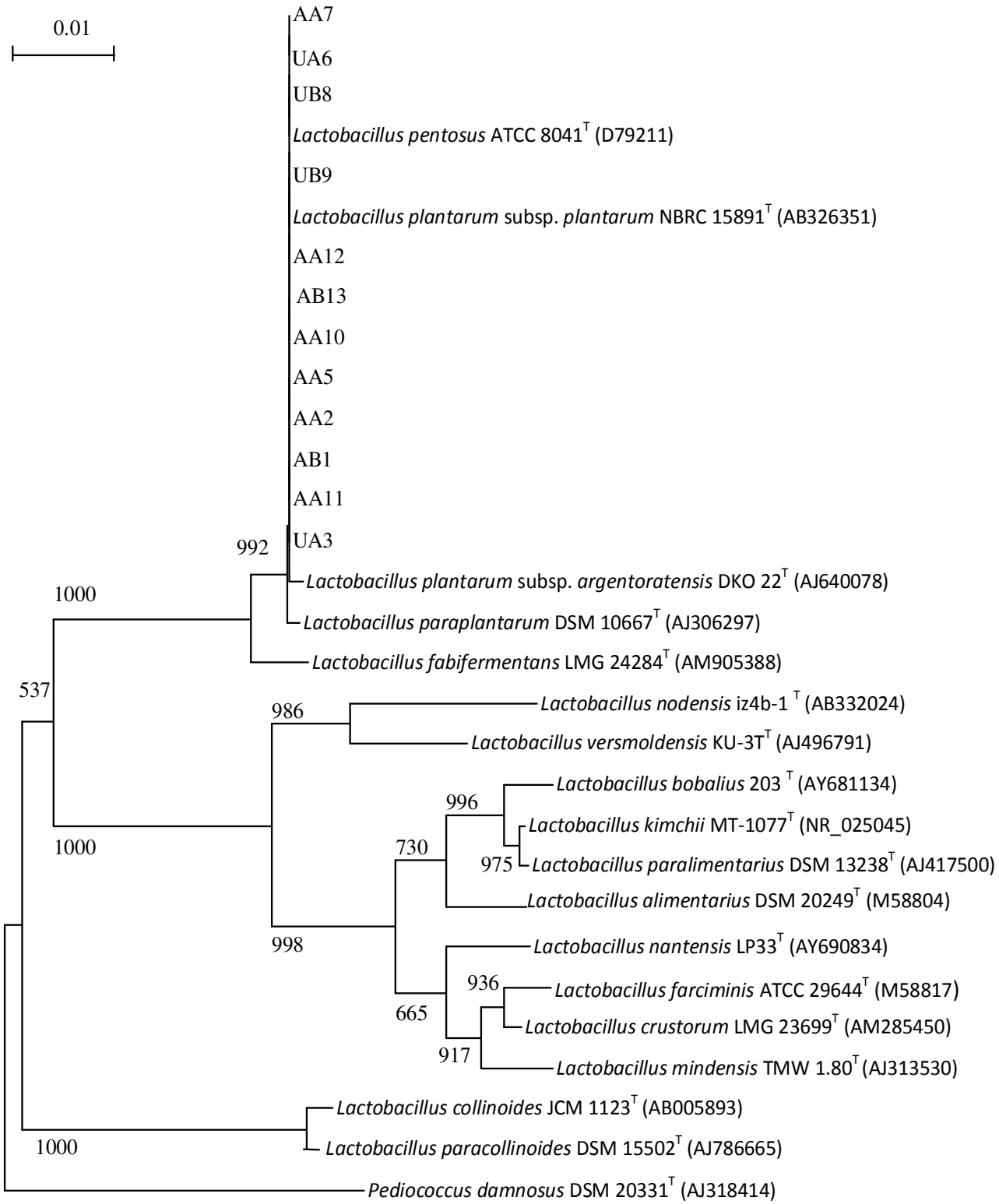

Figure 1. Phylogenetic tree showing the relative positions of isolates as inferred by the neighbor-joining method of complete 16S rDNA sequences. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the accession numbers for all 16S rDNA sequences (between brackets). *P. damnosus* is used as an out group. The bar indicates 1% sequence divergence K_{nuc}. nucleotide substitution rate.

