Malaysian Journal of Microbiology, Vol 18(4) 2022, pp. 398-407
DOI: http://dx.doi.org/10.21161/mjm.211392

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Determination of yeast diversity in fermented Sumbawa mare's milk using internal transcribed spacers (ITS) fragment analysis

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Received 30 December 2021; Received in revised form 26 May 2022; Accepted 14 June 2022

ABSTRACT

Aims: Molecular identification of yeast has been conducted on various fermentation products. However, the identification of yeast in fermented Sumbawa mare's milk based on the genotyping method has not been carried out. This study was aimed to determine the diversity profile of yeasts in fermented Sumbawa mare's milk using phenetic characters and PCR-RFLP analysis technique based on the ITS region.
Methodology and results: Yeast isolates were phenotypically characterized and visualized in a dendrogram using CLAD97 software. Then, the yeast DNA was extracted using heat treatment and amplified using ITS1 and ITS4 primers. The amplicons were analyzed by RFLP using *Hind*III and *Hae*III enzymes. The phylogenetic tree was constructed using MEGA 7.0. Based on the result of grouping by phenetic analysis and PCR-RFLP, the 12 isolates were divided into four groups with different members. The results of the phenetic analysis were divided into group I (all isolates of Dompu), group II (isolate B3, B4, S3), group III (isolate B5) and group IV (isolate S1). The types of yeast that were identified molecularly and represented each group of PCR-RFLP results included in group I were *Kluyveromyces marxianus* D1A and *K. marxianus* D1B, group II: *K. marxianus* D7, group III: *Kazachstania humilis* D4, while milk from Bima and Sumbawa has one yeast species as a member of group IV, namely *Pichia kudriavzevii* B3. *Kluyveromyces marxianus* was the yeast frequently found in Sumbawa fermented mare's milk.
Conclusion, significance and impact of study: Various yeast species as a consortium of the milk samples can contribute to the increasing quality of fermented Sumbawa mare's milk.

Keywords: CLAD97, genotypic analysis, ITS region, mare's milk, phenetic analysis

INTRODUCTION

Sumbawa mare's milk is milk from a mare released in the grasslands on Sumbawa Island, West Nusa Tenggara Province. As a superior product, mare's milk is generally fermented naturally (without the addition of starter cultures). Fermented milk is proven to have several benefits for human health, such as healing bronchitis, wet lungs, typhoid, hypertension and lowering cholesterol. The content of Sumbawa mare's milk is known to inhibit the growth of *Mycobacterium tuberculosis* and pathogenic bacteria. In addition to the nutritional content of mare's milk, the health benefits are also inseparable from the activity of microorganisms, especially LAB (lactic acid bacteria) and yeast. Previous research found that LAB from the genus *Lactobacillus* dominated fermented Sumbawa mare's milk with *Lactobacillus rhamnosus* species, which can potentially be used as a probiotic (Sujaya *et al.*, 2008; Mulyawati *et al.*, 2019). In addition, *L. brevis* and *L. acidophilus*, which are also found in fermented mare's milk, have the potential as probiotics (Shi *et al.*, 2012). Meanwhile, the quality of fermented mare's milk is also influenced by the presence of yeast (Ishii *et al.*, 2014). Genus *Kluyveromyces* and *Saccharomyces* are yeasts commonly found in kefir, which can be used as probiotics (Cassanego *et al.*, 2018). *Kluyveromyces marxianus*, *K. unispora* and *Saccharomyces cerevisiae* were the dominant species in fermented koumiss mare's milk (Mu *et al.*, 2012). *Kluyveromyces marxianus* is also found in fermented mare's milk in Mongolia (Watanabe *et al.*, 2008). Yeast can affect the formation of aroma, texture and nutritional value in the fermentation process of mare's milk (Fleet, 2006; Mu *et al.*, 2012). Yeast is capable of fermenting lactose into alcohol (Choi, 2016). The presence of yeast during milk fermentation will interact synergistically with LAB (Lopandic *et al.*, 2006). Yeast can metabolize lactic acid, a metabolite product of LAB (Jatmiko *et al.*, 2012). It can be concluded that yeast plays a beneficial role in improving the quality (nutritional value) and safety of this milk by inhibiting the growth of undesired organisms (Lopandic *et al.*, 2006). In addition, several yeast species

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

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in fermented mare's milk can act as probiotics (Cassanego *et al.*, 2018).

