DNA barcoding analysis of more than 1000 marine yeast isolates reveals previously unrecorded species

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Abstract

Marine ecosystems are least explored niches for yeast population and diversity. The present study aims to construct a comprehensive DNA barcode library for marine derived yeast species. As, we sequenced ITS gene for 1017 isolates belonging to 157 marine derived yeast species in 55 genera, 28 families, 14 orders, 8 classes of 2 Phyla (*viz*., Ascomycota and Basidiomycota) among which 13 yeast species were barcoded for the first time, we witnessed yeast species of both terrestrial and endemic marine origin. Difficulties were faced in taxonomic sequence validation due to large volume of sequencing trace files, variable length of sequences extracted and lack of reference sequences in public databases. Majority of the sequences (62.24%) were between 600 and 649 bps in length. K2P intra-species distance analysis conducted for selective groups yielded the average of 0.33% which was well within the previously proposed barcode gap value for yeast species (1.59%). ITS gene tree based identification conducted for selective species in Ascomycota and Basidomycota, precisely clustered the same species in single groups. About 60% of the yeast species recorded in the present study was previously unrecorded from the marine environment, among which 16.5% of yeast species were recognised human pathogens. Apart from releasing the barcode data in GenBank, provisions were made to access the entire dataset along with meta-data in the Barcode of life database under the project title "DNA barcoding marine yeast". The present study constitutes the biggest dataset for the collection of marine yeast isolates and its DNA barcodes till date.

Key words: Marine yeast, DNA barcoding, DNA barcode gap, yeast pathogens,

1. Introduction

Fungi in terrestrial and aquatic ecosystems were well studied (Gulis et al., 2009; Raja et al., 2018) compared to marine ecosystems. Yeast are fungi that do not form sexual states upon or within a fruiting body, whose growth predominantly results from fission or budding (Kutzman and Fell, 2015). Yeast are polyphyletic group (Kutty and Philip, 2008) whose unique unicellular growth distinguishes from filamentous fungi. Yeast that required seawater for its growth were defined as marine yeast (Chi, 2012). Various significant marine ecosystem processes such as nutrient cycling, plant material decomposition and marine animal parasitism has been implicated by marine yeast (Jones and Pang, 2012) which are facultative or obligate in marine ecosystems (Jones et al., 2013). Marine yeast can be

parasitic, mutualistic or saprophytic and hence can found associated with various marine invertebrates including crabs, clams, mussels, prawns, oysters or other substrates (de Araujo et al., 1995; Kosawa da Costa et al., 1991; Pagnocca et al., 1989). Usually the yeast species isolated from the surrounding ecosystems are also the one which are most frequently isolated from the organisms occurring in it (de Araujo et al., 1995).

Marine yeast are diverse and largely classified within two major phyla viz., Ascomycota and Basidiomycota (Kurtzman 2011; Boekhout et al. 2011). Majority of marine derived Ascomycetes have terrestrial origin with wide spread phylogenetic diversity (Fell, 2012). Various difficulties existed in nomenclature and taxonomy of marine yeast since its first isolation (Fischer and Brebeck, 1894) recently overcame using molecular taxonomy (Fell, 2012). DNA barcoding have streamlined biological species identification using through sequencing and analysis of short DNA fragment recognised as DNA barcodes for that specific biological group (Hebert et al., 2003). Easy identification of biological species by non-experts for the progress of biological and medical research was the one of the vision behind DNA barcoding. Although the species concept was not generally applicable (Wheeler and Meier, 2000), especially for not obligatory sexually reproducing species such as fungi, species identification is a key step for multitude fields of biology such as ecology, agriculture, biotechnology and medicine to describe the biological interactions with respect to, example; biodiversity assessment, bioremediation and pathology (de Queiroz, 2007).

Internal transcribed spacer (ITS) gene was recognised DNA barcode gene that successfully delineates fungal species (Schoch et al., 2012; Velmurugan et al., 2013; Vu et al., 2019), ITS works even better for yeast species discrimination than filamentous fungi (Vu et al., 2016). Lack of validated data is found to be a setback for yeast research (Bidartondo, 2008). The CBS-KNAW microbial biological resource centre (www.cbs.knaw.nl) contains largest number of validated yeast species collected from all types of natural and human (medical strains) environments which was represented in the large dataset of yeast DNA barcodes recently published (Vu et al., 2016). In the present study we explored marine ecosystems such as mangrove swamps and sediments of continental shelves in northern parts of Indian Ocean for marine yeast diversity using DNA barcodes. We aim to publish sizable amount of marine derived yeast DNA barcodes which compared with taxonomically validated database's DNA barcodes and publish them in the public databases such as GenBank and BOLD. Besides estimating the range of yeast species coverage in such databases, which intent to find yeast species that were not DNA sequenced yet. Our expectation was that even after the generation of large barcode dataset (8669 barcodes for

1351 yeast species) for yeast species (Vu et al., 2016), marine ecosystems could still incubate many specie yet to be barcoded. Objective of the study was to collect as many as marine derive yeast culture as possible for the construction of a comprehensive DNA barcode library synthesis. Studies are rare in exploring large scale marine ecosystems for culturable yeast species.

2. Materials and methods

2.1. Study area and sample collection

Sediment samples were extensively collected between Nov, 2008 and Jan 2013 from two different ecosystems, viz., 1) inter-tidal sediments flats under mangrove trees along the Indian coastline, 2) continental shelf off southeast coast of India.

Inter-tidal sediments under mangrove trees were collected along the Indian coastline from various mangrove environments stretched from Gujarat Kachchh Mangroves in West coast of India to West Bengal Sundraban ecosystem in East coast of India. The efforts involved sampling of 14 mangrove station, named as M1 through M14. Details of year of collection, geographical coordinates, and number of samplings per station are given in the **table S1.** Mangrove sediments were collected mostly at low tide during pre-monsoon seasons. About 50 to 100g of undisturbed sediments were sampled using sterile spatula (in triplicates), transferred to sterile polythene bags (Nasco bags, HiMedia), stored in ice and transported to laboratory. Samples were kept in ice until processed, usually within 48hrs. Mangrove sediments (n=52) derived from 13 mangrove species viz., *Avicennia* sp., *A. ilicifolius, A. alba, A. marina, A. officinalis, Bruguiera cylindrical, Ceriops decandra, Excoecaria agallocha, Pongamia pinnata, Rhizophora mucronata, Sonneratia apectala, S. caseolaris* and *Xylocarpus mekongensis* along 14 sampling stations (named as M1 –M14) (table S1). Sediment pore water salinity was measured through hand-held refractometer (BEXCO, USA). Highest of 35ppt salinity was measured in Parangipettai and Pitchavaram mangroves (M9 and M10, respectively) whereas the station M14; upstream mangroves infringe inland of Sundarbans, West Bangal, where the lowest salinity of 8ppt was recorded.

Sediment samples along Southeast continental self of India were collected during cruise no. 260 of Fishery and Ocean Research Vessel (FORV) Sagar Sampada conducted from 06th to 28th December 2008 using Smith–McIntyre grab which covers an area of 0.2m^2 (Lyla et a., 2012). Sediment were sub-sampled trice from the grab using sterile spatula as described above. Along the Southeast continental shelf, sampling station were fixed along the depth gradients around 30m, 50m, 100m, 150m and 200m at each stations, constituting total

of 32 sampling stations, named from CS1 through CS32 **(Table S1).** The depths of sediment sampling was measured using multi-beam echo sounder (capable of measuring up to 1000m depth) in-built with FORV-Sagar Sampada. Total of 96 sediment samples were collected from 32 sampling sites**.** Salinity of all continental shelf sediments varied between 33 and 35ppt. In all collection sites, ambient seawater (AS) was collected for media preparations. AS was 0.22µl filtered and autoclaved, before using it for media preparations.