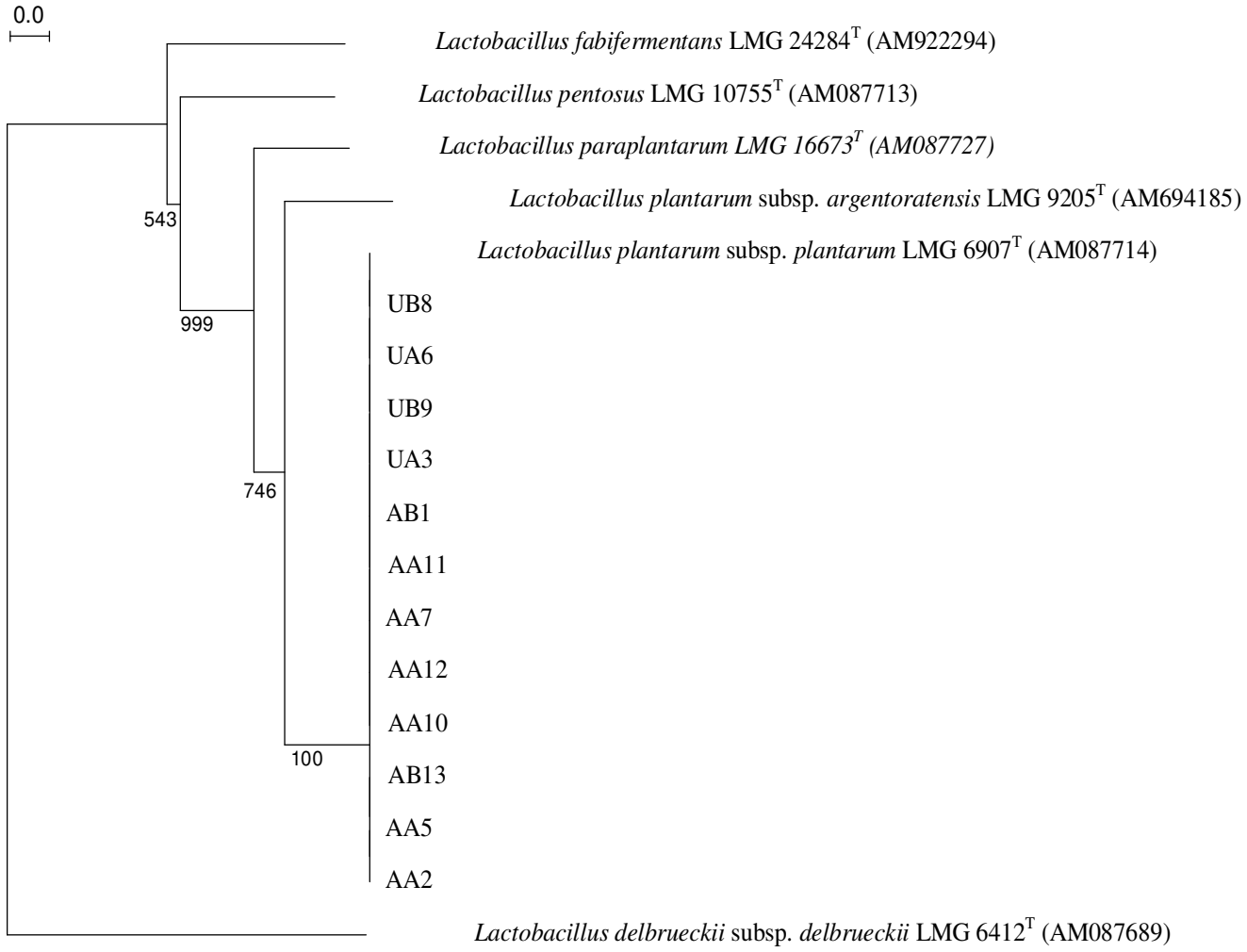

Figure 2. Phylogenetic tree representing the discriminatory power of the *pheS* genes for identification of isolates; the relative positions of isolates as inferred by the neighbor-joining method of complete sequences. References of the type strains used for comparison are given, as well as the accession numbers for all *pheS* genes sequences (between brackets).

