RESEARCH PAPER

BIODIVERSITY OF MARINE YEASTS ISOLATED FROM CORAL SAND IN TRUONG SA ARCHIPELAGO, KHANH HOA PROVINCE, VIETNAM

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ABSTRACT. Truong Sa archipelago of Vietnam are very diverse in microorganisms, however, compared to aquatic microorganisms (sea water, sediment, etc) terrestrial microorganisms (soil, coral sand, etc) has received little attention. This study focuses on assessing the biodiversity of marine yeasts in coral sand samples collected at some islands in Truong Sa archipelago. From nine coral sand samples collected at three islands: Song Tu island (three samples), Sinh Ton island (three samples), Truong Sa island (three samples), twenty – four strains of marine yeasts were isolated. The number of marine yeast strains isolated in Truong Sa island was the highest (ten strains). Sample CS9 had the highest number of strains. These strains were grouped into eight groups based on colony and cell morphology and fourteen groups by DNA fingerprinting. The results showed that there are strains in the same group according to morphology but belong to two different groups according to fingerprinting. Otherwise, some strains have different morphology but are grouped according to fingerprinting. The fourteen yeast strains representing groups by DNA fingerprinting were closely related to fourteen different yeast species and belong to ten yeast genera (*Yamadazyma, Candida, Trichosporon, Saccharomyces, Kodamaea, Rhodotorula, Rhodosporidium, Aureobasidium, Meyerozyma, Pichia*). Among them, the genus Candida accounted for the highest number. This is the first study on marine yeasts in coral sand in Truong Sa archipelago, Vietnam. This study can be a premise for further studies on marine yeast in different fields such as medicine, agriculture, environment, etc.

KEYWORDS: marine yeast, coral sand, DNA fingerprinting, Truong Sa archipelago

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INTRODUCTION

Truong Sa archipelago belongs to Khanh Hoa province, Vietnam, it consists of islands, reefs, banks and shoals made up of biogenic carbonate. Truong Sa archipelago are located in a tropical climate with two seasons, divided into eight island clusters. In this study, three islands with high biodiversity representing the islands of Truong Sa archipelago (in the north – south direction) are selected including: Song Tu, Sinh Ton and Truong Sa. In which, we focus on studying marine yeast diversity in the coral sand ecosystem here. The actual surveys show that the coral sand ecosystem in the Truong Sa archipelago has differences compared to the coastal sandy ecosystems such as: sand grain structure, vegetation, natural and artificial factors. Sand in Truong Sa has diverse structure, which can be in the form of fine sand grains or larger coral fragments that are typical for each island each sampling site. Vegetation here is very limited, extreme weather conditions make it very difficult for natural plants to grow (only a few species have been recorded such as: *Canavalia maritima, Ipomoea pes-caprae* and *Passiflora foetida*) instead of plants grown by humans (mainly *Heliotropium foertherianum, Scaevola taccada, Casuarina equisetifolia, Cocos nucifera*). Due to these differences, microorganisms (including marine yeast) in the coral sand ecosystem in Truong Sa also have species differences. However, their role in the ecosystem may be similar to that of yeast in the soil, including: maintenance of sand structure, contribute to essential ecological processes such as the mineralization of organic material and dissipation of carbon and energy, some yeasts may also play a role in both the nitrogen and sulphur cycles and have the ability to solubilize insoluble phosphates (Botha A. 2011).

Studies on marine microorganisms in the Truong Sa archipelago (Vietnam), including marine yeasts, are limited. Studies on marine microorganisms in the Truong Sa archipelago (Vietnam), including marine yeasts, are limited. So far, there have been only a few studies by Vietnamese scientists on marine bacteria (Do Thi Tuyen et al. 2021), on marine yeasts (Chu Thanh Bình et al. 2019). Chu Thanh Binh et al. (2019) studied yeast in some marine animals of the genus Gastropoda. The yeast identification results showed that they consisted of four genera: *Meyerozyma,*

Aureobasidium, Pichia, Candida. Marine yeasts, defined as the yeasts that are isolated from marine environments, are able to grow better on a medium prepared using seawater rather than freshwater (Chi Z.M. et al. 2010). Marine yeasts were first reported by Fischer and Brebeck (1894) from Atlantic Ocean seawater and identified them as *Torula* sp. and *Mycoderma* sp.; subsequently yeasts have been observed in all oceans of the world, ranging from nearshore environments to oceanic surface waters and deep sea sediments (Kutty and Philip 2008). Many studies also show that marine yeast has very versatile applications. These include industrial, aquaculture, medical and environmental fields (Abdelrahman S. Z. et al. 2014; Anwesha S. and Bhaskar R. 2016; Sarkar et al. 2010, Long Y. et al. 2021, Skjermo J. 1999).