Currently, mare's milk fermentation research focuses on lactic acid bacteria. However, research on the diversity of yeasts in fermented Sumbawa mare's milk is essential to determine the yeast species that contribute to Sumbawa mare's milk fermentation. Several yeast species have a high degree of similarity. The limitations of conventional yeast identification methods (phenetic methods) make yeast species often misidentified or reported as unknown species (Alcoba-Flórez *et al.*, 2007; Karimi *et al.*, 2015).

PCR-RFLP analysis is a simple and reliable analysis to identify two adjacent species in the same genus or same species (different strains) (Ge *et al.*, 2012). Previous studies have proven that yeast analysis in fermented milk products provides a diversity analysis using PCR-RFLP analysis. Restriction enzyme cleavage in the ITS1-5.8S-ITS2 region of yeast rDNA can provide a good profile by providing various fragments for each species (Jatmiko *et al.*, 2012; Angelov *et al.*, 2017; Jatmiko *et al.*, 2019). The ITS-5.8S rDNA region appears to help detect genetic variability among yeast species, which is valuable for taxonomic and species identification purposes (Fadda *et al.*, 2013). In addition, the PCR-RFLP analysis has succeeded in classifying LAB isolates in fermented Sumbawa mare's milk (Mulyawati *et al.*, 2019). Therefore, the molecular identification of yeasts in Sumbawa mare's milk has received significant attention. In the interest of obtaining novel potential non-LAB organisms, identifying yeast species in fermented Sumbawa mare's milk in combination with phenetic and genetic characters (PCR-RFLP) was conducted. Identifying yeast species from fermented Sumbawa mare's milk is essential so that further development can be carried out on the role of yeast in fermented milk.

MATERIALS AND METHODS

Preparation of yeast culture

The yeast isolates used were a culture collection from the Microbiology Laboratory, Faculty of Mathematics and Natural Science, Universitas Brawijaya, obtained from previous research. The yeasts were isolated from three samples, namely Bima, Dompu and Sumbawa (Siallagan, 2020). The 15 isolates consisted of 12 isolates from previous research (7 isolates from Dompu, 3 isolates from Bima and 2 isolates from Sumbawa) and the rest for controls. In addition, two reference isolates for phenetic characteristics (*S. cerevisiae* and *Candida albicans*) and *K. marxianus* (D6) were used as the positive control for DNA amplification. First, the yeast isolates from Sumbawa mare's milk were re-cultured in YPD agar (20 g/L peptones, 10 g/L yeast extract, 20 g/L glucose and 15 g/L agar) using the streak plate method. Then, the cultures were incubated at 30 °C for 2 to 3 days.

Phenetic characterization

The phenetic characterization of yeast was based on the morphological characteristics of the colony (such as shape, size, edge, elevation, surface, texture and color), cell (including shape and size) and biochemical test (catalase and oxidase test). The yeast cells were stained using methylene blue to determine the cell's morphology (Reis *et al.*, 2013). The isolates characterized as yeast were further characterized based on biochemical tests (catalase and oxidase test). The catalase test was carried out using 3% hydrogen peroxide (H_2O_2) and the oxidase test using an oxidase test strip (Bryer, 2016). The biochemical test was conducted in triplicates. The yeast characters obtained were analysed using CLAD97 software (Rahardi *et al.*, 2012) with reference isolates of *S. cerevisiae* and *C. albicans* to determine the similarity level among isolates.

Isolation of yeast DNA by heat treatment

A single yeast colony was taken using a loop and suspended in a microtube containing 200 µL of sterile ddH_2O. First, the yeast suspension was homogenized using a vortex (Scientific Industries, Inc., United States) for one min. The suspension was then heated in a water bath at 95 °C for 20 min. Next, the yeast suspension was centrifuged with a speed of 10,000× *g* at 4 °C for 5 min. Finally, the supernatant was transferred into a new microtube. It was stored at –20 °C and be ready to be used as a DNA template (Mulyawati *et al.*, 2019).