2.2.Isolation of yeast-like cells

Two types of enrichment media was used. Either one or both (for most sampling stations sampled after 2010) of the enrichment media were used for yeast cell enrichment. Briefly, after homogenising the sediment samples within the collection bag, yeast cells were enriched by adding one gram of sediments to 100ml of Yeast extract/Malt extract (YM) broth (3 g malt extract, 3 g yeast extract, 10 g dextrose, and 5 g peptone, in 1 L AS) and/or 2) GPY (2% glucose, 1% peptone, 0.5% yeast extract in 100ml AS) supplemented with an antibiotic cocktails (300 mg L^{-1} penicillin, 300 mg L^{-1} streptomycin, and 250 mg L^{-1} sodium propionate) and/or 0.02% of chloramphenicol to inhibit bacterial growth. For sediments sampled after 2010, we used both enrichment media to increase yeast diversity in isolation. The enrichment broth with sediment samples in a 250 mL Erlenmeyer flasks were shaken on a rotary shaker at 150 rpm, incubated at 17-20 ºC (higher temperature was found to increase filamentous fungal growth later in agar plates) for 2-3 days. Autoclaved AS was used as inoculum in the control plates. Following incubation, 100µl to 1000µl (based on the turbidity of the broth) was spread over YM and/or GYP agar plates (composition as mentioned above with addition of 1.5% agar) in triplicates from corresponding broth cultures. The remaining broth was conserved at 4ºC with the over lay of mineral oil for future use, like in case no colonies or filamentous fungi over grows the incubated plates. The plates were incubated for 10-20 days or until colonies appears and were monitored every 24 hours. Prolonged incubation period with concurrent removal of fast growing yeast-like colonies were adopted to facilitate the maximum recovery yeast-like species including slow growing colonies. Care was also taken to stop the incubation when the probability of mould over growth was higher.

Microscopic analysis of yeast-like colonies was started from the minimum of 5 days of incubation by methylene blue staining (analysed for single type of shape and to ensure no bacterial cells were associated) and purified by streaking on to fresh agar (YM or GYP) plates (to avoid over growth on other colonies or to save from rapidly growing moulds). GYP media was autoclaved twice (at 100ºC for 30min) during two consecutive days to reduce

mould contaminations (Gadanho and Sampaio, 2005). Gradually one representative morphotype of each different colony per sample (i.e., when the yeast colonies were <50 numbers or when the mould was completely absent or scarce in the agar plates) were twice streaked on to corresponding enrichment agar plates for purification. In other cases (when yeast colonies were >50 or dense mould growth in the agar plates), representative number of colonies (40-70 colonies) were randomly selected and purified twice.

For continental shelf sediments, enrichments cultures as described above was done on the ship board Microbiology laboratory, FORV-Sagar Sampada. After 2 sub-culturing of yeast-like cells, the isolates were grown in 1mL of GPY broth (in duplicates) in 2mL microcentrifuge tubes (in duplicates) in the shaker (120 rpm) for 2-3days. The cultures in one tube was used for DNA isolation and other was cryopreserved (culture volume increased to 1.5ml using 2% GPY broth, 10% glycerine) in marine microbial culture collection centre, Centre of Advanced Study in Marine Biology, Annamalai University. There was total of 1398 colonies (916 colonies from mangrove sediments and 482 colonies from continental shelf sediments) for molecular analysis. Each purified cultures were numbered under DBMY (DNA Barcoding Marine Yeast) acronym. We also tried to cluster the colonies based on macro- and micro-morphological features as described by Yarrow (1998) and Kurtzman et al. (2010).

2.3.DNA extraction, polymerase chain reaction and DNA sequencing

Yeast like cells grown in the 2mL microcentrifuge was centrifuged at 8000 X g for five minutes to recover the cells. DNA was isolated from the pellets using GeneiPure Yeast DNA preparation kit (GeNei, India) or DNeasy blood and tissue kit (Qiagen, India) following manufacturer's instruction. Eluted DNA in the elution buffer was stored at -20ºC. In PCR, the Internal Transcribed Spacer (ITS) gene amplified includes partial sequences of 18S ribosomal RNA gene; complete sequences of internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2 and partial sequences of 28S ribosomal RNA gene. The ITS primers; ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) (White et al., 1990) was used for amplification. PCR were run on a Thermal cycler 130045GB (GeNei, India) with following conditions; 4 min at 94 ºC, followed by 30 cycles of 30 s at 94 ºC, 40 s at 48 ºC for annealing and 90 s at 72 ºC, with a final extension at 72 ºC for 7 minutes. PCR amplicon were separated by 1.5% agarose gel electrophoresis. Amplicons were two way sequenced using commercial sequencing services of Macrogen (South Korea) or Bioserve Biotachnologies Pvt. Ltd. (India).

2.4.DNA sequence analysis

Forward and reverse sequences were assembled using BioEdit ver. 5.0 (Hall, 1999). Sequences were aligned using CLUSTAL X (Larkin et al., 2007) and were adjusted manually in MEGA X (Kumar et al., 2018). DNA sequences were compared with GenBank sequences using BLAST algorithm (Altschul et al., 1997) and species threshold cut off of 98.41% (Vu et al., 2016) was used for yeast species delamination. The species that were barcoded for the first time (i.e., when species threshold is <98%) was identified using search similarity values of BLAST analysis and by individually searching GenBank for ITS sequences of respective taxa.

The Neighbor-Joining method (Saitou and Nei, 1987) was used for tree based identification using the reference sequences extracted from GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) (Felsenstein, 1985) were indicated as circles next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated (complete deletion option). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).The NJ trees were visualized in interactive Tree of Life (iTOL) database (Letunic and Bork, 2019).

DNA sequences produced from the present study was made available through GenBank KJ706221-KJ707237. Entire dataset produced in the present study could be accessed through Barcode of life database under the project title “DNA barcoding marine yeast” containing unique tag, “DBMY” or through a digital object identifier; http://dx.doi.org/10.5883/DS-MYIC .

3. Results and discussion

3.1.Composition of marine yeast species revealed through BLAST analysis

Out of 1398 isolates selected for molecular analysis, 1017 (72.74%) were successfully sequenced. The BLAST analysis reveals that ITS gene sequences belonged to 157 marine derived yeast species, 55 genera, 28 families, 14 orders, 8 classes (viz., Agaricostilbomycetess, Cystobasidiomycetes, Dothideomycetes, Exobasidiomycetes, Microbotryomycetes, Saccharomycetes, Tremellomycetes, Ustilaginomycetes) in 2 Phyla

(*viz*., Ascomycota and Basidiomycota). Two species (*viz*., *Curvibasidium cygneicollum* (J.P. Sampaio, 2004), and *Hasegawazyma lactosa* (T. Hasegawa, 1959)) cannot be classified in to any defined families, hence were placed under *incertae sedis*. Among total number of sequences (n=1017) produced, 52.01% (n=529) of sequences were contributed by Ascomycota and remaining 47.99% (n=488) sequences were contributed by Basidiomycota.