source. As slowly diverging molecule, 16S rRNA is not able to reveal significant differences between recently diverged species, such as *L. plantarum, L. paraplantarum* and *L. pentosus* due to the high gene sequence similarity (Torriani et al., 2001; Naser et al., 2007). 16S rRNA gene sequence analysis can be indicated as only belonging to a group, not to a definite species. Thus, the identification based on 16S rRNA gene may be misleading if closely related species are analyzed. PCR using species-specific oligonucleotides designed based on phylogenetic molcular markers could be a useful approach, since these molecules are ubiquitous and relatively highly conserved. For this purpose, 16S ribosomal DNA sequences are not suitable because of the high identity value (99%) shared by *L. plantarum* and *L. pentosus*. Consequently, the definition of phylogenetic distances is also not feasible by such a classical approach for *L. plantarum* group species (Mori et al., 1997; Torriani et al., 2001).

At the subspecies level, the neighbor-joining tree based on the *pheS* gene sequences showed that, *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis* were clearly differentiated from each other (Figure 3). Accuracy allows the distinction between intraspecific variation and interspecies divergence in the selected loci (Meyer and Paulay, 2005). The *pheS* (382-455 nt) as one partial gene sequences were applied as alternative genomic markers alternative tool for the rapid and reliable identification of different species of the genus *Lactobacillus* (Massi et al., 2004; Nasser et al., 2007). Naser et al. (2007) examined that, the analysis of *pheS* and *rpoA*

