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THESIS

DIVERSITY OF PHYLLOPLANE YEASTS AND ELUCIDATION OF THEIR BIOCONTROL MECHANISMS ON PLANT PATHOGENIC FUNGI

RUNGLUK KAEWWICHIAN

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Microbiology)
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Rungluk Kaewwichian 2013: Diversity of Phylloplane Yeasts and Elucidation of Their Biocontrol Mechanisms on Plant Pathogenic Fungi. Doctor of Philosophy (Microbiology), Major Field: Microbiology, Department of Microbiology. Thesis Advisor: Professor Savitree Limtong, Dr.Eng. 241 pages.

Eighty five samples of rice leaves, 65 samples of corn leaves and 94 samples of sugarcane leaves were collected from 28 districts in nine provinces of Thailand. Yeast isolation from phylloplane was carried out by an enrichment technique. A total of 441 yeast strains were collected from the 244 leaf samples including 156 yeast strains from rice leaves, 126 yeast strains from corn leaves and 159 yeast strains from sugarcane leaves. On the basis of the D1/D2 domain of the large subunit rRNA gene sequences analysis and phylogeny 155 yeast strains from rice leaves were identified as 32 known species in 18 genera belonging to Ascomycota i.e. *Candida*, *Clavispora*, *Cyberlindnera*, *Debaryomyces*, *Kodamaea*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Wickerhamomyces*, *Yamadazyma* and *Yarrowia*, and Basidiomycota i.e. *Cryptococcus*, *Pseudozyma*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon*, 123 strains from corn leaves were identified as 39 known species in 17 genera belonging to Ascomycota i.e. *Candida*, *Cyberlindnera*, *Geotrichum*, *Hanseniaspora*, *Kluyveromyces*, *Kodamaea*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora*, *Wickerhamomyces* and *Yamadazyma*, and Basidiomycota i.e. *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus*, and 146 strains from sugarcane leaves were identified as 36 known species in 19 genera belonging to Ascomycota i.e. *Candida*, *Cyberlindnera*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Lachancea*, *Lodderomyces*, *Metschnikowia*, *Meyerozyma*, *Millerozyma*, *Pichia*, *Torulaspora* and *Wickerhamomyces*, and Basidiomycota i.e. *Hannaella*, *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces*. Eight strains were similar to undescribed species. Moreover, nine strains were found to represent six novel species and they were proposed on the basis of polyphasic taxonomy as *Metschnikowia saccharicola* sp. nov., *Metschnikowia lopburiensis* sp. nov., *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov., *Wicherhamomyces siamensis* sp. nov. and *Nakazawaea siamensis* sp. nov. The prevalent species were *Rhodotorula taiwanensis*, *Meyerozyma caribbica* and *Candida tropicalis* but with low frequencies, 14.3, 12.0 and 10.9% of total strains, respectively. Individual leaf sample was generally accommodated by two yeast species (56.5, 60.0 and 48.9% of total sample of rice, corn and sugarcane phylloplane, respectively).

One hundred and forty eight strains in 40 species, four strains of an undescribed species and two strains of a novel species showed the capability to produce IAA cultivated in yeast extract peptone dextrose broth supplemented with 0.1% L-tryptophan. *Rhodosporidium fluviale* DMKU-RK253 produced the highest IAA concentration of 417.8 mg/L.

Only one strain namely *Torulaspora globosa* DMKU-RK547 significantly inhibited the three pathogenic fungi of rice and its biocontrol activity seem to be based on the production of siderophore and volatile compounds. The nutrient concentrations had significant different effect on fungal growth inhibition by *T. globosa* DMKU-RK547 and its living cells are necessary for the biological control activity.

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Student's signature Thesis Advisor's signature

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April 2013

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DIVERSITY OF PHYLLOPLANE YEASTS AND ELUCIDATION OF THEIR BIOCONTROL MECHANISMS ON PLANT PATHOGENIC FUNGI

INTRODUCTION

The external surface of plant leaves which is usually referred to as the phylloplane or phyllosphere has been recognized as an important habitat for microorganisms (Phaff and Starmer, 1987; Fonseca and Inácio, 2006). The phylloplane has been found to be colonized by members of both basidiomycete and ascomycete yeasts (Nakase *et al*., 2001; Fonseca and Inácio, 2006; Glushakova *et al*., 2007; Slavikova *et al*., 2009; Landell *et al*., 2010). Although most common phylloplane yeasts are yeast species in basidiomycete genera such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo *et al*., 1998; Nakase *et al*., 2001; Fonseca and Inácio, 2006; Slavikova *et al*., 2009; Glushakova and Chernov, 2010), various ascomycete yeast species have also been detected on the phylloplane i.e. *Debaryomyces hansenii*, *Hanseniaspora uvarum, Kazachstania barnetii*, *Metschnikowia lopburiensis, M. pulcherrima, M. saccharicola, Pichia membranifaciens, Saccharomyces cerevisiae* and various *Candida* species including *Candida aechmeae*, *C. olephila, C. chumphonensis, C. mattranensis* and *C. vrieseae* (Glushakova *et al*., 2007; Slavikova *et al*., 2009; Glushakova and Chernov, 2010; Landell *et al*., 2010; Koowadjanakul *et al*., 2011; Kaewwichian *et al*., 2012).

Thailand is agricultural country because climatic and soil conditions permit the cultivation of a wide range of crops. Rice is the major crop while corn and sugarcane are the other crops regularly cultivate. One major cultivation problem of these crops is destructive diseases cause by various plant pathogenic fungi. To control of these fungal diseases chemical fungicides are usually applied. However, using large amount of fungicides may harmful to the farmers and sometime cause negative impacts on plants. Moreover, public concerns about fungicide residues and development of fungicide resistant strains of pathogens have suggested searching for environmental friendly means for controlling of fungal disease of these crops. Biocontrol is considered to be one of the

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environmental friendly approaches for fungal plant diseases control. In recent years more interest has paid to utilize microbial antagonists to control plant pathogenic fungi.

Yeast is one of the most important microorganisms for industrial application. However, a few attempts are focusing on agricultural application especially using as biocontrol agents, plant growth promoters and biopreservation agents. Various yeast species have been found to be the effective biocontrol agents of plant and postharvest pathogenic fungi. Yeasts which have been studied for their efficiency as biocontrol agents were derived from various habitats, especially from plants and habitats related to plants, for example: fruits, inner tissue of medicinal plant, phylloplane and rhizosphere (Calvente *et al.*, 1999; El Mehalawy, 2004; Zhang *et al.*, 2009; Rosa *et al.*, 2010). However, some other biocontrol yeast species could be obtained from the other habitats such as *Rhodosporidium paludigenum* was derived from marine habitat (Wang *et al.*, 2010), *Rhodosporidium toruloides* and *Rhodosporidium dacryoidum* were isolated from seawater and *Cryptococcus humicola* was isolated from soil (Filonow *et al.*, 1996).

In Thailand there were some reports on using yeasts such as, *Candida guilliermondii, Candida tropicalis, Candida utilis, Debaryomyces hansenii, Hensenula saturnus* and *Pichia membranaefaciens* (Sourha, 1997; Sangwanich, 2004; Niamjang, 2006) as biocontrol agents of postharvest pathogenic fungi. However, most yeasts used in the studies were obtained from culture collections. Only a few works on isolation and selection of biocontrol yeasts from natural habitats in Thailand was carried out in Thailand.

Due to only a few studies on phylloplane yeasts biodiversity, isolation and selection of biocontrol yeasts in Thailand were carried out, therefore this work is proposed. The aims of this work are to investigate diversity of yeasts on phylloplane of rice, corn and sugarcane, determination of their potential to be biocontrol agents of rice, corn and sugarcane pathogenic fungi and study on mechanisms for controlling of plant pathogenic fungi of the potent biocontrol yeast strains.

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OBJECTIVES

1. To investigate diversity of yeasts on phylloplane of rice, corn and sugarcane by enrichment isolation and identification on the basis of molecular taxonomy and phylogenetic analysis.

2. To explore and characterize of new yeast species obtained from phylloplane of rice, corn and sugarcane by polyphasic taxonomy.

3. To determine the Indole-3-acetic acid production by phylloplane yeast

4. To determine the ability to be biocontrol agent of rice, corn and sugarcane pathogenic fungi of phylloplane yeasts.

5. To study mechanisms for controlling of plant pathogenic fungi of the biocontrol yeast strain.

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LITERATURE REVIEW

1. Definition, Classification and Nomenclature of Yeasts

1.1 Definition of Yeasts

The English word yeast and its equivalents in many other languages are based on words meaning "foam" and "to rise" because of direct references to the fermentation processes that produce beer and bread. For this reason, yeasts are often thought of as fermentative ascomycete fungi similar to *Saccharomyces cerevisiae*. In fact, it is not uncommon in some areas of molecular biology to treat the words "yeast" and "*Saccharomyces*" as synonyms. From the discovery of the nature of yeasts found that some taxa are basidiomycetes has widened perception. As a consequence, yeasts as fungi that asexually reproduce by budding or fission, which results in growth that is comprised mainly of single cells. Therefore, in summary of yeast is defined as ascomycetes or basidiomycetes that generally characterized by budding or fission as the primary means of asexual reproduction, and have sexual states that are not enclosed in fruiting bodies (Kurtzman *et al*., 2011b). Yeast cells can be globose, subglobose, ellipsoid, ovoid, obovoid, cylindrical, botuliform, bacilliform, elongate, apiculate, ogival, lunate, or triangular. In the ascomycetous yeasts, the site of meiosis is the ascus, where the haploid generation of ascospores is formed. In the basidiomycetous yeasts, reduction division is restricted to either the teliospore, or the basidium on which the haploid basidiospores are formed externally (Kurtzman *et al*., 2011b).

1.2 Classification of Yeasts

On the basis of classification scheme as described by using a famous monograph entitled "The Yeasts, a Taxonomic Study" fifth edition (Kurtzman *et al*., 2011a). In this edition, yeasts were classified into the Phylum Ascomycota and Phylum Basidiomycota. The outline of ascomycetous yeast classification that is based on the classification of the Mycota presented by Hibbett *et al*. (2007), and on the multigene analyses reported by Kurtzman and Robnett (2003, 2007, 2010), Kurtzman and Suzuki

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(2010) and Kurtzman *et al*., (2007, 2008) are distributed in two Subphylum; Taphrinomycotina and Saccharomycotina. The classification system of ascomycetous yeasts is shown in Table 1. The basidiomycetous yeasts are classified to be three Subphylum namely Pucciniomycotina, Agaricomycotina and Ustilaginomycotina (Boekhout *et al*., 2011) is shown in Table 2.

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Table 1 Classification of ascomycetous yeasts

Class	Order	Family	Genus
Subphylum Taphrinomycotina			
Neolectomycetes	Neolectales	Neolectaceae	*Neolecta* (T)
Pneumocystidomycetes	Pneumocystidales	Pneumocystidaceae	*Pneumocystis* (T)
Schizosaccharomycetes	Schizosaccharomycetales	Schizosaccharomycetaceae	*Schizosaccharomyces* (T)
Taphrinomycetes	Taphrinales	Protomycetaceae	*Burenia* (T)
			Protomyces (T)
			Protomycopsis (T)
			Saitoella (A)
			Taphridium (T)
			Volkartia (T)
		Taphrinaceae	*Lalaria* (A)
Subphylum Saccharomycotina			
Saccharomycetes	Saccharomycetales	Ascoideaceae	*Ascoidea* (T)
		Cephaloascaceae	*Cephaloascus* (T)

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Table 1 (Continued)

Class	Order	Family	Genus
		Debaryomycetaceae	*Debaryomyces* (T)
			Kurtzmaniella (T)
			Lodderomyces (T)
			Meyerozyma (T)
			Millerozyma (T)
			Priceomyces (T)
			Scheffersomyces (T)
			Schwanniomyces (T)
			Spathaspora (T)
			Wickerhamia (T)
			Yamadazyma (T)
		Dipodascacea	*Dipodascus* (T)
			Galactomyces (T)
			Geotrichum (A)
		Endomycetaceae	*Endomyces* (T)
			Helicogonium (T)
			Phialoascus (T)

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Table 1 (Continued)

Class	Order	Family	Genus
		Lipomycetaceae	*Dipodascopsis* (T)
			Lipomyces (T)
			Myxozyma (A)
		Metschnikowiaceae	*Aciculoconidium* (A)
			Clavispora (T)
			Hyphopichia (T)
			Kodamaea (T)
			Metschnikowia (T)
		Phaffomycetaceae	*Komagataella* (T)
			Phaffomyces (T)
		Pichiaceae	*Brettanomyces* (A)
			Dekkera (T)
			Kregervanrija (T)
			Pichia (T)
			Saturnispora (T)

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Table 1 (Continued)

Class	Order	Family	Genus
		Saccharomycetaceae	*Cyniclomyces* (T)
			Eremothecium (T)
			Kazachstania (T)
			Kluyveromyces (T)
			Lachancea (T)
			Nakaseomyces (T)
			Naumovozyma (T)
			Saccharomyces (T)
			Tetrapisispora (T)
			Torulaspora (T)
			Vanderwaltozyma (T)
			Zygosaccharomyces (T)
			Zygotorulaspora (T)
		Saccharomycodaceae	*Hanseniaspora* (T)
			Kloeckera (A)
			Saccharomycodes (T)
		Saccharomycopsidaceae	*Saccharomycopsis* (T)

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Table 1 (Continued)

Class	Order	Family	Genus
		Trichomonascaceae	*Blastobotrys* (A)
			Sugiyamaella (T)
			Trichomonascus (T)
			Wickerhamiella (T)
			Zygoascus (T)
		Wickerhamomycetaceae	*Barnettozyma* (T)
			Lindnera (*Cyberlindnera*) (T)
			Starmera (T)
			Wickerhamomyces (T)
	Saccharomycetales		*Ambrosiozyma* (T)
	(incertae sedis)		*Ascobotryozyma* (T)
			Babjeviella (T)
			Botryozyma (A)
			Candida (A)
			Citeromyces (T)
			Coccidiascus (T)
			Kuraishia (T)
			Macrorhabdus (A)

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Table 1 (Continued)

Class	Order	Family	Genus
			Magnusiomyces (T)
			Nadsonia (T)
			Nakazawaea (T)
			Ogataea (T)
			Pachysolen (T)
			Peterozyma (T)
			Saprochaete (A)
			Schizoblastosporion (A)
			Sporopachydermia (T)
			Starmerella (T)
			Trigonopsis (A)
			Wickerhamia (T)
			Yarrowia (T)

(A) = anamorphic genus, (T) = teleomorphic genus

Source: Kurtzman *et al*. (2011a)

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Table 2 Classification of basidiomycetous yeasts

Class	Order	Family	Genus
Subphylum Pucciniomycotina			
Agaricostilbomycetes	Agaricostilbales		*Agaricostilbum* (T)
			Bensingtonia (pro parte) (A)
			Chionosphaera (T)
			Kondoa (T)
			Kurtzmanomyces (A)
			Sterigmatomyces (A)
	Spiculogloeales		*Sporobolomyces* (pro parte) (A)
Cystobasidiomycetes	Cystobasidiales		*Cystobasidium* (T)
			Occultifur (T)
			Rhodotorula (pro parte) (A)
	Erythrobasidiales		*Bannoa* (A)
			Erythrobasidium (A)
			Rhodotorula (pro parte) (A)
			Sporobolomyces (pro parte) (A)
	Naohideales		*Naohidea* (T)
	Incertae sedis		*Cyrenella* (A)
			Rhodotorula (pro parte) (A)
			Sakaguchia (T)

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Table 2 (Continued)

Class	Order	Family	Genus
			Sporobolomyces (pro parte) (A)
Microbotryomycetes	Leucosporidiales		*Leucosporidiella* (A)
			Leucosporidium (pro parte) (T)
			Mastigobasidium (T)
	Microbotryales		*Rhodotorula hordea* (A)
	Sporidiobolales		*Rhodosporidium* (T)
			Rhodotorula (pro parte) (A)
			Sporobolomyces (pro parte) (A)
			Sporidiobolus (T)
	Incertae sedis		*Bensingtonia yamatoana* (A)
			Colacogloea (T)
			Curvibasidium (T)
			Kriegeria (T)
			Leucosporidium (pro parte) (T)
			Rhodotorula (pro parte) (A)
			Sporobolomyces (pro parte) (A)
Mixiomycetes	Mixiales		*Mixia*
Subphylum Agaricomycotina			
Tremellomycetes	Cystofilobasidiales		*Cryptococcus* (pro parte) (A)

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Table 2 (Continued)

Class	Order	Family	Genus
			Cystofilobasidium (T)
			Guehomyces (A)
			Itersonilia (A)
			Mrakia (T)
			Mrakiella (A)
			Tausonia (A)
			Xanthophyllomyces (T)
			Udeniomyces (A)
	Filobasidiales		*Bullera* (pro parte) (A)
			Cryptococcus (pro parte) (A)
			Filobasidium (T)
	Tremellales		*Auriculibuller* (T)
			Bullera (pro parte) (A)
			Bulleribasidium (T)
			Bulleromyces (T)
			Cryptococcus (pro parte) (A)
			Cuniculitrema (T)
			Derxomyces (A)
			Dioszegia (A)

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Table 2 (Continued)

Class	Order	Family	Genus
			Fellomyces (A)
			Fibulobasidium (T)
			Filobasidiella (T)
			Kockovaella (A)
			Kwoniella (T)
			Papiliotrema (T)
			Sirobasidium (T)
			Tremella (T)
			Trimorphomyces (T)
	Trichosporonales		*Bullera* (pro parte) (A)
			Cryptococcus (pro parte) (A)
			Cryptotrichosporon (A)
			Trichosporon (A)
	Incertae sedis		*Cryptococcus* (pro parte) (A)
			Holtermannia (T)
Subphylum Ustilaginomycotina			
Exobasidiomycetes	Entylomatales		*Tilletiopsis* (pro parte) (A)
	Exobasidiales		*Acaromyces* (A)

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Table 2 (Continued)

Class	Order	Family	Genus
			Meira (A)
	Malasseziales		*Malassezia* (A)
	Georgefischerales		*Tilletiaria* (T)
			Tilletiopsis (pro parte) (A)
	Microstromatales		*Sympodiomycopsis* (A)
			Rhodotorula (pro parte) (A)
	Incertae sedis		*Tilletiopsis albescens*
Ustilaginomycetes	Ustilaginales		*Farysizyma* (A)
			Pseudozyma (A)

(A) = anamorphic genus, (T) = teleomorphic genus, (pro parte) is placed after the name of a synonym to indicate that some, but not all, individuals identified as this species are synonymous with the accepted species.

Source: Kurtzman *et al*. (2011a)

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1.3 Nomenclature of Yeasts

Rules for taxonomy of the yeasts and other fungi fall under of the International Code of Nomenclature of algae, fungi, and plants (ICN). The Nomenclature Section held just before the 18th International Botanical Congress in Melbourne, Australia in July 2011 produced several changes to the way scientific name of new plants, algae, and fungi. For the first time the Code will allow for the electronic publication of names of new taxa because the effective publication being a fundamental requirement of the *Code* for acceptance of any nomenclatural act. As an alternative, publication online in Portable Document Format (PDF) in a publication with an International Standard Serial Number (ISSN) or International Standard Book Number (ISBN) will be permitted. In attempt to make the publication of new names more correct and efficient, the requirement for a Latin diagnosis or description was changed to allow either English or Latin for the publication of a new name. Both of these latter changes will take effect on 1 January 2012. The nomenclatural rules for fungi will see several important changes, the most important of which is probably the adoption of the principle of "one fungus, one name." (Miller *et al*., 2011). Since 2004, the online database namely MycoBank has become increasingly used by mycologists to register new fungal names and associated data, such as descriptions and illustrations. On registration, MycoBank issues a unique number which can be cited in the publication where the name appears. This number is also used by the nomenclatural database Index Fungorum and serves as a Life Science Identifier (LSID). The Nomenclature Section in Melbourne approved a new rule in the *Code* whereby, on or after 1 January 2013, the publication of a new fungal name must include a citation of an identifier issued by a recognized repository in order to be validly published i.e. to have any status under the *Code* (McNeill and Turland, 2011). However, two other repositories had been developed in anticipation of the need for official repositories. These were Index Fungorum and Fungal Names. The present, MycoBank, Index Fungorum, and Fungal Names recommended as official nomenclatural repositories for 2013 (Redhead and Norvell, 2012).

From gene sequence comparisons, strains that represent new species are easily recognized. However, some would argue against description of a new species based on a single strain. The argument is that a single strain does not reflect the genetic variation that

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might be found in a species, and that little can be learned of the ecology of a species when only a single strain is available. Nevertheless, nearly one-third of described yeast species are based on a single strain. If these species had not been described, much less would be known about the phylogenetic diversity of the yeasts. From the point of understanding diversity among the yeasts, description of single strain species is to be supported, although descriptions based on multiple strains are preferred. Further, it is recommended that the description should be based on multigene analysis to lessen the possibility that the strain represents a hybrid of known species (Kurtzman *et al*., 2011b).

2. Diversity and Ecology of Yeasts on Phylloplane

2.1 Nutrients in phylloplane

The external surface of plant leaves is usually referred to as the phylloplane or phyllosphere has been recognized as an important habitat for microorganisms (Phaff and Starmer, 1987; Fonseca and Inácio, 2006). On the phylloplane, nutrients are made available to microbes either endogenously from plant exudates or exogenously from compounds found in materials that land on the plant surface (Andrews and Harris, 2000). Molecules leaching from the phylloplane include a variety of organic and inorganic compounds, such as sugars, organic acids, amino acids, methanol and various salts. The abundance of such nutrients varies with plant species, leaf age and growing conditions. Exogenous nutrient sources, such as aphid honeydew and pollen, have been associated with dramatic increases in the microbial carrying capacities of some leaves (Fonseca and Inácio, 2006). Typically, there is a higher carbon (C) to nitrogen (N) ratio encountered on leaf surfaces (Fonseca and Inácio, 2006). While inorganic nutrients are leached to the surface, organic substances which are mostly simple sugars like glucose, fructose, and sucrose, account for the majority of nutrients exuded from leaf tissues (Tukey, 1971).

Some yeasts isolated from various natural environments, including the phylloplane, are tolerant of low nutrient concentrations in general and nitrogen in particular (Kimura *et al*., 1998). All yeasts require a source of carbon and nitrogen for maintenance and growth, whereas mineral and vitamin needs vary considerably among species (Deak, 2006). Rodrigues *et al.* (2006) reported that yeasts are able to utilize

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various carbon sources for development but preferentially metabolize simple sugars and polysaccharides and other macromolecules like proteins and lipids. Yeasts also metabolize inorganic nitrogen (i.e. ammonium salts) and organic nitrogen sources (i.e. amino acids and urea) for growth. However, in the common absence of such significant but ephemeral nutrient sources, plant leaves are still usually colonized by large microbial populations. Nutrients released to the leaf surface should thus be adequate to support microbial growth and depletion of sugars on the phylloplane by bacteria and yeasts has been demonstrated (Dik *et al*., 1992). These data suggest that at least certain populations of phylloplane microbes are nutrient-limited, which would have an impact on the overall carrying capacity of the leaf. Carbon compounds alone or both carbon and nitrogen compound were shown to be limiting factors for bacteria and yeast populations on leaves. Moreover, probably the plant surfaces are enriched in methanol produced inside the leaves and emitted primarily through stomata (Fall and Benson, 1996). Nemecek-Marshall *et al*. (1995) suggested that the majority (94-97%) of methanol exited the leaves of four investigated plant species via stomata. It is likely that leaf methanol supports an abundant population of epiphytic bacterial methylotrophes (Fall and Benson, 1996). The known yeast species, which are able to grow considerably with methanol as a sole carbon source, belong to ascomycetous yeast species (Kurtzman and Fell, 1998; Barnett *et al*., 2000).

2.2 Diversity of phylloplane yeast

The phylloplane constitutes a sizeable terrestrial habitat populated by numerous microbial species. Bacteria are often the earliest colonists of young leaves, possibly the most abundant. Fungi are also active phylloplane colonizers, most of which are yeasts or rapidly sporulating hyphomycetes such as *Aureobasidium pullulans* (Andrews and Harris, 2000). Yeast isolated from various phylloplane, are tolerant of low nutrient concentrations in general (Kimura *et al.*, 1998). The phylloplane has been found to be colonized by members of both basidiomycetous and ascomycetous yeasts (Nakase *et al*., 2001; Fonseca and Inacio, 2006; Glushakova *et al*., 2007; Slavikova *et al*., 2009; Landell *et al*., 2010). Although most common phylloplane yeasts have been reported so far are yeast species in basidiomycetes genera, various ascomycetes species have also been detected on the phylloplane.

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Jager *et al.* (2001) determined bacteria, filamentous fungi, and yeasts on the mango phylloplane by isolation using indirect leaf impression, leaf washing and dilution plating methods. The phylloplane microbial community was qualitatively and quantitatively related to leaf age, position in the tree canopy, seasonality, and chemical spraying. Filamentous fungi and yeasts were more abundant during winter and spring, whereas bacterial population densities increased during autumn. Community density and diversity increased progressively with leaf age. Gram-positive bacteria exceeded gram negative bacteria. The most common fungal genera isolated were *Cladosporium* and *Alternaria.* Yeasts prevalent in the mango phylloplane were of the genera *Cryptococcus* and *Sporobolomyces.*

Whipps *et al.* (2008) reviewed the diversity of phyllosphere microbial populations. The microbial communities of the phyllosphere are diverse, supporting numerous genera of bacteria, filamentous fungi, yeasts, algae and in some situations protozoans and nematodes. Epiphytic filamentous fungi, yeasts and bacteria may arrive on the leaf surface through insect-, atmosphere-, seed- or even animal-borne sources. Tree buds, seeds of annual plants and the waste from previous crops are likely to be the most important sources for the colonization of new plants and leaves as they are a major source of bacteria already adapted to the phylloplane. Yeasts are the major epiphytic fungal group in the phylloplane with filamentous fungi largely occurring as dormant spores rather than active mycelia except on older leaves. Culturable yeast populations can range between 10 and 10^{10} CFU g^{-1} leaf. The diversity of culturable yeasts appears to be mostly limited to the genera *Cryptococcus*, *Sporobolomyces* and *Rhodotorula*.

Pozo *et al.* (2011) evaluated yeast species richness at both the plant community and plant species levels in the mountainous forest area in southern Spain, and also explores possible correlations between the incidence of different yeast species in nectar and their reported tolerance to high sugar concentrations, and between yeast diversity and pollinator composition. Yeast species occurring in a total of 128 field collected nectar samples from 24 plant species were identified by sequencing the D1/D2 region of the large subunit rRNA gene, and rarefaction-based analyses were used to estimate yeast species richness at the plant community and plant species levels, using nectar drops as elemental sampling units. The results shown that 216 yeast strains

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belonging to 12 species, consist of both ascomycetous and basidiomycetous yeasts. *Metschnikowia reukaufii* and *Metschnikowia gruessii* were found 84.7% of the total strains. The other yeast included species in the genera *Rhodotorula*, *Cryptococcus*, *Sporobolomyces* and the black yeasts namely *A. pullulans* and *Lecythophora hoffmannii*. The richness accumulation curves were quite similar for the nectar drop and plant species approaches, but the two approaches yielded different expected richness estimates. Expected richness was higher for plant species-based than for nectar drop-based analyses, showing that the coverage of nectar yeast species occurring in the region would be improved by sampling additional host plant species.

