Isolation of marine yeasts from coastal waters of northeastern Taiwan

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ABSTRACT: Twelve seawater samples were collected from the coastal marine waters of northeastern Taiwan, and 109 yeast cultures were isolated from the samples. Isolates were first classified by phenotype and then into 9 groups according to restriction fragment length polymorphism (RFLP) analysis and the sequencing of 5.8S-ITS ribosomal DNA. The results showed that *Candida tropicalis* was the most frequently recovered yeast found in the coastal waters of northeastern Taiwan. Other species found in this study included *C. glabrata*, *Saccharomyces yakushimaensis*, *Kazachstania jiainicus*, *Kodamaea ohmeri*, *Pichia anomala*, *Issatchenkia orientalis*, and *Hanseniaspora uvarum*. The biodiversity of yeast species was determined from the south to north of the northeastern coastal waters.

KEY WORDS: Marine yeasts · *Candida tropicalis* · Taiwan

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INTRODUCTION

Yeasts are primarily used by the food industry in the production of alcohol and carbon dioxide, which are important to the brewing, alcohol distillation, and baking industries (Yanagida et al. 2002). Yeasts can also be applied to various other fields, such as in the production of biofuel, biocontrol of fungi in agriculture, and other applications in biotechnology (Passoth & Schnürer 2003, Hill et al. 2006, Matsushika et al. 2008).

Yeasts are widely distributed in the terrestrial environment, including plants and soil, as well as in wine and various foods (Nisiotou & Gibson 2005, Bhadra et al. 2007, Nyanga et al. 2007, Lee et al. 2008, Liu et al. 2008, Rao et al. 2008). However, yeasts with different metabolic attributes have been reported to occur in aquatic environments, such as oceans and seas, estuaries, lakes, and rivers (Kutty & Philip 2008).

Marine yeasts are ubiquitous in the marine environment. They are frequently found in the digestive tract of marine organisms and in seawater and beach sand (van Uden & Branco 1963, Taysi & van Uden 1964, Kawakita & van Uden 1965, Fell 1967, Vogel et al. 2007, Kutty & Philip 2008). It is therefore considered that the factors affecting the distribution of marine

yeasts include currents, migration of marine organisms, and contamination from terrigenous sources (van Uden & Branco 1963, Fell 1967, Vogel et al. 2007, Kutty & Philip 2008).

Indigenous marine yeasts need to grow on or in a marine substrate. However, salinity tolerance does not distinguish marine species from terrestrial species, because some terrestrial species can grow in sodium chloride concentrations exceeding those normally present in the sea (Yamagata & Fujita 1970, Kutty & Philip 2008). Previous studies reported that marine yeasts do not belong to a specific genus or group, but are represented by a wide variety of well-known genera, such as *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Hansenula*, *Rhodotorula*, *Saccharomyces*, *Trichosporon*, and *Torulopsis* (Kutty & Philip 2008).

Recently, various molecular techniques have been applied to discriminate or identify yeast species. Restriction fragment length polymorphism (RFLP) analysis is a simple method of comparing the molecular profiles of DNA sequences that provides information on composition without analyzing the DNA sequences. RFLP and sequence analysis of the 5.8S rDNA gene and its flanking internal transcribed spacer regions, collectively called 5.8S-ITS, has been proposed for yeast

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identification purposes due to its high discriminative capacity, relative ease of manipulation, and high reproducibility (Nisiotou & Gibson 2005). Strains isolated in this study were therefore classified and identified using this method.

The distribution of marine yeasts along the coastal areas of northeastern Taiwan have not yet been studied. The purpose of this study was therefore to identify and enumerate the yeast species along the coastal areas of northeastern Taiwan. Based on our results, a hypothetical pathway of microbial spatial transportation is presented, and further information is provided to gain insight into the distribution of yeasts in this area.