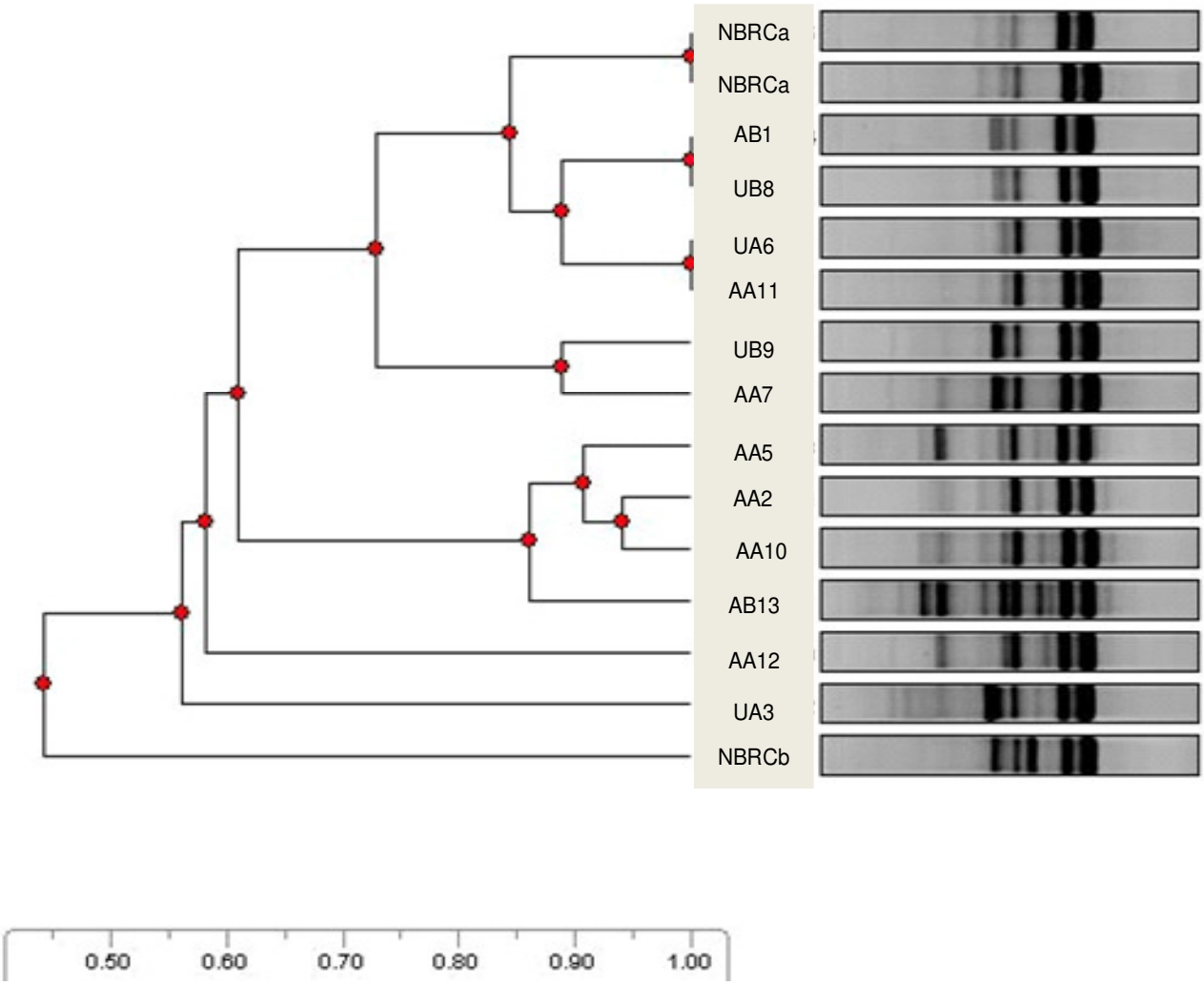

Figure 3. Dendogram among twelve isolates and two reference strain generated through RAPD data using Primer 1. Symbols represent code of isolates; NBRC a: *L. plantarum* subsp. *plantarum*. NBRC b *L. plantarum* subsp. *argentoratensis*

gene sequences effectively allows closely related *Lactobacillus* species to be differentiated at a higher discrimination level than possible with 16S rRNA gene sequence comparisons.

For this case, it can be concluded that all of isolates were *L. plantarum* subsp. *plantarum*. But the comparison results with the other analyses, that is, specific differences on fermenting much kind of carbohydrates by API 50 CHL and RAPD pattern of DNA finger printing are useful information for predicting that some of this isolates might have different strains. RAPD is a technique for rapidly detecting genomic polymorphisms, utilizing a single short oligonucleotide primer of arbitraty sequence in polymerase chain reaction. RAPD-PCR fingerprinting successfully identified strains at the species level (Maurier and Grimont., 1993; Nishitani et al., 2004; Stephan et al., 1994). RAPD clustering of isolate UA3 were in parallel with the physiological characteristic of API results. This isolate had three differences on sugar fermentation profile compare with reference strain *L. plantarum* subsp. *plantarum* NBRC 15891. The subgroup consisting AA2, AA5, AA10 and AB13 were having the same API profile, although just differ from reference strain on using L-rhamnose. Isolate AA11 which have two differences on carbohydrate profiles, that is, positive reaction of L-rhamnose and inulin only could be separated in different sub cluster by using primer 6.

In general, these three methods of genotypic characterization may not be able to identify the amylolytic capability of LAB. Therefore, physiological analysis remains to be done to find out the specific characteristics. The facts that within species group, different genes may yield