Limtong and Koowadjanakul (2012) determined yeasts isolated from phylloplane of various plant species collected in Thailand. In total 114 yeast strains and 10 strains of a yeast-like fungus were identified on the basis of the D1/D2 region of the LSU rRNA gene sequence similarity, 98 yeast strains were identified to be of 36 yeast species in 18 genera belonging to Ascomycota namely *Candida*, *Clavispora, Cyberlindnera, Debaryomyces, Hanseniaspora, Hyphopichia, Kazachstania, Kluyveromyces, Kodamaea, Lachancea, Metschnikowia, Meyrozyma, Pichia, Starmerella, Torulaspora* and *Wickerhamomyces* and to Basidiomycota namely *Sporidiobolus* and *Trichosporon.* Three strains were found to represent two novels *Candida* species namely *C. sirachaensis* and *C. sakaeoensis*. Ten strains of yeast-like fungus were identified as *Aureobasidium pullulans* of the phylum Ascomycota. The prevalent species was *C. tropicalis* with a low frequency of isolation (14.3 %). Yeast species which were reported to isolate from phylloplane in various country were shown in Table 3.

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Table 3 Yeasts have been reported to isolate from phylloplane

Genus	Species	Reference
Basidiomycetous yeast		
Bullerra	*B. arundinariae*	Fungsin *et al*., 2002
	B. begoniae	Nakase *et al*., 2004
	B. panici	Fungsin *et al*., 2003a
	B. penniseticola	Takashima and Nakase, 1998
	B. sakaeratica	Fungsin *et al*., 2003b
	B. setariae	Nakase *et al*., 2004
	B. siamensis	Fungsin *et al*., 2003a
Cryptococcus	*C. aerius*	Pozo *et al*., 2011
	C. amylolyticus	Inácio *et al*., 2005
	C. armeniacus	Inácio *et al*., 2005
	C. cistialbidi	Inácio *et al*., 2005
	C. diffluens	Pozo *et al*., 2011
	C. filicatus	Golubev and Samaio, 2009
	C. laurentii	Slavikova *et al*., 2009
	C. tibetensis	Wang *et al*., 2007
	C. victoriae	Pozo *et al*., 2011
	C. uzbekistanensis	Pozo *et al*., 2011
Derxomyces	*D. amylogenes*	Liu *et al*., 2011
	D. bambusicola	Liu *et al*., 2011
	D. corylopsis	Liu *et al*., 2011
Diozegia	*Di. athyri*	Wang *et al*., 2008a
	Di. buhagiarii	Inácio *et al*., 2005
	Di. butyracea	Wang *et al*., 2008a
	Di. catarinonii	Inácio *et al*., 2005
	Di. fristingensis	Inácio *et al*., 2005
	Di. takashimae	Inácio *et al*., 2005
	Di. xingshanensis	Wang *et al*., 2008a
Kockovaella	*K. sacchari*	Takashima and Nakase, 1998
Laphrina	*L. arrabidae*	Inácio *et al*., 2004
	L. carpini	Inácio *et al*., 2004

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Table 3 (Continued)

Genus	Species	Reference
	L. inositophila	Inácio *et al*., 2004
	L. kurtzamanii	Inácio *et al*., 2004
	L. veronaeram-bellii	Inácio *et al*., 2004
Pseudozyma	*P. graminicola*	Golubev *et al*., 2007
	P. hubeiensis	Wang *et al*., 2006
	P. shanxiensis	Wang *et al*., 2006
Rhodotorula	*R. colostri*	Pozo *et al*., 2011
	R. glutinis	Slavikova *et al*., 2009
	R. graminis	Fonsceca and Inácio, 2006
	R. minuta	Fonsceca and Inácio, 2006
	R. mucilaginosa	Fonsceca and Inácio, 2006; Pozo *et al*., 2011
Sporidiobolus	*S. ruineniae*	Limtong and Koowadjanakul, 2012
Sporobolomyces	*Sp. blumeae*	Takashima and Nakase, 2000
	Sp. fushanensis	Nakase *et al*., 2005
	Sp. nylandii	Takashima and Nakase, 2000
	Sp. poonsookiae	Takashima and Nakase, 2000
	Sp. roseus	Fonsceca and Inácio, 2006; Pozo *et al*., 2011
	Sp. ruberrimus	Fonsceca and Inácio, 2006
	Sp. vermiculatus	Takashima and Nakase, 2000
Tilletiopsis	*T. derxii*	Takashima and Nakase, 2001
	T. oryzicola	Takashima and Nakase, 2001
	T. penniseti	Takashima and Nakase, 2001
Trichosporon	*T. asahii*	Limtong and Koowadjanakul, 2012
Ascomycetous yeast		
Candida	*C. aechmeae*	Landell *et al*., 2010
	C. amphixiae	Limtong and Koowadjanakul, 2012
	C. apicola	Limtong and Koowadjanakul, 2012
	C. chumphonensis	Koowadjanakul *et al*., 2011
	C. etchellsii	Limtong and Koowadjanakul, 2012

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Table 3 (Continued)

Genus	Species	Reference
	C. glabrata	Limtong and Koowadjanakul, 2012
	C. jaroonii	Limtong and Koowadjanakul, 2012
	C. maltosa	Limtong and Koowadjanakul, 2012
	C. mattranensis	Koowadjanakul *et al*., 2011
	C. metapsilosis	Limtong and Koowadjanakul, 2012
	C. nivariensis	Limtong and Koowadjanakul, 2012
	C. olephila	Glushakova *et al.*, 2007
	C. parapsilosis	Limtong and Koowadjanakul, 2012
	C. potacharoeniae	Limtong and Koowadjanakul, 2012
	C. rugosa	Limtong and Koowadjanakul, 2012
	C. sakaeoensis	Limtong *et al*., 2012
	C. sirachaensis	Limtong *et al*., 2012
	C. sorboxylosa	Limtong and Koowadjanakul, 2012
	C. stigmatis	Limtong and Koowadjanakul, 2012
	C. tropicalis	Limtong and Koowadjanakul, 2012
	C. trypodendroni	Limtong and Koowadjanakul, 2012
	C. vrieseae	Landell *et al*., 2010
Clavispora	*C. lusitaniae*	Limtong and Koowadjanakul, 2012
Cyberlindnera	*C. rhodanensis*	Limtong and Koowadjanakul, 2012
Debaryomyces	*D. hansenii*	Fonseca and Inacio, 2006
	D. nepalensis	Limtong and Koowadjanakul, 2012
Hanseniaspora	*H. guilliermondii*	Limtong and Koowadjanakul, 2012
	H. opuntiae	Limtong and Koowadjanakul, 2012
	H. thailandica	Limtong and Koowadjanakul, 2012
	H. uvarum	Slavikova *et al*., 2009
Hyphopichia	*H. burtonii*	Limtong and Koowadjanakul, 2012
Kazachstania	*K. barnetii*	Slavikova *et al*., 2009
	K. siamensis	Limtong and Koowadjanakul, 2012
Kluyveromyces	*K. marxianus*	Limtong and Koowadjanakul, 2012
Kodamaea	*K. ohmeri*	Limtong and Koowadjanakul, 2012
Lachancea	*L. thermotolerans*	Limtong and Koowadjanakul, 2012
Metschnikowia	*M. gruessii*	Pozo *et al.*, 2011

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Table 3 (Continued)

Genus	Species	Reference
	M. koreensis	Limtong and Koowadjanakul, 2012
	M. lopburiensis	Kaewwichian *et al*., 2012
	M. pulcherrima	Fonseca and Inacio, 2006
	M. reukaufii	Pozo *et al*., 2011
	M. saccharicola	Kaewwichian *et al*., 2012
Meyrozyma	*M. guilliermondii*	Limtong and Koowadjanakul, 2012
Ogataea	*O. allantospora*	Peter *et al*., 2007
	O. cecidiorum	Glushakova and Chernov, 2010
	O. nitratoaversa	Peter *et al*., 2008
	O. phyllophila	Koowadjanakul *et al*., 2011
Pichia	*P. anomala*	Slavikova *et al*., 2009
	P. kudriavzevii	Limtong and Koowadjanakul, 2012
	P. manshurica	Limtong and Koowadjanakul, 2012
	P. membranifaciens	Slavikova *et al*., 2009
Saccharomyces	*S. cerevisiae*	Slavikova *et al*., 2009
Starmerella	*S. meliponinorum*	Limtong and Koowadjanakul, 2012
Torulaspora	*T. delbrueckii*	Limtong and Koowadjanakul, 2012
	T. maleeae	Limtong *et al*., 2008
	T. pretoriensis	Limtong and Koowadjanakul, 2012
Wickerhamomyces	*W. edaphicus*	Limtong and Koowadjanakul, 2012

2.3 Ecology of phylloplane yeast

Yeasts participate of the complex processes in ecosystems. In the food chain model, yeasts are primarily decomposers. Generally, yeasts are among the earlier colonizers of nutrient rich substrates, where they are followed by a succession of organisms that degrade dead organic matter. Along with their role in the transformation of nutrients, they can engage in intimate relationships with other organisms as mutualists, competitors, parasites, or pathogens. Yeasts are widely distributed throughout all biomes of the world. They have been found in the upper levels of the atmosphere, in the deepest

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parts of the oceans, in aquifers under the sea, in ancient glacial ice, and are abundant throughout the phylloplane (Starmer and Lachance, 2011).

The occurrence of yeast in association with plants has been acknowledged for many years. Their ability to utilize an overabundance of organic compounds through respiratory processes greatly the range of ecological niches that they may colonize on sugar rich substrates such as fermenting fruit juices. Many compounds such as pentoses, polyols, organic acids, aromatic compounds and amino acids, which specific yeasts can assimilate, are either products of plant metabolism or products of other microorganisms from precursors in plants. For examples of plant habitats that have been investigated for their yeast inhibitants include the nectar of flowers, tree exudates or slime fluxes, the necrotic tissues of cacti, plant surfaces e.g. leaves, flowers, fruits and bark and aerial organs of plants e.g. stems, fruits or flowers (Fonseca and Inácio, 2006).

Starmer and Lachance (2011) suggested that although the yeasts present on leaves are regarded either epiphytes (commensals) or saphrophytes (decomposers), some species may act as protective mutualists under some circumstances because of their negative influence on potential pathogens. Under experimental conditions by Fokkema *et al.* (1983) showed that the naturally occurring phylloplane yeasts prevented establishment of pathogens. The mechanism for this competitive inhibition is probably the consumption of carbohydrates by the saprophytes and thus the yeast serves as a nutrient sink. Competition for space could also be a factor. However, because only a small proportion (1 to 10%) of the leaf surface is colonized by leaf microbes, space may or may not be important (Macauley and Waid, 1981).

Phylloplane yeasts may changes from one type of ecological interaction to another as its habitat changes. For example, epiphytes inhabiting on leaves of deciduous trees are joined by other fungi and become saprophytes when the leaves drop and start to decay in the upper layer of the forest floor. The subsequent process of nutrient recycling in the saprophytic channel of the ecosystem is an important indirect role of many fungi that are associated with leaves. Fungal decomposers are thought to drive the global carbon cycle and phylloplane yeasts ultimately participate in that process in one way or another. However, the role of phylloplane yeasts in soil processes may not be significant

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because many only join the upper layers of soil and do not persist in soil after leaves fall (di Menna, 1959).

2.4 Phylloplane yeast isolation methods

Several methods have been used for detection or isolation of microorganisms from phylloplane including yeast. Standard direct detection methods, mainly in studies before the 1980s such as optical microscopy directly on leaves, optical microscopy on leaf impression (i.e. in nail varnish pellicles or acetate adhesive tape) and scanning electronic microscopy. In these methods, there are difficulties in detecting small yeast cells or small cell densities against the background. Moreover, it is not usually possible to distinguish between different species with similar cell morphologies, or between viable, viable but non culturable and dead cells. Enumeration of microbial populations associated with leaves relies almost exclusively on indirect methods involving culture of the organisms on artificial agar media such as plating of leaf washing, plating of washed leaves, plating of leaf macerates and leaf impression onto solid media. Leaves are usually cut into small pieces and submerged in a washing solution (e.g. water or Ringer solution), which is then shaken (i.e. with the aid of a vortex, rotary shaker or sonicator). The resulting suspension is then serially diluted and aliquots are plated onto appropriate media. The culture medium was added antibacterial or antibiotic such as chloramphenicol is used to prevent bacterial growth for isolation of fungi or yeast (Fonseca and Inacio, 2006). Moreover, the spore-fall method was cultivation method for yeast isolation from the phylloplane, which is specific for ballistoconidiogenic yeasts. Leaf samples are fixed under the lid of a Petri dish, suspended above the agar surface, and colonies develop from discharged spores that fall on the culture medium (Nakase and Takashima, 1993).

In recent years several molecular methods have been used for studying the diversity of phylloplane microorganism without cultivation steps. The main organisms have been bacteria, but in a few cases yeasts and dimorphic fungi were also included. For example, denaturing gradient gel electrophoresis (DGGE) of DNA fragment amplified with universal primers for ribosomal DNA (rDNA) regions has been used to study bacterial population in the phylloplane of citrus fruit. Among several organisms detected, some DNA bands were assigned to be *Aureobasidium pullulans* (Yang *et al.,* 2001).

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Bramwell *et al.* (1995) compared a range of DNA extraction protocols for microbial cells on leaf samples for this type of study. Moter and Göbel (2000) used a different molecular technique, fluorescence in situ hybridization (FISH), has been used to detection and identification of microorganisms in their natural environments. The rationale underlying FISH is that, under appropriate hybridization conditions, the annealing between complementary sequences in the oligonucleotide fluorochrome-labelled probe and target molecules can be detected by fluorescence microscopy in intact cells. FISH has already been used for detecting and enumerating *Aureobasidium pullulans* on apple leaves (Andrews *et al*., 2002), *Taphrina deformans* on peach leaves (Tavares *et al*., 2004) and *Cryptococcus* sp. nov. and other yeasts on leaves of different Mediterranean plant species (Fonseca and Inacio, 2006).

3. Yeast for Agricultural Utilization

Yeasts are benefitted for humanity because they have wide-ranging fundamental and industrial importance in scientific, food, medical and agricultural disciplines. For example traditional industrial feature of yeasts include their primary roles in many food fermentations such as beer, cider, wine, sake, distilled spirit, bakery product, cheese, sausage, and other fermented foods. Other long-standing industrial processes that involve yeasts are the production of fuel ethanol, single cell protein, industrial enzymes, and small molecular weight metabolites (Johnson and Echavarri-Erasun, 2011). Some application of yeasts are shown in Table 4.

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Table 4 Some application of yeasts*

Application	Example
Traditional Yeast Fermentations	Beer, wine, sake, soy sauce, other food fermentations
Environmental biotechnology	Bioremediation, pollutant degradation
Food and feed ingredients	Enzymes, flavors, pigments, amino acids, organic acids
Bioenergy	Bioethanol, biodiesel
Biocontrol	Crop protection, food and feed safety, probiotics
Biocatalysis	Pharmaceuticals, chiral chemical intermediates, biotransformations
Biomedical research	Drug discovery, drug resistance and metabolism, elucidation of disease mechanisms
Heterologous protein production	Protein pharmaceuticals, enzymes, hormones, vaccines, toxins
Fundamental biological research	Molecular and cellular biology, genomics, functional genomics, pathway engineering, systems biology mechanisms

*adapted from Johnson and Echavarri-Erasun (2011).

Yeasts are microorganisms that very important in several biotechnological processes and they are the most important microorganism for industrial application. However, a few attempts are applying on agricultural application especially using as biological control agents, plant growth promoters and biopreservation agents.

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3.1 Yeasts as biological control agents

At present, yeast products for controlling of fungi causing post harvest diseases have already reached on the market such as Aspire, Candifruit, Nexy, Shemer, Sporodex and YieldPlus (Table 5).

Table 5 Commercially biological control products to manage plant pathogenic and postharvest pathogenic fungi

Product name	Yeast	Fungal disease	Type*	Reference
Aspire	*Candida oleophila* strain I-182	*Penicillium digitatum*	2	Droby *et al*., 2002
Candifruit	*Candida sake* strain CPA-2	*Penicillium expansum*	2	Morales *et al*., 2008
Nexy	*Candida oleophila* strain O	*Botrytis cinerea*	2	Jijakli *et al*., 1993
		Penicillium expansum	2	
Shemer	*Metschnikowia fructicola*	*Penicillium digitatum*	2	Karabulut *et al.*, 2003, 2004
		Penicillium italicum	2	
		Penicillium expansum	2	
		Botrytis cinerea	1, 2	
		Rhizopus stolonifer	1, 2	
		Aspergillus niger	2	
Sporodex	*Pseudozyma flocculosa* strain PF-A22	*Blumeria graminis*	1	Jarvis *et al*., 2007
		Sphaerotheca fusca	1	
YieldPlus	*Cryptococcus albidus*	*Botrytis cinerea*	2	Fravel, 2005
		Colletotrichum spp.	2	
		Penicillium spp.	2	
		Rhizopus spp.	2	

* Type 1: the commercially biological control products to manage plant pathogenic fungi, 2: the commercially biological control products to manage postharvest pathogenic fungi.

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Other yeast species both in ascomycota and basidiomycota have been found to be the effective biological control agents of plant pathogenic and postharvest pathogenic fungi in laboratories and field studies (Table 6).

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Table 6 Examples of yeast as biological control agents applied to reduce plant pathogenic and postharvest pathogenic fungi

Yeast	Fungal disease	Type*	Assessment Utilized	Reference
Ascomycota				
Candida membranifaciens	*Penicillium expansum*	2	Apple fruit	Gholamnejad *et al*., 2010
Candida oleophila	*Penicilium digitatum*	2	Grape fruit	Droby *et al.*, 2002
Candida sake	*Penicillium expansum*	2	Apple fruit	Teixidó *et al.*, 1998
Candida sake	*Cercospora beticola*	1	Sugar beet	Ziedan and Farrag, 2011
Candida sake	*Erysiphe betae*	1	Sugar beet	Ziedan and Farrag, 2011
Candida valida	*Rhizoctonia solani*	1	Sugar beet	El-Tarabily, 2004
Metschnikowia pulcherrima	*Botrytis cinerea*	2	Apple fruit	Saravanakumar *et al.*, 2009
Metschnikowia fructicola	*Botrytis cinerea*	1,2	Strawberry	Karabulut *et al*., 2004
Ogataea polymorpha	*Botrytis cinerea*	2	Apple fruit	Fiori *et al.*, 2008
Ogataea polymorpha	*Monilinia fructicola*	2	Apple fruit	Fiori *et al.*, 2008
Pichia albicans	*Cercospora beticola*	1	Sugar beet	Ziedan and Farrag, 2011
Pichia albicans	*Erysiphe betae*	1	Sugar beet	Ziedan and Farrag, 2011
Pichia anomala	*Botryodiplodia theobromae*	2	Guava fruit	Hashem and Alamri, 2009
Pichia anomala	*Botrytis cinerea*	2	Apple fruit	Haissam, 2011
Pichia anomala	*Penicillium expansum*	2	Apple fruit	Haissam, 2011
Pichia fermentans	*Monilinia fructicola*	2	Apple fruit	Giobbe *et al.*, 2007
Pichia guilliermondii	*Rhizopus nigricans*	2	Tomato fruit	Zhao *et al*., 2008

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Table 6 (Continued)

Yeast	Fungal disease	Type*	Assessment Utilized	Reference
Pichia membranifaciens	*Botrytis cinerea*	1	Grapevine	Masih and Paul, 2002
Saccharomyces cerevisiae	*Cercospora beticola*	1	Sugar beet	Ziedan and Farrag, 2011
Saccharomyces cerevisiae	*Erysiphe betae*	1	Sugar beet	Ziedan and Farrag, 2011
Saccharomycopsis schoenii	*Penicillium digitatum*	2	Orange fruit	Pimenta *et al.*, 2008
Saccharomycopsis schoenii	*Penicillium expansum*	2	Orange fruit	Pimenta *et al.*, 2008
Saccharomycopsis schoenii	*Penicillium italicum*	2	Orange fruit	Pimenta *et al.*, 2008
Torulaspora globosa	*Colletotrichum sublineolum*	2	Sorghum	Rosa *et al.*, 2010
Basidiomycota				
Cryptococcus albidus	*Botrytis cinerea*	1	Bean and tomato plants	Elad *et al.*, 1994
Cryptococcus albidus	*Sclerotinia sclerotiorum*	1	Bean	Reeleder, 2004
Cryptococcus flavescens	*Fusarium graminearum*	1	Wheat	Schisler *et al*., 2002
Cryptococcus nodaensis	*Fusarium graminearum*	1	Wheat	Khan *et al*., 2004
Cryptococcus laurentii	*Penicillium expansum*	2	Apple fruit	Guo-Zheng *et al.*, 2003
Rhodosporidium diobovatum	*Botrytis cinerea*	2	Tomato	Utkhede and Mathur, 2006
Rhodosporidium paludigenum	*Alternaria alternata*	2	Cherry tomato fruit	Wang *et al*., 2008c
Rhodosporidium paludigenum	*Alternaria alternata*	2	Chinese winter jujube	Wang *et al.*, 2009
Rhodotorular glutinis	*Botrytis cinerea*	1	Bean and tomato plants	Elad *et al.*, 1994
Rhodotorular glutinis	*Penicillium expansum*	2	Apple fruit	Guo-Zheng *et al.*, 2003

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Table 6 (Continued)

Yeast	Fungal disease	Type*	Assessment Utilized	Reference
Rhodotorular glutinis	*Botrytis cinerea*	2	Apple fruit	Zhang *et al.*, 2008
Rhodotorular glutinis	*Penicillium expansum*	2	Pear fruit	Zhang *et al.*, 2009
Rhodotorular glutinis	*Rhizoctonia solani*	1	Sugar beet	El-Tarabily, 2004
Rhodotorula mucilaginosa	*Penicillium expansum*	2	Apple fruit	Gholamnejad *et al*., 2010
Rhodotorula mucilaginosa	*Botrytis cinerea*	2	Apple fruit	Li *et al.*, 2011
Rhodotorula mucilaginosa	*Penicillium expansum*	2	Apple fruit	Li *et al.*, 2011
Sporidiobolus pararoseus	*Botrytis cinerea*	2	Strawberry	Huang *et al.*, 2012
Trichosporon asahii	*Rhizoctonia solani*	1	Sugar beet	El-Tarabily, 2004
Trichosporon pullulan	*Penicillium expansum*	2	Apple fruit	Guo-Zheng *et al.*, 2003

* Type 1: yeast as biological control agents applied to reduce plant pathogenic fungi, 2: yeast as biological control agents applied to reduce postharvest pathogenic fungi.

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In Thailand there were some reports on using yeasts as biocontrol agents of plant pathogenic and postharvest pathogenic fungi in vitro and in vivo i.e. *Rhodotolula* sp. and *Hensenula saturnus* were tested controlling for stem end rot of mango fruit. *Pichia membranaefaciens, Candida tropicalis* and *Hensenula saturnus* were used to control fruit rot of banana (Sourha, 1997). *Candida guilliermondii* and *Saccharomycopsis fibuligera* were found to be the antagonistic yeasts for controlling crown rot of banana fruit cv. Hom Tong, caused by *Lasiodiplodia theobromae* (Sangwanich, 2004). *Candida utilis, Candida tropocalis, Debaryomyces hansenii* and *Pichia* sp. were observed to inhibit spore germination and germ tube elongation of *Penicilium digitatum*, a green mold causal disease of citrus fruit cv. Sai-Numphaung (Niamjang, 2006). *Pichia guilliermondii* strain R13 was tested to control *Colletotrichum capsici*, a anthracnose pathogen in chilli fruit after harvest (Chanchaichaovivat *et al.*, 2008).

Yeasts which have been studied for their efficiency as biological control agents were derived from various habitats. Most of yeast strains that were applied to control post harvest disease were often isolated from sources, which occur disease. For example *Rhodotorula glutinis* was isolated from red delicious apple fruit. Rhodotorulic acid produced by this strain improved the biological control of blue rot caused by *Penicillium expansum* in apples fruit. (Calvente *et al.*, 1999). *Trichosporon pullulans, Cryptococcus laurentii* and *Rhodotorula glutinis* were isolated selectively from the surface of apple fruit. These three antagonistic yeasts were used to control *Penicillium expansum* in harvested apple fruits (Guo-Zheng *et al.*, 2003). *Metschnikowia pulcherrima* strain MACH1 was isolated from the carposphere of apple fruit. It was tested for their efficacy against *Botrytis cinerea* in vitro and in vivo on apples fruit (Saravanakumar *et al.*, 2009). *Candida membranifaciens* and *Rhodotorula mucilaginosa* were isolated from the surface of apple healthy fruits collected from organic production orchards. They were used to control of apple blue mold (Gholamnejad *et al.*, 2010). *Sporidiobolus pararoseus* was biocontrol agent of *Botrytis cinerea* on post-harvest strawberry fruits. It was isolated from a healthy leaf of strawberry (*Fragaria ananassa*) (Huang *et al.*, 2012).

However, some yeast strains used as biocontrol agents were isolated from other sources not from the sources that they would used to control diseases such as *Candida glabrata, Candida maltose, Candida slooffii, Rhodotorula rubura* and

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Trichosporon cutanum were obtained from rhizosphere of maize plant. They were used as biocontrol agents for wilt disease of kidney bean caused by *Fusarium oxysporum* (El Mehalawy, 2004). *Pichia fermentans* was isolated from wine must, it antagonised *Monilinia fructicola* causing brown rot on apple fruit (Giobbe *et al.*, 2007). *Rhodotorula glutinis* was derived from surface of strawberries fruit. It was biological control agent for controlling blue mold decay of pears fruit (Zhang *et al.*, 2009). *Tolulaspora globosa* was biocontrol agent for controlling *Colletotrichum sublineolum* in sorghum. It was isolated from rhizosphere of sugarcane (Rosa *et al.*, 2010). *Rhodosporidium paludigenum*, which was used to control gray mold of cherry tomatoes fruit, was obtained from the south East China Sea (Wang *et al.*, 2010). *Pichia albicans* and *Candida sake* were isolated from the surface of orange fruits and *Saccharomyces cerevisiae* was obtained from Arab company for medicines and medicinal plant. They were used as biocontrol agents for controlling foliar diseases on sugar beet plants (Ziedan and Farrag, 2011).

3.2 Plant growth-promoting yeasts and production of plant growth regulators

The production of plant growth regulators (PGRs) such as auxins, gibberellins, and cytokinins that directly promote plant growth. Indole-3-acetic acid (IAA), an important auxin plant growth regulator, has been shown to have potent physiological regulatory properties, such as delaying the ripening and softening of fruits, inhibiting postharvest physiological disorders and inducing defense responses in plant tissues (Rojo *et al*., 2003). IAA is a common product of L-tryptophan metabolism by several microorganisms including yeast (Nasser *et al.*, 2005; Xin *et al*., 2009). The production of indole-3-acetic acid (IAA) was report to be one mechanism it has been reported for controlling of phytopathogenic fungi. Martinez Noel *et al.* (2001) showed that the supply of IAA to excised potato leaves resulted in a reduced severity of the disease caused by *P. infestans*. Nasser *et al.* (2005) reported that the yeast *Williopsis saturnus*, an endophytic yeast in maize roots, have the capacity to produce IAA and indole-3-pyruvic acid (IPYA). Yu and Zheng (2007) found that indole-3-acetic acid enhances the biocontrol of *Penicillium expansum* and *Botrytis cinerea* on pear fruit by *Cryptococcus laurentii*. Yu *et al.* (2008) evaluated the effect of a yeast antagonist *Cryp. laurentii* and a plant regulator indole-3-acetic acid (IAA) on inhibition of *Botrytis cinerea* infection in harvested apple fruit. The results showed that the combined treatment with *Cryp. laurentii* and IAA at 20

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μg/ml was a more effective approach to reduce the gray mold rot in apple wounds than the *Cryp. laurentii* alone. Therefore, combination of *Cryptococcus laurentii* with IAA, which integrated the dual biological activity from the antagonistic yeast and plant regulator, might be developed to be a useful approach to control gray mold in harvested apple fruit. Yu *et al.* (2009) had recently described that IAA could reinforce the biocontrol efficacy of *Cryptococcus laurentii* in inhabiting blue mold (*Penicillium expensum*) of apple fruit by induction of the natural resistance of the fruit.