MATERIALS AND METHODS

Sampling. Seawater samples were collected from 12 stations, from south to north (Stns 1 to 12), each 8 to 15 km apart, located along 122 km of coastline in northeastern Taiwan (Fig. 1). On 7 January 2008, a 2 l seawater sample (from 10 to 15 cm depth) was collected at each station by walking along the shoreline. Each sample was collected in a sterilized bottle, which was rinsed twice with local seawater before use. Samples were analyzed within 5 h of acquisition at these stations. In addition to isolating yeasts, the salt concentration and pH of the seawater were measured. To measure the salt concentration, the seawater was placed onto the sensor of a model SK-5S salt meter (Sato Keiryoki). The pH was measured by placing seawater directly onto the sensor of a model B-112 compact pH meter (Horiba).

Isolation procedure. Isolation was performed using the methods described by Kodama (1999). Seawater samples were filtered with sterilized 0.45 µm mixed cellulose ester membrane filters (ADVANTEC). After filtration, the membrane filters were laid on isolation medium (IM) plates (Table 1) and incubated at 25°C for 10 d. Yeast colonies were selected from IM plates and purified by replating on IM agar plates. Colonies were reselected and initially stained with methylene blue to check their shape. Only cultures with a single shape were selected. The selected strains were stored at –80°C in YM-DMSO medium (Table 1).

Classification and identification. Yeast cells were grown aerobically in YM medium (Table 1) at 25°C. Total genomic DNA was extracted and purified from 5 ml cultures using a DNA/RNA extraction kit (Viogene). Polymerase chain reaction (PCR) was carried out using a Takara Ex Taq gene amplification

Fig. 1. Sampling area (slanted lines) with locations of sampling Stations 1–12

PCR kit and performed on a Gene Amp PCR System 9700 (PerkinElmer) following the methods described by Nisiotou & Gibson (2005). The 5.8S-ITS rDNA region was amplified using the primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Nisiotou & Gibson 2005). RFLP analysis of 5.8S-ITS rDNA was carried out with the methods described by Osorio-Cadavid et al. (2008). In this study, 2 restriction enzymes, *HinfI* (G/ANTC) and *HaeIII* (GG/CC) (Osorio-Cadavid et al. 2008), were used for grouping.

For sequence analysis of 5.8S-ITS rDNA, the PCR products were purified with a Clean/Gel Extraction Kit (BioKit) and then directly sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems). Sequence homologies were examined by comparing the obtained sequences to those in the DNA Data Bank of Japan (DDBJ) (www.ddbj.nig.ac.jp/) using BLAST.

Table 1. Media used

Isolation medium		YM-DMSO medium		YM medium	
Sucrose	20 %	Dimethyl sulfoxide	10 %	Polypeptone	0.5 %
Polypeptone	3 %	Polypeptone	0.5 %	Yeast extract	0.3 %
Yeast extract	3 %	Yeast extract	0.3 %	Malt extract	0.3 %
Chloramphenicol	100 ppm	Malt extract	0.3 %	D-glucose	1 %
Agar	1.5 %	Deionized water		Deionized water	
Filtered seawater					
pH	5.6				

Table 2. Identification of marine yeasts from seawater collected in east Taiwan. Stn: Sampling station; *P.*: *Pichia*; *C.*: *Candida*; *K.*: *Kodamaea*; *H.*: *Hanseniaspora*; *S.*: *Saccharomyces*; *Kaz.*: *Kazachstania*; *T.*: *Torulaspora*; *I.*: *Issatchenkia*. –: no culture observed

