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Taxonomic Approach for Species Diversity of Yeasts and Yeasts-like Fungi through D1/D2 Region of Large Subunit Ribosomal DNA Sequences

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Abstract

The identification of yeasts or yeasts-like fungi and verify their diversity are principal aspect for bioindustry and ecosystem sustainability. Taxonomic approach provides identification tool to ensure the taxonomic position of yeasts and yeasts-like fungi which definitely set to utilization concerns. The aim of this study is to understanding the taxonomic position of yeasts and yeasts-like fungi from the distinctive of its sequences relationship. Yeasts and yeasts-like fungi strains were isolated through various culture dependent methods from natural resources samples of Karimun Besar Island, Province of Riau Islands, Indonesia. The identification process was performed through amplifying the accurate DNA-based in D1/D2 region of large subunit (26S) ribosomal DNA. As the result, a total of 85 isolates of yeasts and yeasts-like fungi were obtained with 16 closest related taxa through phylogenetic tree construction. Ascomycetous was the predominating group representing 91% of the total isolates sequences followed by Basidiomycetous (8%) and Zygomycetous (1%). The black yeasts (yeasts-like) known as *Aureobasidium melanogenum* was predominant species with represent to 54% of total isolates and present in particular habitat. Taxonomically, there are six isolates are represent to be novel taxa candidates which pretend to enhance genetic resources of yeasts and yeasts-like fungi especially from Indonesia. In addition, this information provides specific technique to reach specific yeasts or yeasts-like fungi species in nature by managing the sample collection and culture methods.

How to Cite

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INTRODUCTION

Yeasts have been exploited for many decades by scientist. They are important microbes for bioindustry and ecosystem services. They have essential roles on nutrient cycling (Botha, 2011), food (Aidoo *et al.*, 2006), bioenergy (Buijs *et al.*, 2013; Nielsen *et al.*, 2013), health (Chi *et al.*, 2009; Leathers *et al.*, 2015), biological control (Pantelides *et al.*, 2015), and environmental indicator (Vogel *et al.*, 2007). However the number of yeasts and yeasts-like species that already studied, cultivated and preserved are about 1% from expected in nature (Fell *et al.*, 2000). Therefore, many studies are needed to unravel the biodiversity and its ecological roles for bioindustry. Discovering biodiversity and elucidating the taxonomic position of yeasts and yeasts-like fungi are necessary for conservation, quality control, and ecological monitoring (Xu, 2016). Obviously, the biodiversity studies cover construction of taxonomical richness as an exhaustive mapping of community structure and predict their roles in ecosystem (Takashima *et al.*, 2012).

Culture dependent technique may preserve the isolate for further analysis to discover many physiological characters and explore its bioindustry prospect. Thus, the taxonomic identity of the isolate is a compulsory to carry out. In polyphasic identification, taxa position of yeasts are commonly using some criteria based on septal morphology, cell wall composition, chemical characteristic, and conserve genes analysis (Fell *et al.*, 2000; Kurtzman *et al.*, 2011). Earlier study, identification of yeasts was only used conventional method based on morphological and chemical analyses. Those methods took many time and expensive (Fell *et al.*, 2000; Xu, 2016). Recently, rapid identification method is carried out on DNA sequence amplification which supported by the emergence of scientific community such GenBank for database comparison. Based on conserve genes database, we are able to mapping the taxonomical position of our isolates. The conserve genes are more stable through mutation factor rather than other genes.

There are some conserve genes as marker to distinguish fungi taxa position such as mitochondrial and nuclear rDNA (White *et al.*, 1990; Kurtzman & Robnett, 1998). The evolve time of those genes is useful for determining taxonomic position of certain microorganism (White *et al.*, 1990). In yeasts and yeasts-like identification, region of D1/D2 on Large Subunit rDNA was commonly in used (Kurtzman & Robnett, 1998; Fell *et al.*, 2000). That region has divergence fragment around ± 600 bp and sufficient for identify yeasts and yeasts-like species. Meanwhile, Scorzetti *et al.* (2002) emphasis the combination of some conserve genes like internal transcribed spacer (ITS) was required to analyze certain characteristics and perhaps able to describe a novel species.