There are many methods to study yeast diversity, DNA fingerprinting is the method that allows to evaluate diversity with high reliability (Sam C. et al., 2014; Christopher D. C. et al., 2014; Konstantina A. et al. 2020;). Satellite DNA usually does not code for genes and is less prone to mutations and changes. Therefore, they are often used as powerful tools in classification. Using primers designed according to the sequence of satellite DNA in PCR reaction. The amplified satellite DNA fragments are then electrophoresed for size comparison (Mauricio Ramirez-Castrillon et al. 2014; Sam C. et al. 2014). The similarity of the band spectra represents the closeness of the yeast strains. Fingerprinting technique based on satellite DNA for taxonomy to species. Strains with the same band spectrum will belong to the same species, strains with different band spectrum will probably belong to different species depending on the degree of difference. Therefore, in this study, the morphological classification method combined with the DNA fingerprinting method will provide a more complete and reliable assessment of the diversity of yeast present in coral sands in the Truong Sa archipelago area, Khanh Hoa province, Vietnam.

MATERIALS AND METHODS

Materials

Sample

Nine coral sand samples were collected on three islands in the Truong Sa archipelago (three samples per each island (Table 1).

Samples were collected at locations spread around the sandy shores of the islands (Fig. 1.). GPS location coordinates were recorded from each sampling location.

Table 1. List of samples

No.	Place	Coral sand samples code	Time	Temperature
01	Song Tu island (11°25′55′′N 114°18′00′′E)	CS1, CS2, CS3	November 2021	32°C
02	Sinh Ton island (9°53′7′′N 114°19′46′′E)	CS4, CS5, CS6	November 2021	32°C
03	Truong Sa island (8°38′30′′N 111°55′55′′E)	CS7, CS8, CS9	October 2021	33°C
Total		9		