Stn	Salt Cont. (%)	pH	Strain No.	Species	Stn	Salt Cont. (%)	pH	Strain No.	Species	Stn	Salt Cont. (%)	pH	Strain No.	Species
1	2.9	8.2	TL0101	<i>P. anomala</i>	4	3.1	8.2	TL3001	<i>P. anomala</i>	8	3.2	8.2	NT1102	<i>T. delbrueckii</i>
			TL0201	<i>P. anomala</i>				TL3002	<i>P. anomala</i>				NT1103	<i>C. tropicalis</i>
			TL0202	<i>P. anomala</i>				TL3003	<i>P. anomala</i>				NT1104	<i>C. tropicalis</i>
			TL0301	<i>C. tropicalis</i>				TL3004	<i>P. anomala</i>				NT1105	<i>C. tropicalis</i>
			TL0302	<i>C. tropicalis</i>	5	2.6	8.2	TL1901	<i>Kaz. Jaienicus</i>	9	3.4	8.1	NT0201	<i>C. tropicalis</i>
			TL0401	<i>C. tropicalis</i>				TL1903	<i>Kaz. Jaienicus</i>				NT0202	<i>C. tropicalis</i>
			TL0501	<i>C. tropicalis</i>				TL1501	<i>C. tropicalis</i>				NT0203	<i>C. glabrata</i>
			TL0502	<i>C. tropicalis</i>				TL1502	<i>C. tropicalis</i>				NT0204	<i>C. glabrata</i>
			TL0601	<i>C. tropicalis</i>	6	3.2	7.8	TL1503	<i>C. tropicalis</i>	10	3.0	8.2	NT0301	<i>C. glabrata</i>
			TL0701	<i>K. ohmeri</i>				TL1601	<i>C. tropicalis</i>				NT0401	<i>C. tropicalis</i>
			TL0702	<i>K. ohmeri</i>				TL1602	<i>C. tropicalis</i>				NT0402	<i>I. orientalis</i>
			TL0801	<i>K. ohmeri</i>				TL1603	<i>C. tropicalis</i>				NT0403	<i>I. orientalis</i>
			TL0802	<i>K. ohmeri</i>	7	2.9	8.2	TL1604	<i>C. tropicalis</i>	11	3.6	8.2	NT0405	<i>I. orientalis</i>
			TL0803	<i>K. ohmeri</i>				TL1701	<i>C. tropicalis</i>				NT0406	<i>I. orientalis</i>
			TL0901	<i>P. anomala</i>				TL1702	<i>C. tropicalis</i>				NT0407	<i>I. orientalis</i>
			TL0902	<i>P. anomala</i>				P11	<i>P. anomala</i>				NT1401	<i>C. tropicalis</i>
			TL0903	<i>P. anomala</i>	12	2.6	8.3	P12	<i>P. anomala</i>	12	2.6	8.3	NT1403	<i>C. tropicalis</i>
			TL1001	<i>P. anomala</i>				P31	<i>C. tropicalis</i>				NT1404	<i>I. orientalis</i>
			TL1002	<i>P. anomala</i>				P32	<i>C. tropicalis</i>				NT1405	<i>C. tropicalis</i>
			TL1003	<i>P. anomala</i>				P33	<i>C. tropicalis</i>				NT1406	<i>C. tropicalis</i>
2	3.0	8.2	TL1101	<i>P. anomala</i>	7	2.9	8.2	P34	<i>C. tropicalis</i>	11	3.6	8.2	NT1407	<i>C. glabrata</i>
			TL1201	<i>P. anomala</i>				P35	<i>C. tropicalis</i>				NT1408	<i>C. tropicalis</i>
			TL2701	<i>H. uvarum</i>				NT1106	<i>C. tropicalis</i>				NT1409	<i>C. tropicalis</i>
			TL2702	<i>H. uvarum</i>				NT1107	<i>C. tropicalis</i>				NT1410	<i>C. tropicalis</i>
			TL0602	<i>C. tropicalis</i>				NT1108	<i>C. tropicalis</i>				–	–
			TL2801	<i>S. yakushimaensis</i>				NT1109	<i>C. glabrata</i>				NT0501	<i>I. orientalis</i>
3	3.0	8.3	TL2502	<i>S. yakushimaensis</i>	7	2.9	8.2	NT1110	<i>C. tropicalis</i>	12	2.6	8.3	NT0502	<i>I. orientalis</i>
			TL2503	<i>S. yakushimaensis</i>				NT1111	<i>C. glabrata</i>				NT0503	<i>I. orientalis</i>
			TL2504	<i>S. yakushimaensis</i>				NT0101	<i>C. tropicalis</i>				NT0504	<i>I. orientalis</i>
			TL1301	<i>P. anomala</i>				NT0102	<i>C. tropicalis</i>				NT0505	<i>C. tropicalis</i>
			TL1401	<i>P. anomala</i>				NT0103	<i>H. uvarum</i>				NT0506	<i>C. glabrata</i>
			TL1403	<i>P. anomala</i>				NT0104	<i>C. tropicalis</i>				NT0507	<i>I. orientalis</i>
			TL1801	<i>P. anomala</i>				NT0105	<i>C. glabrata</i>				NT0508	<i>C. glabrata</i>
			TL1802	<i>P. anomala</i>				NT0106	<i>C. glabrata</i>				NT1501	<i>I. orientalis</i>
			TL1803	<i>P. anomala</i>				NT0107	<i>C. tropicalis</i>				NT1503	<i>I. orientalis</i>
			TL1804	<i>P. anomala</i>				NT0108	<i>C. tropicalis</i>				–	–
			TL2901	<i>S. yakushimaensis</i>				NT1101	<i>C. tropicalis</i>