Ecological and taxonomic yeasts in some area of Indonesia have been studied but not in outer islands yet (Sjamsuridzal *et al.*, 2010). It considers to exploring the occurrence yeasts and yeasts-like in outer islands in Indonesia such Karimun Besar Island. This island is part of Kepulauan Riau Province as border of Indonesian country between Malaysia and Singapore. In this study, to understand the taxonomic position of the yeasts and yeasts-like fungi, we explore them by molecular approach to construct their diversity through the phylogenetic tree. The diversity of yeasts and yeasts-like fungi are necessary for industry, ecosystem management and genetic resources database from Indonesian natural resources.

METHODS

Sampling sites

Samples were collected from four locations there i.e. Jantan mount (N: 01^0 06' 341'' E: 103^0 21' 609''), Betina mount (N: 01^0 05' 260'' E: 103^0 21' 250''), mangrove on Musodo bay (N: 1^0 7' 33,7'' E: 103^0 22' 20,5'') and mangrove on Setimbul bay N: 01^0 6' 457'' E: 103^0 19' 499'') at March-April 2015. Kind of samples such as soils, leaf, leaf liters, decay woods were collected in male and female mount. Otherwise, sediment samples were collected from mangrove.

Isolation method and preservation

Isolation was conducted in Biosystematic of Yeast Laboratory-Indonesian Culture Collection (InaCC), Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences (LIPI). The isolation of samples were performed with various methods such as direct plating, serial dilution, balistospores-fall, millipore vacuum, and incubated into enrichment media. The isolation process was performed by following Sumerta and Kanti (2016) steps. The pure isolates were maintained in deep freezing -80^0 C with 10% glycerol and 5% trehalose. Some of this isolates were deposited in InaCC for public collection (http://inacc.biologi.lipi.go.id/).

Molecular identification

Amplification of the D1/D2 region of

Large Subunit ribosomal DNA (26S) was performed by using primer NL1 5'-CATATCAATAAGCGAAAAG-3' dan NL4 5'-GGTCCGTGTTTCAAGACGG-3' (Kurtzman & Robnett, 1998). The PCR procedure was conducted by following Packeiser *et al.* (2013) protocol with some modification. The 48 hours yeasts and yeasts-like colony were extracted using 20 µL lysis buffer (20 mM Tris-HCl; 5 mM EDTA; 400 mM NaCl; 0,3% SDS) and homogenized with 50 µL NFW (nuclease free water). Extraction was executed by boiling to 98^{0}C for 10 minutes and the supernatant was used to DNA template after microsentrifuged. Mix PCR for amplification was contained 0,5 µL 10 pmol primer respectively; 12,5 µL GoTaq Green Master Mix (Promega); 10,5 µl NFW; and 1 µL DNA template as to 25 µL final volume. PCR condition was followed 95^0 C initial denaturation and then 30 cycles of 95^0 C denaturation for 30 seconds, 55^0 C annealing for 30 seconds, and 72^0 C elongation for 60 seconds. Final elongation at 72^{0}C for 5 minutes. PCR product was visualized by electrophoresis 1% agarose then sequenced. Data of sequences were compared with the database in genBank/DDBJ/EmbL using BLASTn.

Phylogenetic tree analysis

As the taxonomic dependent analysis, sequences data were compared with known type strains. The MAFFT 7.304 (Katoh & Standley, 2013) was operated for multiple alignment then modified manually with MEGA 7.0 (Tamura *et al.*, 2013). The phylogenetic position was constructed by Neighbor-Joining method and Kimura 2-parameter model with partial deletion. Bootstraps value generated by 1000 replication to determine evolutionary distance. Finnaly, the phylogenetic tree was enhanced in iTOL (https://itol.embl.de).