From each yeast species, minimum for 6 isolates were sequenced. Maximum of 19 sequences were produced from isolates of *Scheffersomyces ergatensis* (Santa María, 1971), *Naganishia vishniacii* (Vishniac & Hempfling, 1979) and *Aureobasidium* sp. (Viala & G. Boyer, 1891). Each of the yeast species *viz*., *Aureobasidium mansonii* (Cooke W.B., 1962), *Bandonia marina* (Uden & Zobell, 1962), *Candida carvajalis* (James S. A., 2009), *Candida insectamans* (D.B. Scott, Van der Walt & Klift, 1972), *Debaryomyces mycophilus* (Thanh, Van Dyk & M.J. Wingf., 2002), *Scheffersomyces shehatae* (H.R. Buckley & Uden, 1964) collected from various mangroves and continental shelf stations were sequenced for 13 times and 1 species (*Pichia kudriavzevii* (Boidin, Pignal & Besson, 1965)) was sequenced for 12 times. List of species recovered and their respective sampling stations were given in **table 1.** *Candida* spp. was the most speciose genera (n=33 species) followed by *Rhodotorula* spp. (n=13 species).

Table 1: List of marine derived yeast species and its respective sampling station. Colonies that could not be identified to species level was indicated by "sp."

No. of Sp.	Family	Genera	Species	Sampling source
1	Agaricostilbaceae	*Sterigmatomyces*	*elviae*	CS23, M5, CS25, CS5, CS6.
2			*halophilus*	M5, M6, M7, CS28, CS26.
3	Buckleyzymaceae	*Buckleyzyma*	*kluyveri-nielii*	CS4, M6, M7, M8, M9.
4			*salicina*	CS25, M7, M10, M11, M12, M13.
5	Chionosphaeraceae	*Cystobasidiopsis*	*lactophilus*	M3, CS31, M2, CS19.
6	Chrysozymaceae	*Chrysozyma*	*griseoflava*	M9, CS13, M9, CS26, M1.
7		*Fellozyma*	*inositophila*	M4, M5, CS5, CS30, CS31.
8		*Hamamotoa*	*lignophila*	M2, CS3, CS13, M10, M11.
9			*singularis*	M1, CS16, M2, CS29, M4.
10		*Oberwinklerozyma*	*yarrowii*	M10, CS14, CS27, M2.
11		*Pseudohyphozyma*	*bogoriensis*	M11, CS15, CS28, M3.
12			*buffonii*	M14, CS17, M13, CS28.
13			*pustula*	CS27, M14, CS11, M8, M9.
14		*Slooffia*	*cresolica*	CS28, M10, CS18, CS26, CS27.
15			*pilati*	M12, M13, M14, M8., CS8

16			*tsugae*	M13, CS27, CS15, CS16.
17		*Trigonosporomyces*	*hylophilus*	CS25, CS26, CS27, M8, M9.
18		*Udeniozyma*	*ferulica*	M4, CS32, CS20, M5, M6.
19		*Vonarxula*	*javanica*	M3, CS4, CS14, M11.
20	Cystobasidiaceae	*Cystobasidium*	*pallidum*	M12, CS26, CS14, CS15.
21			*pinicola*	M13, M14, CS25, CS26, CS27.
22			*slooffiae*	M13, M14, CS25, CS26, CS27.
23	Cystofilobasidiaceae	*Cystofilobasidium*	*macerans*	M5, M9, CS28, CS29.
24		*Filobasidium*	*globisporum*	CS19, M1, M11, CS16, M14.
25			*stepposum*	M5, CS7, M3, CS31, M6.
26			*uniguttulatum*	M4, M5, CS26, CS27, CS28.
27	Debaryomycetaceae	*Debaryomyces*	*fabryi*	M9, CS29, CS30.
28			*hansenii*	M10, CS11, CS12, CS13.
29			*mycophilus*	M13, CS13, M10, CS11, CS12, CS29, M11, CS19, CS27, CS28, M8.
30			*nepalensis*	M1, CS30, M2, CS18, M5.
31			*pseudopolymorphus*	CS27, CS28, CS32, M12, CS29, M10.
32			*subglobosus*	M14, CS28, CS16, CS15.
33		*Millerozyma*	*acaciae*	M1, CS30, CS31.
34		*Scheffersomyces*	*coipomoensis*	M13, CS29, CS11, CS25, CS26.
35			*shehatae*	M12, M1, CS31, CS30, CS30, CS29, CS28, CS27, CS20, CS19, CS11.
36		*Yamadazyma*	*scolyti*	M11, CS27, CS13, CS14, CS15.
37			*triangularis*	CS20, CS6, M1, M2, M3.
38	Dipodascaceae	*Galactomyces*	*geotrichum*	M1, M2, M11, CS11.
39		*Yarrowia*	*lipolytica*	CS18, CS4, M9, M10, M11.
40	Erythrobasidiaceae	*Erythrobasidium*	*elongatum*	M13, CS29, CS3, CS13.
41	Filobasidiaceae	*Cryptococcus*	*adeliensis*	M12, M7, CS7, M3.
42			*albidus*	M11, CS25, CS13, CS14.
43			*diffluens*	CS24, CS26, M8, CS25.
44			*magnus*	CS25, CS28, CS29, M10, CS26.
45			*oeirensis*	CS4, CS27, M9, CS25.
46			*randhawai*	M1, M2, M3, M4, CS22, CS20.
47			sp.1	M6, CS31, CS21, M3.
48			*uzbekistanensis*	M14, M9, CS26, CS27, CS28.
49		*Naganishia*	*globosa*	CS20, CS13, M4, M5, M10.
50			*liquefaciens*	M2, M3, CS31, CS32, CS3.
51			*vishniacii*	CS25, CS28, M10, CS26, CS25, CS27, M14, M3, M4, M5, M6, CS11, CS29, CS30, M8, M9.
52	Heterogastridiaceae	*Colacogloea*	*diffluens*	CS29, CS18, CS19, M13, M14.
53			*eucalyptica*	CS27, CS28, M12, M13.
54			*falcata*	CS24, CS25, CS26, M11, M12.
55			*foliorum*	CS31, CS32, M10, M11.