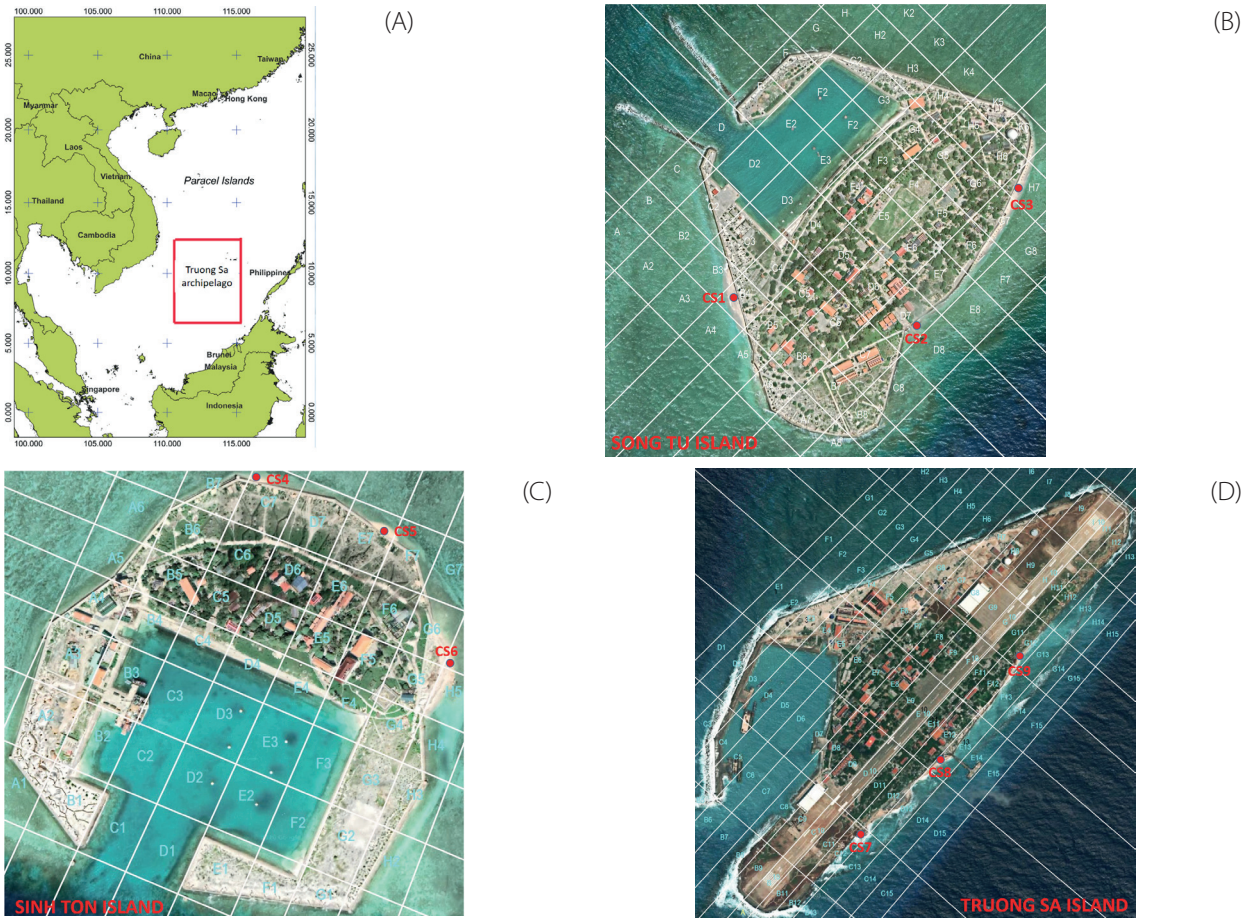

Fig. 1. (A) Map showing the location of Truong Sa archipelago in Vietnam; (B) Sampling locations at Song Tu island; (C) Sampling locations at Sinh Ton island; (D) Sampling locations at Truong Sa island

Fig. 2. Sampling in Truong Sa island (October 2021)

Media

Glucose Yeast Peptone Agar medium (GYPA medium, Glucose – 20g, Yeast extract – 5g, Peptone – 10g in 1000ml, pH 7.5 ± 0.2) with seawater. Sterilize by autoclaving at 121°C for 15 minutes. Mix well and pour into sterile Petri plates (Ausubel et al. 1994).

Methods

Sampling and physiochemical analysis: Coral sand samples were collected from the islands under conditions preventing the ingress of exogenous microbial contamination. Coral sand samples were scooped with a sterilized spoon at a depth of about 20 cm at the sandy shores of the islands. Then, the samples were stored in sterilized plastic boxes, sealed and stored at 4oC until transported to the laboratory for several weeks. The moisture and pH of the samples were measured in the laboratory using a moisture and pH meter (Tekamura DM15). The salinity of coral sand is determined by: (1) Drying 100g of sample in an oven at 105°C; (2) grind the sample; (3) Add deionized water to dissolve the sample; (4) Stir continuously with a magnetic stirrer for 5 minutes then allow to settle for 10 minutes; (5) Determine the salinity of the above solution using a hand-held refractometer.

Isolation method: The standard dilution method was used to count and isolate yeast in coral sand samples. A series of dilutions (using sterile 1.5 M NaCl solution) was prepared for each sample from the stock suspension, 1 g coral sand in 99 mL of 1.5M NaCl solution. Aliquots, 0.1 mL, from various dilutions (10–1 to 10–4) were spread on the surfaces of plates containing the GYPA medium. The plates were sealed and incubated at 30°C for a week. Three replicate plates were prepared for each dilution.

The total colony numbers were counted, and colony-forming units (CFU) per gram of sample were calculated. Three replicate plates of dilutions containing countable numbers of colonies (100–200) were pooled. Various colonies (colony shapes, sizes, colors, margins, texture, etc. and cell shapes and sizes) were sub-cultured, purified and maintained for further study (Kurtzman J.W. et al. 2011).

Grouping method: the strains were cultivated on GYPA medium, at 30°C in 5 days. Then they were differentiated by their macro and micro-morphologies according to Kurtzman J.W. et al. (Kurtzman J.W. et al. 2011)

DNA extraction: the experiment was carried out using Zymo Research Kit (USA). The genomic DNA is confirmed by gel electrophoresis by running it on 1% gel in 0.5 × TBE buffer. Gel images were observed by U-Genius 3 gel documentation system.