PCR-RFLP analysis

The ITS1-5,8S-ITS2 region was amplified using a forward primer ITS1 (5'-TCCGTAGGTGAACCTGCG G-3') and a reverse primer ITS4 (5'-TCCTCCGCTTATTGATAT GC-3'). The PCR mix containing 25 µL GoTaq Green Master Mix (2×), 2.5 µL of each primer, 15 µL ddH_2O was transferred to a PCR tube and 5 µL DNA sample was added to each PCR tube. The solution was homogenized and put into a thermal cycler (Eppendorf, Germany). The PCR program consisted of 35 cycles (denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and elongation at 72 °C for 1 min), an initial denaturation at 94 °C for 5 min and an additional cycle at 72 °C for 5 min as a final extension. After incubation, 6 µL of the PCR product was put into a 1.5% agarose gel well containing 1 µL of GelRed and then electrophoresed in 1× TBE buffer with an electric voltage of 100 V for 25 min (Artati, 2013; Mulyawati *et al.*, 2019). The gel electrophoresis results were visualized using UV Transilluminator (Vilber Lourmat, Germany). The size of the DNA fragment was determined by comparing its relative mobility to the 100 bp ladder (size range: 100*S.*1500 bp, Promega) (Jatmiko *et al.*, 2019).

A total volume of 10 µL was used for RFLP analysis, which consisted of 9 µL of amplicons, 0.5 µL of buffer digest mix (Multi-CORE, Promega) and 0.5 µL of restriction enzyme. In addition, *Hind*III and *Hae*III

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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Table 1: Grouping of yeasts based on ITS-PCR result and PCR-RFLP analysis.

Group	Samples origins	Number of isolates	Sequences length (bp)		
			ITS-PCR	*Hind*III	*Hae*III
I	Dompu	1	800, 590	600, 590, 200	700, 550, 450, 90, 50
II	Dompu	5	800	600, 200	700, 550, 90, 50
III	Dompu	1	740	740	460, 280
IV	Bima	3	500	500	400, 100
	Sumbawa	2			

(Promega) enzymes were used to cut specific yeast fragments separately. The mixture was incubated at 37 °C for 3 h. The electrophoresis method of the RFLP product was carried out, similar to the electrophoresis method of the PCR product.

Molecular identification of yeast

The amplicons from RFLP cluster representatives were sent for sequencing at the 1st BASE DNA Sequencing Service, Malaysia. First, the nucleotide sequences obtained were analysed using Sequence Scanner v 1.0 software. After that, the DNA sequences of the two primers were merged using BioEdit v 7.0.5.3 software. Next, DNA sequence alignment was performed using the Basic Local Alignment Search Tool (BLAST) by looking at the sequence homology. Several isolates that correlated with the species found were searched in NCBI with specifications, particularly ITS regions and lengths ranging from 500 to 800 bp. Next, the gene sequences of the isolates identified as reference strains and the outgroup strain (*Schizosaccharomyces pombe* CBS:1062) were aligned using MEGA v 7.0. Finally, the Neighbor-Joining method and the Tamura-Nei Model were used to construct a phylogenetic tree with a bootstrap of 1000 (Mbuk *et al.*, 2016).

RESULTS AND DISCUSSION

Diversity of yeasts based on phenetic character

From a phenetic study of 19 isolates, 12 isolates were characterized as yeast, while the other seven isolates were identified as bacteria. The 12 yeast isolates had quite diverse phenetic characteristics (Figure 1). The isolates from Dompu had the same morphological characteristics (HTU1). These phenetic differences were caused by differences in cell shape, texture, configuration and optical characteristics of isolates from Dompu (D1, D2, D4, D5, D6, D7 and D11) compared to other isolates. Isolates B3, B4 and S3 also had the same characteristics, which had 100% similarity (HTU2). The separation of the three isolates from isolates B5 and S1 was caused by differences in configuration, elevation, optical characteristics of the colonies and cell shape among isolates. Meanwhile, HTU3 B5 and S1 isolates had 90.24% similarity. The difference between the two isolates was caused by each elevation and optical characteristics. Then, the reference isolates, namely *C. albicans* and *S. cerevisiae*, were hypothesized to have