Fig. 2. 5.8S-ITS rDNA RFLP patterns of *Hae*III and *Hin*FI digests from Groups A to I. Lane M, size marker; H, *Hae*III-digested patterns; F, *Hin*FI-digested patterns

The evolutionary distance (Knuc value) and similarity values of representative strains of each group were generated using the software CLUSTAL W (<http://clustalw.ddbj.nig.ac.jp/top-j.html>), and a phylogenetic tree was constructed by the neighbor-joining method with the TreeView program (v.1.66).

RESULTS

Analyses of seawater samples revealed salt concentrations from 2.5 to 3.6 ‰ and pH values from 8.1 to 8.3 (Table 2). The high salinity and moderately alkaline conditions indicated that the collected waters rarely mixed with riverine water.

In total, 109 cultures were isolated from the seawater samples. The number of isolates from each site is shown in Table 2. The 109 isolates were classified into 9 groups (A to I) based on cell morphology, size of the 5.8S-ITS PCR products, and the results of 5.8S-ITS rDNA RFLP analysis (Fig. 2, Table 3).

To identify the isolates, representative strains were randomly selected from each group, and 5.8S-ITS

rDNA sequencing analysis was carried out. The results identified Group A isolates as *Candida tropicalis*, Group B as *Pichia anomala*, Group C as *Issatchenkia orientalis*, Group D as *C. glabrata*, Group E as *Kodamaea ohmeri*, Group F as *Saccharomyces yakushimaensis*, Group G as *Hanseniaspora uvarum*, Group H as *Kazachstania jiainicus*, and Group I as *Torulaspora delbrueckii* (Table 3). The sequences determined in this study have been deposited in the DDBJ database under accession numbers AB467289 to AB467306, and AB469378 to AB469381. The evolutionary distance of marine yeasts was analyzed phylogenetically, as shown in Fig. 3.

Yeast cultures were isolated from the seawater samples of all 12 sampling stations, except for Stn 11, with differences in abundance and diversity evident among the stations (Table 2). For samples at Stns 1, 7, and 10, a comparatively greater number of yeast colonies was observed and isolated after incubation (Table 2).

The most frequently recovered species, *Candida tropicalis* and *C. glabrata*, were found in 7 and 5 of the 12 stations, respectively. *Saccharomyces yakushimaensis* was only found at Stns 3 and 4, while *Issatchenkia orientalis* was only found at Stns 10 and 12. *Pichia anomala* was not found at the northern sampling stations (7 to 12). Species such as *Torulaspora delbrueckii* and *Kazachstania jiainicus* were only observed at specific stations (Table 2).