56			*philyla*	CS5, CS6, CS11, M9, M10.
57	incertae sedis	*Curvibasidium*	*cygneicollum*	CS28, CS31, CS32, M8, M9.
58		*Hasegawazyma*	*lactosa*	M1, M2, M3, CS30, CS12.
59	Kondoaceae	*Kondoa*	*aeria*	M12, M13, M11, CS18, CS19.
60			*malvinella*	CS28, CS29, CS30, M7, M8.
61	Kriegeriaceae	*Phenoliferia*	*glacialis*	CS28, CS29, CS18, CS19, M6, M5.
62			*psychrophenolica*	CS5, CS26, CS27, M5, M6.
63	Leucosporidiaceae	*Leucosporidium*	*fellii*	CS6, CS31, CS32, M1.
64			*scottii*	CS18, CS15, CS16, M5, M6.
65	Metschnikowiaceae	*Metschnikowia*	*noctiluminum*	CS15, CS16, CS30, M4, M5.
66	Microsporomycetaceae	*Microsporomyces*	*pini*	CS28, CS29, CS5, CS14, M3, M4.
67	Microstromataceae	*Microstroma*	*bacarum*	CS29, CS30, M3, M2.
68	Phaffomycetaceae	*Wickerhamomyces*	*anomalus*	CS31, CS17, CS18, M2, M3.
69			*subpelliculosus*	CS29, CS6, M13, M14.
70	Pichiaceae	*Nakazawaea*	*pomicola*	CS22, M6, CS31, CS21, M3.
71		*Pichia*	*cecembensis*	CS32, M14, CS31, CS18.
72			*farinosa*	CS11, CS27, CS28, M9.
73			*fermentans*	CS25, CS29, CS30, CS31, M9.
74			*guilliermondii*	CS6, CS11, CS25, M14.
75			*jadinii*	CS30, CS31, CS32, M1, M2.
76			*kluyveri*	CS27, CS28, CS29, M14, M1.
77			*kudriavzevii*	M8, M5, M11, M10, CS6, CS5, CS29, CS28.
78			*subpelliculosa*	CS28, CS29, CS30, M3, M12.
79	Ruineniaceae	*Ruinenia*	*dracophylli*	CS26, CS27, M4, M11.
80			*rubra*	CS11, CS28, M5, M10.
81	Saccharomycetaceae	*Candida*	*albicans*	CS19, M14, M1, CS16, M2.
82			*boidinii*	M12, CS28, M10, CS26.
83			*boleticola*	CS26, CS31, M12, CS23.
84			*carpophila*	CS27, M14, M3, M4, M5.
85			*catenulata*	CS5, CS6, M14, M13, CS31.
86			*carvajalis*	M3, M14, M11, M10, CS31, CS28, CS27, CS26, CS26, CS25.
87			*dubliniensis*	CS27, CS28, M5, M9, M10.
88			*duobushaemulonii*	CS6, CS7, CS26, M6, M8.
89			*ergatensis*	CS5, CS25, M7, CS32, CS22, M4, CS24, CS4, M6, M8, M9, M10, CS18, CS19, CS20, CS21.
90			*fermenticarens*	CS28, CS29, CS18, M7, M8.
91			*fragi*	CS28, CS26, CS27, M14, M13.
92			*glaebosa*	CS31, CS26, CS27, M10, M11.
93			*haemulonii*	CS29, CS30, M5, M6.
94			*humilis*	CS26, CS27, CS28, M8, M9.
95			*hungchunana*	CS11, CS25, M9, M10, M11.
96			*insectamans*	M8, M5, M2, M13, M10, CS32, CS31, CS30, CS28.
97			*intermedia*	CS4, CS32, CS6, M11, M12.

98			*magnoliae*	CS22, CS32, CS3, M13, M14.
99			*molendinolei*	M12, M11, CS26, CS27.
100			*nivariensis*	CS30, CS31, CS32, M1, M3.
101			*oceani*	CS28, CS29, M6, M7.
102			*orthopsilosis*	CS26, CS27, M3, M7.
103			*palmioleophila*	M13, CS21, M5, CS30.
104			*parapsilosis*	M2, M3, M5, CS29, CS31.
105			*quercitrusa*	M14, M9, M1, CS31, CS32.
106			*railenensis*	CS28, CS2, M10, CS26.
107			*rugosa*	M7, CS31, M8, CS27, CS32.
108			*santamariae*	CS6, CS26, CS26, M8.
109			sp.1	CS3, M14, CS22, M6.
110			*spencermartinsiae*	CS3, CS25, CS26, M9.
111			*viswanathii*	CS1, CS2, M12, CS28.
112			*xylopsoci*	CS3, M13, CS21, M5.
113			*zeylanoides*	CS2, CS5, CS13, M7, M9.
114		*Hanseniaspora*	*guilliermondii*	CS3, CS4, CS6, M14.
115			*occidentalis*	M2, CS30, M3, CS31, CS9.
116			*opuntiae*	CS6, CS29, CS30, M6.
117			*uvarum*	CS10, CS25, CS28, CS29, M10.
118		*Issatchenkia*	*orientalis*	CS11, CS12, M3, CS19.
119		*Kazachstania*	*aerobia*	CS5, CS2, CS25, M7.
120			*exigua*	CS32, CS22, M4, CS24.
121			*humilis*	CS1, CS4, CS5, M14.
122		*Kluyveromyces*	*dobzhanskii*	CS6, CS26, CS27, CS31, M1.
123			*lactis*	M4, CS24, CS4, M6.
124			*marxianus*	M7, M8, M9, CS5.
125		*Saccharomyces*	*bayanus*	M12, CS28, M14, CS22.
126			*cerevisiae*	M6, CS31, CS21, M3.
127			*kudriavzevii*	M8, CS2, CS23, M5.
128		*Torulaspora*	*delbrueckii*	CS2, M10, CS26, CS25.
129			*franciscae*	CS23, M5, CS25, CS5.
130		*Williopsis*	*saturnus*	M1, M2, M3, M4, CS25, M7.
131	Saccharomycopsidaceae	*Saccharomycopsis*	*fibuligera*	M13, M14, M9, CS32.
132	Saccotheciaceae	*Aureobasidium*	*pullulans*	M12, M13, CS22, M4.
133			*mansoni*	M6, M14, M13, M11, M10, CS32, CS31, CS28, CS27, CS26, CS25.
134			sp.1	CS16, M13, CS27, CS15, M14, CS22, M6, M11, CS28, CS25, M10, M7, CS7.
135	Sakaguchiaceae	*Sakaguchia*	*dacryoidea*	M6, CS27, CS20, M7.
136	Sporidiobolaceae	*Rhodosporidiobolus*	*fluvialis*	M7, CS7, M3, CS31.
137		*Rhodosporidium*	*diobovatum*	M2, M3, M4, CS7.
138		*Rhodotorula*	*araucariae*	M14, M9, M1, CS3, CS4, CS5.
139			*babjevae*	M12, M13, CS1, CS4.
140			*dairenensis*	M12, CS28, M10, M11.

141			*glutinis*	M13, M14, M9, CS32, CS26.
142			*graminis*	M5, CS3, CS4, CS5, CS6.
143			*ingeniosa*	M7, M8, M9, CS8, CS28.
144			*kratochvilovae*	CS3, M7, CS24, M6, CS26.
145			*mucilaginosa*	CS22, CS11, CS25, M6, M7.
146			*nothofagi*	M3, M4, M5, CS7, CS8.
147			*paludigena*	CS1, M9, CS28, CS25.
148			*sphaerocarpa*	M1, CS1, CS2.
149			*toruloides*	CS22, M6, CS31, CS21.
150		*Sporobolomyces*	*beijingensis*	M3, CS2, CS31, CS18.
151			*koalae*	CS9, CS27, CS28, M8.
152			*oryzicola*	M12, CS20, CS28, CS29.
153			*roseus*	CS29, CS6, M7, M8.
154	Symmetrosporaceae	*Symmetrospora*	*foliicola*	M6, M7, CS26, CS11.
155			*gracilis*	M1, CS16, M3, CS29.
156	Tetragoniomycetaceae	*Bandonia*	*marina*	M9, M4, M3, M2, M13, M12, M11, M10, M1, CS28, CS25.
157	Ustilentylomataceae	*Ustilentyloma*	*graminis*	M11, CS19, CS27.