4. Mechanisms of yeast as biocontrol agents

Various yeasts species have been shown to effectively control of postharvest pathogenic fungi in the field. However, the mechanisms of yeasts have not been fully elucidated. Various mechanisms have been reported to play a significant role in the biocontrol activity of these antagonistic yeasts on plant pathogenic fungi. They are included competition for nutrients and space (Saligkarias *et al*., 2002; Vero *et al*., 2002; Bencheqroun *et al.*, 2007; Zhao *et al*., 2008; Zhang *et al.*, 2008, 2009; Rosa *et al*., 2010; Li *et al.*, 2011), production of antifungal diffusible and volatile metabolites (Walker *et al*., 1995; Calvente *et al.*, 1999; Hofte *et al*., 2004; Santos and Marquina, 2004; Rosa *et al*., 2010; Huang *et al.*, 2012), production of cell wall degrading enzymes such as β-1,3-glucanase and chitinase (Chanchaichaovivat *et al*., 2008; Saravanakumar *et al.*, 2009; Zhang *et al.*, 2010), induction of host resistance (Wilson *et al*., 1994; Droby *et al*., 2002; El Ghaouth *et al*., 2003; Zhao *et al*., 2008), and mycoparasitism (Wisniewski *et al*., 1991; Arras *et al*., 1998; El Ghaouth *et al*., 1998).

4.1 Competition for nutrients and space

They were few reports indicated that the main of mechanism of yeast as biocontrol agents is competition for nutrients and space (Saligkarias *et al*., 2002; Vero *et al*., 2002). This is based on ability of yeast to develop quickly on the surface of leaves, fruits and flowers, especially in the presence of sugar so their capacity to rapidly colonize and multiply in plant surface and subsequently to compete with the pathogens for nutrients and space. The rapid reproduction of yeast helps them to outcompete fungal pathogens. For example, Spadaro *et al.* (2002) found that during cocultivation on a

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synthetic medium *Metschnikowia pulcherrima* completely inhibited spore germination of *Botrytis cinerea.* However, culture filtrate and autoclaved cell suspension of *Metschnikowia pulcherrima* failed to reduce spore germination and was ineffective in reducing the lesions on apple fruits caused by *Botrytis cinerea*, *Penicillium expansum*, or *Monilia* sp. This result supporting that living cells are necessary for effective biocontrol and nutrient competition could be the main mechanism rather than other mechanism in vitro. Bencheqroun *et al.* (2007) selected a yeast-like fungus, *Aureobasidium pullulans* strain Ach1-1, for controlling blue mold caused by *Penicillium expansum* on stored apple fruit. This study was evidence that competition for apple nutrients, most particularly amino acids, may be a main mechanism of the biocontrol activity of *Aureobasidium pullulans* strain Ach1-1. Zhao *et al*. (2008) found that *Pichia guilliermondii* could acclimatize itself to the tomato fruit wounds and occupied the living space quickly. The results indicated that *Pichia guilliermondii* antagonized *Rhizopus nigricans* because of competition for nutrients and space on wounds appeared to play a role in the activity of the biocontrol and could be one of the mechanisms. Zhang *et al.* (2008; 2009) observed that *Rhodotorula glutinis* has potential to control of postharvest gray mold decay and blue mold decay on pear fruits caused by *Botrytis cinerea* and *Penicillium expansum*, respectively. They suggested that the competition for nutrients and space maybe the mechanism of yeast strains because they rapidly colonize in pear tissues and competes with the pathogen for nutrients. Rosa *et al.* (2010) reported that the competition for space and nutrients, killer toxin production and altering the hyphal morphology are mechanisms utilized by *Torulaspora globosa* antagonized *Colletotrichum sublineolum*, a causal agent of sorghum disease. The possible modes of action of *Rhodotorula mucilaginosa* was used to control postharvest gray mold, blue mold and natural decay development of apples were based on competition for space and nutrients with pathogens (Li *et al.*, 2011).

4.2 Antibiosis

Some yeast species may produce antimicrobial compounds, such as killer toxins (Walker *et al*., 1995; Hofte *et al*., 2004). Walker *et al.* (1995) reported the potential ability of killer yeasts to inhibit pathogenic fungi. These killer yeasts were shown to strongly inhibit the vegetative growth of *Heterobasidion annosum*, *Rhizoctonia solani*, *Fusarium equiseti*, and a range of plant pathogenic fungi. Santos and Marquina

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(2004) showed that *Pichia membranifaciens* controlled the causal agent of grey mold disease in grape (*Botrytis cinerea*) by producing a killer toxin. Rosa *et al*. (2010) found that *Torulaspora globosa* controlled the anthracnose disease in sorghum and they exhibited killer activity against two sensitive strains, suggesting that killer toxin production was the one of mechanisms utilized by the yeast in the biocontrol.

Payne *et al.* (2000) reported that *Saccharomyces cerevisiae* and *Debaryomyces* sp. significantly reduced radial growth of selected mold and stain fungi in vitro, solely through the liberation of volatile antifungal compounds. Huang *et al*. (2012) found that the volatile organic compounds (VOCs) from the cultures of *Sporidiobolus pararoseus* strain YCXT3 on yeast extract peptone dextrose agar were highly effective in inhibiting both the conidial germination and the mycelial growth of *Botrytis cinerea*, the causal agent of strawberry gray mold disease.

Some yeast species are also able to produce siderophores, the low-molecular weight compounds, which act as iron-chelating ligands. These compounds are capable of stealing the iron (III) ions result in that inhibition of fungal growth. Siderophores production by *Rhodotorula glutinis* strains improved the biological control of blue rot caused by *Penicillium expansum* in postharvested apples (Calvente *et al.*, 1999).

4.3 Cell wall degrading enzymes and mycoparasitism

Some yeast strains produce hydrolytic enzymes, i.e. β-glucanases and chitinases, which destroy other fungal cell walls. Saravanakumar *et al.* (2009) showed that *Metschnikowia pulcherrima* strain MACH1 has the ability to secrete chitinase in liquid media containing apple juice and the enzyme could be involved in the postharvest biological control of *B. cinerea*. *Pichia guilliermondii* strain R13 produced hydrolytic enzymes including β-1,3-glucanases and chitinase in solid and liquid medium and the activities of these enzymes were the highest when the *Colletotrichum capsici* hyphal cell wall was used as the carbon source, rather than laminarin or glucose. Thus, secretion of hydrolytic enzymes of *Pichia guilliermondii* strain R13 was one of the modes of action to suppress chilli anthracnose causal by *Colletotrichum capsici* (Chanchaichaovivat *et al*.,

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2008). Zhang *et al.* (2010) reported that production of β-1,3-glucanases and chitinase were important modes of action of yeast like fungus, *Aureobasidium pullulans* PL5, antagonized postharvest pathogens of fruits.

4.4 Induction of host resistance

Activation of host resistance has been found to relate the production of phenylalanine ammonia lyase, phytoalexins, peroxidases, and ethylene in plant tissues. For example, In apple wounds, *Aureobasidium pullulans* caused transient increases in β-1,3-glucanase, chitinase, and peroxidase activities (Ippolito *et al*., 2000). Zhao *et al*. (2008) investigated ability of *Pichia guilliermondii* to control *Rhizopus nigricans* on tomato fruit during storage. The fruit inoculated with *Pichia guilliermondii* demonstrated changes in peroxidase, polyphenoloxidase, superoxide dismutase, catalase, phenylalanine ammonia-lyase, chitinase and β-1,3-glucanase activities, all of which were correlated to the induction of resistance. This result suggested that tomato fruit was capable of responding to the yeast *Pichia guilliermondii*, which could activate defensive enzymes and thereby induce host disease resistance.

5. Important Agronomic crops in Thailand and their diseases cause by pathogenic fungi

Thailand is agricultural country because climatic and soil conditions permit the cultivation of a wide range of crops. Thai agriculture exports are very successful internationally. Rice is the country's most important crop. Thailand is a major exporter in the world rice market. Other agricultural commodities produced include corn, cassava, sugarcane, pineapple, soybean, mungbean and groundnut (Office of Agricultural Economics, 2012). In 2012 value export of rice is 135,000 million baht which value export of raw sugar and sugar is 109,000 million baht and value export of corn is 2,661 million baht (Office of Agricultural Economics, 2012).

Rice is the major crop grown in the north, central and northeast of Thailand (31.1 million acre) especially Buri Ram, Kamphaeng Phet, Nakhon Ratchasima, Nakhonsawan,

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Phichit, Phitsanulok, Roi Et, Suphan Buri, Surin and Ubon Ratchathani province while corn and sugarcane are other crops regularly grown in the north, central and northeast of Thailand (5.9 million acre). Most corn is grown in Loei, Nakhon Ratchasima, Nan, Phetchabun and Tak province. Sugarcane is grown in Kanchanaburi, Khon Kaen, Nakhon Ratchasima, Nakhonsawan and Suphan Buri province (Office of Agricultural Economics, 2012).

One problem of cultivation of these crops is destructive diseases cause by various plant pathogenic fungi. Fungal diseases of rice especially rice blast disease, caused by the fungus *Pyricularia grisea*, is one of the most common and most severe diseases of rice in Thailand. Large lesions of gray centers bordered by brown to dark red usually develop. Chlorophyll in the parts which attack by the pathogen is disappeared so photosynthesis and rice production yield are reduced (Mekwatanakarn *et al*., 2007). The other fungal diseases of rice were shown in Table 7

Table 7 List of rice diseases caused by fungi

Diseases	Pathogenic fungi
Bakanae	*Fusarium moniliforme*
Black kernel	*Curvularia lunata*, *Cochliobolus lunatus* (teleomorph)
Blast (leaf, neck [rotten neck], nodal and collar)	*Pyricularia grisea* (*Pyricularia oryzae*), *Magnaporthe grisea* (teleomorph)
Brown spot	*Cochliobolus miyabeanus*, *Bipolaris oryzae* (anamorph), *Helminthosporium oryzae*
Leaf scald	*Microdochium oryzae* (*Rhynchosporium oryzae*)
Narrow brown leaf spot	*Cercospora janseana* (*Cercospora oryzae*)

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Table 7 (Continued)

Diseases	Pathogenic fungi
Seedling blight	*Cochliobolus miyabeanus*, *Curvularia* spp., *Fusarium* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*, *Athelia rolfsii* (teleomorph)
Sheath rot	*Sarocladium oryzae* (*Acrocylindrium oryzae*)
Sheath blight	*Rhizoctonia solani*
Sheath spot	*Rhizoctonia oryzae*
Stem rot	*Magnaporthe salvinii*

Source: Bureau of Rice Research and Development (2008)

Fungi disease of corn such as downy mildew caused by *Peronosclerospora sorghi*, is an important corn disease in many tropical areas, although symptoms are vary depending on plant age, prevailing climatic conditions and host germplasm. Northern leaf blight caused by *Exserohilum turcicum* is another corn disease. The general characteristic symptoms are elliptical shaped, gray-green to tan colored lesions on the leaves (Makhawin *et al.*, 2005). In addition, the corn diseases caused by fungal were shown in Table 8.

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Table 8 List of corn diseases caused by fungi

Diseases	Pathogenic fungi
Brown spot, Black spot, Stalk rot	*Physoderma maydis*
Charcoal rot	*Macrophomina phaseolina*
Diplodia ear rot, Stalk rot, Seed rot, Seedling blight	*Diplodia maydis*
Sorghum downy mildew	*Peronosclerospora sorghi* (*Sclerospora sorghi*)
Fusarium kernel, root and stalk rot, seed rot and seedling blight	*Fusarium moniliforme*, *Gibberella fujikuroi* (teleomorph)
Leaf spots	*Curvularia lunata*
Northern corn leaf blight, White blast, Crown stalk rot, Stripe	*Setosphaeria turcica*, *Exserohilum turcicum* (anamorph) (*Helminthosporium turcicum*
Northern corn leaf spot, Helminthosporium ear rot	*Cochliobolus carbonum*, *Bipolaris zeicola* (anamorph) (*Helminthosporium carbonum*)
Sheath blight	*Rhizoctonia solani*
Smut	*Ustilago zeae* (*Ustilago maydis*)
Southern corn leaf blight and stalk rot	*Cochliobolus heterostrophus*, *Bipolaris maydis* (anamorph) (*Helminthosporium maydis*)
Southern rust	*Puccinia polysora*
Stem rot	*Fusarium moniliforme*, *Macrophomina phaseolina*

Source: Department of Agriculture, Ministry of Agriculture and Cooperative (2004)

Ekasingh *et al.* (2004) surveyed on sugarcane diseases in six provinces of the central region of Thailand and reported that there were five diseases caused by pathogenic fungi including smut caused by *Ustilago scitaminae*, red rot of midrib caused by *Colletotrichum falcatum*, rust caused by *Puccinia melanocephala*, downy mildew caused by *Peronosclerospora sacchari* and ring spot caused by *Leptosphaeria sacchari*.

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Moreover, the other sucarcane diseases caused by fungal which were reported in Thailand were shown in Table 9.

Table 9 List of sugarcane diseases caused by fungi

Diseases	Pathogenic fungi
Banded sclerotial (leaf) disease	*Thanatephorus cucumeris* (*Pellicularia sasakii*), *Rhizoctonia solani* (anamorph)
Brown spot	*Cercospora longipes*
Eye spot	*Bipolaris sacchari* (*Helminthosporium sacchari*)
Marasmius sheath and shoot blight	*Marasmiellus stenophyllus* (*Marasmius stenophyllus*)
Red rot	*Glomerella tucumanensis* (*Physalospora tucumanensis*), *Colletotrichum falcatum* (anamorph)
Root rots	*Marasmius sacchari*, *Pythium arrhenomanes*, *Pythium graminicola*, *Rhizoctonia* sp., Unidentified Oomycete
Sheath rot	*Cytospora sacchari*

Source: Philippe *et al*. (2000)

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MATERIALS AND METHODS

1. Sample Collection

Green and undamaged leaves of three agronomic crops (rice: *Oryza sativa* Linn., corn: *Zea mays* Linn. and sugarcane: *Saccharum officinarum* Linn.) were collected and placed in plastic bags, sealed and transferred in ice-box to laboratory. The samples were kept at 8°C until subjected for yeast isolation.

2. Yeast isolation

Yeast was isolated by an enrichment technique using yeast extract malt extract (YM) broth (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose) supplemented with 250 mg/L sodium propionate and 200 mg/L chloramphenicol (Limtong *et al.*, 2007). Three grams of cut leaves, derived from cutting few leaves to the size that can be put into a 250 mL Erlenmeyer, was added into 50 mL enrichment broth in the flask and incubated on a rotary shaker at 30 ± 3°C for 2 days. A loopful of the enriched culture was streaked on YM agar supplemented with 25 mg/L sodium propionate and 20 mg/L chloramphenicol. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10% v/v glycerol and maintained at -80°C.

3. Identification of yeasts by DNA sequencing and phylogenetic analysis

3.1 Sequencing of the D1/D2 region of the large subunit (LSU) rRNA gene, the internal transcribed spacer (ITS) region, the actin gene (ACT1) and the elongation factor 2 gene (EF2).

3.1.1 DNA extraction was carried out by the method of Lachance *et al*. (1999) with slightly modification. Cells grown on YM agar at 25°C for 24-48 h were suspended in 50 µl of sterile distilled water in steriled 1.5 ml microfuge tube, and kept at -20°C for 30 min. Then, the microfuge tube containing cell suspension was put into a

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boiling water (100°C) for 10-15 min, after that immediately transferred to -20°C. After keeping for 10-30 min, it was subjected to centrifugation at 8,000 rpm for 5 min. The supernatant containing yeast DNA was used as DNA template for amplification of the D1/D2 region of the LSU rRNA gene and the ITS region.

3.1.2 Amplification of the D1/D2 region of the LSU rRNA gene was performed by the method of Kurtzman and Robnett (1998) with slightly modification using forward primer NL1 and reverse primer NL4. The ITS region was amplified with primers, ITS1 and ITS4 following the method of White *et al.* (1990). The ACT1 gene was amplified with primers, ACT1 and ACT2, and EF2 gene was amplified with primers, EFIIF1 and EFIIR2 following the method of Diezmann *et al.* (2004). The primer sequences were shown in Table 10. The PCR reaction consists of 3 µl of 10X PCR buffer, 2.4 µl of 25 mM $MgCl_2$, 2.4 µl of 2.5 mM dNTP mixtures, 0.9 µl of each of 10 pmol NL1 and NL4, 0.15 µl of Taq DNA polymerase (Fermentas, USA), 3 µl of DNA template solution, and 17.25 µl of sterile distilled water. PCR amplification operated with GeneAmp PCR System 9700 (PE Applied Biosystems, USA) was initially performed by predenaturation at 94 °C for 5 min. Then, 30 PCR cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, were carried out, and the reaction was completely performed by final extension at 72 °C for 10 min. The PCR product was examined by agarose gel electrophoresis.

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Table 10 Primers used for PCR amplification and sequencing

Region/ gene	Primer	Sequence	Reference
D1/D2	NL1 (forward)	5' GCATATCAATAAGCGGAGGAAAAG 3'	Kurtzman and Robnett (1998)
	NL4 (reverse)	5' GGTCCGTGTTTCAAGACGG 3'	
ITS	ITS1 (forward)	5' TCCGTAGGTGAACCTGCGG 3'	White *et al.* (1990)
	ITS4 (reverse)	5' TCCTCCGCTTATTGATATGC 3'	
ACT1	ACT1 (forward)	5' TACCCAATTGAACACGGTAT 3'	Diezmann *et al.* (2004)
	ACT2 (reverse)	5' TCTGAATCTTTCGTTACCAAT 3'	
EF2	EFIIF1 (forward)	5' AAGTCTCCAAACAAAGCATAAC 3'	Diezmann *et al.* (2004)
	EFIIR2 (reverse)	5' GGGAAAGCTTGACCACCAGTAGC 3'	

3.1.3 Purification PCR product by using Gel/PCR DNA Fragments Extraction Kit (Geneaid), according to the manufacturer's protocol. Visualization of the purified PCR product was performed by electrophoresis using 1.2% agarose gel in 1X TBE buffer and stained with ethidium bromide and observed under UV illuminator.

3.1.4 The purified products were submitted to Macrogen Inc. (Korea) for sequencing with primers NL1 and NL4 for the D1/D2 region of the LSU rRNA gene; ITS1 and ITS4, for the ITS region; ACT1 and ACT2, for the actin gene; and EFIIF1 and EFIIR2, for EF2 gene (Table 10).

Note: In this study, 250 yeast strains were analysed at the NITE Biological Resource Center (NBRC), Japan. The DNA extraction and the amplification of the D1/D2 region of the LSU rRNA gene and the ITS region were carried out same as above description. PCR products were purified with AMpure (Beckman Coulter) following the manufacturer's protocol and directly used for sequence analysis. The sequences were determined using the BigDye Terminator version 3 (Applied Biosystems) on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) following the manufacturer's protocol.

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3.2 Identification

The sequence generated was applied to identify the yeast strain at species level, comparing with all currently available sequences of the D1/D2 region of the LSU rRNA gene of yeasts in GenBank using BLAST (basic local alignment search tool) homology search program (Altschul *et al*., 1997). Principle of identification based on the suggestion of Kurtzman and Robnett (1998) that yeast strains with 0-3 nucleotide differences are conspecific or sister species, yeast strains with 4-5 nucleotide substitutions are sister species or different species and yeast strains showing nucleotide substitutions greater than 6 are usually different species.

3.3 Phylogenetic Analysis

Phylogenetic analysis was carried out using the multiple alignment program CLUSTAL_X version 1.81 (Thompson *et al.*, 1997). A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), using the neighbor joining method (Saitou and Nei, 1987) by MEGA software version 5.0 (Tamura *et al*., 2011). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Felsenstein, 1985).

4. Polyphasic taxonomy of the new yeast species

In this study, all yeast strains identified by sequence analysis of the D1/D2 region of the LSU rRNA gene and phylogenetic analysis as the new yeast species were determined for their characteristics which are important in conventional taxonomy; including morphological, biochemical and physiological characteristics and chemotaxonomy.

Moreover, new yeast species were analyzed other genes viz. the ITS region, the ACT1 gene and the EF2 gene for comparing with their closet species.

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4.1 Morphology

Study on yeasts morphology was performed by the methods of Yarrow (1998) in The Yeast, A Taxonomic Study, 4th edition. There were included mode of vegetative reproduction and cell morphology, cultural characteristics on solid medium and liquid medium, filamentation and ascospores formation etc.

4.1.1 Mode of vegetative reproduction and cell morphology

The active yeast cells cultivated on YM agar at 25 °C for 24-48 h were inoculated into YM broth and incubated at 25 °C for 3-5 days. The culture was examined microscopically. The results, such as mode of vegetative reproduction, cell shapes and cell size were recorded.

4.1.2 Cultural characteristics on solid medium and in liquid medium

Yeast culture were inoculated on YM agar and YM broth and incubated at 25°C for 7 days. The characteristics of colony on YM agar such as color, size, surface, margin, and texture were recorded. The culture characteristics in YM which were observed are formation of sediment (compact, coherent, flocculent or mucous), a ring, islets or a pellicle.

4.1.3 Filamentation

The formation of pseudomycelium and true mycelium were examined using slide culture method. Glass slide in Petri dish with U-shaped glass rod (on a filter paper) and a tweezers wrapped with aluminium foil were sterilized at 160-170°C for 30-60 min. Sterilized glass slide was removed from Petri dish by sterilized tweezers and was dipped into molten 50-55°C PDA, then placed on U-shaped glass rod in the Petri dish and allowed agar to solidify. After drying of agar surface, yeast was inoculated by a streaking one line with a tiny loop (light inoculum), a sterilized cover slip was placed on the

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inoculated line and incubated at 25°C. The formation of pseudomycelium and true mycelium were examined under microscope at a few day's intervals until 3 weeks.

4.1.4 Ascospores formation

The active culture grown on YM agar were streaked on YM agar, Acetate agar, corn meal agar, Gorodkowa agar and 5% malt extract agar. Mode of ascus formation and shape, surface structure, number per ascus and size of ascospores were examined under microscope after incubation at 25°C for 7, 14, 21 and 28 days.

4.2 Biochemical and physiological characteristics

Determine biochemical and physiological characteristics by the methods of Yarrow (1998) as follows. In all cases, the active yeast cells were prepared on YM agar at 25°C for 24-48 hour.

4.2.1 Fermentation of carbohydrates

The active yeast cells were inoculated into carbohydrate fermentation medium inserted with Durham tubes containing series of carbohydrate, including glucose, galactose, sucrose, maltose, lactose, trehalose, raffinose, xylose and cellobiose. The concentration of carbohydrates used was 2 % (w/v), except 4 % (w/v) for raffinose. The tubes were incubated at 25°C for 4 weeks. The Durham fermentation tubes were inspected everyday for the first week and weekly until 4 weeks for gas accumulation in the Durham tube. Fermentation result was report depending on the times to fill the Durham tube with gas and the amount of accumulating gas according to the method of Yarrow (1998); +, strongly positive (insert filled within 7 days); l, delayed positive (insert rapidly filled, but only after more than 7 days); s, slowly positive (insert slowly filled after more than 7 days); w, weakly positive (the insert is not fully filled with gas); less than 1/3 full is often considered weak, whereas greater than 1/3 full is positive); -, negative (no accumulation of gas in the insert).

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4.2.2 Assimilation of carbon compounds

Light suspension of the active yeast cells were prepared in sterile distilled water. Then a drop of yeast cell suspension was inoculated into carbon assimilation medium and incubated at 25 °C for 4 weeks. The growth was examined weekly as turbidity of culture broth by placing the test tube on a white paper on which 0.75 mm thick lines drawn with India ink are present. The degree of growth was recorded as 3+ (lines are completely obscured), 2+ (lines observed are diffuse band), 1+ (lines are indistinguishable but have blurred edges), and +/- (the results of these are doubtful). Finally, the results was interpreted as described by Yarrow (1998); +, positive, either a 2+ or a 3+ reading after 1 week, or 2 weeks; l, delayed positive (latent), either a 2+ or 3+ reading develops rapidly, but after 2 weeks or longer; s, slow positive, a 2+ or 3+ reading develops slowly over a period exceeding 2 weeks; w, weakly positive, a 1+ reading; -, negative.

In this study, carbon compounds, including D-glucose, D-galactose, L-sorbose, cellobiose, lactose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, inulin, soluble starch, D-arabinose, L-arabinose, D-ribose, L-rhamnose, D-xylose, meso-erythritol, galactitol, D-glucitol, glycerol, myo-inositol, D-mannitol, ribitol, xylitol, ethanol, methanol, D-glucono-δ-lactone, citric acid, DL-lactic acid, succinic acid, D-gluconic acid, galacturonic acid, D-glucuronic acid, α-methyl-D-glucoside, salicin, D-glucosamine, N-acetyl-D-glucosamine, 2-keto-D-gluconate, 5-keto-D-gluconate, arbutin, hexeadecane, propane-1,2-diol, butane-2,3-diol and arabinitol were used.

4.2.3 Assimilation of nitrogen compounds

The starved inoculum was prepared by suspending of yeast cells grown on YM agar in YCB (Becto-yeast carbon base) broth without nitrogen source, and incubated at 25 °C for 1 week. Then, the starved inoculum was dropped on surface of YCB agar containing each of various nitrogen compounds; potassium nitrate, sodium nitrite, ethylamine hydrochloride, cadaverine dihydrochloride and lysine and incubated at 25 °C. The colony development was inspected every two days until two weeks. The

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degree of growth was recorded as 3+, 2+, 1+, +/- and – by comparison of the colony size with the positive (YCB agar plus ammonium sulfate) and the negative controls (YCB agar without nitrogen source).

4.2.4 Production of starch-like substances

The production of starch-like substance was determined according to the method described by Wicherham (1951). After assimilation test finished, Lugol's solution was added to glucose assimilation medium and ammonium sulfate assimilation medium. A positive result was indicated by the development in the culture of a color varying from dark blue to green.

4.2.5 Growth in vitamin free medium

The active yeast cells were suspended in sterile distilled water and inoculated in vitamin requirement test medium and incubated at 25°C for 4 weeks. The growth was weekly examined and recorded to be +3, +2, +1, +/- and – by reading as described in assimilation of carbon compounds test.

4.2.6 Acid production from glucose

The production of acid from glucose was determined according to the method of van der Walt (1970). The yeast culture was streaked onto slants or Petri dish of Custer's chalk agar and incubated at 25°C for 2 weeks. The result was recorded as positive if the chalk dissolved.

4.2.7 Tolerance of cycloheximide

Cycloheximide resistant was determined according to Whiffen (1948). This experiment was carried out in liquid Bacto-yeast nitrogen base with D-glucose (basal medium), supplement with cycloheximide to a final concentration of either 100

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ppm or 1000 ppm. The inoculation and detection were done in the same way as for the carbon assimilation test.

4.2.8 Growth in high osmotic pressure media

The active yeast cells were inoculated on 50 % glucose agar, 60 % glucose agar and 10 % sodium chloride plus 5 % glucose broth, and incubated at 25°C for 4 weeks. The growth was weekly examined.

4.2.9 Growth at various temperatures

The active yeast cells were inoculated into the series of YM broth, and incubated at 20, 25, 30, 35, 37, 40, 42 and 45 °C for 3 weeks. The growth of culture tested was weekly inspected and recorded.

4.2.10 Hydrolysis of urea

The active yeast cells were inoculated on Christensen's urea agar and the same medium without urea which was designated as the control, and incubated at 25 °C for 4 days. The result was examined daily, and the positive result was recorded when the medium colour changed to deep pink colour.

4.2.11 Diazonium blue B color reaction

The active yeast cells were inoculated on YM agar in Petri dish by the simple streak technique, and incubated at 25 °C for 10-14 days, followed by 16 h at 55-60 °C in incubator. The culture was allowed to cool at 25 °C, and then flood with cold Diazonium Blue B (DBB) reagent. If the culture colour turns black red within 2 min, the result was recorded as positive.