Table 3. Classification of 109 marine yeasts found in coastal waters of northeastern Taiwan

Group	Species	Number
A	<i>Candida tropicalis</i>	45
B	<i>Pichia anomala</i>	25
C	<i>Issatchenkia orientalis</i>	13
D	<i>C. glabrata</i>	10
E	<i>Saccharomyces yakushimaensis</i>	5
F	<i>Kodamaea ohmeri</i>	5
G	<i>Hanseniaspora uvarum</i>	3
H	<i>Kazachstania jiainicus</i>	2
I	<i>Torulaspora delbrueckii</i>	1
	Total	109

DISCUSSION

Relatively few studies have investigated marine yeasts, and this group of Mycota is still poorly understood (Kutty & Philip 2008). To the best of our knowledge, this is the first report on the distribution of marine yeasts in waters off the coast of northeastern Taiwan. As shown in Table 2, the number of yeast cul-

Fig. 3. Phylogenetic relationships of representative strains from each group. The tree was constructed using the neighbor-joining method. Scale bar: 0.1 nucleotide substitution per site

tures recovered from each station differed. These differences might be due to geographical, oceanological, or biological factors. More analyses are necessary to clarify this point.

Several decades ago, marine-occurring yeasts were isolated from estuarine and coastal sediments in western Taiwan (Cheng & Lin 1977). The genera of yeasts classified included *Saccharomyces*, *Torulopsis*, *Debaryomyces*, *Endomycopsis*, *Pichia*, *Kloeckera*, and *Rhodotorula*. However, many rivers converge into the continental shelf sea of the Taiwan Strait between the densely populated areas of western Taiwan and northeastern China. Therefore, microbiological contamination may have occurred at the near-shore areas of western Taiwan, rather than from the Pacific Ocean off eastern Taiwan.

The genera *Saccharomyces* and *Pichia* have been found in both western and northeastern coastal waters of Taiwan (Tien & Wang 2004, present study). Only *Candida*, the most frequently recovered genus in this study, was isolated from seawater along the east coast.

In the present study, *Candida tropicalis* was the most frequently isolated yeast species found in the coastal waters of northeastern Taiwan. Species such as *C. tropicalis* and *Rhodotorula rubra* have been found to

occur widely in the marine environment. *C. tropicalis* has been found in the Indian Ocean waters and in the intestines of marine animals distributed in the Pacific and Atlantic Oceans (Kutty & Philip 2008, Wang et al. 2008). In addition, *C. tropicalis* was among the most frequently isolated yeasts found in beach sand at 3 bathing beaches in South Florida (Vogel et al. 2007) and in bivalve shellfish from Long Island Sound, USA (Buck et al. 1977). Based on such studies, *C. tropicalis* can therefore be considered as one of the yeast species that is widely distributed in the marine environment.

Fell (1967) reported that the Somali Current had a significant effect on yeast abundance. In our study, the Kuroshio Current is a strong western boundary current that originates east of the Philippines, flows northward off eastern Taiwan, and then turns northeastward off Taiwan and past Japan via the Okinawa Trough (Liu et al. 2000). The current is therefore considered one of the forces that controls the distribution of yeasts. For example, some species found in this study showed interesting connections to the location where their type strains were found in previous studies. A novel yeast species, *Kazachstania jiainicus*, was isolated from forest soil in Jain, Hualein (Lee et al. 2008), which is far from our sampling stations along the eastern coast

of Taiwan. *Saccharomyces yakushimaensis* is another interesting case; it was initially isolated from soil on Yaku Island off southern Kyushu in Japan (Mikata et al. 2001). The Kuroshio Current flows northward off the east coast of Taiwan and reaches Yaku Island 1100 km away (Noda et al. 1998, Liu et al. 2000). Therefore, we consider that marine yeasts could be shifted by the Kuroshio Current and drift in aerosols with sea wind.

As previously described, yeasts were also frequently isolated from marine organisms (Kawakita & van Uden 1965, Fell 1967, Buck et al. 1977, Kutty & Philip 2008). Marine organisms are therefore suggested as another mechanism for controlling the distribution of yeasts. However, more evidence is needed to support this hypothesis.

In conclusion, our results provide information on the distribution and taxonomy of marine yeasts in the coastal waters of northeastern Taiwan. The results indicate that *Candida tropicalis* was the most frequently recovered yeast collected from the sampling stations. Future work will focus on investigating characteristics of these isolates, such as salt tolerance, alcohol production ability and carbohydrate fermentation, and their applications to food fermentation and other industries.

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