RESULT AND DISCUSSION

Occurrence of yeasts and yeasts-like fungi

Isolation process of pure cultures was very challenging due to luxurious growth of filamentous fungi. Particularly, isolation of yeasts from soil was very difficult even with adding fungi growth suppressing chemical. Various techniques were carried out to obtain yeasts that has special characters for biofuel and product development as well as to verify its physiological characters to predict its roles in natural habitat. Introduction of enriched media was intended to obtain yeasts and yeasts-like fungi that have ability to ferment glucose, xylan, and xylose. Hence, it also expected as prescreening process to obtain important isolates for bioproduct development and to study its ecological roles.

Occurrence of yeasts and yeasts-like fungi were observed in all types of samples. There are 85 isolates were collected mostly from leaf liters (Table 1). Fourteen isolates from leaf liters and decay woods were shown as candidate to hydrolyze organic material such xylan and xylose. Yeasts and yeasts-like fungi have cellulolytic enzymes that able to decomposing those organic material (Souza *et al.*, 2013; Kanti, 2015). Those abilities would be important for second generation biofuel research (Morais *et al.*, 2013). In other hands, isolation techniques have crucial role to gain particular isolates. Millipore vacuum filtration was the great method to collect yeasts in leaf liters sample. Each sample from natural sources has differences in physic-chemical and biological characteristic. Hence, special isolation techniques were required to culture under artificial condition. As yeasts and yeasts-like fungi have many industrial interests, we have to develop specific technique for success isolation of targeted yeasts and yeasts-like fungi.

Identification and phylogenetic analysis

Verification of species richness and abundance of yeasts and yeasts-like fungi were conducted by molecular approach. The 85 isolates were delineated by phylogenetic tree of sequences of D1/D2 region LSU ribosomal DNA (Figure 1). That region was quite variable and difficult to align (Kurtzman & Robnett, 1998). The bootstraps value with 1000 replication was performed to solve it. As the result, the monophyletic tree was constructed with high bootstrap support (>50%) among each clades. Otherwise, the bootstrap value under 50% was healed to showed significant level among each branch. It convinced significant distinct of each other clades.

The phylogenetic tree delineates the relationship among yeasts and yeasts-like fungi with their type strains to propose certain position. Most of isolates represent to Ascomycetous 91%, Basidiomycetous (8%) and only 1% member of Zygomycetous (Figure 1).

In total, they were classified to 16 closest related species with strong bootstraps. The strong bootstraps were provided by high homology among 99-100% in similarity analysis result. Thirteen isolates were recognized to true yeasts which closely related to *Saccharomyces cerevisiae, Candida glabrata, Schwanniomyces polymorphus, Cyberlindera fabianii, Kodamaea ohmeri, Candida orthopsilopsis, Sporobolomyces poonsokiae, Kazachtania*

africana, Wickerhamomyces rabaulensis, Pichia kudriavsevii, Pseudozyma hubeiensis, Bullera formosensis, and *Kluveromyces aestuarii.* Three species were belonged to yeasts-like fungi i.e. *Aureobasidium melanogenum, Aureobasidium thailandense,* and *Umbelopsis ovata.* In other hands, there are 6 isolates were showed poor similarity analysis result. It was displayed on Figure 1 which were labeled to A and B. The tree on those labels was pruned to Figure 2 with some additional closely type strains. Isolates number Y15Kr022, Y15Kr023, Y15Kr024, Y15Kr026, and Y15Kr027 were 92% closely related to *Candida ghanaensis* CBS: 8798 (acc. number KY106464). They were clustered to strong sister clade (Figure 2A)

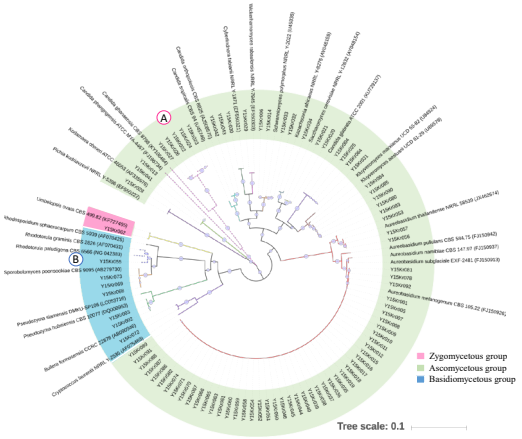

Figure 1. The Neighbor-Joining phylogenetic tree of yeast and yeast-like fungi diversity in Karimun Besar island. It showed closely related species among Karimun Island's yeasts isolates and type strains. The big grey dots mean strong bootstraps.