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4.3 Chemotaxonomy: Ubiquinone System

The ubiquinone or coenzyme Q system in yeast cells was determined by the methods of Yamada and Kondo (1973). Cells grown in 250 ml of YPD broth on a rotary shaker at 25 °C for 24-48 h were harvested by centrifugation at 8,000 rpm for 15 min, and washed twice with distilled water. For saponification, 12.5 ml of distilled water, 45 ml of methanol, 6 g of sodium hydroxide, 1.5 g of pyrogallol and glass beads were added and mixed with cells harvested. The mixture was refluxed in a heating mantle for 45 min. Ubiquinone was extracted from the saponified cell suspension in hexane and purified by thin layer chromatography with silica gel plate (F254TLC Merck, Germany) using hexane and diethyl ether (85:15) as mobile phase. The ubiquinone type was identified by high performance liquid chromatography (HPLC) using a Cosmosil (5C18; Waters) 4.6x250 mm column and methanol/2-propanol (2:1) at 1 ml/ min as the elution system, with spectrophotometric detection (275 nm wavelength), according to the method of Kuraishi *et al.*(1985).

4.4 Percentage of GC content

The DNA base composition was determined by HPLC as described by Tamaoka and Komagata (1984) using the DNA-GC kit (Yamasa Shoyu Co., Japan).

5. Determination of indole-3-acetic acid production by phylloplane yeast

Production of indole-3-acetic acid (IAA) by the phylloplane yeasts were determined by the method of Xin *et al.* (2009). The active yeast cells was inoculated in 5 mL of yeast extract peptone dextrose (YPD) broth (1% yeast extract, 2% peptone and 2% dextrose) supplemented with 0.1% (w/v) L-tryptophan in a test tube and incubated at room temperature on a shaker 150 rpm for 7 days. The cells in 1.5 ml of culture broth was pelleted by centrifugation at 8,000 rpm for 5 min. One ml of supernatant was mixed with one ml of Salkowski reagent (12 g/l $FeCl_3$ and 7.9 M of H_2SO_4) (Glickmann and Dessaux, 1994), and the intensity of pink color developing in the mixture after 30 min was quantified by spectrophotometer at wavelength 530 nm. Cell pellets was dried at

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100°C overnight and weighed for normalizing IAA production as mg of IAA/g dry cell weight. Similarly, pink color was also developed for a series of IAA standard solutions to establish a stand curve.

6. Determination of biological control activity against plant pathogenic fungi of phylloplane yeasts

To determine the biological control activity against rice and corn pathogenic fungi of the phylloplane yeasts isolated in this study. Eight pathogenic fungi causing rice and corn were used as tests microorganisms throughout the experiments. There were as follows.

1) *Fusarium moniliforme,* the causal agent of bakanae diseases of rice
2) *Helminthosporium oryzae,* the causal agent of brown spot diseases of rice
3) *Rhizoctonia solani,* the causal agent of sheath blight diseases of rice
4) *Bipolaris maydis,* the causal agent of southern leaf blight of corn
5) *Bipolaris zeicola,* the causal agent of northern leaf spot of corn
6) *Exserohilum turcicum,* the causal agent of northern leaf blight of corn
7) *Fusarium moniliforme* the causal agents of stem rot of corn
8) *Macrophomina phaseolina* the causal agents of stem rot of corn

Evaluation of the biological control of plant pathogenic fungi by phylloplane yeasts in vitro were investigated by the method of Rosa *et al.* (2010) with slight modification. Each pathogenic fungus and yeast were grown side by side on agar in Petri dish was used. Potato dextrose agar (PDA) (39 g potato dextrose agar in 1000 ml distilled water) was inoculated with 5 mm diameter disk of an actively growing fungal mycelium (5 days at 25°C on PDA agar) near the dish edge. A loop of the active yeast culture (1-2 days at 25°C on YM agar) was inoculated at the opposite edge. PDA inoculates with only fungi or yeast was used as the controls. The dual cultures in PDA dishs were incubated at 25°C for 7 days and the pathogen mycelial growth reduction was measured and recorded. Yeast strain that reduced pathogen mycelial growth was selected to evaluate of

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mechanisms for controlling of plant pathogenic fungi. The percentage of inhibition of the fungal growth was calculated as follow

% Inhibition = (Diameter of control fungal colony - Diameter of fungal colony grown with yeast) x 100/ Diameter of control fungal colony

7. Evaluation of mechanisms for controlling of plant pathogenic fungi of the selected biological control yeast

To evaluate mechanisms for controlling of plant pathogenic fungi of the selected biological control yeast various characteristics were determined in vitro.

7.1 Evaluation of antagonism on solid medium

The method of Rosa *et al.* (2010) was used. The pathogenic fungus and yeast were grown side by side on PDA and incubated at 25°C for 7 days. The fungal mycelium was microscopically observed to assess the possible mycelium damage caused by yeast. Mycelium disks (5 mm in diameter) from different parts were removed from the dish in which the yeast had inhibited the fungal growth. The mycelia were studied microscopically at 400X magnification. The mycelia were photographed and their morphology was compared with the morphology of the pathogenic fungus growing alone on PDA.

7.2 Production of antifungal volatile compounds

To determine the antifungal volatile compounds production by the selected biological control yeast the method described by Rosa *et al.* (2010) was used. The Petri dishes that has partition in the middle, which prevents non-volatile exudates produced by the yeast from reaching the fungus through the culture medium was used. The same amount of PDA medium was poured into each side of the dish. The PDA dish was inoculated by actively growing yeast on one side and a 5 mm diameter disk of an actively growing fungal mycelium on another one. After 7 days of incubation at 25°C, the

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mycelium growth was compared with that of dish on which only fungus was inoculated without yeast inoculation on another side.

7.3 Siderophores production

To detect siderophores production by the selected biological control yeast. Chrome Azurol S (CAS) Blue Agar was prepared according to Schwyn and Neilands (1997) using 60.5 mg CAS dissolved in 50 ml water distilled, deionized, and mixed with 10 ml iron (III) solution (1 mM FeCl .6H_2O, 10 mM HCl).This solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml water with stirring. The resultant dark blue liquid was autoclaved at 121°C for 15 min. Also autoclaved a mixture of 750 ml water, 15 g agar, 30.24 g Pipes, and 12 g of a solution of 50% (w/w) NaOH to raise the pH to the pKa of Pipes (pKa 6.8). The dye solution was finally poured along the glasswall and agitated with enough care to avoid foaming. A loop of the active yeast culture (1-2 days at 25°C on YM agar) was inoculated on CAS medium and incubated at 25°C for 10 days, in the dark. The medium color change (from blue to purple or yellow) indicates siderophore production.

7.4. Detection of killer activity

The yeast strain was characterized for the production of killer toxin by the method of Ceccato-Antonini *et al.* (2004). Cell suspension of the killer sensitive strain, *Rhodotorula sloffiae* (CBS 7095), was prepared to the concentration 10^5 cells/ml, after cultivation for 24 h at 30°C in YPD broth. A volume of 100 µl of the sensitive strain was spread over the buffered YPD-methylene blue agar (pH 4.3-4.7) and after drying the investigating yeast strain was inoculated. The agar plate was incubated at 30°C for 5 days. If the investigating yeast strain has capacity to produce killer toxin an inhibition halo was observed surrounding the colony of yeast.

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7.5 Production of hydrolytic enzymes (β-glucanase and chitinase)

Method of Saligkarias *et al.* (2002) was used to determine the production of lytic enzymes. A loop of the active yeast cells (1-2 days at 25°C on YM agar) were transferred to 50 ml of yeast extract peptone dextrose (YPD) broth with cell wall of fungal in 250 ml Erlenmeyer flask and incubated at room temperature for 3 days on a rotary shaker 150 rpm. The culture broth was removed and centrifuged at 10,000g for 5 min. The supernatant was recovered and analyzed for β-glucanase and chitinase activities.

To evaluate β-1,3-glucanase activity, the colorimetric quantification of glucose (reducing sugars) released from laminarin was determined by the method of Miller (1959). The reaction was performed by mixing 200 μl of the sample, 50 μl of sodium acetate buffer (0.1 M, pH 5.0) and 250 μl of laminarin (4 mg/ml) and incubated at 37°C for 1 h. Then, the reaction was stopped by adding 250 μl of dinitrosalicylic acid reagent to 250 μl of the reaction and the reducing sugars was determined.

Chitinase production was evaluated by estimating the release of N-acetylglucosamine (NAG) from the substrate colloidal chitin. A volume of 100 μl of the sample was mixed with 200 μl of Mcllvaine buffer (50 mM citric acid; 100 mM sodium phosphate; pH 6.0) and 100 μl of 0.01% w/v colloidal chitin (prepared in the same buffer). After incubation at 37°C for 15 min, the reaction was stopped by adding 250 μl of dinitrosalicylic acid reagent to 250 μl of the reaction and the reducing sugar was determined by the method of Miller (1959).

7.6. Effect of nutrient concentration on yeast-fungal pathogen interaction

To examine the effect of nutrients on mycelia growth reduction by yeast strain the method of Zhang *et al.* (2010) with slight modification was used. The dual inoculation of yeast and the pathogens was performed as follows aliquots of 10 μl of yeast suspension (the active yeast cells 1-2 days at 25°C on YM agar were suspend in sterile distilled water) was pipetted into each side of the dish containing different

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concentrations of PDA (39, 19.5, 9.7, and 3.9 g/L) and after 1 h, on another side inoculated with a 5 mm diameter disk of an actively growing fungal mycelium (5 days at 25°C on PDA agar). After 7 days of incubation at 25°C, the pathogen mycelium growth reduction was determined by measuring diameter of pathogenic fungal colony and recorded.

7.7 Effect of yeast cell suspension, culture filtrate and autoclaved yeast cell suspension on pathogic fungal growth

The dual inoculation of yeast cell suspension, culture filtrate and autoclaved yeast cell suspension with the pathogens was performed on PDA according to the procedures described by Spadaro *et al.* (2002) with slight modification. Yeast cell suspension was prepared by suspended active yeast cells (1-2 days at 25°C on YM agar) in YM broth. After 2 days of incubation at room temperature, yeast cell suspension was removed and centrifuged at 8,000 rpm for 5 min. Yeast cell were washed with sterile distilled water two times and suspended in sterile distilled water. Culture filtrate was prepared by filterd yeast cell in YM broth after 2 days of incubation at room temperature and collected culture filtrate. Autoclaved yeast cell suspension was prepared by autoclaved yeast cell suspension (2 days at room temperature in YM broth). Aliquots of 10 µl of each suspension were pipetted into each side of the dish. After 1 h, inoculated with a 5 mm diameter disk of an actively growing fungal mycelium (5 days at 25°C on PDA agar) on another side. After 7 days of incubation at 25°C, the pathogen mycelium growth reduction was determined by measuring diameter of pathogenic fungal colony and recorded.

8. Statistical analysis

Homogeneity of variances was tested by Levene's test ($P = 0.05$). When the variances were considered homogenous, data were subjected to analysis of variance (ANOVA) using SPSS (version 16.0) and statistical significance was assessed at the level of $P < 0.05$. Duncan's Multiple Range Test was used to separate the means.

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RESULTS AND DISCUSSION

1. Sample collection and yeast isolation

In this study, eighty five samples of rice leaves were collected from 14 districts in seven provinces (Ang Thong, Lop Buri, Nakhon Ratchasima, Nakhon Sawan, Phra Nakhon Si Ayutthaya, Pathum Thani and Suphan Buri) in central and north-eastern parts of Thailand in February, March and May 2010 (Table 11). Sixty five samples of corn leaves were collected from 8 districts in four provinces (Lop Buri, Nakhon Ratchasima, Nakhon Sawan and Phetchabun) in central and north-eastern parts of Thailand in September 2009 and March and May 2010 (Table 12). Ninety four samples of sugarcane leaves were collected from 15 districts in seven provinces (Ang Thong, Lop Buri, Nakhon Ratchasima, Nakhon Sawan, Phra Nakhon Si Ayutthaya, Suphan Buri and Uthai Thani) in central and north-eastern parts of Thailand in September 2009 and February, March and May 2010 (Table 13).

Table 11 Rice leaves collection and number of yeast isolation

Province	Location	Date	Sample No.	No. of yeast strain
Phra Nakhon Si Ayutthaya	Bang Pa In district	3/2/2010	1-7	14
	Bang Ban district	3/2/2010	8-9 and 40-41	10
	Sena district	3/2/2010	10-15	14
	Phak Hai district	3/2/2010	16-24	19
Ang Thong	Samko district	3/2/2010	34-39	12
Pathum Thani	Samkhok district	3/2/2010	42-51	18
Suphan Buri	Si Prachan district	3/2/2010	25-33	17
	Doem Bang Nang Buat district	22/3/2010	52-58	10
Nakhon Sawan	Krok Phra district	23/3/2010	59-66	11

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Table 11 (Continued)

Province	Location	Date	Sample No.	No. of yeast strain
	Chum Saeng district	23/3/2010	67-69	5
Lop Buri	Ban Mi district	5/5/2010	70-76	11
Nakhon Ratchasima	Mueang district	6/5/2010	77-79	4
	Chok Chai district	6/5/2010	80-82	6
	Khon Buri district	6/5/2010	83-85	5

Table 12 Corn leaves collection and number of yeast isolation

Province	Location	Date	Sample No.	No. of yeast strain
Nakhon Ratchasima	Sikhio district	28/9/2009	1-10	15
	Khon Buri district	6/5/2010	39-41	8
	Pak Chong district	6/5/2010	42-65	48
Nakhon Sawan	Chum Saeng district	23/3/2010	11-12	3
	Takhli district	5/5/2010	15-29	31
Phetchabun	Si Thep district	5/5/2010	13-14	6
Lop Buri	Chai Badan district	5/5/2010	30-35	10
	Tha Luang district	5/5/2010	36-38	5

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Table 13 Sugarcane leaves collection and number of yeast isolation

Province	Location	Date	Sample No.	No. of yeast strain
Nakhon Ratchasima	Sikhio district	28/9/2009	1-5	7
	Dan khun Thot district	5/5/2010	72-74	5
	Mueang district	6/5/2010	75-80	8
	Chok Chai district	6/5/2010	81-88	14
	Khon Buri district	6/5/2010	89-94	15
Phra Nakhon Si Ayutthaya	Phak Hai district	3/2/2010	6-16	16
Suphan Buri	Dan Chang district	22/3/2010	17-21	11
Uthai Thani	Ban Rai district	22/3/2010	22-24	7
	Lan Sak district	22/3/2010	25-26	4
Nakhon Sawan	Takhli district	5/5/2010	35-46	18
Phetchabun	Bueng Sam Phan district	5/5/2010	27-31	8
	Wichian Buri district	5/5/2010	32-34	4
Lop Buri	Nong Muang district	5/5/2010	47-55	14
	Khok Samrong district	5/5/2010	56-60	8
	Tha Luang district	5/5/2010	61-71	20

A total of four hundred and forty one yeast strains were isolated by the enrichment technique from the 244 leaf samples including 156 yeast strains from rice leaves, 126 yeast strains from corn leaves and 159 yeast strains from sugarcane leaves (Table 14).

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Table 14 Number of yeast from three crops phylloplane obtained by enrichment isolation

Leaves	Number of sample	Number of sample with yeast	Number of yeast (strains)
Rice	85	85	156
Corn	65	65	126
Sugarcane	94	94	159
Total	244	244	441

2. Identification of yeasts by molecular technique

A total of 441 yeast strains which were isolated from phylloplane of rice, corn and sugarcane were identified by molecular taxonomy based on the analysis of the D1/D2 region of the LSU rRNA gene sequences similarities according to a guideline of Kurtzman and Robnett (1998) that yeast strains with 0-3 nucleotide differences are conspecific or sister species, yeast strains with 4-5 nucleotide substitutions are sister species or different species and yeast strains showing nucleotide substitutions greater than 6 are usually different species. Therefore, a strain showing 0-3 nucleotide substitutions from the type strain of the closest species could be designed as the conspecific or sister species, yeast strains with 4-5 nucleotide substitutions are sister species or new species so should be investigate mutigene for identification and yeast strain showing greater than six from the type strain of the closest species could be designed as the novel species. Undescribed species are the species that the D1/D2 region of the LSU rRNA gene sequences was deposited in the GenBank without description. A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980), using the neighbor-joining method (Saitou and Nei, 1987). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates; Felsenstein, 1985). The yeast strains which are in the same position on phylogenetic tree are conspecific or sister species and yeast strains form cluster with the closest species are usually different species.

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2.1 Identification of yeast strains isolated from rice leaves samples

Identification results of 156 yeast strains from rice leaves revealed that 155 yeast strains were identified to be 32 known species in 18 genera (Table 15 and 16). Twenty-three species were in Phylum Ascomycota (13 genera) viz. *Candida* (9 species), *Clavispora* (1 species), *Cyberlindnera* (2 species), *Debaryomyces* (1 species), *Kodamaea* (1 species), *Lachancea* (1 species), *Metschnikowia* (1 species), *Meyerozyma* (2 species), *Millerozyma* (1 species), *Pichia* (1 species), *Wickerhamomyces* (1 species), *Yamadazyma* (1 species) and *Yarrowia* (1 species), and nine species were in Phylum Basidiomycota (5 genera) viz. *Cryptococcus* (4 species), *Pseudozyma* (2 species), *Rhodotorula* (1 species), *Sporobolomyces* (1 species) and *Trichosporon* (1 species). One strain was found to be represent a novel *Metschnikowia* species. Phylogenetic positions of known species in ascomycete yeast and basidiomycete yeast obtained from rice phylloplane were shown in Figure 1, 2 and 3, respectively. In phylogenetic tree all strains identified as known yeast species were in the same positions with the type strains of known yeast species. All strains identified to be ascomycete yeast species were distributed in 14 phylogenetically distinct clades including *Clavispora, Cyberlindnera, Debaryomyces, Kodamaea, Lachancea, Lodderomyces*, *Metschnikowia, Meyerozyma, Millerozyma, Nakaseomyces, Pichia, Wickerhamomyces, Yamadazyma* and *Yarrowia* clades while *Candida catenulate* and *Candida rugosa* were in unaffiliated clade. Basidiomycete yeast species were distributed in 4 phylogenetically distinct clades including *Bulleromyces Rhodosporidium Sporidiobolus* and *ovoides* clades. *Pseudozyma aphidis* and *Pseudozyma antarctica* were in Ustilaginales.

The dominant yeast species which were isolated from rice phylloplane (85 samples) was *Rhodotorula taiwanensis*, although only 36 strains of this species were isolated from 36 samples, which was calculated to be a frequency of isolation (Number of plant leaf sample from which that species was isolated/ number of plant leaf sample examined × 100) as 23.04%. Twenty five strains of *Candida tropicalis* were isolated from 25 samples, giving a frequency of isolation of 16.03%. Nineteen strains of *Cyberlindnera fabianii* were isolated from 19 samples, represented a 12.18% frequency of isolation (Table 16).

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Table 15 Identification of yeast strains isolated from phylloplane of rice and their GenBank accession numbers of the D1/D2 region of LSU rRNA gene

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
1	DMKU-RK30	*Wickerhamomyces ciferrii* (U74587)	2/567	*Wickerhamomyces ciferrii* (A)	AB772427
1	DMKU-RK31	*Sporobolomyces blumeae* (AY213010)	0/573	*Sporobolomyces blumeae* (B)	AB772428
2	DMKU-RK32	*Cyberlindnera fabianii* (U73573)	2/570	*Cyberlindnera fabianii* (A)	AB772429
2	DMKU-RK33	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772430
3	DMKU-RK34	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772431
4	DMKU-RK35	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772432
4	DMKU-RK36	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772433
5	DMKU-RK37	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772434
5	DMKU-RK38	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772435
5	DMKU-RK39	*Sporobolomyces blumeae* (AY213010)	1/573	*Sporobolomyces blumeae* (B)	AB772436
6	DMKU-RK40	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772437
6	DMKU-RK41	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772438
7	DMKU-RK42	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772439
7	DMKU-RK43	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772440
8	DMKU-RK44	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772441
8	DMKU-RK45	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772442
9	DMKU-RK47	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772443
9	DMKU-RK48	*Candida pseudolambica* (U71063)	1/559	*Candida pseudolambica* (A)	AB772444
10	DMKU-RK49	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772445

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Table 15 (Continued)

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
10	DMKU-RK51	*Trichosporon asahii* (AF105393)	0/597	*Trichosporon asahii* (B)	AB772446
11	DMKU-RK52	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772447
11	DMKU-RK53	*Candida catenulata* (U45714)	0/465	*Candida catenulate* (A)	AB772448
11	DMKU-RK54	*Cryptococcus rajasthanensis* (AM262324)	0/597	*Cryptococcus rajasthanensis* (B)	AB772449
12	DMKU-RK56	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772450
12	DMKU-RK57	*Candida membranifaciens* (U45792)	0/542	*Candida membranifaciens* (A)	AB772451
13	DMKU-RK58	*Rhodotorula taiwanensis* (GU646863)	1/551	*Rhodotorula taiwanensis* (B)	AB741522
13	DMKU-RK59	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772452
13	DMKU-RK60	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772453
14	DMKU-RK61	*Metschnikowia koreensis* (AF296438)	0/512	*Metschnikowia koreensis* (A)	AB772454
14	DMKU-RK62	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772455
15	DMKU-RK63	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772456
15	DMKU-RK64	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772457
16	DMKU-RK67	*Kodamaea ohmeri* (U45702)	0/493	*Kodamaea ohmeri* (A)	AB772458
16	DMKU-RK69	*Trichosporon asahii* (AF105393)	0/597	*Trichosporon asahii* (B)	AB772459
17	DMKU-RK70	*Metschnikowia koreensis* (AF296438)	0/477	*Metschnikowia koreensis* (A)	AB772460
17	DMKU-RK71	*Debaryomyces nepalensis* (U45839)	0/570	*Debaryomyces nepalensis* (A)	AB772461
18	DMKU-RK72	*Trichosporon asahii* (AF105393)	0/597	*Trichosporon asahii* (B)	AB772462
18	DMKU-RK73	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772463
19	DMKU-RK75	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772465

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Table 15 (Continued)

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
19	DMKU-RK76	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB772466
20	DMKU-RK77	*Trichosporon asahii* (AF105393)	0/597	*Trichosporon asahii* (B)	AB772467
20	DMKU-RK78	*Debaryomyces nepalensis* (U45839)	0/570	*Debaryomyces nepalensis* (A)	AB772468
20	DMKU-RK79	*Kodamaea ohmeri* (U45702)	0/493	*Kodamaea ohmeri* (A)	AB772469
21	DMKU-RK80	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772470
22	DMKU-RK81	*Candida catenulata* (U45714)	1/465	*Candida catenulate* (A)	AB772471
22	DMKU-RK82	*Yarrowia lipolytica* (U40080)	1/504	*Yarrowia lipolytica* (A)	AB772472
23	DMKU-RK84	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772473
23	DMKU-RK85	*Rhodotorula taiwanensis* (GU646863)	0/529	*Rhodotorula taiwanensis* (B)	AB772474
23	DMKU-RK86	*Pseudozyma aphidis* (AJ235303)	3/588	*Pseudozyma aphidis* (B)	AB772475
24	DMKU-RK90	*Rhodotorula taiwanensis* (GU646863)	3/572	*Rhodotorula taiwanensis* (B)	AB772476
24	DMKU-RK91	*Metschnikowia koreensis* (AF296438)	0/512	*Metschnikowia koreensis* (A)	AB772477
25	DMKU-RK92	*Rhodotorula taiwanensis* (GU646863)	0/555	*Rhodotorula taiwanensis* (B)	AB772478
26	DMKU-RK94	*Candida jaroonii* (DQ404493)	0/548	*Candida jaroonii* (A)	AB772479
26	DMKU-RK95	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772480
27	DMKU-RK98	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772481
27	DMKU-RK99	*Cryptococcus flavescens* (AB035042)	0/570	*Cryptococcus flavescens* (B)	AB772482
28	DMKU-RK100	*Rhodotorula taiwanensis* (GU646863)	1/559	*Rhodotorula taiwanensis* (B)	AB741523
28	DMKU-RK101	*Candida jaroonii* (DQ404493)	0/548	*Candida jaroonii* (A)	AB772483
28	DMKU-RK102	*Trichosporon asahii* CBS 2479 (AF105393)	0/597	*Trichosporon asahii* (B)	AB772484

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Table 15 (Continued)

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
29	DMKU-RK103	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772485
29	DMKU-RK104	*Candida tropicalis* (U45749)	1/570	*Candida tropicalis* (A)	AB772486
29	DMKU-RK105	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772487
30	DMKU-RK106	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772488
31	DMKU-RK107	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772489
31	DMKU-RK108	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772490
32	DMKU-RK109	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772491
32	DMKU-RK110	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772492
33	DMKU-RK112	*Pseudozyma antarctica* (AJ235302)	0/588	*Pseudozyma antarctica* (B)	AB772493
34	DMKU-RK113	*Rhodotorula taiwanensis* (GU646863)	1/509	*Rhodotorula taiwanensis* (B)	AB741527
34	DMKU-RK114	*Cyberlindnera fabianii* (U73573)	0/569	*Cyberlindnera fabianii* (A)	AB772494
35	DMKU-RK117	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772495
35	DMKU-RK118	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772496
36	DMKU-RK121	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772497
36	DMKU-RK122	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772498
37	DMKU-RK123	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772499
37	DMKU-RK124	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772500
38	DMKU-RK127	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772503
39	DMKU-RK128	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772504
39	DMKU-RK129	*Cryptococcus flavescens* (AB035042)	0/548	*Cryptococcus flavescens* (B)	AB772505

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Table 15 (Continued)

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
39	DMKU-RK130	*Trichosporon asahii* AF105393	0/597	*Trichosporon asahii* (B)	AB772506
40	DMKU-RK131	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772507
40	DMKU-RK132	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772508
40	DMKU-RK133	*Pichia kudriavzevii* (U76347)	1/564	*Pichia kudriavzevii* (A)	AB772509
41	DMKU-RK134	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772510
41	DMKU-RK135	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772511
41	DMKU-RK136	*Wickerhamomyces ciferrii* (U74587)	2/567	*Wickerhamomyces ciferrii* (A)	AB772512
42	DMKU-RK137	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772513
42	DMKU-RK138	*Candida jaroonii* (DQ404493)	0/548	*Candida jaroonii* (A)	AB772514
43	DMKU-RK140	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772515
44	DMKU-RK141	*Cryptococcus flavescens* (AB035042)	0/570	*Cryptococcus flavescens* (B)	AB772516
44	DMKU-RK142	*Debaryomyces nepalensis* (U45839)	1/570	*Debaryomyces nepalensis* (A)	AB772517
45	DMKU-RK144	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772518
46	DMKU-RK145	*Candida tropicalis* (U45749)	1/570	*Candida tropicalis* (A)	AB772519
47	DMKU-RK146	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772520
48	DMKU-RK148	*Rhodotorula taiwanensis* (GU646863)	1/550	*Rhodotorula taiwanensis* (B)	AB772521
48	DMKU-RK149	*Meyerozyma guilliermondii* (U62311)	0/570	*Meyerozyma guilliermondii* (A)	AB772522
49	DMKU-RK150	*Rhodotorula taiwanensis* (GU646863)	1/533	*Rhodotorula taiwanensis* (B)	AB772523
49	DMKU-RK151	*Pichia rhodanensis* (U73571)	1/573	*Pichia rhodanensis* (A)	AB772524
49	DMKU-RK152	*Candida tropicalis* (U45749)	0/538	*Candida tropicalis* (A)	AB772525

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Table 15 (Continued)