3.2.Character based identification and BLAST analysis

The recovered ITS sequence length ranged between 542bps to 891bps (**Fig. 1**). Majority (62.24%) of the sequences (633 sequences belonging to 81 species) were between 600 and 649 bps. Minimum length between 552 and 599 bps was recovered only for 6 sequences. All remaining sequences retrieved were above or equal to 600bp length. List of 1017 barcodes with their respective species match in the GenBank reference library (with percentage of similarity, its accession numbers and its taxonomy) was given in **table S2.**

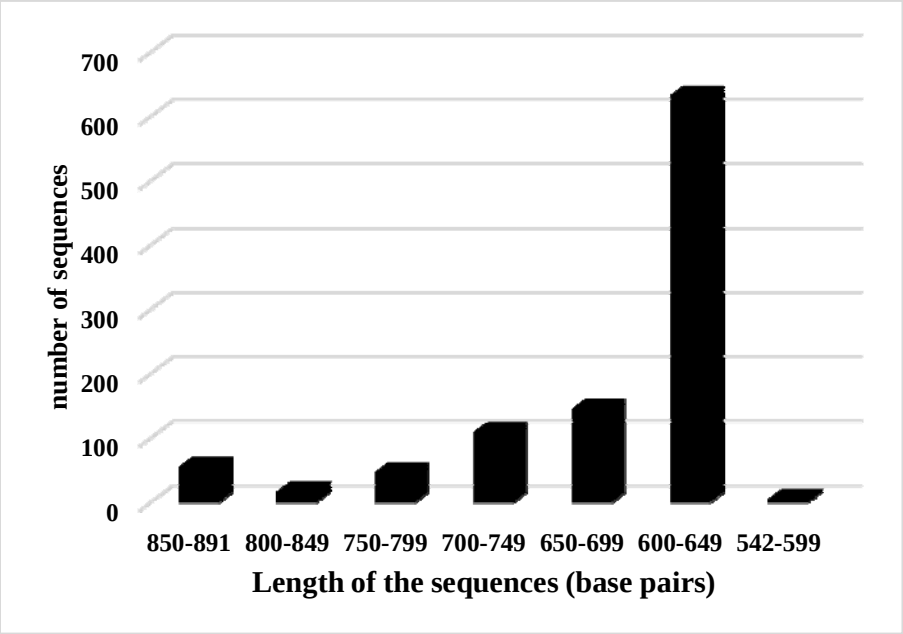

Fig. 1: Variations in sequence length (bps) distributed among 1017 ITS gene sequences. More than 60% of the sequences were between 600 and 649 bps.

BLAST analysis revealed that 13 yeast species were barcoded for the first time (**table 2**) and individual search of the ITS gene of those species in GenBank did not yield any results, indicating those species were first time sequenced. Even though *Candida* spp. was specious genera, the ITS gene sequences of *C. carvajalis* (n=15), C. *duobushaemulonii* (n=6) and *C. haemulonis* (n=6) was barcoded for the first time. Even though previous extensive study barcoded 1351 yeast species producing 8669 barcodes (Vu et al., 2016), these 13 species was not included, may be because the previous studies was concentrating on human pathogenic yeast species.

Table 2: List of marine derived yeast species barcoded for the first time with its respective species authority and corresponding GenBank accession numbers.

No.	Family and species	Authority	Acc. Nos.
1	Family: Buckleyzymaceae *Buckleyzyma salicina*	B.N. Johri & Bandoni	KJ706369, KJ706528, J706687, KJ706846, KJ707005, KJ707163
2	Family: Chrysozymaceae *Chrysozyma griseoflava*	Nakase & M. Suzuki	KJ706363, KJ706522, J706681, KJ706840, KJ706999, KJ707157
3	*Trigonosporomyces hylophilus*	Van der Walt, D.B. Scott & Klift	KJ706323, KJ706482, J706641, KJ706800, KJ706959, KJ707117
4	Family: Cystobasidiaceae *Cystobasidium pallidum*	Lodder	KJ706324, KJ706483, J706642, KJ706801, KJ706960, KJ707118
5	Family: Erythrobasidiaceae *Erythrobasidium elongatum*	R.G. Shivas & Rodr. Mir.	KJ706362, KJ706521,KJ706680, KJ706839, KJ706998, KJ707156
6	Family: Heterogastridiaceae *Colacogloea falcata*	Nakase, Itoh & M. Suzuki	KJ706367, KJ706526,KJ706685, KJ706844, KJ707003, KJ707161
7	Family: Saccharomycetaceae *Candida carvajalis*	S.A. James, E.J. Carvajal, C.J. Bond, K. Cross, N.C. Núñez	KJ706236, KJ706395,KJ706554, KJ706713, KJ706872,KJ707031, KJ707176, KJ707185,KJ707194, KJ707203, KJ707212,KJ707221, KJ707230
8	*Candida duobushaemulonii*	Cend.-Bueno, Kolecka, Alastr.-Izq., Gómez-López, Cuenc.-Estr. & Boekhout	KJ706233, KJ706392,KJ706551, KJ706710, KJ706869, KJ707028
9	*Candida haemulonis*	Uden & Kolip.	KJ706229, KJ706388,KJ706547, KJ706706, KJ706865, KJ707024
10	*Kazachstania aerobia*	F.Y. Bai & Y.M. Cai	KJ706291, KJ706450,KJ706609, KJ706768, KJ706927, KJ707085
11	Family: Saccotheciaceae *Aureobasidium mansonii*	Castell.	KJ706380, KJ706539,KJ706698, KJ706857, KJ707016,KJ707174, KJ707183, KJ707192,KJ707201, KJ707210, KJ707219,KJ707228
12	Family: Sporidiobolaceae *Rhodosporidiobolus fluvialis*	Fell, Kurtzman, Tallman & J.D. Buck	KJ706317, KJ706476,KJ706635, KJ706794, KJ706953, KJ707111
13	*Rhodosporidiobolus sphaerocarpa*	S.Y. Newell & Fell	KJ706316, KJ706475,KJ706634, KJ706793, KJ706952, KJ707110

Among the 13 species barcoded for the first time, minimum of 0.25 K2P distance and 0.024 nucleotide diversity was recorded for *Candida carvajalis* (number of isolates sequenced = 13) (fig. 2). Maximum of 0.37 K2P distance was recorded for *Buckleyzyma salicina* (n=6), *Trigonosporomyces hylophilus* (n=6) and *Rhodosporidiobolus sphaerocarpa* (n=6) (where maximum of nucleotide diversity of 0.036 was recorded). Overall intra-species K2P distance average was 0.33% which was well less than 1.59% for yeast species threshold

(Vu et al., 2016), indicating all isolates clustered representing individual species. Values of nucleotide diversity directly proportionated the K2P distances (Fig. 2).

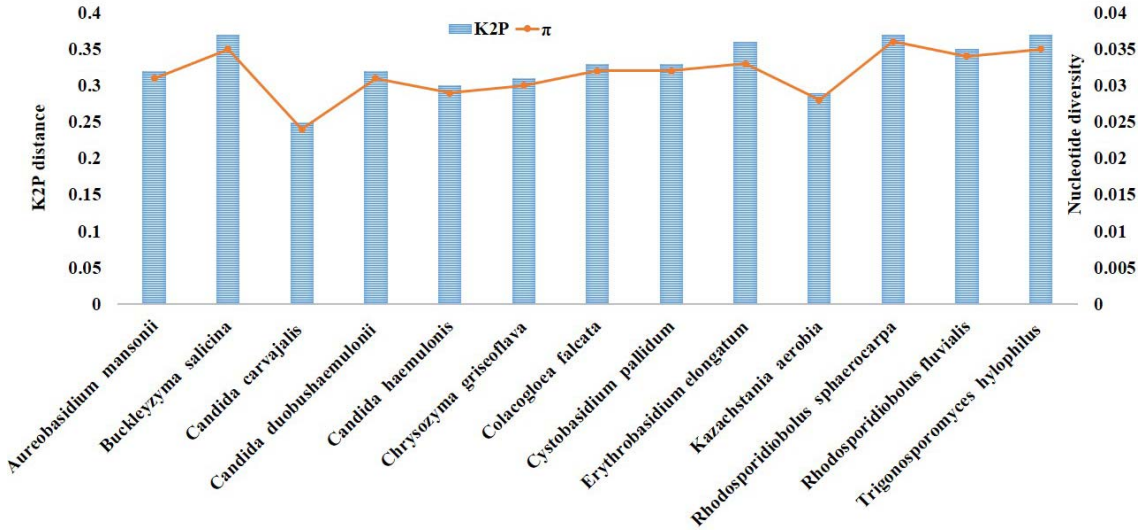

Fig. 2: Intra-species average K2P distance and nucleotide diversity (π) of ITS gene sequences of 13 species barcoded for the first time.