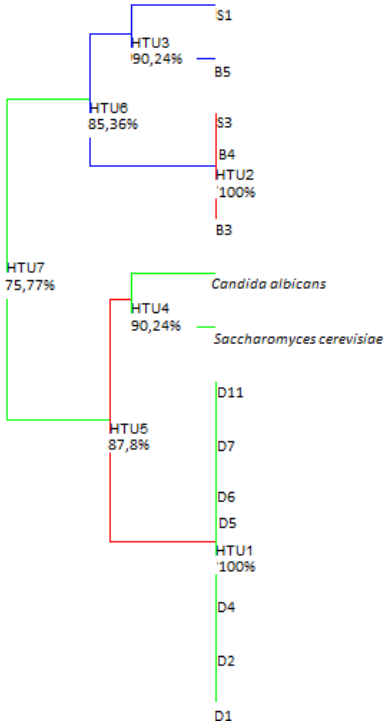

Figure 1: The similarity of yeast isolates from Sumbawa horse milk based on phenetic characters.

one common ancestor (HTU4) with a similarity of 90.24%. *Saccharomyces cerevisiae* was classified as oxidase-negative and catalase-positive, while *C. albicans* had oxidase-positive and catalase-positive. Meanwhile, the 12 yeast isolates tested positive for oxidase and catalase tests. The oxidase and catalase tests on *K. marxianus*, *K. humilis*, *P. kudriavzevii* as those used in this study showed the same results as previous studies (Miyasaka *et al.*, 2004; Pankiewicz and Jamroz, 2010; Chi *et al.*, 2015; Dahiru *et al.*, 2018; Public Health England, 2019).

Diversity profile of yeast based on PCR-RFLP

The diversity profile of yeast from Sumbawa fermented mare's milk has been identified based on the clustering of PCR results and PCR-RFLP analysis (Table 1). The PCR results succeeded in dividing 12 isolates into four groups based on differences in DNA band size, namely group I with 800 and 590 bp, group II with 800 bp, group III with 750 bp and group IV with a DNA band size of 500 bp.

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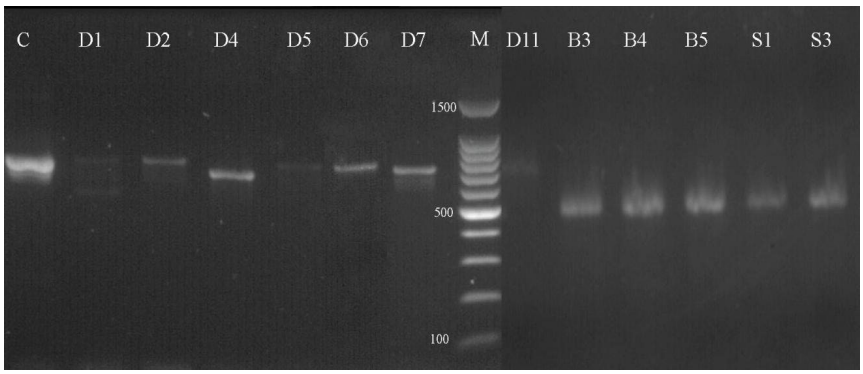

Figure 2: PCR amplification of ITS region. C: Control (*Kluyveromyces marxianus*), M: Marker.

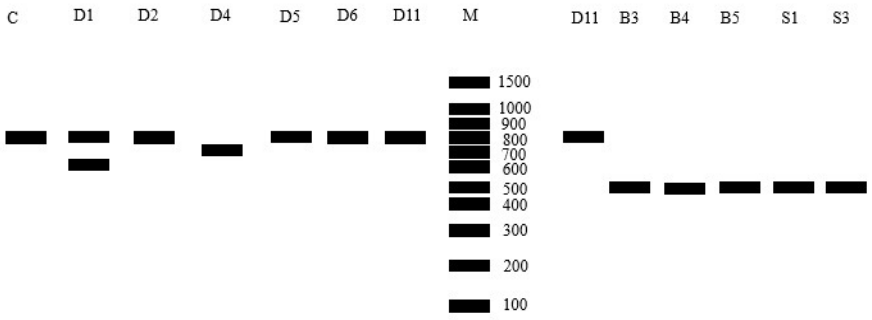

Figure 3: Representative diagram of PCR amplification of ITS region. C: Control (*Kluyveromyces marxianus*), M: Marker.