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
49	DMKU-RK153	*Debaryomyces nepalensis* (U45839)	3/570	*Debaryomyces nepalensis* (A)	AB772526
50	DMKU-RK155	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772527
50	DMKU-RK156	*Debaryomyces nepalensis* (U45839)	0/550	*Debaryomyces nepalensis* (A)	AB772528
51	DMKU-RK157	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772529
51	DMKU-RK158	*Debaryomyces nepalensis* (U45839)	1/533	*Debaryomyces nepalensis* (A)	AB772530
52	DMKU-RK161	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772532
53	DMKU-RK163	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772534
54	DMKU-RK164	*Candida rugosa* (U45727)	3/484	*Candida rugosa* (A)	AB772535
55	DMKU-RK165	*Candida glabrata* (U44808)	1/581	*Candida glabrata* (A)	AB772536
55	DMKU-RK166	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772537
56	DMKU-RK167	*Cryptococcus rajasthanensis* (AM262324)	0/597	*Cryptococcus rajasthanensis* (B)	AB772538
56	DMKU-RK168	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772539
57	DMKU-RK169	*Cryptococcus* aff. *laurentii* (AM931019)	0/597	*Cryptococcus* aff. *laurentii* (B)	AB772540
58	DMKU-RK171	*Cryptococcus* aff. *laurentii* (AM931019)	0/597	*Cryptococcus* aff. *laurentii* (B)	AB772541
58	DMKU-RK172	*Rhodotorula taiwanensis* (GU646863)	1/551	*Rhodotorula taiwanensis* (B)	AB741524
59	DMKU-RK215	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772542
59	DMKU-RK216	*Meyerozyma guilliermondii* (U62311)	0/518	*Meyerozyma guilliermondii* (A)	AB772543
60	DMKU-RK219	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772544
61	DMKU-RK221	*Meyerozyma guilliermondii* (U62311)	0/570	*Meyerozyma guilliermondii* (A)	AB772545
62	DMKU-RK224	*Cryptococcus rajasthanensis* (AM262324)	0/597	*Cryptococcus rajasthanensis* (B)	AB772546

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Table 15 (Continued)

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
63	DMKU-RK226	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772547
64	DMKU-RK227	*Rhodotorula taiwanensis* (GU646863)	1/560	*Rhodotorula taiwanensis* (B)	AB772548
64	DMKU-RK228	*Meyerozyma guilliermondii* (U62311)	1/570	*Meyerozyma guilliermondii* (A)	AB772549
65	DMKU-RK229	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772550
65	DMKU-RK230	*Cryptococcus* aff. *laurentii* (AM931019)	0/597	*Cryptococcus* aff. *laurentii* (B)	AB772551
66	DMKU-RK231	*Rhodotorula taiwanensis* (GU646863)	0/551	*Rhodotorula taiwanensis* (B)	AB772552
67	DMKU-RK235	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772553
68	DMKU-RK237	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772554
68	DMKU-RK238	*Millerozyma koratensis* (DQ404484)	0/569	*Millerozyma koratensis* (A)	AB772555
69	DMKU-RK240	*Meyerozyma caribbica* (EU348786)	0/547	*Meyerozyma caribbica* (A)	AB772556
69	DMKU-RK241	*Millerozyma koratensis* (DQ404484)	0/569	*Millerozyma koratensis* (A)	AB772557
70	DMKU-RK272	*Millerozyma koratensis* (DQ404484)	0/569	*Millerozyma koratensis* (A)	AB772558
70	DMKU-RK273	*Meyerozyma caribbica* (EU348786)	0/533	*Meyerozyma caribbica* (A)	AB772559
71	DMKU-RK274	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772560
71	DMKU-RK275	*Lachancea thermotolerans* (U69581)	1/571	*Lachancea thermotolerans* (A)	AB772561
72	DMKU-RK277	*Candida hainanensis* (EU284103)	18/449	New species (A)	AB697756
73	DMKU-RK279	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772562
74	DMKU-RK280	*Debaryomyces nepalensis* (U45839)	1/570	*Debaryomyces nepalensis* (A)	AB772563
74	DMKU-RK281	*Meyerozyma caribbica* (EU348786)	0/517	*Meyerozyma caribbica* (A)	AB772564
75	DMKU-RK282	*Meyerozyma caribbica* (EU348786)	0/533	*Meyerozyma caribbica* (A)	AB772565

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Table 15 (Continued)

Rice leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
75	DMKU-RK283	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772566
76	DMKU-RK284	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772567
77	DMKU-RK434	*Candida ruelliae* (AM262326)	2/521	*Candida ruelliae* (A)	AB772568
78	DMKU-RK435	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772569
78	DMKU-RK436	*Candida terebra* (U45784)	0/531	*Candida terebra* (A)	AB772570
79	DMKU-RK437	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772571
80	DMKU-RK460	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772573
80	DMKU-RK461	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772574
81	DMKU-RK462	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772575
81	DMKU-RK463	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772576
82	DMKU-RK464	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772577
82	DMKU-RK465	*Yamadazyma mexicanum* (U45790)	0/531	*Yamadazyma mexicanum* (A)	AB772578
83	DMKU-RK481	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772579
84	DMKU-RK482	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772580
84	DMKU-RK483	*Candida rugosa* (U45727)	1/485	*Candida rugosa* (A)	AB772581
85	DMKU-RK484	*Clavispora lusitaniae* (AJ539561)	1/517	*Clavispora lusitaniae* (A)	AB772582
85	DMKU-RK485	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772583

Remark: (A), Ascomycota (B), Basidiomycota

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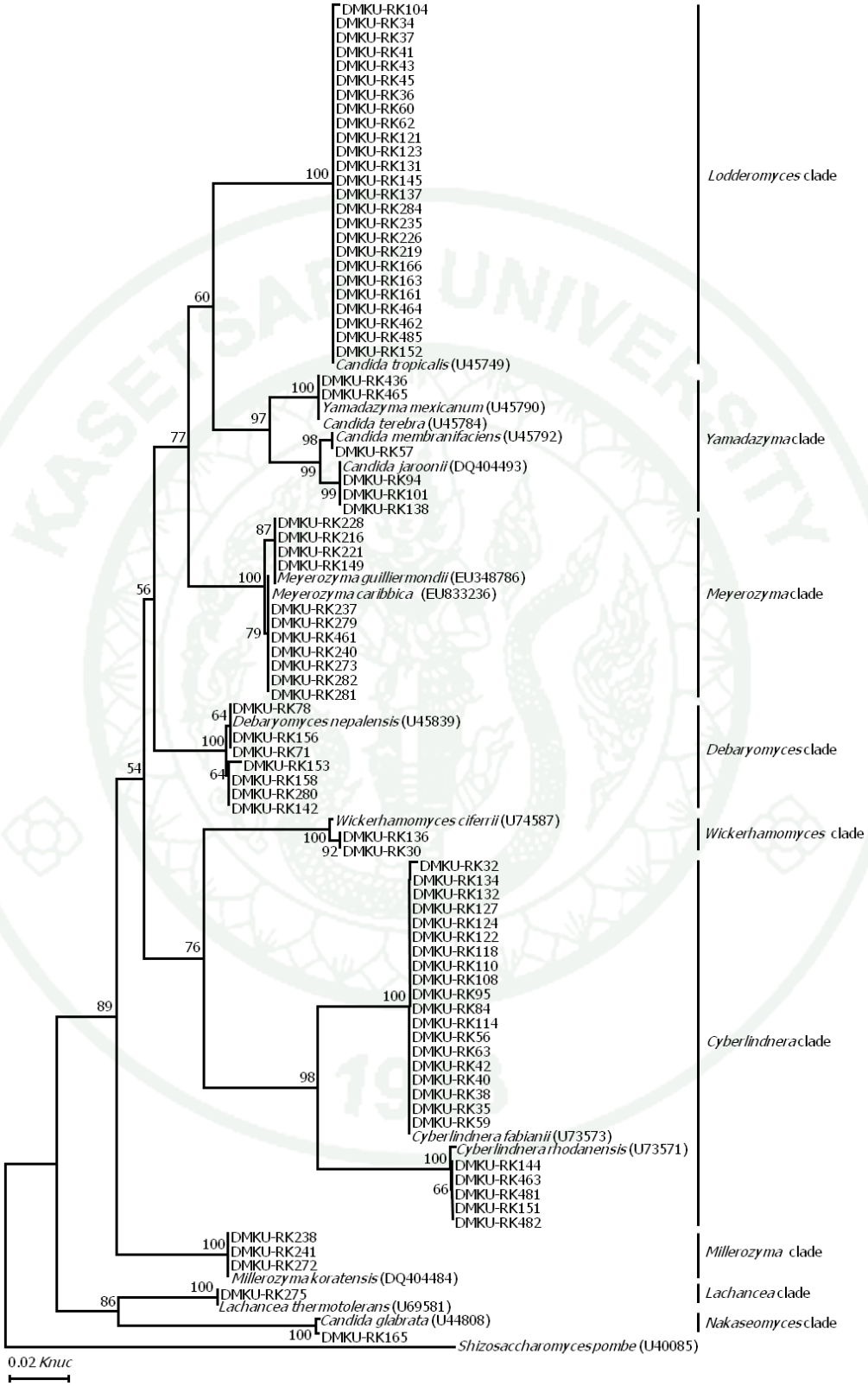


Figure 1 Phylogeny of ascomycete yeasts isolated from rice phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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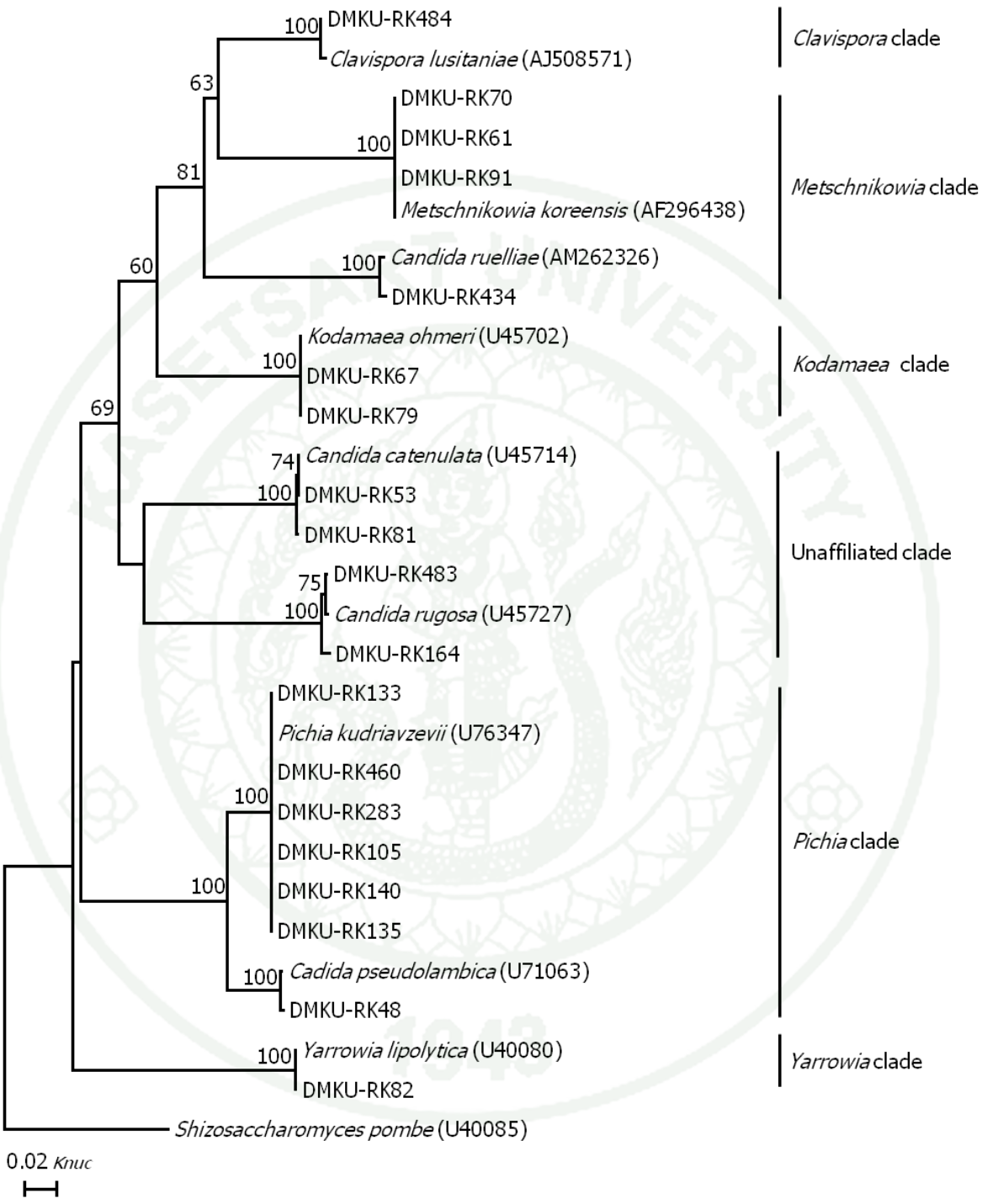


Figure 2 Phylogeny of ascomycete yeasts isolated from rice phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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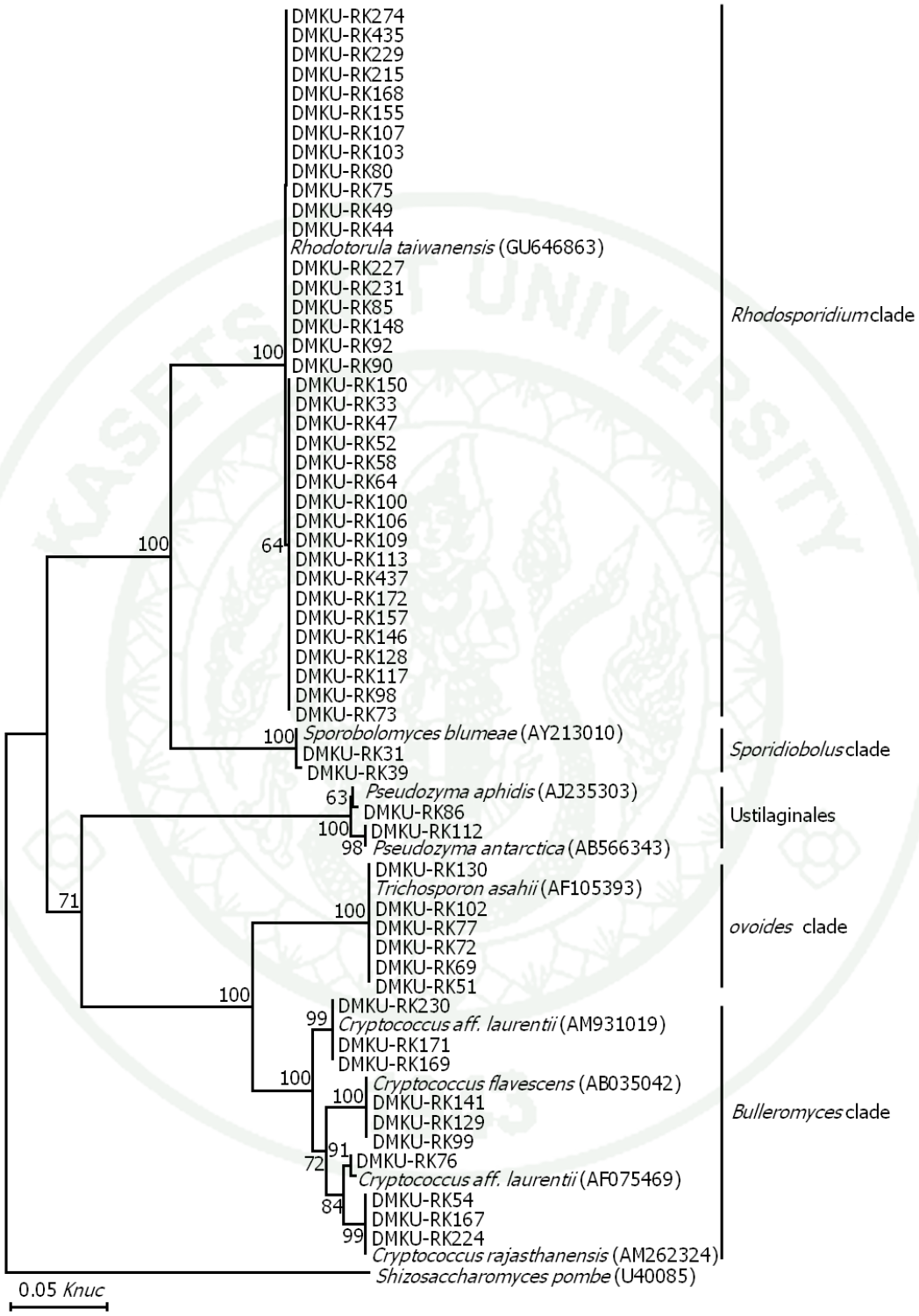


Figure 3 Phylogeny of basidiomycete yeasts isolated from rice phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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Table 16 Yeasts from rice phylloplane and their frequency of isolation

Species	No. of leaves sample with this yeast species	Frequency of isolation (%)*
Known species		
Ascomycota		
Candida catenulata	2	1.28
Candida glabrata	1	0.64
Candida jaroonii	3	1.92
Candida membranifaciens	1	0.64
Candida pseudolambica	1	0.64
Candida ruelliae	1	0.64
Candida rugosa	2	1.28
Candida terebra	1	0.64
Candida tropicalis	25	16.03
Clavispora lusitaniae	1	0.64
Cyberlindnera fabianii	19	12.18
Cyberlindnera rhodanensis	5	3.21
Debaryomyces nepalensis	7	4.49
Lachancea thermotolerans	1	0.64
Kodamaea ohmeri	2	1.28
Metschnikowia koreensis	3	1.92
Meyerozyma caribbica	7	4.49
Meyerozyma guilliermondii	4	2.56
Millerozyma koratensis	3	1.92
Pichia kudriavzevii	6	3.85
Wickerhamomyces ciferrii	2	1.28
Yamadazyma mexicanum	1	0.64
Yarrowia lipolytica	1	0.64
Basidiomycota		
Cryptococcus flavescens	3	1.92
Cryptococcus aff. *laurentii*	3	1.92
Cryptococcus laurentii	1	0.64
Cryptococcus rajasthanensis	3	1.92
Pseudozyma antarctica	1	0.64
Pseudozyma aphidis	1	0.64
Rhodotorula taiwanensis	36	23.04
Sporobolomyces blumeae	2	1.28
Trichosporon asahii	6	3.85
New species		
Metschnikowia lopburiensis sp. nov.	1	0.64

* Number of plant leaf sample from which that species was isolated/ number of plant leaf sample examined ×100

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2.2 Identification of yeast strains isolated from corn leaves samples

Identification of 126 yeast strains from corn leaves revealed that 123 yeast strains were identified to be 39 known species in 17 genera (Table 17 and 18). Twenty-eight species were in Phylum Ascomycota (12 genera) viz. *Candida* (11 species), *Cyberlindnera* (1 species), *Geotrichum* (1 species), *Hanseniaspora* (3 species), *Kluyveromyces* (1 species), *Kodamaea* (1 species), *Meyerozyma* (2 species), *Millerozyma* (2 species), *Pichia* (3 species), *Torulaspora* (1 species), *Wickerhamomyces* (1 species) and *Yamadazyma* (1 species), and eleven species were in Phylum Basidiomycota (5 genera) viz. *Cryptococcus* (4 species), *Rhodosporidium* (3 species), *Rhodotorula* (1 species), *Sporobolomyces* (1 species) and *Sporidiobolus* (2 species). Two strains were similar to *Candida* sp. 05-7-186T which was undescribed species in Ascomycota and one strain was found to be represent a novel *Yamadazyma* species. Phylogenetic positions of known species in ascomycete yeast and basidiomycete yeast obtained from corn phylloplane were shown in Figure 4, 5 and 6, respectively. In phylogenetic tree all strains identified as known yeast species were in the same positions with the type strains of known yeast species. All known ascomycete yeast species were distributed in 15 phylogenetically distinct clades including *Cyberlindnera, Hanseniaspora, Galactomyces, Kodamaea, Kluyveromyces, Kurtzmaniella, Lodderomyces-Spathospora, Metschnikowia, Meyerozyma, Millerozyma, Nakaseomyces, Pichia, Torulaspora, Wickerhamomyces* and *Yamadazyma* clades while *C. catenulate, C. rugosa, C. mesorugosa* and *C. sorboxylosa* were in unaffiliated clade. Known basidiomycete yeast were distributed in four phylogenetically distinct clades including *Bulleromyces, Kwoniella, Rhodosporidium* and *Sporidiobolus* clades.

The dominant yeast species was *Kodamaea ohmeri, Meyerozyma caribbica* and *Rhodotorula taiwanensis,* although only 10 strains of each species were isolated from 30 samples, represented a 7.94% frequency of isolation of each species. Eight strains of each species namely *Candida tropicalis, Hanseniaspora opuntiae, Pichia kudriavzevii* and *Rhodosporidium fluviale* were isolated from 32 samples, giving a frequency of isolation of each species 6.35%. Seven strains of *Cryptococcus laurentii* were isolated from 7 samples, represented a 5.56% frequency of isolation (Table 18).

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Table 17 Identification of yeast strains isolated from phylloplane of corn and their GenBank accession numbers of the D1/D2 region of LSU rRNA gene

Corn leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
1	DMKU-RK1	*Candida quercitrusa* (U45831)	3/567	*Candida quercitrusa* (A)	AB773291
1	DMKU-RK3	*Candida sorboxylosa* (U62314)	0/337	*Candida sorboxylosa* (A)	AB773293
2	DMKU-RK4	*Candida quercitrusa* (U45831)	3/567	*Candida quercitrusa* (A)	AB773294
3	DMKU-RK5	*Candida quercitrusa* (U45831)	2/565	*Candida quercitrusa* (A)	AB773295
4	DMKU-RK6	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773296
5	DMKU-RK7	*Candida* sp. 05-7-186T (FJ537069)	0/495	Undescribed species (A)	AB773297
5	DMKU-RK8	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773298
6	DMKU-RK9	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB773299
6	DMKU-RK10	*Cryptococcus flavescens* (AB035042)	0/570	*Cryptococcus flavescens* (B)	AB773300
7	DMKU-RK11	*Candida pseudointermedia* (U44816)	0/518	*Candida pseudointermedia* (A)	AB773301
7	DMKU-RK12	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773302
8	DMKU-RK13	*Cryptococcus flavescens* (AB035042)	0/570	*Cryptococcus flavescens* (B)	AB773303
9	DMKU-RK18	*Cryptococcus flavescens* (AB035042)	0/570	*Cryptococcus flavescens* (B)	AB773304
10	DMKU-RK21	*Candida* sp. 05-7-186T (FJ537069)	0/495	Undescribed species (A)	AB773306
10	DMKU-RK23	*Cryptococcus flavescens* (AB035042)	2/570	*Cryptococcus flavescens* (B)	AB773308
11	DMKU-RK242	*Rhodotorula taiwanensis* (GU646863)	1/570	*Rhodotorula taiwanensis* (B)	AB741529
11	DMKU-RK243	*Cryptococcus* aff. *laurentii* (AM931019)	0/597	*Cryptococcus* aff. *laurentii* (B)	AB773309
12	DMKU-RK245	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB773310
13	DMKU-RK263	*Sporobolomyces nylandii* (AB279629)	0/527	*Sporobolomyces nylandii* (B)	AB773311
13	DMKU-RK264	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB773312
13	DMKU-RK265	*Millerozyma koratensis* (DQ404484)	0/530	*Millerozyma koratensis* (A)	AB773313
14	DMKU-RK266	*Rhodosporidium paludigenum* (AF363640)	0/574	*Rhodosporidium paludigenum* (B)	AB773314
14	DMKU-RK267	*Cryptococcus laurentii* (AF075469)	1/566	*Cryptococcus laurentii* (B)	AB773315

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Table 17 (Continued)

Corn leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
14	DMKU-RK268	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773316
15	DMKU-RK304	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB773317
15	DMKU-RK305	*Candida mesorugosa* (FJ768919)	2/453	*Candida mesorugosa* (A)	AB773318
15	DMKU-RK306	*Rhodosporidium fluviale* (AF070422)	2/555	*Rhodosporidium fluviale* (B)	AB773319
16	DMKU-RK307	*Sporobolomyces nylandii* (AF387123)	0/546	*Sporobolomyces nylandii* (B)	AB773320
16	DMKU-RK308	*Kodamaea ohmeri* (U45702)	0/475	*Kodamaea ohmeri* (A)	AB773321
17	DMKU-RK310	*Geotrichum phurueaensis* (AB498990)	0/545	*Geotrichum phurueaensis* (A)	AB773323
17	DMKU-RK311	*Meyerozyma guilliermondii* (U62311)	0/570	*Meyerozyma guilliermondii* (A)	AB773324
17	DMKU-RK312	*Candida tropicalis* (U45749)	0/548	*Candida tropicalis* (A)	AB773325
18	DMKU-RK313	*Candida tropicalis* (U45749)	0/548	*Candida tropicalis* (A)	AB773326
19	DMKU-RK315	*Rhodotorula taiwanensis* (GU646863)	1/551	*Rhodotorula taiwanensis* (B)	AB773328
19	DMKU-RK317	*Kodamaea ohmeri* (U45702)	0/476	*Kodamaea ohmeri* (A)	AB773329
20	DMKU-RK318	*Candida glabrata* (U44808)	1/543	*Candida glabrata* (A)	AB773330
20	DMKU-RK319	*Candida tropicalis* (U45749)	0/548	*Candida tropicalis* (A)	AB773331
20	DMKU-RK320	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB773332
21	DMKU-RK321	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB773333
21	DMKU-RK322	*Candida orthopsilosis* (FJ746056)	0/570	*Candida orthopsilosis* (A)	AB773334
22	DMKU-RK323	*Rhodotorula taiwanensis* (GU646863)	1/572	*Rhodotorula taiwanensis* (B)	AB773335
22	DMKU-RK324	*Candida orthopsilosis* (FJ746056)	1/553	*Candida orthopsilosis* (A)	AB773336
23	DMKU-RK325	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB773337
23	DMKU-RK326	*Wickerhamomyces edaphicus* (AB436763)	2/573	*Wickerhamomyces edaphicus* (A)	AB714244
24	DMKU-RK327	*Hanseniaspora opuntiae* (AJ512453)	0/550	*Hanseniaspora opuntiae* (A)	AB773338
24	DMKU-RK328	*Kodamaea ohmeri* (U45702)	1/493	*Kodamaea ohmeri* (A)	AB773339
25	DMKU-RK330	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB773341
25	DMKU-RK331	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773342

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Table 17 (Continued)

Corn leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
26	DMKU-RK332	*Rhodotorula taiwanensis* (GU646863)	0/508	*Rhodotorula taiwanensis* (B)	AB773343
26	DMKU-RK333	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773344
27	DMKU-RK334	*Rhodosporidium toruloides* (AF070426)	0/573	*Rhodosporidium toruloides* (B)	AB773345
27	DMKU-RK335	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB773346
28	DMKU-RK336	*Rhodosporidium toruloides* (AF070426)	0/573	*Rhodosporidium toruloides* (B)	AB773347
28	DMKU-RK337	*Cryptococcus laurentii* (AF075469)	1/546	*Cryptococcus laurentii* (B)	AB773348
29	DMKU-RK338	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB773349
30	DMKU-RK368	*Sporidiobolus ruineniae* (AF070438)	0/552	*Sporidiobolus ruineniae* (B)	AB773350
30	DMKU-RK369	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773351
31	DMKU-RK371	*Kluyveromyces marxianus* (U94924)	0/545	*Kluyveromyces marxianus* (A)	AB773352
32	DMKU-RK372	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis*	AB773353
32	DMKU-RK373	*Cryptococcus* aff. *laurentii* (AM931019)	0/570	*Cryptococcus* aff. *laurentii* (B)	AB773354
33	DMKU-RK375	*Meyerozyma guilliermondii* (U62311)	1/533	*Meyerozyma guilliermondii* (A)	AB773355
34	DMKU-RK378	*Meyerozyma caribbica* (EU348786)	0/533	*Meyerozyma caribbica* (A)	AB773356
34	DMKU-RK379	*Millerozyma acaciae* (U45767)	2/548	*Millerozyma acacia* (A)	AB773357
35	DMKU-RK380	*Rhodosporidium fluviale* (AF070422)	1/555	*Rhodosporidium fluviale* (B)	AB773358
35	DMKU-RK381	*Cryptococcus flavescens* (AB035042)	2/558	*Cryptococcus flavescens* (B)	AB773359
36	DMKU-RK402	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB773360
37	DMKU-RK404	*Rhodosporidium fluviale* (AF070422)	1/554	*Rhodosporidium fluviale* (B)	AB773362
37	DMKU-RK405	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB773363
38	DMKU-RK406	*Rhodosporidium fluviale* (AF070422)	1/574	*Rhodosporidium fluviale* (B)	AB773364
38	DMKU-RK407	*Candida rugosa* (U45727)	1/466	*Candida rugosa* (A)	AB773365
39	DMKU-RK472	*Rhodosporidium fluviale* (AF070422)	1/574	*Rhodosporidium fluviale* (B)	AB773366
39	DMKU-RK473	*Pichia galeiformis* (U75738)	0/560	*Pichia galeiformis* (A)	AB773367
39	DMKU-RK474	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB773368