3.3. Tree based identification

Tree based identification for all 157 species in the present study was not practical and involves construction of mega tress which will be difficult to curate. Hence, the species of selective genera in phylum Ascomycota (*Cryptococcus* spp.) and Basidomycota (*Colacogloea* spp.) was employed to test the tree based identification. For constructing NJ tree of *Cryptococcus* spp., we used maximum of 3 sequences produced in the present study belonging to 5 selective species against the corresponding available GenBank reference sequences. All selected species in the *Cryptococcus* spp. precisely clustered its corresponding species in the single clade (**Fig. 3**). This indicates the efficacy of ITS gene sequences in precisely delineating the corresponding yeast species. The overall K2P pairwise distance of the *Cryptococcus* spp. was 0.7% which is well within the proposed ITS cut off of 1.59% for yeast species (Vu et al., 2016).

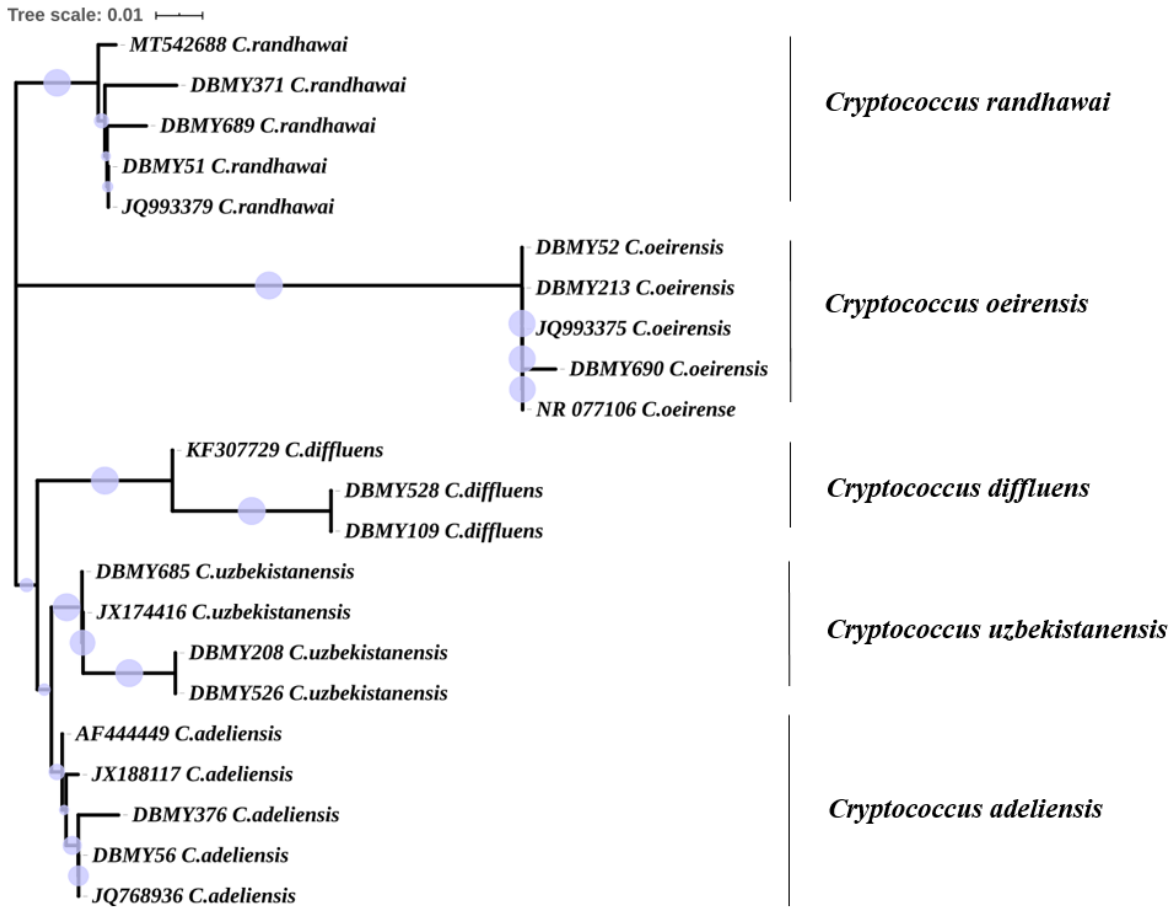

Fig. 3: NJ tree based identification of *Cryptococcus* spp. spp. (phylum: Ascomycota, class: Saccharomycetes). The acronym "DBMY" denotes the sequences produced in the present study. The acronym other "DBMY" denotes the accession number of GenBank reference sequences.

Colacogloea spp. and its respective reference sequences from the GenBank precisely clusters the individual species in single clade differentiating from other species of the same genera demonstrating the efficacy of ITS gene in species differentiation (**Fig. 4**). The overall kimura-2 parametric distance was 1.4% which is well within the cut-off value proposed by Vu et al. (2016).