DNA fingerprinting: PCR reaction contained Master mix (Intron) 1x10µl, Primer MST1 (IDT) 5' – GTG GTG GTG GTG GTG – 3' 2 µl, H2O PCR (deionized water) 11 µl, DNA template 2 µl with 35 cycles. Amplification was programmed as follows: 2 min 94°C; 40s denaturation at 94°C; 60s at 52°C; 120s at 72°C; 10 min at 72°C (Mauricio Ramirez-Castrillon et al., 2014).

Amplification and sequencing of the D1-D2 region of the large-subunit RNA gene: Primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman C. P. and C. J. Robnett, 1997) were used to amplify this region. PCR was performed in a total reaction volume of 50 µl consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates (0.2 mM each), 1.2 U of *Taq* DNA polymerase, 0.4 µM (each) of the NL 1 primers and NL4 primers, 2 µl (1 to 5 ng) of DNA template, and add enough PCR water to 50 µl. PCR was carried out using the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min), and extension (72°C for 1 min); and a final extension step at 72°C for 3 min. A negative control was performed with each run by replacing the template DNA with PCR water in the PCR mixture (Shiang N.L. et al. 2006).

Identification of yeast by the D1-D2 region of the large-subunit RNA gene: A total of 14 strains were examined. Species were identified by searching databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Analysis of samples collected from Truong Sa archipelago

The results of moisture, salinity, and pH testing of coral sand samples are shown in Table 2.

Table 2. Moisture, salinity, and pH of coral sand samples

No.	Sample	Moisture	Salinity	pH
01	CS1	12,1%	9,7‰	7,78
02	CS2	10,9%	10,2‰	8,02
03	CS3	12,5%	10,2‰	7,57
04	CS4	11,7%	11,1‰	8,44
05	CS5	12,1%	11,0‰	8,12
06	CS6	11,8%	9,8‰	8,01
07	CS7	12,8%	10,0‰	7,85
08	CS8	12,3%	9,8‰	8,23
09	CS9	12,7%	9,9‰	8,25
	Average	*12,1%*	*10,2‰*	*8,03*

Isolation results

Twenty-four yeast strains were isolated from nine coral sand samples collected from Truong Sa archipelago (Table 3).

Grouping based on characteristics of colonies and cells

Colony and cells morphology showed marine yeasts of various colors (milky white, cream white, red, dark brown, black), colonies smooth or wrinkled, cells spherical or short rods, reproducing by budding. Accordingly, twenty-four yeast strains were divided into eight groups (Table 4).

DNA fingerprinting

DNA of twenty-four yeast strains was extracted and PCR with primer MST1 (primer sequences were given in the methods).

According to the results in Fig. 4, twenty-four yeast strains were divided into fourteen groups based on fingerprinting bands (Table 5).

On the basis of grouping by DNA fingerprinting, the study results are considered larger than the comparison at the island scale (Fig. 5).

Table 3. List of yeast strains

No.	Samples	Yeast strains code	Total per sample	Total per island
01	CS1	Y1	1	5
02	CS2	Y2, Y3,	2	
03	CS3	Y4, Y5	2	
04	CS4	Y6, Y7, Y8, Y9, Y10	5	9
05	CS5	Y11, Y12	2	
06	CS6	Y13, Y14	2	
07	CS7	Y15	1	10
08	CS8	Y16, Y17, Y18	3	
09	CS9	Y19, Y20, Y21, Y22, Y23, Y24	6	
Total			24	

Table 4. Grouping of yeasts by colonies and cells

Group	Strains code	Characteristic	
		Colony	Cell
01	Y1, Y2, Y3, Y6	Milky white, spongy	Spherical cells, separated, reproduce by budding
02	Y9, Y10, Y16	White, smooth	Spherical cells, separated, reproduce by budding
03	Y4, Y5, Y7	Creamy white, spongy	Spherical cells, arranged in chains
04	Y8, Y13, Y18	Red, wet, viscous	Spherical cells, reproduce by budding
05	Y11, Y12, Y22, Y23, Y24	Dark brown, wet, viscous	Rod and spherical cells, arranged in chains
06	Y14	Black, wet, viscous	Rod cells, arranged in chains
07	Y17, Y19, Y20, Y21	White, wrinkled surface	Rod cells, arranged in chains
08	Y15	Creamy white, spongy	Spherical cells, separated, reproduce by budding