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Table 17 (Continued)

Corn leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
40	DMKU-RK475	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB773369
40	DMKU-RK476	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB773370
40	DMKU-RK477	*Kodamaea ohmeri* (U45702)	0/446	*Kodamaea ohmeri* (A)	AB773371
41	DMKU-RK479	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB773372
41	DMKU-RK480	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB773373
42	DMKU-RK503	*Cryptococcus laurentii* (AF075469)	1/563	*Cryptococcus laurentii* (B)	AB773374
43	DMKU-RK504	*Candida quercitrusa* (U45831)	3/544	*Candida quercitrusa* (A)	AB773375
43	DMKU-RK505	*Candida pseudointermedia* (U44816)	0/518	*Candida pseudointermedia* (A)	AB773376
44	DMKU-RK509	*Rhodosporidium paludigenum* (AF363640)	0/574	*Rhodosporidium paludigenum* (B)	AB773378
44	DMKU-RK510	*Meyerozyma caribbica* (EU348786)	0/534	*Meyerozyma caribbica* (A)	AB773379
45	DMKU-RK511	*Sporidiobolus pararoseus* (AF189977)	2/552	*Sporidiobolus pararoseus* (B)	AB773380
45	DMKU-RK512	*Wickerhamomyces edaphicus* (AB436763)	2/573	*Wickerhamomyces edaphicus* (A)	AB714245
45	DMKU-RK513	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB773381
46	DMKU-RK514	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB773382
46	DMKU-RK515	*Candida pseudointermedia* (U44816)	0/518	*Candida pseudointermedia* (A)	AB773383
46	DMKU-RK516	*Candida quercitrusa* (U45831)	3/567	*Candida quercitrusa* (A)	AB773384
47	DMKU-RK519	*Cryptococcus heveanensis* (AF075467)	1/578	*Cryptococcus heveanensis* (B)	AB773385
48	DMKU-RK520	*Hanseniaspora opuntiae* (AJ512453)	0/550	*Hanseniaspora opuntiae* (A)	AB773386
49	DMKU-RK521	*Hanseniaspora opuntiae* (AJ512453)	0/546	*Hanseniaspora opuntiae* (A)	AB773387
49	DMKU-RK522	*Pichia kudriavzevii* (U76347)	0/542	*Pichia kudriavzevii* (A)	AB773388
50	DMKU-RK523	*Candida glabrata* (U44808)	1/581	*Candida glabrata* (A)	AB773389
50	DMKU-RK524	*Candida catenulata* (U45714)	0/439	*Candida catenulata* (A)	AB773390
51	DMKU-RK525	*Hanseniaspora guilliermondii* (U84230)	0/552	*Hanseniaspora guilliermondii* (A)	AB773391
51	DMKU-RK526	*Meyerozyma guilliermondii* (EU348786)	0/570	*Meyerozyma guilliermondii* (A)	AB773392
52	DMKU-RK528	*Rhodosporidium paludigenum* (AF363640)	0/574	*Rhodosporidium paludigenum* (B)	AB773393

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Table 17 (Continued)

Corn leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
53	DMKU-RK529	*Cyberlindnera rhodanensis* (U73571)	0/465	*Cyberlindnera rhodanensis* (A)	AB773394
53	DMKU-RK530	*Pichia kudriavzevii* (U76347)	0/542	*Pichia kudriavzevii* (A)	AB773395
53	DMKU-RK531	*Pichia kluyveri* (U75727)	1/568	*Pichia kluyveri* (A)	AB773396
54	DMKU-RK532	*Hanseniaspora thailandica* (AB617997)	1/571	*Hanseniaspora thailandica* (A)	AB773397
54	DMKU-RK533	*Kodamaea ohmeri* (U45702)	0/471	*Kodamaea ohmeri* (A)	AB773398
55	DMKU-RK534	*Hanseniaspora thailandica* (AB617997)	1/572	*Hanseniaspora thailandica* (A)	AB773399
55	DMKU-RK535	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB773400
55	DMKU-RK536	*Kodamaea ohmeri* (U45702)	0/493	*Kodamaea ohmeri* (A)	AB773401
56	DMKU-RK537	*Rhodosporidium fluviale* (AF070422)	1/574	*Rhodosporidium fluviale* (B)	AB773402
56	DMKU-RK538	*Candida sojae* (U71070)	2/532	*Candida sojae* (A)	AB773403
57	DMKU-RK539	*Rhodosporidium fluviale* (AF070422)	1/574	*Rhodosporidium fluviale* (B)	AB773404
57	DMKU-RK540	*Candida conglobata* (U45789)	0/504	*Candida conglobata* (A)	AB773405
58	DMKU-RK541	*Hanseniaspora opuntiae* (AJ512453)	1/572	*Hanseniaspora opuntiae* (A)	AB773406
58	DMKU-RK542	*Pichia galeiformis* (U75738)	0/560	*Pichia galeiformis* (A)	AB773407
59	DMKU-RK543	*Hanseniaspora opuntiae* (AJ512453)	0/547	*Hanseniaspora opuntiae* (A)	AB773408
59	DMKU-RK544	*Yamadazyma philogaea* (U45765)	0/530	*Yamadazyma philogaea* (A)	AB773409
60	DMKU-RK545	*Rhodosporidium fluviale* (AF070422)	1/553	*Rhodosporidium fluviale* (B)	AB773410
60	DMKU-RK546	*Hanseniaspora opuntiae* (AJ512451)	0/572	*Hanseniaspora opuntiae* (A)	AB773411
61	DMKU-RK547	*Torulaspora globosa* (U72166)	3/573	*Torulaspora globosa* (A)	AB773412
61	DMKU-RK548	*Candida* sp. TrB1-1 (AY562397)	3/530	New species (A)	AB734047
62	DMKU-RK549	*Hanseniaspora guilliermondii* (U84230)	0/550	*Hanseniaspora guilliermondii* (A)	AB773413
62	DMKU-RK550	*Kodamaea ohmeri* (U45702)	0/466	*Kodamaea ohmeri* (A)	AB773414
63	DMKU-RK551	*Hanseniaspora opuntiae* (AJ512451)	0/572	*Hanseniaspora opuntiae* (A)	AB773415
63	DMKU-RK552	*Kodamaea ohmeri* (U45702)	0/493	*Kodamaea ohmeri* (A)	AB773416
64	DMKU-RK553	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB773417

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Table 17 (Continued)

Corn leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
64	DMKU-RK554	*Kodamaea ohmeri* (U45702)	0/493	*Kodamaea ohmeri* (A)	AB773418
65	DMKU-RK555	*Hanseniaspora opuntiae* (AJ512453)	0/550	*Hanseniaspora opuntiae* (A)	AB773419
65	DMKU-RK556	*Kodamaea ohmeri* (U45702)	0/493	*Kodamaea ohmeri* (A)	AB773420

Remark: (A), Ascomycota (B), Basidiomycota

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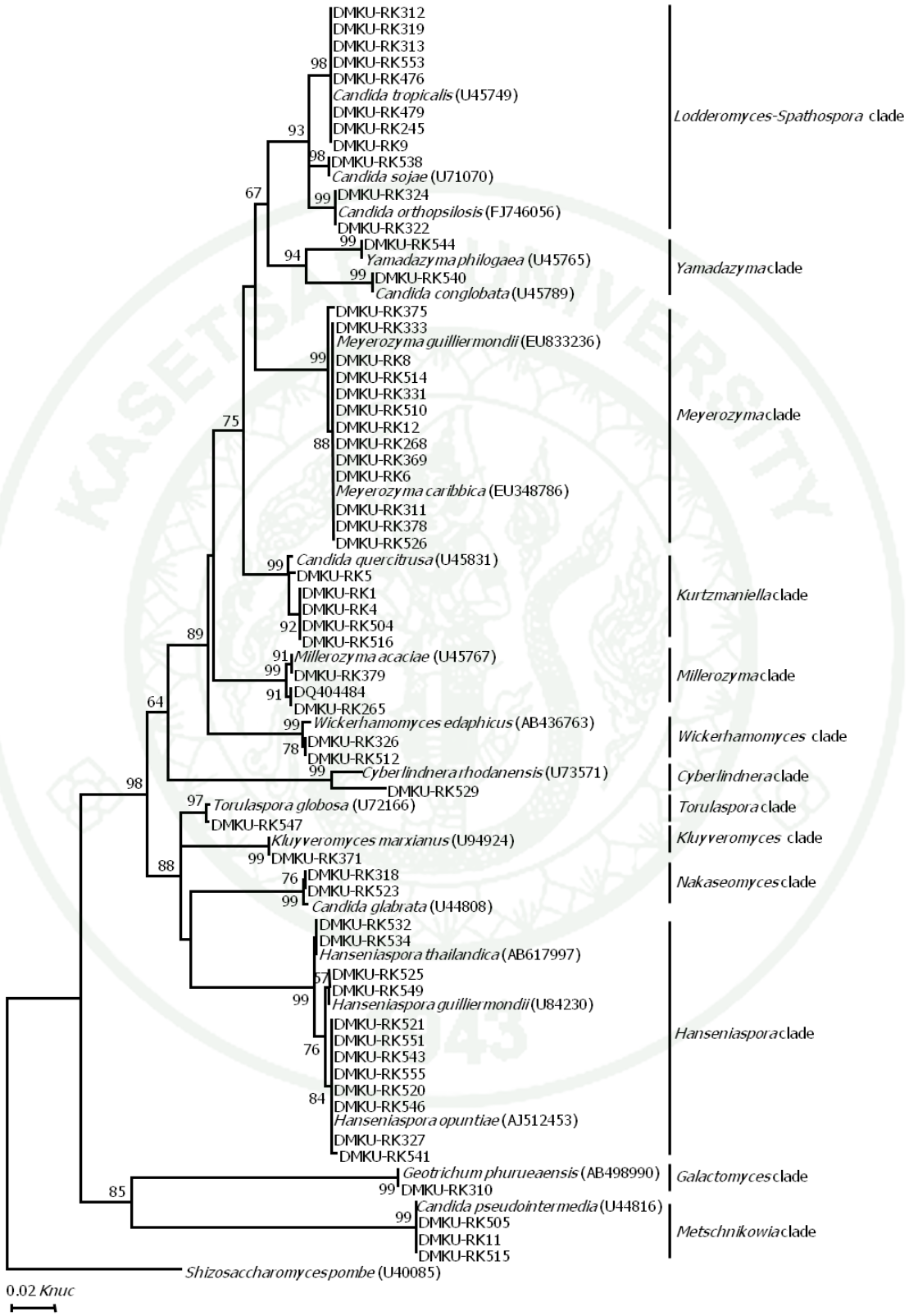


Figure 4 Phylogeny of ascomycete yeasts isolated from corn phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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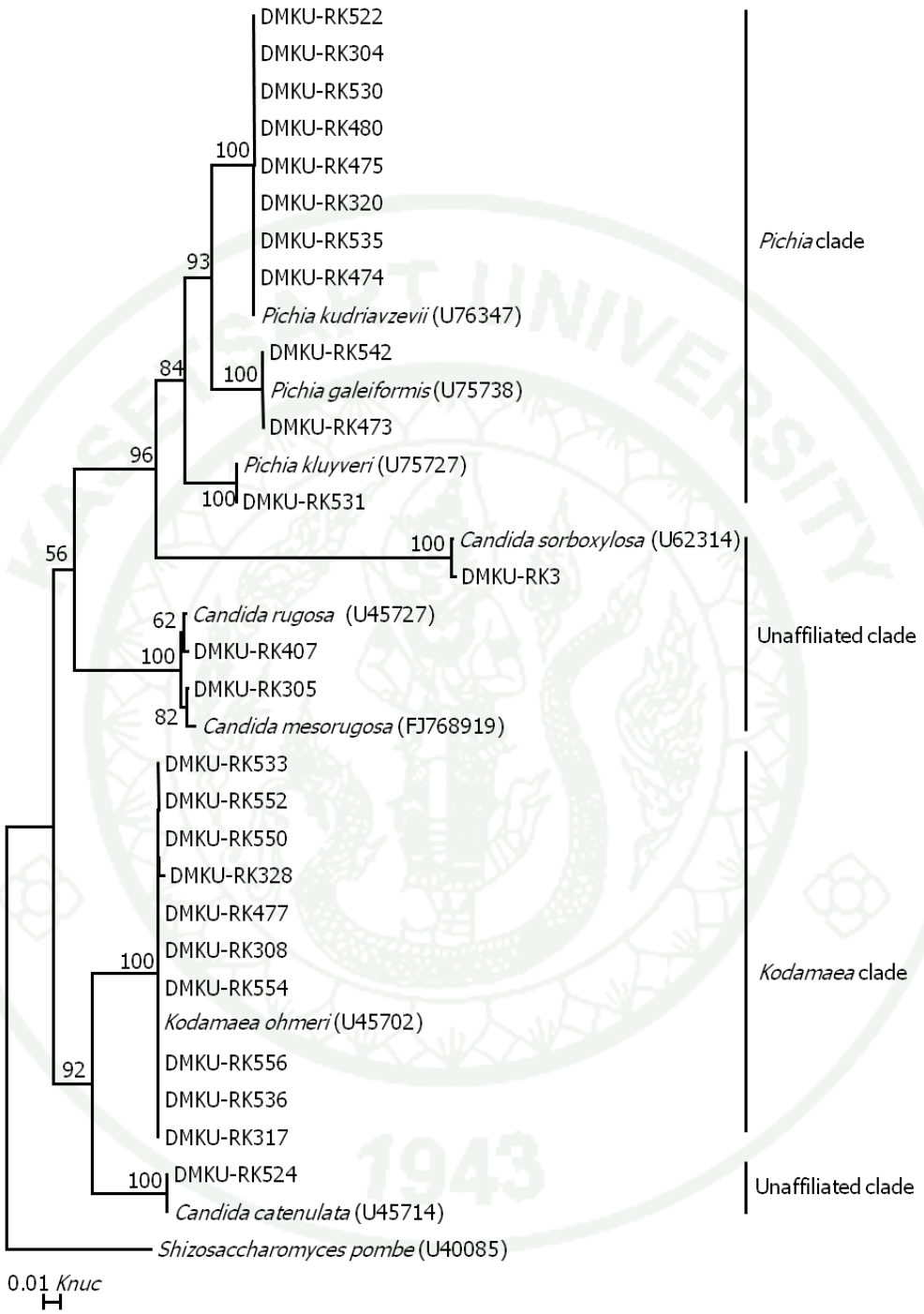


Figure 5 Phylogeny of ascomycete yeasts isolated from corn phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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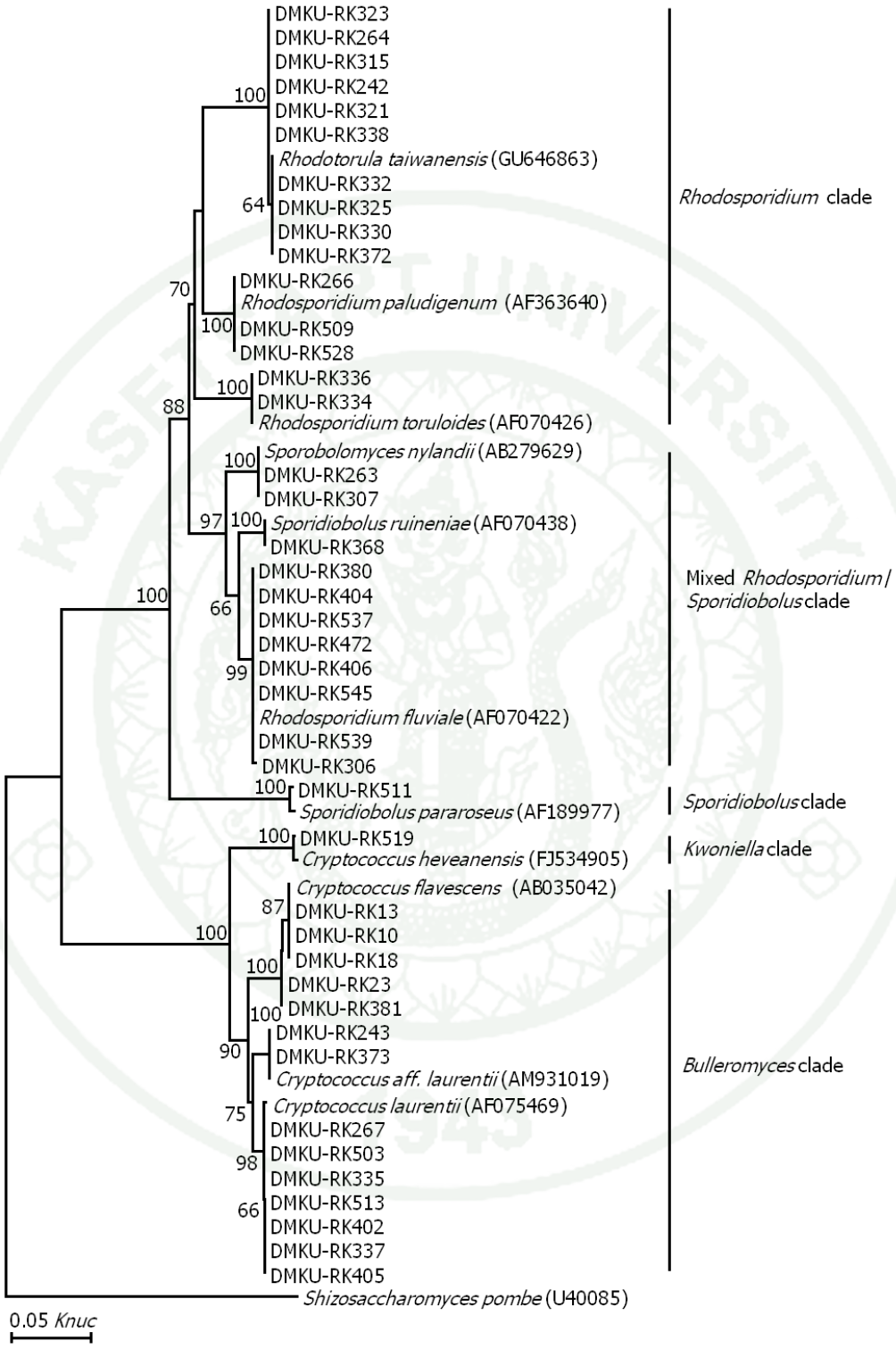


Figure 6 Phylogeny of basidiomycete yeasts isolated from corn phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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Table 18 Yeasts from corn phylloplane and their frequency of isolation

Species	No. of leaves sample with this yeast species	Frequency of isolation (%)*
Known species		
Ascomycota		
Candida catenulata	1	0.79
Candida conglobata	1	0.79
Candida glabrata	2	1.59
Candida pseudointermedia	3	2.38
Candida quercitrusa	5	3.97
Candida tropicalis	8	6.35
Candida mesorugosa	1	0.79
Candida orthopsilosis	2	1.59
Candida rugosa	1	0.79
Candida sojae	1	0.79
Candida sorboxylosa	1	0.79
Cyberlindnera rhodanensis	1	0.79
Geotrichum phurueaensis	1	0.79
Hanseniaspora guilliermondii	2	1.59
Hanseniaspora opuntiae	8	6.35
Hanseniaspora thailandica	2	1.59
Kluyveromyces marxianus	1	0.79
Kodamaea ohmeri	10	7.94
Meyerozyma caribbica	10	7.94
Meyerozyma guilliermondii	3	2.38
Millerozyma acaciae	1	0.79
Millerozyma koratensis	1	0.79
Pichia galeiformis	2	1.59
Pichia kluyveri	1	0.79
Pichia kudriavzevii	8	6.35
Torulaspora globosa	1	0.79
Wickerhamomyces edaphicus	2	1.59
Yamadazyma philogaea	1	0.79
Basidiomycota		
Cryptococcus flavescens	5	3.97
Cryptococcus heveanensis	1	0.79
Cryptococcus aff. *laurentii*	2	1.59
Cryptococcus laurentii	7	5.56
Rhodosporidium fluviale	8	6.35
Rhodosporidium paludigenum	3	2.38
Rhodosporidium toruloides	2	1.59
Rhodotorula taiwanensis	10	7.94

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Table 18 (Continued)

Species	No. of leaves sample with this yeast species	Frequency of isolation (%)*
Sporidiobolus pararoseus	1	0.79
Sporidiobolus ruineniae	1	0.79
Sporobolomyces nylandii	2	1.59
Undescribed species		
Similar to *Candida* sp. 05-7-186T	2	1.59
New species		
Yamadazyma phyllophila sp. nov.	1	0.79

* Number of plant leaf sample from which that species was isolated/ number of plant leaf sample examined ×100

2.3 Identification of yeast strains isolated from sugarcane leaves samples

Identification of 159 yeast strains from sugarcane leaves revealed that 146 yeast strains were identified to be 36 known species in 19 genera (Table 19 and 20). Twenty-three species were in Phylum Ascomycota (13 genera) viz. *Candida* (10 species), *Cyberlindnera* (2 species), *Debaryomyces* (1 species), *Hanseniaspora* (1 species), *Kluyveromyces* (1 species), *Lachancea* (1 species), *Lodderomyces* (1 species), *Metschnikowia* (1 species), *Meyerozyma* (1 species), *Millerozyma* (1 species), *Pichia* (1 species), *Torulaspora* (1 species) and *Wickerhamomyces* (1 species), and thirteen species were in Phylum Basidiomycota (6 genera) viz. *Hannaella* (1 species), *Cryptococcus* (4 species), *Rhodosporidium* (2 species), *Rhodotorula* (3 species), *Sporidiobolus* (1 species) and *Sporobolomyces* (2 species). Three strains were similar to two undescribed species in Ascomycota and three strains were similar to *Rhodosporidium* sp. APSS 849 which was undescribed species in Basidiomycota. Seven strains were found to be represent four novels species in *Metschnikowia*, *Yamadazyma*, *Wickerhamomyces* and *Nakazawaea* clade. Phylogenetic positions of known species in ascomycete yeast and basidiomycete yeast obtained from sugarcane phylloplane were shown in Figure 7, 8 and 9, respectively. In phylogenetic tree all strains identified as known yeast species were in the same positions with the type strains of known yeast species. Know ascomycete yeast species

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were distributed in 16 phylogenetically distinct clades including *Candida tanzawaensis, Cyberlindnera, Debaryomyces, Hanseniaspora, Kluyveromyces, Kurtzmaniella, Lachancea, Lodderomyces-Spathospora, Metschnikowia, Meyerozyma, Millerozyma, Nakaseomyces, Pichia, Torulaspora, Wickerhamomyces* and *Yamadazyma* clades while *C. rugosa* and *C. mesorugosa* were in unaffiliated clade. Known basidiomycete yeast species were distributed in four phylogenetically distinct clades including *Bulleromyces, Kwoniella, Rhodosporidium* and *Sporidiobolus* clades.

The dominant yeast species was *Meyerozyma caribbica*, although only 36 strains of this species were isolated from 36 samples, represented a 22.64% frequency of isolation. Seventeen strains of *Rhodotorula taiwanensis* were isolated from 17 samples, giving a frequency of isolation of 10.69%. Fifteen strains of *Candida tropicalis* were isolated from 15 samples, represented a 9.43% frequency of isolation (Table 20).