The high bootstraps emphasized they were quite different than closest related species and supposed to be novel taxa. Meanwhile isolate Y15Kr055 was delineated a distinct branch with *Rodotorula* clade (Figure 2B). Based on its similarity analysis result, it was 96% closely related to *Rhodotorula paludigena* CBS 6566 (acc. number NG_042383). That's mean Y15Kr055 could be recognized to novel species in *Rhodotorula* group. On these cases, further study should be conducted to ensure those claims such amplify another conserve regions, and chemotaxonomy test. Internal transcribed spacer (ITS) is one of common region that can be used to recognize them into species level (Scorzetti *et al.*, 2002).

Molecular approaches analyses are effective and efficient method for identification than phenotypic approaches. Earlier studies showed that phenotypic characters of yeasts are variable and difficult to define genera level than filamentous fungi (Takashima *et al.*, 2012). Combine many approaches able to representing dimorphism among yeasts and filamentous fungi life histories (Fell *et al.*, 2000). In some cases, phenotypic identification does not linier with genotype approach (Kurtzman & Suzuki, 2010). As well found in genera of *Aureobasidium* (Zalar *et al.*, 2008; Peterson *et al.*, 2013). For example, Rich *et al.* (2016) study reported that *A. pullulans* strains which delineated by phylogenetic tree showed they have distinct clade with each color formation and organic material production such as xylanase, feruloyl esterase, and pullulan.

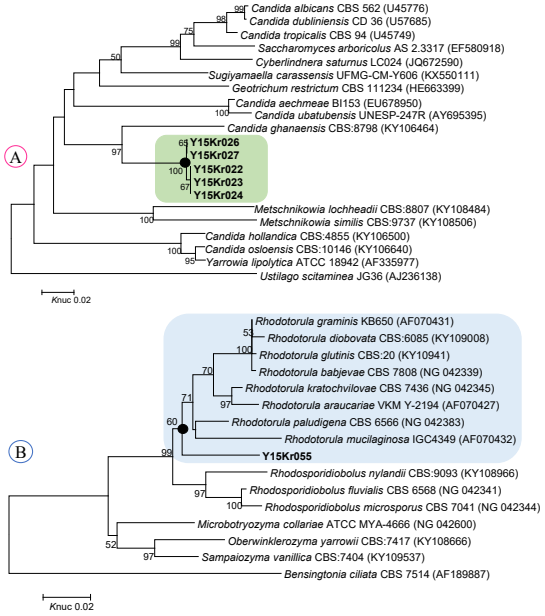

Figure 2. Pruned phylogenetic trees of Karimun Besar Island isolates were supposed to novel taxa. A) Strong sister clade of Karimun Besar isolates from *Candida ghanaensis* CBS: 8798; B) Separately branch of Y15Kr055 with *Rhodotorula* clade.