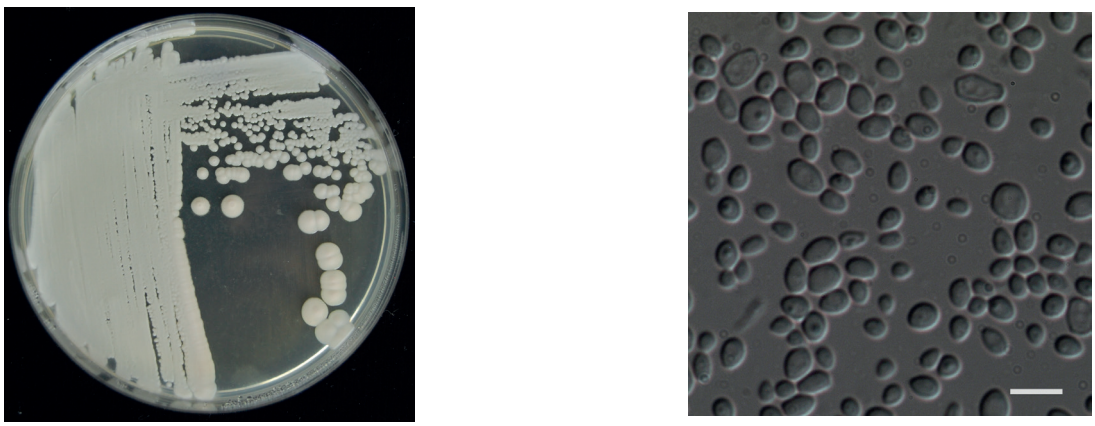

Fig. 3. Colony (left) and cell (right) of marine yeast strain Y10 (Cell images were taken under an optical microscope at 400x)

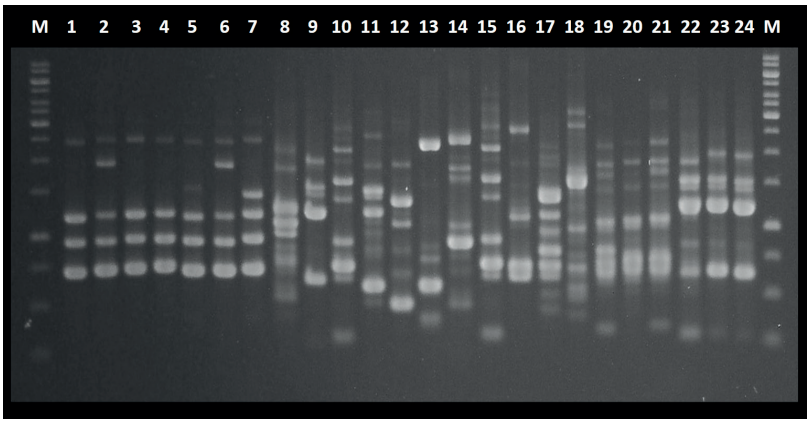

Fig. 4. Profile of fingerprinting of yeasts isolated from coral sand

Table 5. Grouping of yeasts by DNA fingerprinting

Group	Strains code	Group	Strains code
1	Y1, Y3, Y4	8	Y12
2	Y2, Y6	9	Y13
3	Y5, Y7	10	Y14
4	Y8	11	Y16
5	Y9, Y22, Y23, Y24	12	Y17
6	Y10, Y15	13	Y18
7	Y11	14	Y19, Y20, Y21

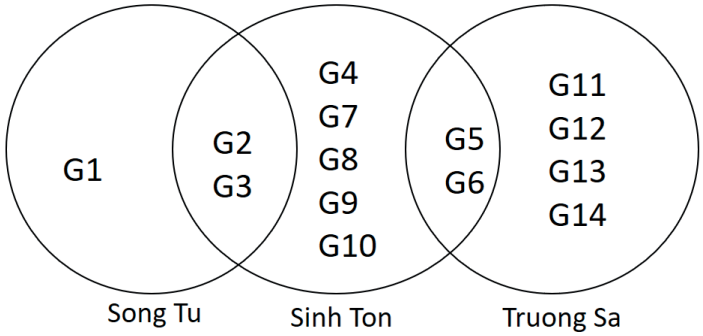

Fig. 5. Compare yeast groups among three islands

Identification of strains by the D1-D2 regions sequence analysis

Based on the results of DNA fingerprinting analysis, fourteen strains representing fourteen groups were selected for identification. BLAST search results have shown species closely related to yeast strains isolated in the study (Table 6).