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Table 19 Identification of yeast strains isolated from phylloplane of sugarcane and their GenBank accession numbers of the D1/D2 region of LSU rRNA gene

Sugarcane leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions / total nt	Identification result	
				Species	GenBank accession no.
1	DMKU-RK14	*Candida quercitrusa* (U45831)	1/565	*Candida quercitrusa* (A)	AB772038
1	DMKU-RK15	*Candida* sp. BG01-8-20-001B-C1 (AY242320)	1/565	Undescribed species (A)	AB772039
2	DMKU-RK16	*Candida succicola* (GU810536)	20/490	New species (A)	AB697752
3	DMKU-RK24	*Candida succicola* (GU810536)	20/490	New species (A)	AB697753
4	DMKU-RK26	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772040
4	DMKU-RK27	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772041
5	DMKU-RK28	*Candida orthopsilosis* (FJ746056)	1/581	*Candida orthopsilosis* (A)	AB772042
6	DMKU-RK65	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB741526
7	DMKU-RK66	*Metschnikowia koreensis* (AF296438)	0/512	*Metschnikowia koreensis* (A)	AB772043
8	DMKU-RK173	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772044
9	DMKU-RK174	*Rhodotorula taiwanensis* (GU646863)	1/551	*Rhodotorula taiwanensis* (B)	AB772045
9	DMKU-RK175	*Cryptococcus rajasthanensis* (AM262324)	0/597	*Cryptococcus rajasthanensis* (B)	AB772046
9	DMKU-RK176	*Debaryomyces nepalensis* (U45839)	1/570	*Debaryomyces nepalensis* (A)	AB772047
10	DMKU-RK179	*Candida mesorugosa* (FJ768919)	2/482	*Candida mesorugosa* (A)	AB772048
11	DMKU-RK180	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772049
12	DMKU-RK181	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772050
13	DMKU-RK182	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772051
13	DMKU-RK183	*Meyerozyma caribbica* (EU348786)	0/510	*Meyerozyma caribbica* (A)	AB772052
14	DMKU-RK184	*Meyerozyma caribbica* (EU348786)	0/536	*Meyerozyma caribbica* (A)	AB772053
15	DMKU-RK185	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772054
15	DMKU-RK186	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772055
16	DMKU-RK187	*Meyerozyma caribbica* (EU348786)	0/533	*Meyerozyma caribbica* (A)	AB772056

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Table 19 (Continued)

Sugarcane leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
16	DMKU-RK188	*Rhodotorula taiwanensis* (GU646863)	1/574	*Rhodotorula taiwanensis* (B)	AB772057
17	DMKU-RK189	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772058
17	DMKU-RK190	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772059
18	DMKU-RK191	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB741530
18	DMKU-RK192	*Cryptococcus laurentii* (AF075469)	2/597	*Cryptococcus laurentii* (B)	AB772060
18	DMKU-RK193	*Torulaspora delbrueckii* (U72156)	0/573	*Torulaspora delbrueckii* (A)	AB772061
19	DMKU-RK196	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772062
19	DMKU-RK197	*Cryptococcus flavescens* (AB035042)	0/570	*Cryptococcus flavescens* (B)	AB772063
20	DMKU-RK198	*Candida succicola* (GU810536)	20/490	New species (A)	AB697754
20	DMKU-RK199	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772064
21	DMKU-RK200	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772065
21	DMKU-RK201	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772066
22	DMKU-RK202	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772067
22	DMKU-RK203	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772068
22	DMKU-RK204	*Cryptococcus flavescens* (AB035042)	0/570	*Cryptococcus flavescens* (B)	AB772069
23	DMKU-RK206	*Candida* sp. 05-7-186T (FJ537069)	0/495	Undescribed species (A)	AB772070
24	DMKU-RK207	*Sporobolomyces nylandii* (AB279629)	0/572	*Sporobolomyces nylandii* (B)	AB772071
24	DMKU-RK208	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772072
24	DMKU-RK209	*Candida xylopsoci* (AY013719)	1/560	*Candida xylopsoci* (A)	AB772073
25	DMKU-RK210	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772074
25	DMKU-RK211	*Rhodotorula taiwanensis* (GU646863)	0/539	*Rhodotorula taiwanensis* (B)	AB772075
26	DMKU-RK212	*Meyerozyma caribbica* (EU348786)	0/533	*Meyerozyma caribbica* (A)	AB772076
26	DMKU-RK214	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772078
27	DMKU-RK247	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB741528
27	DMKU-RK248	*Cryptococcus laurentii* (AF075469)	3/596	*Cryptococcus laurentii* (B)	AB772079

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Table 19 (Continued)

Sugarcane leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
28	DMKU-RK249	*Sporobolomyces carnicolor* (AY158641)	0/515	*Sporobolomyces carnicolor* (B)	AB772080
28	DMKU-RK250	*Hannaella* aff. *coprosmaensis* (FN428908)	1/597	*Hannaella* aff. *coprosmaensis* (A)	AB772081
29	DMKU-RK253	*Rhodosporidium fluviale* (AF070422)	2/532	*Rhodosporidium fluviale* (B)	AB772082
29	DMKU-RK254	*Candida diddensiae* (U45750)	9/493	New species (A)	AB734046
30	DMKU-RK255	*Cryptococcus rajasthanensis* (AM262324)	0/597	*Cryptococcus rajasthanensis* (B)	AB772083
31	DMKU-RK256	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772084
32	DMKU-RK257	*Meyerozyma caribbica (EU348786)*	0/570	*Meyerozyma caribbica* (A)	AB772085
33	DMKU-RK259	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772087
34	DMKU-RK261	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772088
34	DMKU-RK262	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772089
35	DMKU-RK286	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772090
35	DMKU-RK287	*Meyerozyma caribbica* (EU348786)	0/540	*Meyerozyma caribbica* (A)	AB772091
36	DMKU-RK288	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772092
37	DMKU-RK290	*Candida nivariensis* (AF313362)	0/580	*Candida nivariensis* (A)	AB772094
38	DMKU-RK292	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772095
38	DMKU-RK293	*Cryptococcus heveanensis* (FJ534905)	1/599	*Cryptococcus heveanensis* (B)	AB772096
39	DMKU-RK294	*Rhodotorula mucilaginosa* (AF070432)	0/566	*Rhodotorula mucilaginosa* (B)	AB772097
39	DMKU-RK295	*Meyerozyma caribbica* (EU348786)	0/536	*Meyerozyma caribbica* (A)	AB772098
40	DMKU-RK297	*Millerozyma koratensis* (DQ404484)	0/569	*Millerozyma koratensis* (A)	AB772099
41	DMKU-RK299	*Candida rugosa* (U45727)	3/450	*Candida rugosa* (A)	AB772100
42	DMKU-RK300	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772101
42	DMKU-RK301	*Candida michaelii* (AY520329)	0/523	*Candida michaelii* (A)	AB772102
43	DMKU-RK302	*Cryptococcus heveanensis* (AB557813)	1/599	*Cryptococcus heveanensis* (B)	AB772103
43	DMKU-RK303	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772104
44	DMKU-RK340	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772105

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Table 19 (Continued)

Sugarcane leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
45	DMKU-RK342	*Rhodosporidium paludigenum* (AF363640)	0/527	*Rhodosporidium paludigenum* (B)	AB772107
45	DMKU-RK343	*Candida michaelii* (AY520329)	0/530	*Candida michaelii* (A)	AB772108
46	DMKU-RK344	*Meyerozyma caribbica* (EU348786)	0/538	*Meyerozyma caribbica* (A)	AB772109
47	DMKU-RK345	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB772110
47	DMKU-RK346	*Meyerozyma caribbica* (EU348786)	0/536	*Meyerozyma caribbica* (A)	AB772111
48	DMKU-RK347	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772112
49	DMKU-RK349	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772114
50	DMKU-RK350	*Cryptococcus laurentii* (AF075469)	1/580	*Cryptococcus laurentii* (A)	AB772115
50	DMKU-RK351	*Meyerozyma caribbica* (EU348786)	0/529	*Meyerozyma caribbica* (A)	AB772116
51	DMKU-RK352	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772117
52	DMKU-RK354	*Rhodosporidium* sp. APSS 849 (FM178290)	1/557	Undescribed species (A)	AB772118
52	DMKU-RK355	*Cryptococcus laurentii* (AF075469)	2/597	*Cryptococcus laurentii* (B)	AB772119
53	DMKU-RK356	*Rhodosporidium* sp. APSS 849 (FM178290)	3/561	Undescribed species (A)	AB772120
53	DMKU-RK357	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB772121
54	DMKU-RK359	*Wickerhamomyces ciferrii* (U74587)	4/571	New species (A)	AB714248
55	DMKU-RK360	*Sporobolomyces nylandii* (AB279629)	0/572	*Sporobolomyces nylandii* (B)	AB772122
55	DMKU-RK361	*Lachancea thermotolerans* (U69581)	0/571	*Lachancea thermotolerans* (A)	AB772123
56	DMKU-RK362	*Rhodosporidium fluviale* (AF070422)	2/556	*Rhodosporidium fluviale* (B)	AB772124
56	DMKU-RK363	*Candida mesorugosa* (FJ768919)	2/482	*Candida mesorugosa* (A)	AB772125
57	DMKU-RK365	*Candida dendronema* (U45751)	1/530	*Candida dendronema* (A)	AB772126
58	DMKU-RK366	*Meyerozyma caribbica* (EU348786)	1/570	*Meyerozyma caribbica* (A)	AB772127
59	DMKU-RK382	*Metschnikowia koreensis* (AF296438)	0/512	*Metschnikowia koreensis* (A)	AB772129
59	DMKU-RK383	*Sporidiobolus ruineniae* (AF070434)	0/574	*Sporidiobolus ruineniae* (B)	AB772130
60	DMKU-RK384	*Rhodosporidium* sp. APSS 849 (FM178290)	1/556	Undescribed species (B)	AB772131
60	DMKU-RK385	*Meyerozyma caribbica* (EU348786)	1/570	*Meyerozyma caribbica* (A)	AB772132

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Table 19 (Continued)

Sugarcane leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions/ total nt	Identification result	
				Species	GenBank accession no.
61	DMKU-RK389	*Candida rugosa* (U45727)	1/485	*Candida rugosa* (A)	AB772133
62	DMKU-RK390	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772134
62	DMKU-RK391	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772135
63	DMKU-RK392	*Sporidiobolus ruineniae* (AF070434)	0/575	*Sporidiobolus ruineniae* (B)	AB772136
63	DMKU-RK393	*Candida nivariensis* (AF313362)	1/580	*Candida nivariensis* (A)	AB772137
63	DMKU-RK394	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772138
64	DMKU-RK398	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772139
64	DMKU-RK399	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772140
65	DMKU-RK400	*Sporobolomyces nylandii* (AB279629)	0/572	*Sporobolomyces nylandii* (B)	AB772141
65	DMKU-RK401	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772142
66	DMKU-RK409	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772143
67	DMKU-RK410	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772144
67	DMKU-RK411	*Wickerhamomyces edaphicus* (AB436763)	2/573	*Wickerhamomyces edaphicus* (A)	AB714243
68	DMKU-RK412	*Millerozyma koratensis* (DQ404484)	0/569	*Millerozyma koratensis* (A)	AB772145
68	DMKU-RK413	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772146
69	DMKU-RK415	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772147
70	DMKU-RK416	*Rhodosporidium fluviale* (AF070422)	1/574	*Rhodosporidium fluviale* (B)	AB772148
70	DMKU-RK417	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772149
71	DMKU-RK418	*Millerozyma koratensis* (DQ404484)	0/569	*Millerozyma koratensis* (A)	AB772150
71	DMKU-RK419	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772151
72	DMKU-RK421	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772152
73	DMKU-RK422	*Rhodosporidium fluviale* (AF070422)	1/574	*Rhodosporidium fluviale* (B)	AB772153
73	DMKU-RK424	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772154
74	DMKU-RK425	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772155
74	DMKU-RK426	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772156

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Table 19 (Continued)

Sugarcane leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions / total nt	Identification result	
				Species	GenBank accession no.
75	DMKU-RK427	*Rhodotorula taiwanensis* (GU646863)	1/571	*Rhodotorula taiwanensis* (B)	AB772157
75	DMKU-RK428	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772158
76	DMKU-RK431	*Metschnikowia koreensis* (AB435077)	0/512	*Metschnikowia koreensis* (A)	AB772159
77	DMKU-RK433	*Candida akabanensis*(EU100744)	2/520	*Candida akabanensis* (A)	AB772160
78	DMKU-RK440	*Lodderomyces elongisporus* (U45763)	0/569	*Lodderomyces elongisporus* (A)	AB772161
78	DMKU-RK442	*Millerozyma koratensis* (DQ404484)	0/569	*Millerozyma koratensis* (A)	AB772162
79	DMKU-RK443	*Rhodotorula taiwanensis* (GU646863)	1/519	*Rhodotorula taiwanensis* (B)	AB772163
80	DMKU-RK445	*Candida nivariensis* (AY627305)	0/548	*Candida nivariensis* (A)	AB772164
81	DMKU-RK447	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772166
81	DMKU-RK448	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772167
81	DMKU-RK449	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB772168
82	DMKU-RK451	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772169
83	DMKU-RK452	*Cyberlindnera rhodanensis* (U73571)	2/573	*Cyberlindnera rhodanensis* (A)	AB772170
84	DMKU-RK454	*Rhodotorula taiwanensis* (GU646863)	0/571	*Rhodotorula taiwanensis* (B)	AB772172
84	DMKU-RK455	*Candida nivariensis* (AY627305)	0/548	*Candida nivariensis* (A)	AB772173
85	DMKU-RK458	*Kluyveromyces marxianus* (U94924)	0/545	*Kluyveromyces marxianus* (A)	AB772174
85	DMKU-RK459	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB772175
86	DMKU-RK466	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772176
86	DMKU-RK467	*Candida wickerhamii* (U70243)	11/569	New species (A)	AB772177
87	DMKU-RK468	*Candida nivariensis* (AY627305)	0/548	*Candida nivariensis* (A)	AB772178
87	DMKU-RK469	*Cryptococcus laurentii* (AF075469)	1/597	*Cryptococcus laurentii* (B)	AB772179
88	DMKU-RK470	*Candida* sp. 05-7-186T (FJ537069)	0/495	Undescribed species (A)	AB772180
89	DMKU-RK487	*Cyberlindnera fabianii* (U73573)	0/570	*Cyberlindnera fabianii* (A)	AB772181
90	DMKU-RK488	*Candida nivariensis* (AY627305)	0/548	*Candida nivariensis* (A)	AB772182
90	DMKU-RK489	*Cyberlindnera rhodanensis* (U73571)	1/573	*Cyberlindnera rhodanensis* (A)	AB772183

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Table 19 (Continued)

Sugarcane leaves sample no.	Strain	Closest species (GenBank accession no.)	Nucleotide substitutions / total nt	Identification result	
				Species	GenBank accession no.
91	DMKU-RK490	*Pichia kudriavzevii* (U76347)	0/564	*Pichia kudriavzevii* (A)	AB772184
91	DMKU-RK491	*Candida tropicalis* (U45749)	0/570	*Candida tropicalis* (A)	AB772185
91	DMKU-RK492	*Cyberlindnera rhodanensis* (U73571)	3/573	*Cyberlindnera rhodanensis* (A)	AB772186
92	DMKU-RK493	*Cyberlindnera rhodanensis* (U73571)	0/425	*Cyberlindnera rhodanensis* (A)	AB772187
92	DMKU-RK494	*Rhodotorula sesimbrana* (AF387144)	0/575	*Rhodotorula sesimbrana* (B)	AB772188
92	DMKU-RK495	*Candida rugosa* (U45727)	1/485	*Candida rugosa* (A)	AB772189
93	DMKU-RK496	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772190
93	DMKU-RK497	*Hanseniaspora guilliermondii* (U84230)	0/572	*Hanseniaspora guilliermondii* (A)	AB772191
94	DMKU-RK498	*Rhodosporidium fluviale* (AF070422)	1/574	*Rhodosporidium fluviale* (B)	AB772192
94	DMKU-RK499	*Meyerozyma caribbica* (EU348786)	0/570	*Meyerozyma caribbica* (A)	AB772193
94	DMKU-RK500	*Candida succicola* (GU810536)	20/490	New species (A)	AB697755
94	DMKU-RK501	*Cyberlindnera rhodanensis* (U73571)	1/463	*Cyberlindnera rhodanensis* (A)	AB772194

Remark: (A), Ascomycota (B), Basidiomycota

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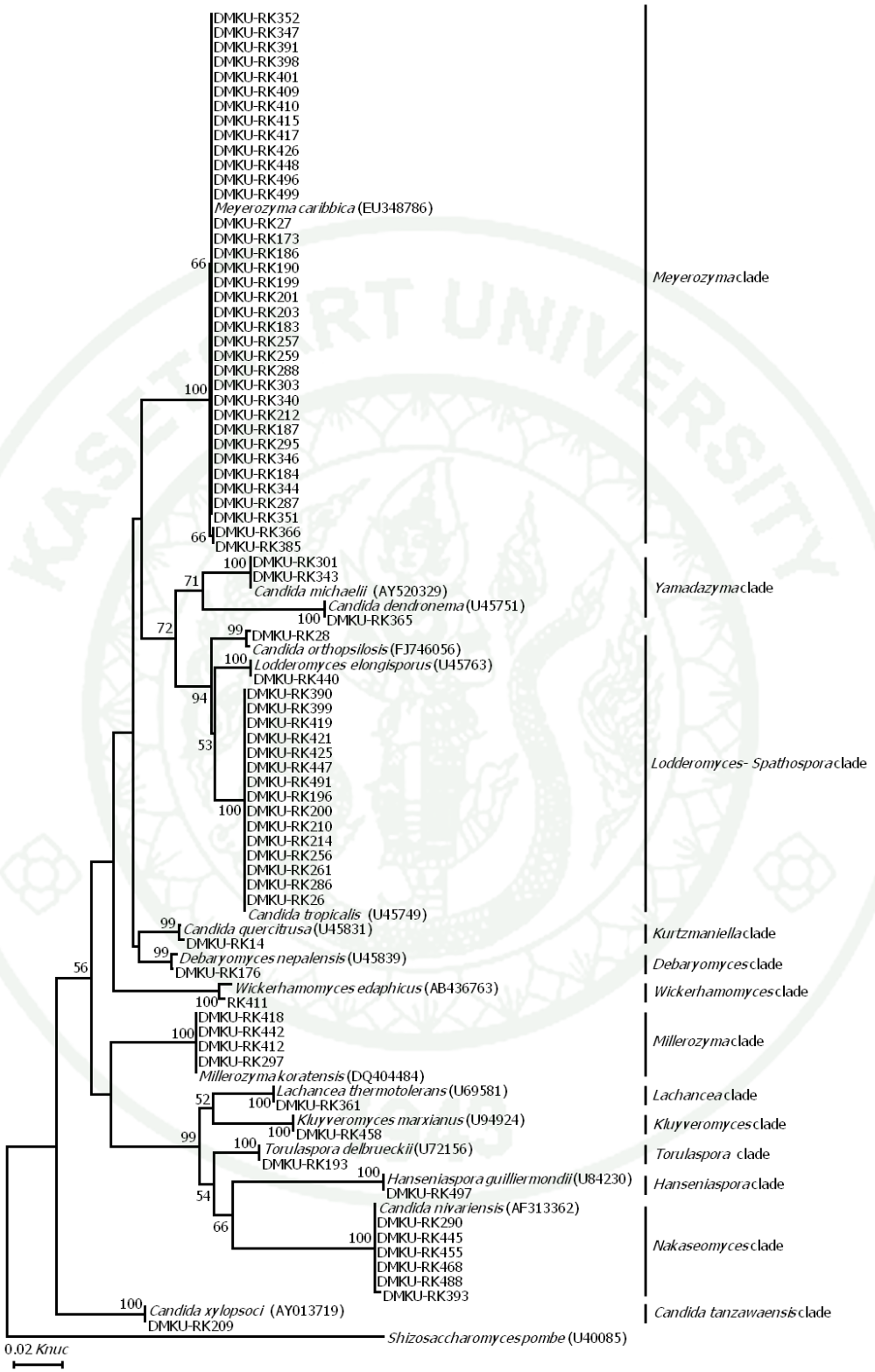


Figure 7 Phylogeny of ascomycete yeasts isolated from sugarcane phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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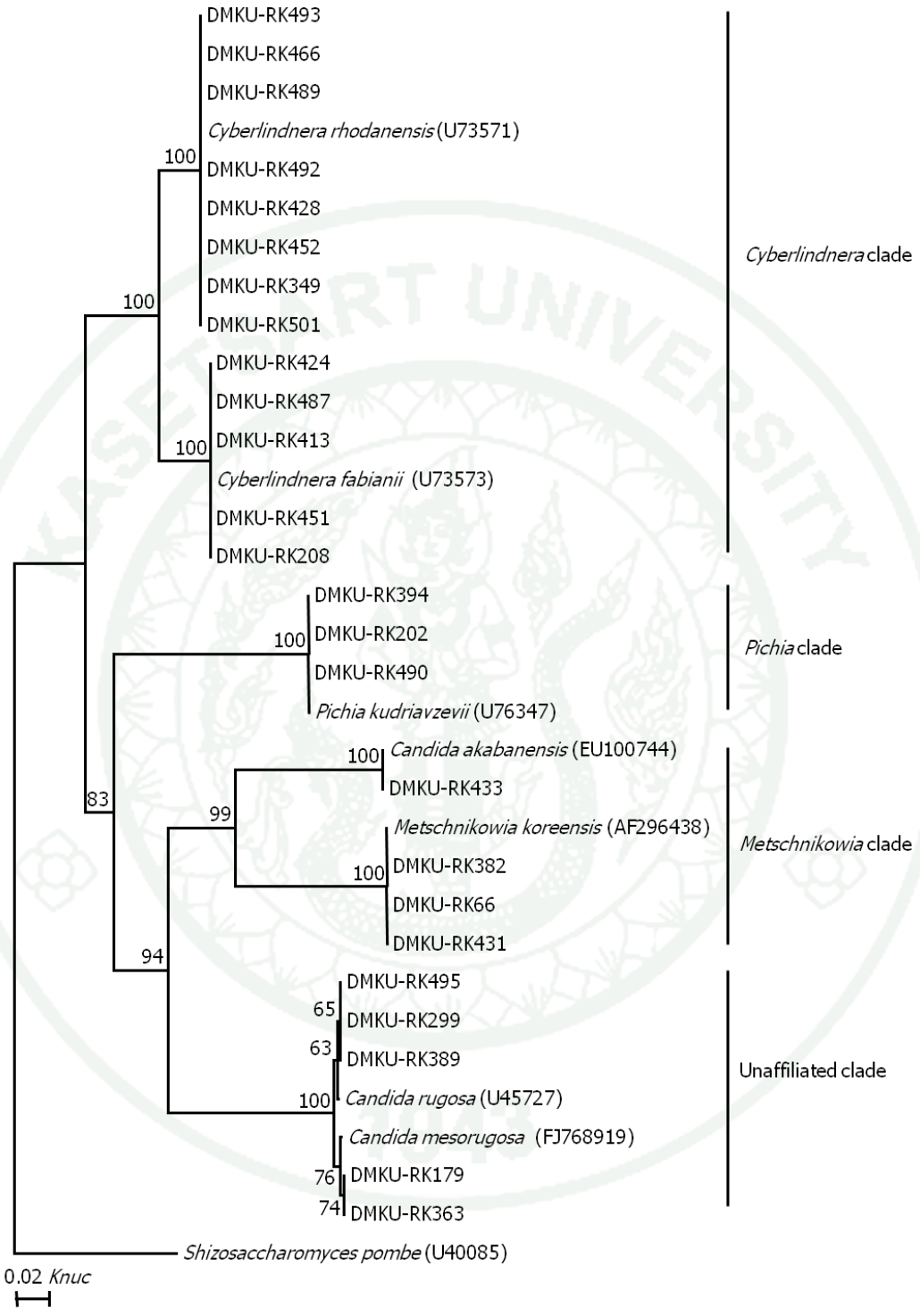


Figure 8 Phylogeny of ascomycete yeasts isolated from sugarcane phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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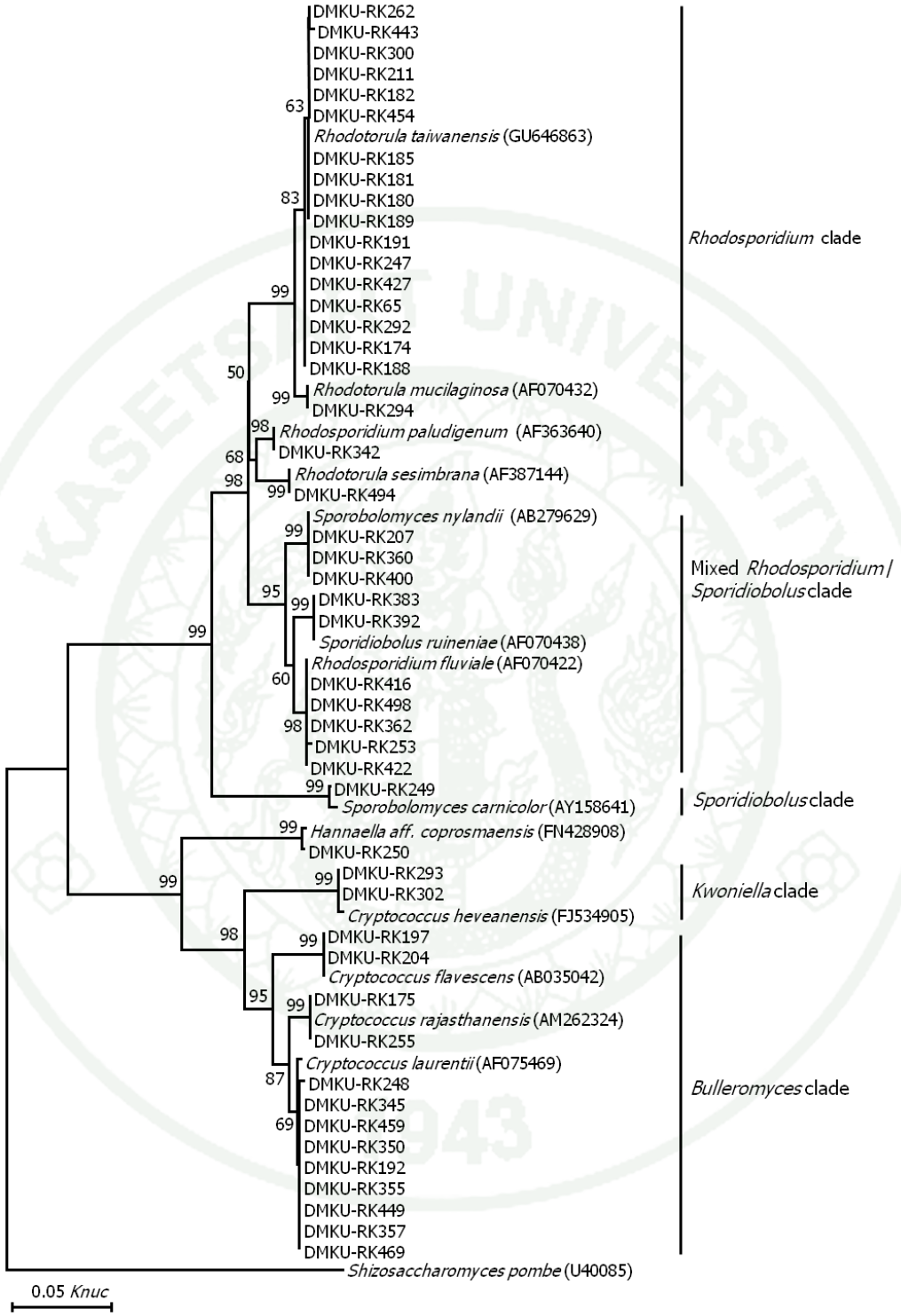


Figure 9 Phylogeny of basidiomycete yeasts isolated from sugarcane phylloplane based on the D1/D2 region of the LSU rRNA gene. *Shizosaccharomyces pombe* was the outgroup in the analysis.

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Table 20 Yeasts from sugarcane phylloplane and their frequency of isolation

Species	No. of leaves sample with this yeast species	Frequency of isolation (%)*
Known species		
Ascomycota		
Candida akabanensis	1	0.63
Candida dendronema	1	0.63
Candida mesorugosa	2	1.26
Candida michaelii	2	1.26
Candida nivariensis	6	3.77
Candida rugosa	3	1.89
Candida orthopsilosis	1	0.63
Candida quercitrusa	1	0.63
Candida tropicalis	15	9.43
Candida xylopsoci	1	0.63
Cyberlindnera fabianii	5	3.14
Cyberlindnera rhodanensis	8	5.03
Debaryomyces nepalensis	1	0.63
Hanseniaspora guilliermondii	1	0.63
Kluyveromyces marxianus	1	0.63
Lachancea thermotolerans	1	0.63
Lodderomyces elongisporus	1	0.63
Metschnikowia koreensis	3	1.89
Meyerozyma caribbica	36	22.64
Millerozyma koratensis	4	2.52
Pichia kudriavzevii	3	1.89
Torulaspora delbrueckii	1	0.63
Wickerhamomyces edaphicus	1	0.63
Basidiomycota		
Hannaella aff. *coprosmaensis*	1	0.63
Cryptococcus flavescens	2	1.26
Cryptococcus heveanensis	2	1.26
Cryptococcus laurentii	9	5.66
Cryptococcus rajasthanensis	2	1.26
Rhodosporidium fluviale	5	3.14
Rhodosporidium paludigenum	1	0.63
Rhodotorula sesimbrana	1	0.63
Rhodotorula taiwanensis	17	10.69
Sporidiobolus ruineniae	2	1.26
Sporobolomyces carnicolor	1	0.63
Sporobolomyces nylandii	3	1.89

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Table 20 (Continued)

Species	No. of leaves sample with this yeast species	Frequency of isolation (%)*
Undescribed species		
Similar to *Candida* sp. BG01-8-20-001B-C1	1	0.63
Similar to *Candida* sp. 05-7-186T	2	1.26
Similar to *Rhodosporidium* sp. APSS 849	3	1.89
New species		
Metschnikowia saccharicola sp. nov	4	2.52
Yamadazyma siamensis sp. nov.	1	0.63
Wickerhamomyces siamensis sp. nov.	1	0.63
Nakazawaea siamensis sp. nov.	1	0.63

* Number of plant leaf sample from which that species was isolated/ number of plant leaf

sample examined ×100

Considering number of yeast species in each leaves sample of all crop in this study resulted that most samples contained two yeast species calculated to be 56.5, 60.0 and 48.9% of total sample of rice, corn and sugarcane phylloplane, respectively (Table 21). The second high number of leaf sample consisted of one yeast species followed by three and four yeast species per one leaf sample as showed in Table 21.