The electrophoresis visualization of the amplification of ITS region can be seen in Figure 2 and Figure 3. According to the visualization, the isolates was divided into four groups, namely group I (isolate D1); group II (isolate D2, D4, D5, D6, D7 and D11); group III (isolate D4); group IV (isolate B3, B4, B5, S1 and S3). The reference isolate used as a positive control was *K. marxianus* D6. Interestingly, electrophoresis visualization of isolate D1 showed two DNA bands (Figure 2). Previous studies reported that no ITS region other than ITS1 had been found to matches the ITS1 primer (Granchi *et al.*, 1999; Pham *et al.*, 2011; Fadda *et al.*, 2013). Therefore, the visualization of the PCR product obtained did not produce a double band as reported in this study. When a BLAST primer was performed on the D1 isolate sequences, no regions matched the ITS1 primer other than the ITS1 region. Therefore, BLAST primer was also carried out further on the NCBI yeast gene database to find suitable sequences other than the ITS region. The result indicated that the ITS1 primer was able to attach to the chromosome 5 sequence in the DNA of the yeast *K. marxianus* CBS6556 at the 1.170.476 bp sequence with a total length of the chromosome 5 sequence 1.391.827 bp. However, the ITS4 primer could not attach to the chromosome 5 sequence. So, if it is true that the ITS1 primer was attached to the sequence of chromosome 5, then the possible sequence length was 221.351 bp. This statement was not following the visualization obtained, which was only around 590 bp. Therefore, the D1 colony was a pure isolate but contained two different yeast species with the same morphology of cells and colonies.

The RFLP analysis in this study resulted in the same group as the amplification results. Furthermore, cleaving DNA bands using restriction enzymes of *Hind*III and *Hae*III also produced groups with the same isolate composition. The *Hind*III enzymes were able to cleave at one DNA site in group II (Figure 4 and Figure 5), while the *Hae*III enzymes were able to cleave at one to three sites in the four groups of isolates (Figure 6 and Figure 7). Thus, the restriction enzymes of *Hind*III and *Hae*III confirmed the presence of four groups of yeast species in Sumbawa mare's milk in the ITS region. However, the *Hind*III enzyme has not cut DNA in groups III and IV.

The dendrogram results did not correlate with the results of the PCR-RFLP. The PCR-RFLP results showed that isolates B3, B4, B5, S1 and S3 should be in the same group. Meanwhile, in this study, there were three groups in the dendrogram which were clustering: B3, B4, S3 for group I; B5 for group II; S1 for group III. Therefore, a possible hypothesis was that the three clusters were composed of different strains. This statement is supported by previous studies, suggesting that the same species had different morphological characteristics. The species *P. kudriavzevii* E20662 has morphological characteristics

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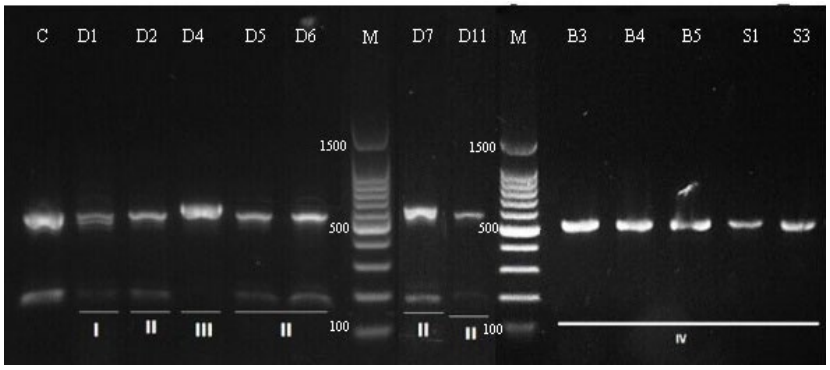

Figure 4: Gel electrophoresis of PCR-amplified ITS-region digested with *Hind*III. C: Control (*Kluyveromyces marxianus*), M: Marker.

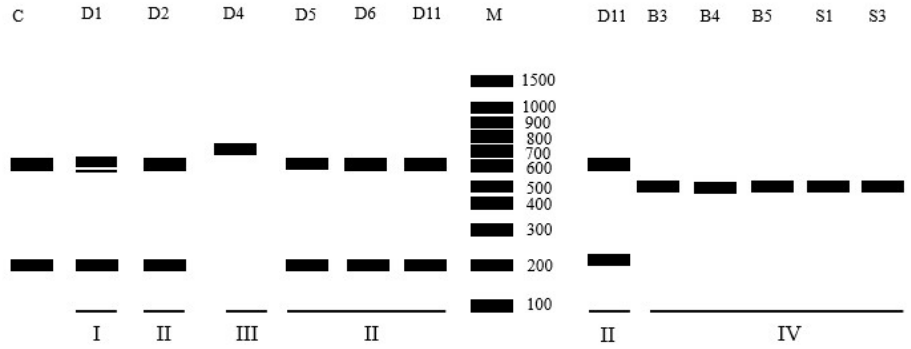

Figure 5: Representative diagram of RFLP result using *Hind*III enzyme. C: Control (*Kluyveromyces marxianus*), M: Marker.