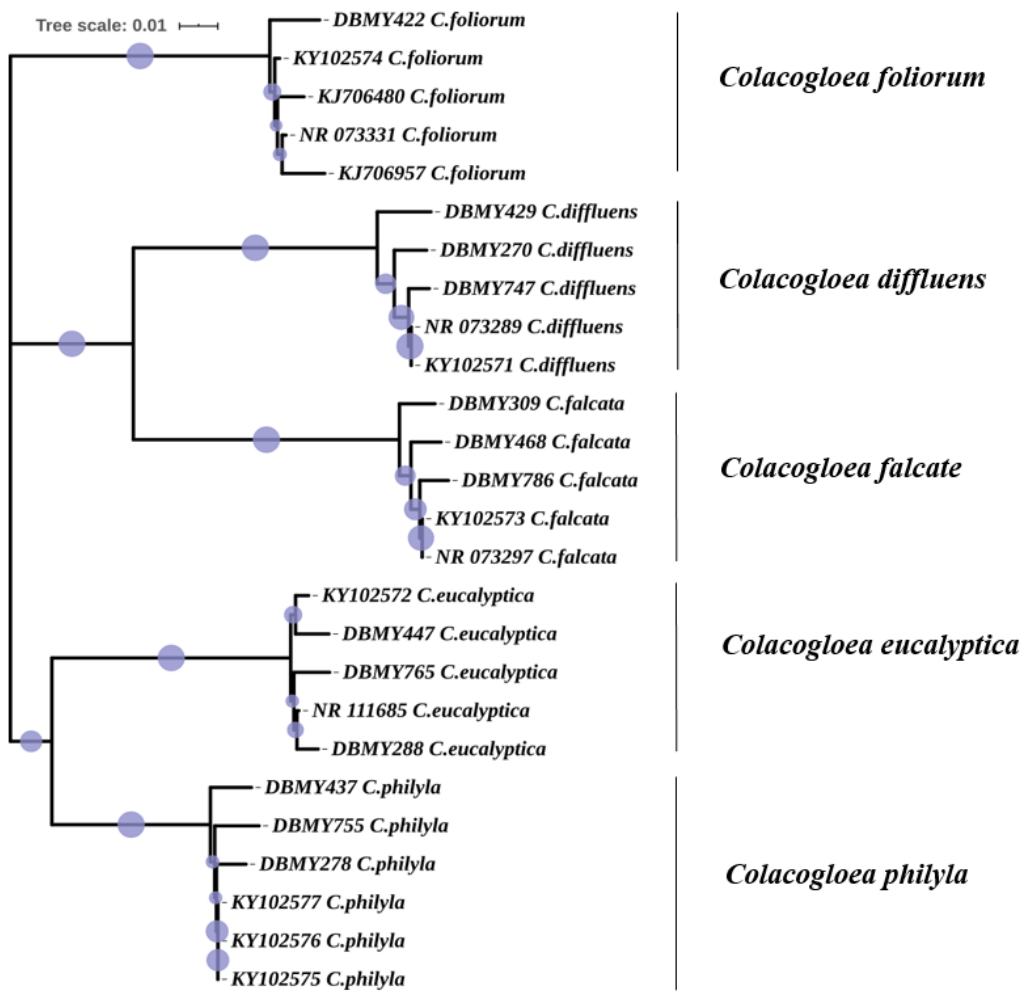

Fig. 4: NJ tree based identification of *Colacogloea* spp. (phylum: Basidiomycota, class: Microbotryomycetes). The acronym "DMBY" denotes the sequences produced in the present study.

3.4. New occurrences of marine derived yeast species

Only 25.5% (n=40) of marine derived yeast species isolated in the present study was previously isolated form marine environment. Example; *Sterigmatomyces halophilus* were known to increase immunity of marine fishes (Reyes-Becerril et al., 2017). *Yarrowia lipolytica* were known for its capability in crude oil degradation (Hassanshahian et al., 2012) and know for dimorphic growth when especially isolated from oil polluted seawater (Zinjarde et al., 1998). Another species isolated in the present study, *Candida oceani*, first isolated from Atlantic hydrothermal vents (Burgaud et al., 2011) was known for its ability to tolerate high hydro-static pressure (Burgaud et al., 2015). *Bandonia marina* reclassified from *Candida marina* (Liu et al., 2015), first isolated from marine environment (Van Uden and

Zobell, 1962) was also known for its hydrocarbonoclastic ability (Itah and Essien, 2005) and was previously isolated from tar balls collected from marine environment (Shinde et al., 2017). Other species (~58%; n=91 species) in the present study was either known to occur in soil, plants and animals (including insects) and their occurrences in the marine environment was previously unrecorded. About 16.6% of recorded yeast species were of potential human pathogens (n=26 species).

3.4.1. **Yeast species previously unrecorded from marine environment**

About 60% of the yeast species recorded in the present study was previously unrecorded from the marine environment. As listing out the previously source of occurrence of 94 yeast species would be tedious, we present here few examples.

Cystobasidiopsis lactophilus was reclassified from *Sporobolomyces lactophilus* (Wang et al., 2015) known to occur in the phyllospheres of coniferous trees (Nakase et al., 1990). The present study reports its occurrences in mangrove sediments were reported for the first time. *Oberwinklerozyma yarrowii* reclassified from *Rhodotorula silvestris* (Wang et al., 2015) was previously known to occur in forest soils (Mašínová et al., 2018) was reported to occur in mangrove sediments for the first time. River run off could be the source of their isolation from the mangrove sediments in the present study. *Slooffia cresolica* reclassified from *Rhodotorula cresolica* (Wang et al., 2015) was known to be a part of soil microbiome (Middelhoven and Spaaij, 1997) and also associated with soils of high oil contaminations (Csutak et al., 2005). The continental shelf sediments from where these strains were isolated in the present study was previously known for hydrocarbon contaminations (Lyla et al., 2012). Similarly, *Candida catenulata* previously isolated from hydrocarbon contaminated sites (Habibi et al., 2017; Babaei et al., 2018) was also isolated in the present study.

Trigonosporomyces hylophilus reclassified from *Candida hylophila* (Wang et al., 2015) was first isolated from tree associated beetles (van der Walt et al., 1971). Their isolation from the mangrove sediments in the present study indicates their possible occurrences in the insects associated with mangrove environments. *Udeniozyma ferulica* and *Vonarxula javanica*, reclassified from *Rhodotorula ferulica* and *Rhodotorula ferulica* (Wang et al., 2015), respectively was known to occur in polluted river waters (Sampaio and Van Uden, 1991). Their occurrences in the mangrove and continental shelf sediments could be attributed to river run offs.

The fact that Debaryomyces mycophilus was first isolated from wood lice (Thanh et al., 2002) opens the possibility of this species also occurring in insects associated with mangrove ecosystems. *Debaryomyces pseudopolymorphus* was widely involved in wine

fermentation and its related processes (Potgieter, 2004; Villena et al., 2006; Arevalo-Villena et al., 2007). The isolation and role of *D. pseudopolymorphus* in mangrove environment is new and unknown. *Scheffersomyces shehatae* was known to occur in degrading woods (Kordowska-Wiater et al., 2017) and in wood digesting insects (Suh et al., 2013) was widely used for bio-ethanol production (Tanimura et al., 2015; Kordowska-Wiater et al., 2017). The association of *S. shehatae* with mangroves and its associated insects could be further explored. *Colacogloea falcatus* reclassified from *Sporobolomyces falcatus* (Wang et al., 2015) were first isolated from dead plant leaves (Nakase et al., 1987). They were also isolated from phylosphere of plants (Nakase et al., 2003; Takashima and Nakase, 2000) and acidic soils (Delavat et al., 2013). Their occurrences in mangrove and continental shelf sediments were new.

Pichia guilliermondii has wide spread occurrences as plant endophytes (Zhao et al., 2010), flora of citrus fruit (Arras et al., 1998), beetle associated (Suh et al., 1998), and in seawage sludges (de Silóniz et al., 2002). Hence the isolation in the present study could be associated with multiple sources. *Kazachstania aerobia*, first isolated from plants (Magalhaes et al., 2011) where recognised as plant associated yeast (Lu et al., 2004). Their occurrences in marine associated sediments were new. *Sporobolomyces koalae* was first isolated from koalas bear (Satoh and Makimura, 2008), and been latter found in other animals like horses (Fomina et al., 2016). Their occurrences in marine related sediments was new.

3.4.2. Potential pathogenic yeast

Among 26 different yeast species recognised as potential human pathogens (Table 3) *Candida* spp. contributes to 42.3% (n=11), followed by *Cryptococcus* spp. (19.2%; n=5). Even though few human pathogens (such as *Candida orthopsilosis, C. viswanathii, Rhodotorula mucilaginosa* and *Sterigmatomyces elviae*) isolated in the present study was previously reported to occur in marine environments (Li et al., 2010; Zaky et al., 2016; Pinheiro et al., 2018; Rasmey et al., 2020), species such as *Candida dubliniensis, C. duobushaemulonii , C. haemulonii, C. nivariensis, C. parapsilosis, C. rugosa, Debaryomyces fabryi* and *Yamadazyma triangularis* were obligate human pathogens (Gasparoto et al., 2009; Ramos et al., 2018; Gade et al., 2020; Ben-Ami et al., 2017; KLi et al., 2014; Mesini et al., 2020; Mloka et al., 2020; Tafer et al., 2016; Kurtzman et al., 2011) whose occurrences outside human host was unknown until now.

Table 3: List of previously known human pathogenic yeast species isolated from the present study.