DISCUSSION

Analysis of samples collected from Truong Sa archipelago

The average values of moisture, salinity, and pH of the samples serve as the basis for establishing the medium and conditions for isolating marine yeast. In this study, marine yeast strains were isolated under conditions similar to their natural habitat conditions. This makes the isolation of yeast more optimal, the assessment of yeast diversity in coral sand is more reliable. Cycil L. M. et al., when studying microorganisms in the Karak salt mine, Pakistan, also analyzed some natural elements of the samples as a basis for establishing the medium and microbial culture conditions (Cycil L. M. et al. 2020).

Isolation results

Table 3 showed that Song Tu island had the smallest number of yeasts, while yeasts in Truong Sa and Sinh Ton islands were similar. This can be partly explained by the geographical position of Song Tu island in the Truong Sa archipelago, which is located in the northern part of the archipelago, where the climatic conditions are harsh, frequent extreme weather events (strong storms, heavy rains, etc.) cause instability of ecosystems, including microorganisms. In contrast, Truong Sa Island is located in the southern part of the archipelago, with a more stable climate that makes the ecosystems here quite diverse. CS9 (in Truong Sa island) was the sample with the highest number of yeasts (six strains). However, these are only results recorded at the isolation step through differences in colony morphology. Cellular or genetic differences should be considered for higher confidence. In similar studies, marine yeasts were often isolated from seawater, sediments and animals (Corey A.H.A. and James B.M., 2019). Chutima K. et al. (2020) isolated forty yeast strains from twenty-five of the forty coral and zoanthid samples in Mu and Khram islands (Chutima K. et al. 2020). Thus, according to the results obtained from this study, the prevalence of yeast

Table 6. List of strains and species identification (% identity with sequences in GenBank)

No.	Yeast strains code	Species identification	% identity
1	Y4	Yamadazyma triangularis	99,67
2	Y2	Candida tropicalis	100
3	Y5	Kodamaea ohmeri	100
4	Y8	Rhodotorula paludigena	100
5	Y24	Trichosporon faecale	100
6	Y15	Candida oceani	99,61
7	Y11	Aureobasidium melanogenum	96
8	Y12	Meyerozyma amylolytica	99,62
9	Y13	Rhodotorula pacifica	98
10	Y14	Aureobasidium namibiae	98
11	Y16	Saccharomyces cerevisiae	100
12	Y17	Candida atlantica	99,62
13	Y18	Rhodosporidium sphaerocarpus	96
14	Y20	Pichia triangularis	99,44

in coral sand was much higher (twenty-four yeast strains in nine coral sand samples) than in marine animals in the study of Chutima Kaewkrajay et al. (forty yeast strains in forty marine animal samples). Vogel C. et al. (2007) studied yeast in the wet and dry sand of three recreational beaches in South Florida. A greater diversity of species (sixteen species) was found in the dry sand above the high tide mark compared with the wet sand in the intertidal zone (eleven species). Densities were also highest in the dry sand relative to wet sand (20-fold higher at Hobie beach, 6-fold higher at Fort Lauderdale Beach and 1.3-fold higher at Hollywood beach) (Vogel C. et al. 2007)

Yeast is a polymorphic microorganism group, characteristics of colonies (color, shape) and cells that can change in the life cycle (Pekka R. et al. 2014; Pincus D. H. et al. 2007; Roger S. et al. 2004). Therefore, it is necessary to use other classification methods (biochemical characteristics, molecular biology) to be able to more accurately assess the biodiversity of yeast.

Grouping based on characteristics of colonies and cells

According to the results of table 4, group 5, includes strains with dark brown, wet, viscous colonies rod and spherical cells, account for the most number (five strains). This is the typical morphology of the black yeast group (Seyedmojtaba S. et al. 2014; Connie F.C.G. 2018; Jerneja Z. et al. 2016). Black yeast is a group of yeasts that are highly adaptable to harsh conditions such as high salt concentration, low pH, high temperature, etc (Claudia S.K. et al. 2022; Vandewalle-Capo M. et al. 2020; Kreusch M.G. et al. 2021). Groups 6 and 8 include only one strain. With polymorphism, the classification of yeasts based on colony and cell morphology is only considered as the first step in classification. As mentioned, in order to have more reliable results, it is necessary to study biochemical and molecular characteristics. In this study, DNA fingerprinting was used to group yeasts at the molecular level.