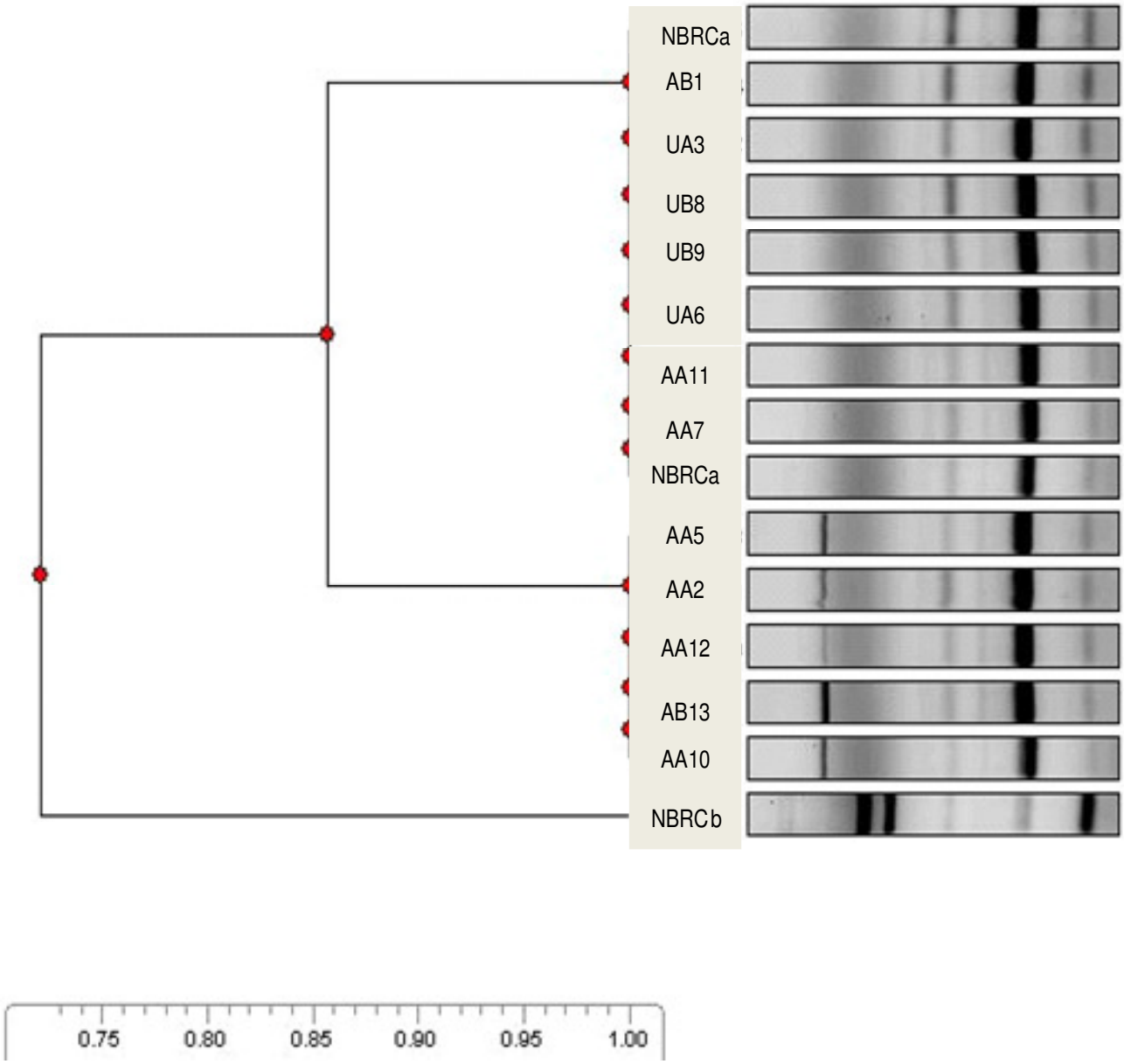

Figure 4. Dendogram among twelve isolates and two reference strain generated through RAPD data using Primer 4. Symbols represent code of isolates; NBRC a: *L. plantarum* subsp. *plantarum*. NBRC b *L. plantarum* subsp. *argentoratensis*

different tree topologies, several factors account for these differences, that is, the level of the information content, the different rate of evolution due to different selection forces on various genes and the length of the partial sequences that are compare (Stiles and Holzapfel, 1997). The groups seem to have no reflection to the time of isolation because strain which were isolated from different time of fermentation belongs to the same sub cluster. Thus, the length of fermentation does not influence the character of isolates. Further evaluation will be undertaken to determine if these isolates are of value for use in cassava fermentation industry.