Table 2: Identification of yeasts based on DNA sequencing of the ITS region.

Group	Number of isolates	Similarity (%)	Identification (gene sequencing)	
			Closely similar to	Accession number
I	1	99.72	*Kluyveromyces marxianus* ICMP 340	MN242725
		98.97	*Kluyveromyces marxianus* CBS:5323	KY103816
II	5	99.98	*Kluyveromyces marxianus* CBS 712	NR_111251
III	1	99.22	*Kazachstania humilis* ABM5099	HG532084
IV	5	99.8	*Pichia kudriavzevii* KGL4A	MG183700

of creamy, granular colonies with ovoid, apiculate and elongated cell shapes (Kadhim *et al.*, 2019). The species *P. kuriavzevii* Mi-1 grown on the same medium and temperature had a white, erose colony morphology, with ovoid and apiculate cell shapes (Chi *et al.*, 2015).

The yeast species in fermented Sumbawa mare's milk from Dompu (group I, II and III) were identified as *K. marxianus* D1A, *K. marxianus* D1B, *K. marxianus* D7 and *K. humilis* D4 (Table 2 and Figure 8). Meanwhile, the fermented Sumbawa mare's milk from Sumbawa and Bima had only one yeast species, namely *P. kudriavzevii* B3. *Kluyveromyces marxianus* can be found in other types of milk, including fermented goat milk in Tajikistan (Qvirist *et al.*, 2016). *Kluyveromyces marxianus* was also found in kumys (Central Asian fermented mare's milk) (Nuratzin *et al.*, 2016), koumiss (fermented mare's milk) from Mongolia (Tang *et al.*, 2020), koumiss from China (Mu *et al.*, 2012), kefir grains (Bolla *et al.,* 2011) and Pecorino di Farindola cheese from sheep in Abruzzo (Tofalo *et al.*, 2014). *Kazachstania humilis* has not been found in any fermented mare's milk before, but it has been detected in kefir grains (De Vuyst *et al.*, 2014). *Kazachstania humilis* is the most representative yeast species in the sourdough (Carbonetto *et al.*, 2020). Meanwhile, *P. kudriavzevii* has been found in curd (Rajawardana *et al.*, 2019), koumiss from China (Mu *et al.*, 2012), cheese from raw milk (Lavoie *et al.*, 2012), fermented cow's milk from West Africa (Jatmiko *et al.*, 2018), fermented cocoa (Wulan *et al.*, 2021) and Hurood cheese from Mongolia (Gao *et al.*, 2017). In a previous study, yeast species found in koumiss were identified as *Candida pararugosa*, *Dekkera anomala*, *Geotrichum* sp.,

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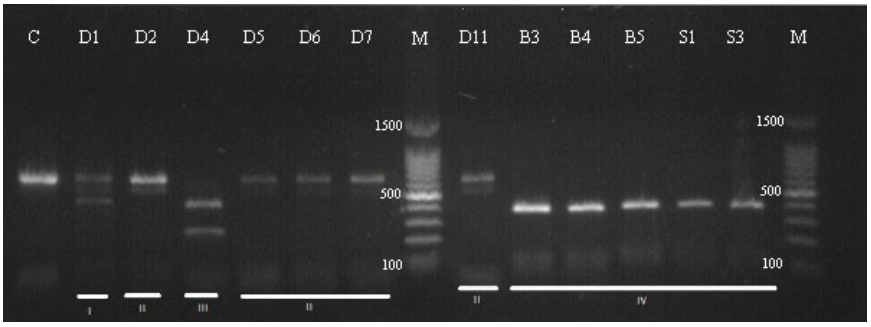

Figure 6: Gel electrophoresis of PCR-amplified ITS region digested with *Hae*III. C: Control (*Kluyveromyces marxianus*), M: Marker.