DNA fingerprinting

From the results in Fig 4, it can be seen that the PCR product multiplied by the primer MST1 of yeast strains has different bands and is very diverse due to the distribution of copy number as well as the position of satellite DNA in the genome. The grouping technique uses satellite DNA-based primers for subspecies resolution. Strains with the same band will definitely belong to the same species, strains with different bands will probably belong to different species depending on the difference (Hilde N. et al. 2014; Dexi B. et al. 2021).

According to published studies, strains with the same bands when multiplied with primer MST1 will be similar in species name even subspecies when classified by 26S rDNA sequencing method (Baleiras Couto M.M. et al. 2005; Melissa L.I. et al. 2011). It can be said that the MST1 primer classification technique is an effective tool, giving the most accurate resolution of the electrophoresis bands. Grouping by fingerprinting makes reading sequences less expensive due to duplication.

Thus, the grouping of yeasts has been rearranged compared to the grouping by characteristics of colonies and cells. There were yeast strains with similar morphology (example Y10 and Y15) that were classified into the same group based on biological classification but had different fingerprinting bands, so they belong to two different groups according to PCR fingerprinting results. Besides, there were strains (example Y3 and Y4) with different morphology, so they were classified into two groups based on their characteristics, but the fingerprinting results showed that they could be in the same taxonomic group.
Considering yeast diversity at the molecular level, Sinh Ton island had the highest yeast diversity (nine yeast strains belonging to nine different groups). Five yeast strains isolated from Song Tu island belong to three different groups. There were groups of yeast that occur on both islands (example groups 2 and 3, groups 4 and 5) while there were groups that occur in only one island (example group 1). Song Tu island and Truong Sa island did not have the same group of yeasts. This can be explained by

geographical distance and differences in climate and soil between the two islands. Sinh Ton Island is located in the middle position compared to Truong Sa Island and Song Tu Island. The weather here is mild, with little volatility, favorable for the formation of sustainable ecosystems.

Identification of strains by the D1-D2 regions sequence analysis

The D1-D2 region gene sequence is commonly used in the identification and classification of yeasts (Shiang N. L. et al., 2006). In this study, the results of yeast identification based on the D1-D2 region gene sequences coincided with species predictions based on colony and cell morphology. The fourteen strains of yeast belonging to ten different genera include: *Yamadazyma, Candida, Trichosporon, Saccharomyces, Kodamaea, Rhodotorula, Rhodosporidium, Aureobasidium, Meyerozyma, Pichia*. In which, the genus Candida accounted for the highest number of species (3/14 species equivalent to 21.4%). Candida is a common yeast genus in marine environments because of its wide salt range and high tolerance to pH fluctuations (Jack W.F. 2012). *Rhodotorula, Rhodosporidium* and *Aureobasidium* were three genera of pigment-producing yeasts. According to many studies, the red pigment yeast group has the potential to biosynthesize many valuable biological compounds such as: squalene, exopolysaccharide, ... (Sara W. et al. 2021; Shakeri S. et. al. 2021).

Thus, the evaluation of yeast diversity by colony and cell morphology combined with DNA fingerprinting and sequencing methods has given a more complete and comprehensive view. Initial assessments have shown that yeast in coral sand in Truong Sa archipelago is diverse in both morphology and genetics. However, on the basis of this study, it is necessary to conduct biochemical studies as well as identify the isolated by other genes yeast strains to get more information about their taxonomy and phylogenetic origin. Thereby, it is possible to predict their roles and correlations in the ecosystem. In addition, this study can be a premise for further studies on marine yeast in many different fields such as medicine, agriculture, environment, etc.

CONCLUSIONS

Song Tu, Sinh Ton, Truong Sa are three representative islands of Truong Sa archipelago selected for research. Nine coral sand samples (three samples per island) were collected at different locations.

Twenty-four yeast strains isolated were morphologically diverse and grouped into eight groups based on their colony and cell morphology.

The results of grouping by DNA fingerprinting divided twenty-four yeast strains into fourteen groups.

The fourteen yeast strains representing groups by DNA fingerprinting were closely related to fourteen different yeast species and belong to ten yeast genera. Among them, the genus Candida accounted for the highest number.

Of the three islands considered in this study, Sinh Ton island has the highest yeast diversity, and Song Tu island has the least diversity. However, if we consider these 3 islands as representative of Truong Sa archipelago, we can evaluate Truong Sa as a place with high diversity of yeast in coral sand. Therefore, further research on biodiversity is needed here to give a fuller picture of the ecosystem.

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