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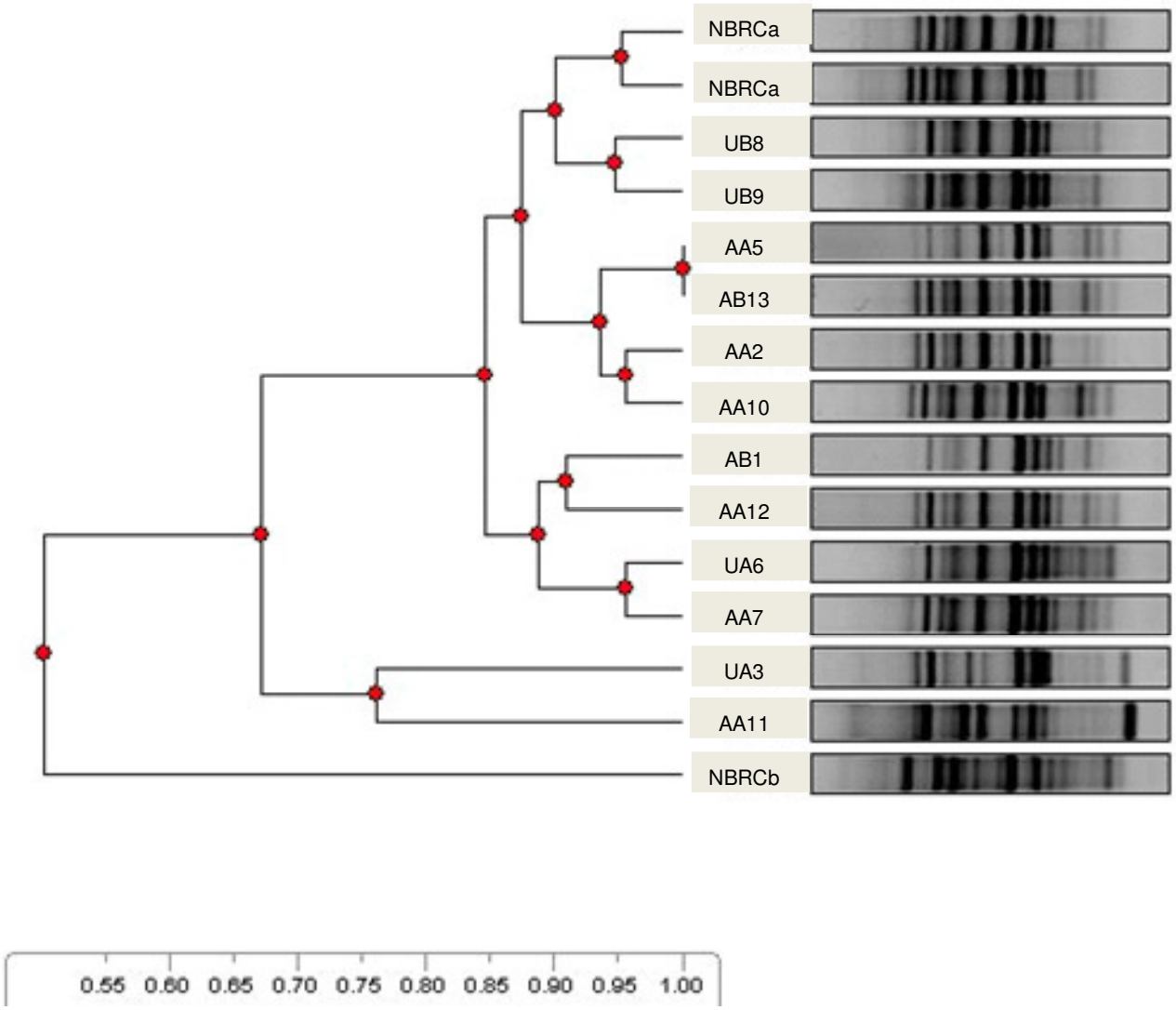

Figure 5. Dendogram among twelve isolates and two reference strain generated through RAPD data using Primer 6. Symbols represent code of isolates; NBRC a: *L. plantarum* subsp. *plantarum*. NBRC b *L. plantarum* subsp. *argentoratensis*

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