As the result, every sample showed different species abundance and species richness (Table 1). Leaf liters sample has most abundance isolates and covered into 10 closely related taxa. The *A. melanogenum* was dominant representing to 54% of total isolates particularly in leaf liters. They were common in every type of samples except soil and decay woods. *Aureobasidium* also called yeasts-like fungi or black yeasts due to they able to change color become blackish which formerly it was white, cream, light pink, or light brown colony (Chi *et al.*, 2009). Actually, they occur in usual or extreme condition such presence in absolute anaerobes substrate (Biedunkiewicz *et al.,* 2013). Also Babic *et al.* (2016) reported that yeast and yeast-like can be found in house hold appliances. Therefore, they can be persisting in

Table 1. Distribution of yeast and yeast-like species based on isolation method and samples

No.	Sample	Isolation method	Yeast species*	Yeast-like fungi species*
1	Leaf	Balistospore fall	*Candida orthopsilopsis* (2)	*A. melanogenum* (2)
		Direct plating	*Kodamaea ohmeri* (1)	*A. melanogenum* (4)
2	Leaf liters	Dilution	*Pseudozyma hubeiensis* (2), *Candida glabrata* (1), and *Schwanniomyces polymorphus* (1)	*A. melanogenum* (10)
		Millipore vacuum	*Sporobolomyces poonsookiae* (3), *Rhodotorula* sp (1), *Bullera formosensis* (1), *Schwanniomyces polymorphus* (1), *C. glabrata* (1)	*Umbelopsis ovata* (1), *A. thailandense* (3), *A. melanogenum* (22)
		Direct plating	*Kodamaea ohmeri* (1)	-
3	Soil	Enriched medium with glucose	*Kasachtania africana* (1)	-
4	Decay woods	Enriched medium with xylose	*Saccharomyces cerevisiae* (2), *Candida* sp. (3), *C. glabrata* (2), and *Wickerhamomyces rabaulensis* (1)	-
		Enriched medium with xylan	*Pichia kudriavsevii* (1), *Cyberlindnera fabianii* (1), *Schwanniomyces polymorphus* (2), and *Candida* sp. (2).	-
5	Sediments	Enriched medium with glucose	*Kluyveromyces aestuarii* (5)	*A. melanogenum* (3)
		Millipore vacuum	-	*A. melanogenum* (5)

* refer to number of isolates

many substrates with specific roles (Bhadra *et al.,* 2008).

Certain taxonomic position serves the economic potency information that can be used to initializing research or industrial purposes. Earlier study showed that several species in this study have been used for many bioindustry interests. For example, *A. melanogenum* isolated from the mangrove ecosystem able to produce biopolymer pullulan (Ma *et al.*, 2015). The basidiomycetous yeast, *P. hubeiensis* has ability to produce mannosylerythritol lipids (MEL), the biosurfactant for cosmetic (Konishi *et al.*, 2011). The phytase producing yeast such *K. ohmeri* is important for food and livestock industry (Li *et al.*, 2008). In energy sector, *A. melanogenum* also able to produces a large amount of long chain alkane oil that can be used for biodiesel production (Liu *et al.*, 2014). Fuel from bioethanol can be conducted to the wild-type strains of *S. cerevisiae* that ferment glucose, mannose, fructose, and galactose from plant hydrolysate by different pathway (Maris *et al.*, 2006). Meanwhile, *P. kudriavsevii* has significance ethanol production during high temperature fermentation (Yuangsaard *et al.*, 2013). Other important Ascomycetous yeast, *Schwanniomyces polymorphus was able to* xylose-fermenting and/or produce xylanase (Morais *et al.*, 2013).

The yeasts and yeasts-like fungi from natural resources of Karimun Island are diverse with many economic and industrial values. The good diversity data facilitates the availability of important yeasts and yeast-like fungi in Indonesia especially in Indonesian public culture collection (InaCC) that we able to access freely for any purposes. In other hands, some strains were taxonomically visible to enhance the genetic database for novel taxa from Indonesian resources. In further, they should be reanalysis for describing their new taxonomic position by using other conserve genes, chemotaxonomy and physiology test.

CONCLUSION

Several taxonomically and economically important yeasts and yeasts-like fungi were isolated from Karimun Besar Island, Indonesia with various culture dependent methods. They were mostly belonging to Ascomycetous yeast which *Aureobasidium melanogenum* found abundance particularly in leaf liters. Several isolates with taxonomic analysis on D1/D1 large subunit ribosomal DNA sequences supposed to candidate for novel taxa that should to reanalysis in further.

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