S. No	Species	Clinical sample	Environmental sample
1	*Cryptococcus adeliensis*	Isolated from clinical samples (Tintelnot and Losert, 2005) and causes meningitis (Rimek et al., 2004)	Antartic algae (Scorzetti et al., 2000) and frequently from antartic terrestrial and marine samples (Duarte et al., 2013) including Antartic moss (Zhang et al., 2014)
2	*Cryptococcus albidus*	Isolated from immune compromised patients (Sugita et al., 2001) and other clinical samples (Lin et al., 1989; Lee et al., 2004)	lake waters (Vadkertiova and Slavikova, 1995) and food samples (Senses-Ergul et al., 2006)
3	*Cryptococcus diffluens*	Causes subcutaneous cryptococcosis (Serda Kantarcioğlu et al., 2007) and dermatitis in humans (Sugita et al., 2003)	Petroleum contaminated environments (Yilmaz and Sayar, 2015; Yalçın et al., 2014)
4	*Cryptococcus magnus*	isolated from leukemia patients (Khan et al., 2011) and in other human clinical samples (Ghajari et al., 2018)	Isolated from grapes (Pantelides et al., 2015), plant leaves (Han et al., 2017) and from some cereals (Han et al., 2015)
5	*Cryptococcus uzbekistanensis*	isolated from immune compromised patients (Powel et al., 2012)	Plant flowers (Hyun and Lee, 2014), and known causative agents of canker in fruit trees (Dehghan-Niri et al., 2015). Also isolated from petroleum contaminated sites (Yalçın et al., 2014; Yılmaz et al., 2015)
6	*Naganishia globosa*	Clinical samples (Pakshir et al., 2018)	Soil (Kim et al., 2018), hyper saline soils (Mokhtarnejad et al., 2016) and lakes (Li et al., 2018)
7	*Naganishia liquefaciens*	Isolated from human skin (Timm et al., 2020) and from hospital environments (de Oliveira Brito et al., 2019)	Soil (Abu-Mejdad et al., 2019), lakes (Li et al., 2018) and from municipal wastes (Selvakumar and Sivashanmugam, 2018)
8	*Pichia farinosa*	Isolated from clinical samples (Adler et al., 2007; Magalhaes et al., 2015)	Halotolerence (Suzuki and Nikkuni, 1989)
9	*Candida albicans*	Numerous clinical samples (Matthews et al., 1987; Asakura et al., 19991; Nolte et al., 1997; Baena-Monroy et al., 2005; Dunkel et al., 2008)	Textile effluents (Vitor and Corso, 2008)
10	*Candida dubliniensis*	Fungal pathogen (Gutierrez et al., 2002), isolated from numerous	-

		clinical samples (Chavasco et al., 2006; Barros et al., 2008; Marcos-Arias et al., 2009; Gasparoto et al., 2009)	
11	*Candida duobushaemulonii*	Emerging pathogen (Boatto et al., 2016; Frías-De-León et al., 2019) and was recently isolated from numerous cases of clinical samples (Fang et al., 2016; Ramos et al., 2018; Gade et al., 2020)	-
12	*Candida haemulonii*	Fungal pathogen (Almeida et al., 2012) and was commonly isolated from clinical samples (Rodero et al., 2002 Khan et al., 2007; Ben-Ami et al., 2017)	-
13	*Candida nivariensis*	pathogen (Alcoba-Flórez et al., 2005; Borman et al., 2008) frequently isolated from various clinical samples (Wahyuningsih et al., 2008; Lockhart et al., 2009; KLi et al., 2014)	-
14	*Candida palmioleophila*	Fungal pathogen (Sugita et al., 1999; Jensen et al., 2011; Pierantoni et al., 2020)	Environmental samples (Nakase et al., 1988), able to decolorize azo dyes (Jafari et al., 2013)
15	*Candida parapsilosis*	Fungal pathaogen (Levy et al., 1998; Tay et al., 2009; Canton et al., 2011; Mesini et al., 2020)	-
16	*Candida rugosa*	fungal pathogen (Pfaller et al., 2006), frequently isolated from various clinical samples (Chaves et al., 2013; Seyom et al., 2020; Mloka et al., 2020)	-
17	*Candida orthopsilosis*	Numerous clinical samples (Yong et al., 2008; Asadzadeh et al., 2009; Canton et al., 2011; Feng et al., 2012)	Marine environment (Rasmey et al., 2020)
18	*Candida spencermartinsiae*	Human pathogen (Shokohi et al., 2018)	Apple fruits (de Garcia et al., 2010) and mangrove ecosystems (Statzell-Tallman et al., 2010)
19	*Candida viswanathii*	Fungal pathogen (Viswanathan and Randhawa, 1959), Various clinical samples (sandhu et al., 1976; Zaragoza et al., 2011; Wong et al., 2020)	Marine samples (Zaky et al., 2016)
20	*Aureobasidium mansoni*	Clinical samples (Krcmery et al., 1994) and were recognised as an emerging pathogens (Bamadhaj et al., 2015)	-
21	*Rhodotorula*	Clinical samples (Cobo et al., 2020)	Flowers and fruits of tropical

	dairenensis		forest (Colla et al., 2010)
22	*Rhodotorula mucilaginosa*	Clinical samples (Galán-Sánchez et al., 1999; 2011; Da Cunha et al., 2009; Falces Romero et al., 2018)	Marine environment (Li et al., 2010)
23	*Sterigmatomyces elviae*	Human skin lesions (Sonck, 1969)	Guts of fishes (Pinheiro et al., 2018)
24	*Filobasidium uniguttulatum*	Meningitis in humans (Pan et al., 2012)	Isolated from decaying woods (Jimenez et al., 1991)
25	*Debaryomyces fabryi*	Human mycotic lesions (Tafer et al., 2016)	-
26	*Yamadazyma triangularis*	Isolated from human lung tissue (Smith & Batenburg-Van der Vegte, 1986; Kurtzman et al., 2011)	-

4. Conclusion

There could be ~3.8 million unknown fungal species (Hawksworth and Lücking 2017) and the environmental selection pressure plays a pivotal role in evolution of new species (Handelsman, 2004, Hibbett, 2016). The present study was first of its kind in exploring large scale of marine ecosystem for culturable yeast species. As a result 1017 barcodes of 147 marine yeast species were produced among which 91 barcodes of 13 species was produced for the first time. The present study documented terrestrial yeast species introduced to marine ecosystem (ex., *Cystobasidiopsis lactophilus, Oberwinklerozyma yarrowii*) and endemic marine species whose occurrences where restricted to specific marine ecosystem (Ex., *Bandonia marina, Candida oceani*). The DNA barcodes were released for public use through GenBank and BOLD databases, which will also improve the yeast barcodes and taxonomy in the public databases. These DNA barcodes will also aid in the documentation and estimation of marine yeast diversity from environmental samples as many metagenomic diversity studies suffers from lack of local library for species identification (Hawksworth, 2001; Handelsman, 2004; Hibbett, 2016).

Our next challenge would be to exploring the biochemical and industrial potential of the isolated strains with continuous expansion of barcode database by venturing into new marine ecosystems. This could be the biggest dataset of marine yeast collection. The yeast barcode data produced could be used to explore taxonomic distribution of specific physiological traits (ex., theromotolerance), species of climate and pathological significance (Robert et al. 2015). Combining yeast barcode data with other traits such as its ability to produce various biotechnologically significant metabolites and industrial products (ex.,

antibiotics) will constitute a useful resource for yeast researchers beyond the taxonomic and identification applications.

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