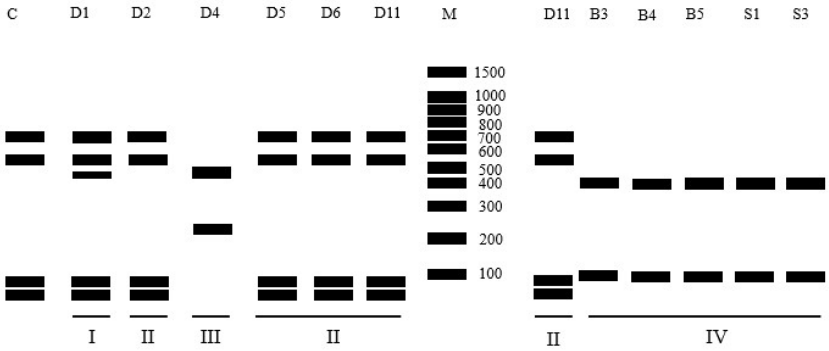

Figure 7: Representative Diagram of RFLP result using *Hae*III enzyme. C: Control (*Kluyveromyces marxianus*), M: Marker.

Issatchenkia orientalis, *Kazachstania unispora*, *K. marxianus*, *Pichia deserticola*, *Pichia fermentans*, *Pichia manshurica*, *Pichia membranaefaciens*, *S. cerevisiae* and *Torulaspora delbrueckii* (Mu *et al.*, 2012).

Referring to the RFLP ITS region analysis in the study, the RFLP profile of *K. marxianus* is not consistently similar to previous studies (Pham *et al.*, 2011; Hesham *et al.*, 2014). The same case was also reported from the RFLP profile of *K. humilis* that showed which was inconsistent with previous studies (Gori *et al.*, 2011; Iosca *et al.*, 2019). The digestion of *Hae*III in *K. marxianus* and *K. humilis* resulted in different restriction patterns. Therefore, the two species found in the study were considered different from those in the database. Meanwhile, the RFLP profile from *Hae*III digestion in *P. kuriavzevii* was consistent (Csutak *et al.*, 2012). Studies on the yeast RFLP profile using *Hind*III have never been conducted.

This study shows that geographical location affects the diversity of the yeast. The three samples came from the same Sumbawa horse species (*Equus caballus*). One of possible reasons for this difference is differences in feed nutrition and environmental conditions that affect the horse's physiology. In addition, other indirect factors include the milking and packaging of the milk specifically for each breeder and hygiene level (Zheng *et al.*, 2018).

Yeast diversity in fermented mare's milk was associated with lactic acid bacteria (LAB). The LAB in sourdough used maltose or sucrose and peptides, while yeasts metabolize glucose and amino acids. Maltose-negative yeasts have a symbiotic mutualism with maltose-positive LAB. The trophic relationship of these two species depends on nutritional mutualism. Glucose broken down from maltose by the LAB enzyme is processed by yeast glycolysis. Furthermore, yeast degrades glucofructan in sourdough, consumes glucose, and produces fructose for LAB. Then, LAB uses fructose as an electron acceptor and then reduces it to mannitol. These yeasts can tolerate high acidity, osmotic pressure and low oxygen levels (Rogalski *et al.*, 2020). In addition, yeasts can also metabolize lactic acid, a metabolite product of LAB (Jatmiko *et al.*, 2012). In previous studies, samples from Dompu contained LAB isolates, namely *L. rhamnosus* and *Lactobacillus plantarum* (positive maltose), which were associated with *K. marxianus* and *K. humilis* (negative maltose). Samples from Bima showed the presence of *L. rhamnosus* (maltose positive) associated with *P. kudriavzevii* (maltose negative) (Tripathi *et al.*, 2006; Miao *et al.*, 2008; Sutejo *et al.*, 2017; Mulyawati *et al.*, 2019; Hwang *et al.*, 2020; Choi *et al.*, 2021).

In addition, the formation of a phylogenetic tree was also performed as a comparison in Figure 8 with the addition of *C. albicans* and *S. cerevisiae* species. The results obtained indicate some differences between the dendrogram results and the phylogenetic tree. The

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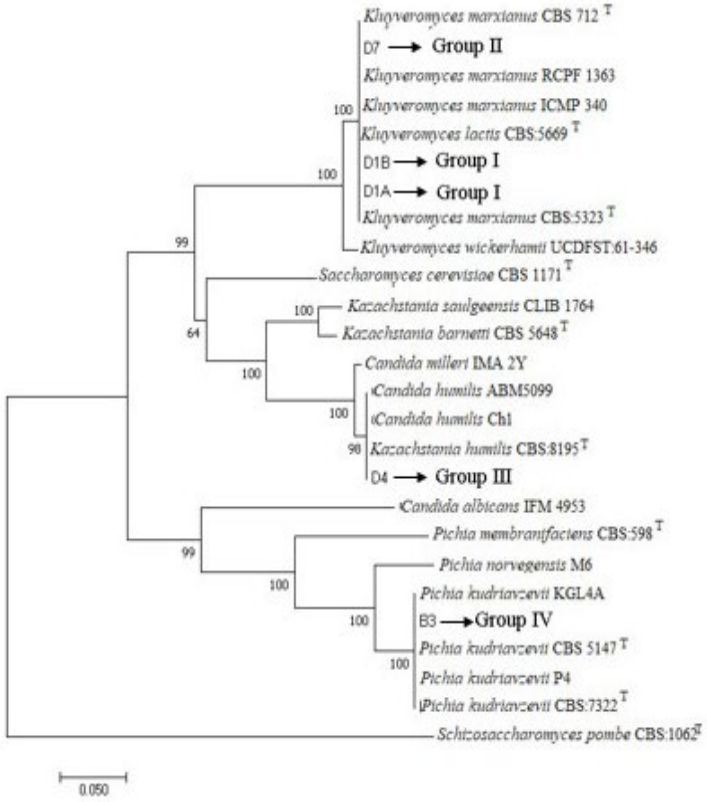

Figure 8: Phylogenetic tree of yeast isolates and reference strains based on ITS region sequences using the Neighbor-Joining method and Tamura-Nei model with 1000× bootstraps.

differences are caused by the more detailed phylogenetic tree giving the percentage of similarity. The phylogenetic tree was able to show the kinship of *S. cerevisiae* which was included in the group genus *Kluyveromyces* and *Kazachstania*. At the same time, *C. albicans* has a closer relationship with the genus *Pichia*. This difference was caused by the number of characters from the phenetic analysis (41 characters) and phylogenetic analysis (630 bp). These differences between species or strains are possibly due to other phenetic characteristics. Therefore, further biochemical tests are needed to increase the validity of the dendrogram.

It was found in this study that one colony consisted of two different strains, namely, isolate D1. Colonies are capable of growing from one or more cells. If only one cell is expected to grow, the other cells are called contaminants. The separation is generally based on colony differences using dilution. In the case of the study, the two strains in one colony had the same morphology, making it difficult to separate them (Davis, 2014).

The drawback of this study is that the yeast diversity index that dominates the Sumbawa mare's milk sample is not yet known. In addition, the biochemical tests performed were not sufficient to distinguish each species. The results of this study contribute to the Indonesian yeast database aimed to improve the quality of Sumbawa mare's milk fermented products. This improvement may be made by conducting further research on the ability and function of yeast in the milk fermentation process.

CONCLUSION

The profile of yeast diversity of fermented Sumbawa mare's milk through PCR-RFLP analysis of the ITS region obtained four groups of yeasts. The fermented Sumbawa mare's milk samples from Dompu obtained yeast species, namely *K. marxianus* D1A, *K. marxianus* D1B, *K. marxianus* D7 and *K. humilis* D4. Meanwhile, the yeast from Sumbawa mare's milk from Bima and Sumbawa was *P. kudriavzevii* B3. In addition, the phenetic analysis of yeast species showed that there were four groups different from the results of the molecular study. Therefore, further research on the potential of each isolate can be carried out on fermented Sumbawa mare's milk products, such as its antimicrobial ability to inhibit the growth of pathogens and its potency as starter cultures.

ACKNOWLEDGEMENTS

We wish to acknowledge PT. Indofood Sukses Makmur Tbk. for funding this research. We also thank Irfan Mustafa S.Si., M.Sc., Ph.D. and Dr. Suharjono, M.S., who have provided valuable suggestions for improving the preparation of the manuscript.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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