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Table 21 Number of yeast species present on each leaves sample

Leaf	Number of yeast species on one leaf sample	Number of leaves sample	% of total sample of each crop
Rice	1	26	30.5
	2	48	56.5
	3	10	11.8
	4	1	1.2
	Total	85	
Corn	1	15	23.1
	2	39	60.0
	3	11	16.9
	4	0	0.0
	Total	65	
Sugarcane	1	39	41.5
	2	46	48.9
	3	8	8.5
	4	1	1.1
	Total	94	

$$\% \text{ of total sample of each crop} = \frac{\text{number of leaves sample}}{\text{number of total leaf sample}} \times 100$$

The results of identification indicated that 96.2% of a total strains (441 strains) which were isolated by the enrichment technique from the three crops phylloplane was known species including ascomycete yeasts 279 strains (63.3 %) and basidiomycete yeasts 145 strains (32.9%). Undescribed species represented 1.8% of all strains and 2.0 % was new yeast species. The dominant basidiomycete yeast species was *Rhodotorula taiwanensis*, but with only 14.3% frequency of isolation and the dominant ascomycete yeast species was *Meyerozyma caribbica*, but with only 12.0% frequency of isolation.

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Six known ascomycete species namely *Candida rugosa*, *Candida tropicalis*, *Cyberlindnera rhodanensis*, *Meyerozyma caribbica*, *Millerozyma koratensis* and *Pichia kudriavzevii*, and three basidiomycete species namely *Cryptococcus flavescens, Cryptococcus laurentii* and *Rhodotorula taiwanensis* were present in all three crops phylloplane. On rice and corn phylloplane five species in both Ascomycota and Basidiomycota namely *Candida catenulate*, *Candida glabrata, Kodamaea ohmeri, Meyerozyma guilliermondii* and *Cryptococcus* aff. *laurentii* were obtained. The other five known yeast species namely *Cyberlindnera fabianii, Debaryomyces nepalensis, Lachancea thermotolerans, Metschnikowia koreensis* and *Cryptococcus rajasthanensis* were found on rice and sugarcane phylloplane. Eight known yeast species namely *Hanseniaspora guilliermondii, Kluyveromyces marxianus, Wickerhamomyces edaphicus, Cryptococcus heveanensis, Rhodosporidium fluviale, Rhodosporidium paludigenum, Sporidiobolus ruineniae* and *Sporobolomyces nylandii* were found on corn and sugarcane phylloplane. The other species presented on only one type of leaf phylloplane (Table 22).

Table 22 Yeast species found in the three crops phylloplane

Yeast species	Rice	Corn	Sugarcane
Ascomycota			
Candida catenulata	√	√	-
Candida glabrata	√	√	-
Candida rugosa	√	√	√
Candida tropicalis	√	√	√
Cyberlindnera fabianii	√	-	√
Cyberlindnera rhodanensis	√	√	√
Debaryomyces nepalensis	√	-	√
Hanseniaspora guilliermondii	-	√	√
Kodamaea ohmeri	√	√	-
Kluyveromyces marxianus	-	√	√
Lachancea thermotolerans	√	-	√
Metschnikowia koreensis	√	-	√

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Table 22 (Continued)

Yeast species	Rice	Corn	Sugarcane
Meyerozyma caribbica	√	√	√
Meyerozyma guilliermondii	√	√	-
Millerozyma koratensis	√	√	√
Pichia kudriavzevii	√	√	√
Wickerhamomyces edaphicus	-	√	√
Basidiomycota			
Cryptococcus flavescens	√	√	√
Cryptococcus heveanensis	-	√	√
Cryptococcus laurentii	√	√	√
Cryptococcus aff. *laurentii*	√	√	-
Cryptococcus rajasthanensis	√	-	√
Rhodosporidium fluviale	-	√	√
Rhodosporidium paludigenum	-	√	√
Rhodotorula taiwanensis	√	√	√
Sporidiobolus ruineniae	-	√	√
Sporobolomyces nylandii	-	√	√

When considered the species which were found from all three crops phylloplane in this study nine species have been reported to be isolated from phylloplane before. *C. rugosa* was reported to be isolated from phylloplane of various plant in Thailand (Limtong and Koowadjanakul, 2012) however it also reported to obtained from human feces, cow dung, rancid butter and soil (Lachance *et al.*, 2011). In this study six strains of *C. rugosa* which were isolated from all three crops phylloplane. *C. tropicalis* was reported to obtain from phylloplane of various plant species, fruits, flowers, soil, water and clinical specimen (Lachance *et al.*, 2011; Limtong and Koowadjanakul, 2012). In this study 48 strains of *C. tropicalis* were found on rice phylloplane (25 strains), corn phylloplane (8 strains) and sugarcane phylloplane (15

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strains). Therefore, *C. tropicalis* seems to be a common yeast species in phylloplane. *C. rhodanensis* was reported to isolate phylloplane in Thailand (Limtong and Koowadjanakul, 2012) and from other sources namely leather or tanning fluid, fermented canaigre (a high tannin plant product) and palm nuts (Kurtzman *et al.*, 2008). *P. kudriavzevii* which was reported to widely distribute in nature often occurring in soil, on fruits and in various natural fermentations, to be a common clinical isolate (Kurtzman, 2011a) and to be found on phylloplane of various plant species in Thailand in the previous study (Limtong and Koowadjanakul, 2012) were also isolated in this study. *Cryp. laurentii* which is known from various sources, such as phylloplane of grasses, palm wine, soil, rhizosphere soil, seawater, decay wood, wood sorrel and clinical sources (Mussa *et al*., 2000; Hong *et al*. 2002; Allen *et al*., 2004; Glushakova and Chernov 2004) was also detected in this study. The other yeast species consisted of *M. caribbica, M. koratensis, Cryp. flavescens* and *R. taiwanensis* have never been isolated from phylloplane. *M. caribbica* was reported to isolate from a variety of substrates that include soil, corn-derived starch, sugar cane and an apparent human infection. The strains of *M. caribbica* have been from various locations in the Western Hemisphere (Vaughan-Martini *et al*., 2005), which suggests that *M. caribbica* is widely distributed geographically (Kurtzman and Suzuki, 2010). *M. koratensis* (*Pichia koratensis*) was reported to obtaine from insect fress (Nakase *et al*., 2007). *Cryp. flavescens* is known from various sources, such as air, kernels of wheat and corn just after harvest, fermenting soy sauce, rhizosphere soil, nectar of the herb white campion, wheat anthers and caterpillar gut (Kurtzman, 1973; Hong *et al*., 2002; Golonka, 2002; Dunlap *et al*., 2007). *R. taiwanensis* was recently isolated from the stems of three *Artemisia argyi* (Huang *et al*., 2011).

Some yeast species isolated from crop phylloplane in this study have been reported by Limtong and Koowadjanakul (2012) to isolate from the phylloplane of various plant species in Thailand before. They included *Candida glabrata, C. jaroonii, C. nivariensis, Clavispora lusitaniae, Debaryomyces nepalensis, Hanseniaspora guilliermondii, Kluyveromyces marxianus, Kodamae ohmeri, Lachances thermotolerans, Hanseniaspora opuntiae, H. thailandica, Metschnikowia koreensis, Meyerozyma guilliermondii, Pichia kudriavzevii* and *Sporidiobolus*

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ruineniae. Strains of many yeast species found in this study were reported to isolate from insects viz. *Candida glabrata, C. dendronema, C. membranifaciens, C. michaelii, C. xylopsoci, Debaryomyces nepalensis, Hanseniaspora guilliermondii, Kluyveromyces marxianus, Kodamae ohmeri, Lachances thermotolerans, Wickerhamomyces ciferrii* and *Yamadazyma* and from other parts of plants including flowers, fruits and tree bark viz. *Candida jaroonii, C. nivariensis, C. tropicalis, C. orthopsilosis, C. quercitrusa, C. sojae, C. sorboxylosa, Clavispora lusitaniae, Cryptococcus flavescens, C. laurentii, Cyberlindnera rhodanensis, C. fabianii, Hanseniaspora opuntiae, H. thailandica, Metschnikowia koreensis, Meyerozyma caribbica, M. guilliermondii, Pichia kudriavzevii, Torulaspora delbrueckii* and *Rhodotorula taiwanensis* (Allen *et al.,* 2004; Cadez and Smith, 2011; Huang *et al*., 2011; Imanishi *et al*., 2008; Kurtzman, 1973, c; Lachance, 2011a, b, c; Lachance *et al*., 2011; Nakase *et al.,* 2010; Jindamorakot *et al.,* 2009; Sipiczki, 2010). The present of these yeast species on the phylloplanes in this study may because of phylloplane is their habitat or may resulted from visiting of insects that carried these yeasts to the phylloplane. Strains of some species obtained in this study were previously reported to be the novel species discovered in Thailand isolated from the other sources viz. *C. jaroonii* (Imanishi *et al*., 2008), *H. thailandica* (Jindamorakot *et al*., 2009), *M. koratensis* (Nakase *et al*., 2007) and *W. edaphicus* (Limtong *et al.*, 2009).

The result of this study revealed that most culturable yeasts obtained from phylloplane of the three agronomic crops in Thailand when the enrichment isolation technique at 30 ± 3°C was used were in the phylum Ascomycota (63.3 %); this is same as the report of Limtong and Koowadjanakul (2012) in contrast with the other investigations, which reported the dominance of basidiomycete yeasts belonging to be genus *Bensingtonia, Bullerra, Cryptococcus, Derxomyces, Diozegia, Kockovaella, Laphrina, Pseudozyma, Rhodotorula, Sporobolomyces, Tilletiopsis* on the phylloplane in the other regions such as Brazil, China, Germany, Portugal, Russia and Taiwan (de Azeredo *et al.,* 1998; Fonseca and Inacio 2006; Glushakova *et al.,* 2007; Nakase *et al*., 2001; Sharma *et al*., 2009). The difference results in this study may be caused from the different technique and incubation temperature employed for isolation. While in the most investigations leaf washing followed by dilution plating technique

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was used for yeast isolation (Fonseca and Inacio 2006), Nakase *et al.* (2001) used the ballistoconidium fall method with YM agar without any antibacterial and antifungal agents for isolation of phylloplane yeast, and they found more basidiomycete yeast species when the incubation temperature was at 23°C than at 30°C. This may due to the fact that most of basidiomycete yeasts grow rather slow and they have fairly low maximum growth temperature in comparison with ascomycete yeasts and the enrichment isolation at high temperature (30 ± 3°C) selects rapid growing yeasts (Nakase *et al.*, 2001; Limtong and Koowadjanakul, 2012). Therefore, more ascomycete yeasts were obtained.

3. Polyphasic taxonomy of six new yeast species

In this study most of yeasts isolated from the external surface of rice leaves, corn leaves and sugarcane leaves were found to be known yeast species and nine strains, including one strain (DMKU-RK277^T) from phylloplane of rice, one strain (DMKU-RK548^T) from phylloplane of corn and seven strains (DMKU-RK16, DMKU-RK24, DMKU-RK198, DMKU-RK254^T, DMKU-RK359^T, DMKU-RK467^T and DMKU-RK500^T) from phylloplane of sugarcane, were found to represent six new yeast species in *Metschnikowia* clade, *Yamadazyma* clade, *Wickerhamomyces* clade and *Nakazawea* clade, respectively. In this study base on the polyphasic taxonomy, the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were described as *Metschnikowia saccharicola* sp. nov., strain DMKU-RK277^T was described as *Metschnikowia lopburiensis* sp. nov., strain DMKU-RK254^T was described as *Yamadazyma siamensis* sp. nov., strain DMKU-RK548^T was described as *Yamadazyma phyllophila* sp. nov., strain DMKU-RK359^T was described as *Wickerhamomyces siamensis* sp. nov. and strain DMKU-RK467^T was described as *Nakazawea siamensis* sp. nov.

3.1 *Metschnikowia* clade

3.1.1 *Metschnikowia saccharicola* sp. nov. (Strains DMKU-RK16, DMKURK24, DMKU-RK198 and DMKU-RK500^T)

Four strains (DMKU-RK16, DMKURK24, DMKU-RK198 and DMKU-RK500^T) were isolated from the external surface of sugarcane by an

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enrichment technique. Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequences of the four strains (DMKU-RK16, DMKU-RK24, DMKURK198 and DMKU-RK500^T) were identical and differed by only two nucleotide substitutions from the two strains of undescribed *Candida* species namely strains HN1-1 and HN1-2(a), however, the strains are not available for further comparison. The sequences of these strains showed 4.1 % nucleotide substitutions (20 nucleotide substitutions and four gaps out of 490 nt) from the type strain of *C. succicola*, their closest known species, in terms of pairwise sequence similarity. The nucleotide sequences of the ITS region of the four strains (DMKU-RK16, DMKU-RK24, DMKURK198 and DMKU-RK500^T) were analyzed and deposited in GenBank as AB697757, AB697758, AB697759 and AB697760, repectively. Their ITS region sequences of all four strains were identical and differed by 12.6 % nucleotide substitutions (37 nucleotide substitutions and one gap out of 294 nt) from type strains of *C. succicola*.

The phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that all strains were in *Metschnikowia* clade. Four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were in the same position and connected to the two undescribed *Candida* species (strains HN1-1 and HN1-2(a)) that closest to them. They connected to *C. succicola*, the closest known species, and form a subclade with *Metschnikowia lunata* though the bootstrap was low (Figure 10).

Cells of the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were globose, subglobose and ovoid (Figure 11a) and proliferated by multilateral budding. Pseudohyphae but not true hyphae were formed (Figure 11b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C.

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On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene and the ITS region, we concluded that the four strains represent a novel *Metschnikowia* species although formation of ascospores was not observed because of the rules under of the International Code of Nomenclature of algae, fungi, and plants (ICN). The nomenclatural rules for fungi were several important changed, the most important of which was probably the adoption of the principle of "one fungus, one name" (Miller *et al*., 2011). The name *Metschnikowia saccharicola* sp. nov. (MB564869) is proposed. The type strain is DMKU-RK500^{T} (= BCC 50735^{T} = NBRC 108904^{T} = CBS 12575^{T}).

In practice, *M. saccharicola* sp. nov. can be distinguished from its closest species *C. succicola*, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also some phenotypic characteristics. *M. saccharicola* sp. nov. did not fermented sucrose and maltose. Ribitol and D-glucono-δ-lactone are assimilated, but D-galactose, hexeadecane and arabinitol are non-assimilated. Growth at 37°C is negative. Acid formation is present while the type strain of *C. succicola* fermented sucrose and maltose. Ribitol (latent), D-glucono-δ-lactone (latent), D-galactose (latent), hexeadecane and arabinitol are assimilated. They grow at 37°C. Acid formation is not present.

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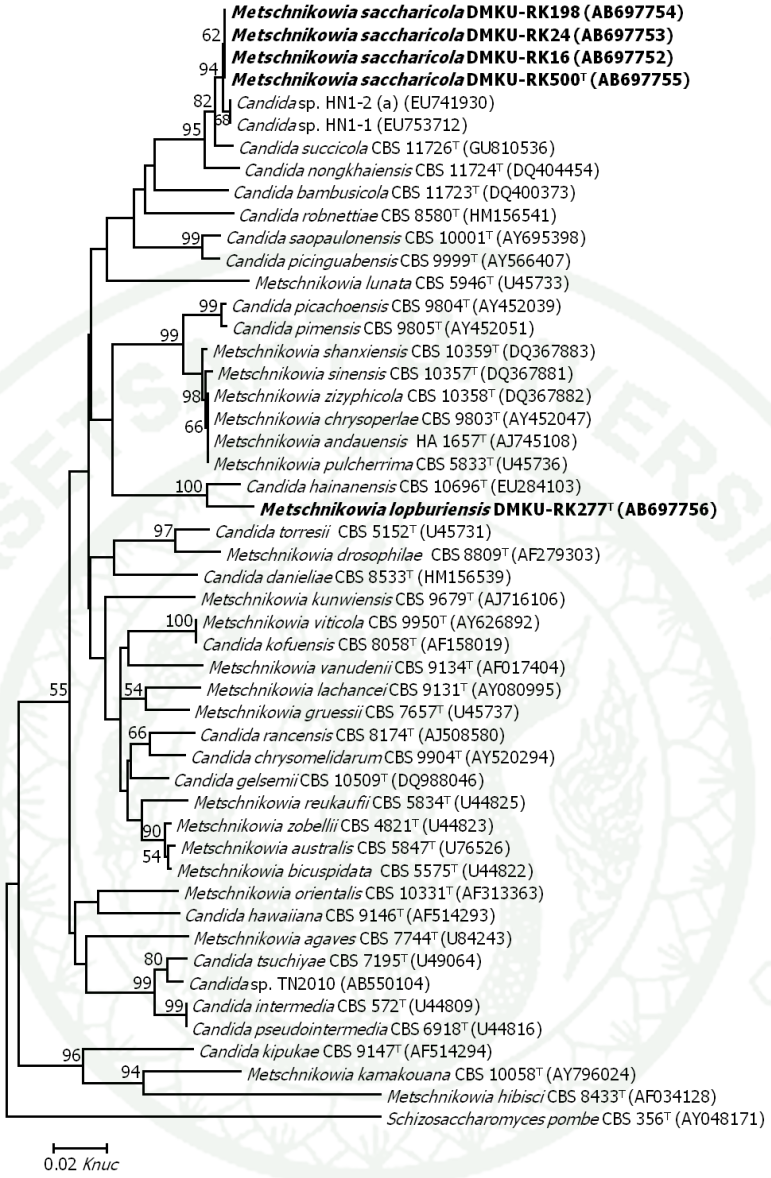


Figure 10 Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Metschnikowia saccharicola* sp. nov. (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^{T}) and *Metschnikowia lopburiensis* sp. nov. (DMKU-RK277^{T}), with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 4.0. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. The numbers in parentheses are GenBank accession numbers. *Schizosaccharomyces pombe* was the outgroup in the analysis.

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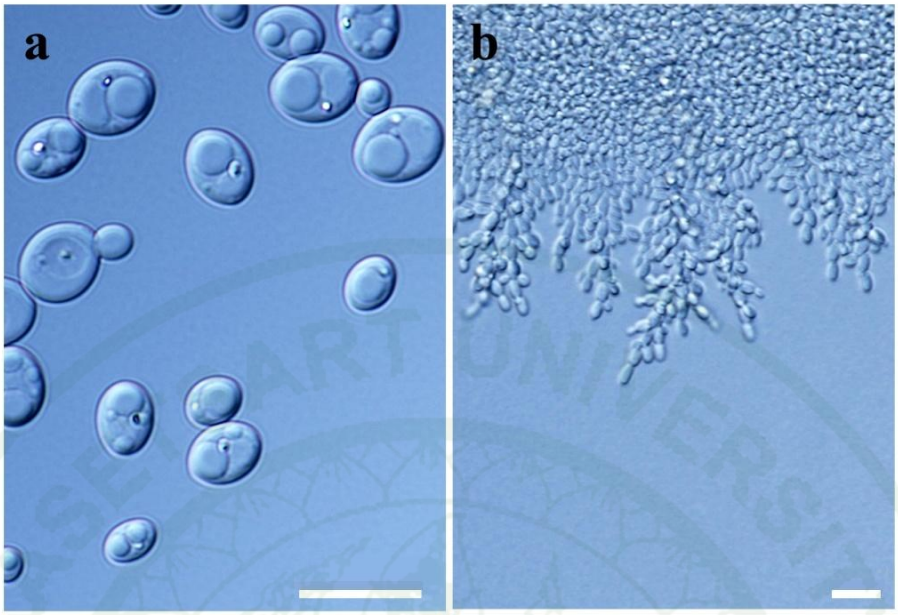


Figure 11 *Metschnikowia saccharicola* sp. nov. (DMKU-RK500^{T}) (a) budding cells in YM broth after 3 days at 25°C (b) pseudohyphae formed on PDA after 3 days at 25°C. Scale bar, 10 μm.

Description of *Metschnikowia saccharicola* Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25°C, cells are globose, subglobose to ovoid (4-7 x 5-8 μm) and occur singly, in pairs or in groups (Figure 11a). Budding is multilateral. Growth on YM agar: After 3 days at 25°C, the streak culture is grayish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25°C (Figure 11b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C. Fermentation of D-glucose and trehalose are positive but negative for D-galactose, sucrose, maltose, lactose, raffinose, xylose and cellobiose. D-glucose, L-sorbose, sucrose, maltose, trehalose, melezitose, D-xylose, ethanol, ribitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, 2-keto-D-gluconic

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acid, D-glucono-δ-lactone, succinic acid, citric acid, xylitol, ethylamine, L-lysine and cadaverine are assimilated, but D-galactose, cellobiose, lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, D-arabinose, L-rhamnose, D-ribose, N-acetyl-D-glucosamine, methanol, glycerol, erythritol, galactitol, salicin, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, 5-keto-D-gluconic acid, DL-lactic acid, inositol, D-glucosamine, arbutin, hexeadecane, propane-1,2-diol, butane-2,3-diol, arabinitol, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is positive (weak). Growth on medium containing 50 % (w/v) glucose and 60 % (w/v) glucose are positive, but 10 % (w/v) sodium chloride/5 % (w/v) glucose and 16 % (w/v) sodium chloride/5 % (w/v) glucose are negative. Growth with 0.01 % cylcloheximide is positive but 0.1 % cylcloheximide is negative. Growth at 25, 30 and 35°C are positive, but at 37, 40, 42 and 45°C are negative. Acid formation is present. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 41.7 mol %. The major ubiquinone is Q-9.

Holotype

DMKU-RK500^{T} is the holotype of *M. saccharicola* (MB564869). The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Nakhon Ratchasima province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50735^{T}; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108904^{T} and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12575^{T}.

Etymology

The species epithet ‘‘saccharicola’’ was chosen because this species was isolated from phylloplane of sugarcane, *S. officinarum* collected in Thailand.

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3.1.2 *Metschnikowia lopburiensis* sp. nov. (DMKU-RK277^T)

The strain DMKU-RK277^T was isolated from the external surface of rice by an enrichment technique. Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of the strain DMKU-RK277^T was closest to *C. hainanensis* but with 4.0 % nucleotide substitutions (18 nucleotide substitutions and three gaps out of 449 nt). The nucleotide sequence of ITS region (AB697761) of strain DMKU-RK277^T and *C. hainanensis* differed by 31.9 % nucleotide substitutions (145 nucleotide substitutions and 51 gaps out of 455 nt).

The phylogenetic tree based on the sequences of the D1/D2 regions of the LSU rRNA gene further demonstrated that strain DMKU-RK277^T connected to *C. hainanensis*, it closest relative in term of pairwise sequence similarity, with 100 % bootstrap support and was near the subclade containing *Metschnikowia pulcherrima*. (Figure 10).

Cells of the strain DMKU-RK277^T was ovoid to ellipsoid (Figure 12) and proliferated by multilateral budding. Pseudohyphae and true hyphae were not formed. Ascospores were not produced on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C.

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene and the ITS region, we concluded that the strains DMKU-RK277^T represent a novel *Metschnikowia* species although formation of ascospores was not observed because of the rules under of the International Code of Nomenclature of algae, fungi, and plants (ICN). The nomenclatural rules for fungi were several important changed, the most important of which was probably the adoption of the principle of "one fungus, one name" (Miller *et al*., 2011). The name *Metschnikowia lopburiensis* sp. nov. (MB564870) is proposed. The type strain is DMKU-RK277^T (= BCC 50731^T = NBRC 108902^T = CBS 12574^T).

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In practice, *M. lopburiensis* sp. nov. can be distinguished from its closest species *C. hainanensis*, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also some phenotypic characteristics. *M. lopburiensis* sp. nov. assimilated succinic acid, D-arabinose (weak) and glycerol (latent) but did not assimilated D-galactose. It grows in vitamin free medium (weak) while the type strain of *C. hainanensis* assimilated D-galactose, glycerol, succinic acid (latent) but did not assimilated D-arabinose. It grows in vitamin free medium.

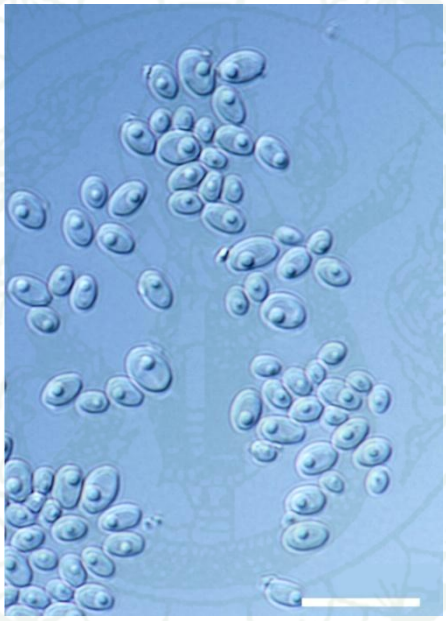


Figure 12 *Metschnikowia lopburiensis* sp. nov. (DMKU-RK277^{T}) budding cells in YM broth after 3 days at 25°C. Scale bar, 10 μm.

Description of *Metschnikowia lopburiensis* Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov

Growth in yeast extract malt extract (YM) broth: After 3 days at 25°C, cells are ovoid to ellipsoid (2-3 x 3-5 μm) and occur singly, in pairs or in groups (Figure 12). Budding is multilateral. Growth on YM agar after 3 days at 25°C, the streak culture is cream, smooth soft to butyrous and has an entire margin. Pseudohyphae and

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true hyphae are not formed in slide culture on PDA. Ascospores were not produced on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C. Fermentation of D-glucose and trehalose (latent) are positive but negative for D-galactose, sucrose, maltose, lactose, raffinose, xylose and cellobiose. D-glucose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melezitose, D-xylose, D-arabinose (weak), N-acetyl-D-glucosamine, ethanol, glycerol (latent), ribitol, D-mannitol, D-glucitol, a-methyl-D-glucoside, salicin, D-gluconic acid, D-glucuronic acid, 2-keto-D-gluconic acid, D-glucono-δ-lactone, succinic acid, citric acid, D-glucosamine, arbutin, xylitol, ethylamine, L-lysine and cadaverine are assimilated, but D-galactose, lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, L-rhamnose, D-ribose, methanol, erythritol, galactitol, D-galacturonic acid, 5-keto-D-gluconic acid, DL-lactic acid, inositol, hexeadecane, propane-1,2-diol, butane-2,3-diol, arabinitol, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is positive (weak). Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose (weak) are positive, but 16 % (w/v) sodium chloride/5 % (w/v) glucose is negative. Growth with 0.01 % cylcloheximide is positive but 0.1 % cylcloheximide is negative. Growth at 25, 30, 35 and 37°C are positive, but at 40, 42 and 45°C are negative. Acid formation is absent. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 51.0 mol %. The major ubiquinone is Q-9.

Holotype

DMKU-RK277^{T} is the holotype of *Metschnikowia lopburiensis* (MB564870). The strain was isolated from phylloplane of rice (*Oryza sativa*) collected from Lop Buri province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50731^{T}; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108902^{T} and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12574^{T}.

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Etymology

The species epithet *lopburiensis* refers to Lop Buri province, Thailand, where the type strain was isolated.

Most of the members of the *Metschnikowia* clade such as *C. nongkhaiensis, C. bambusicola, C. saopaulonensis, C. picinguabensis,* C. *tsuchiyae, C. intermedia* and *C. pseudointermedia* exhibit an association with insect frass, flowers and mosses (Lachance *et al*., 2011; Nakase *et al.*, 2011; Ruivo *et al.*, 2006). In the most recent report, the novel *Candida* species in *Metschnikowia* clade, namely *C. succicola,* the species closest to *M. saccharicola* sp. nov., was isolated from exudate of a tree in Thailand (Nakase *et al*., 2011). *C. hainanensis,* the species closest to *M. lopburiensis* sp. nov., and *C. heveicola* were isolated from flowers and tree sap collected, respectively from tropical forest in Yunnan and Hainan, China (Wang *et al.,* 2008b). *C. danieliae, C. pseudoflosculorum, C. robnettiae* and *C. eppingiae* were reported to isolate from gum produced by trees, and flowers in Thailand and Guyana (Groenewald *et al*., 2011). Therefore, plants seem to be the habitat of numerous yeast species in the *Metschnikowia* clade.

3.2 *Yamadazyma* clade

3.2.1 *Yamadazyma siamensis* sp. nov. (DMKU-RK254^T)

The strain DMKU-RK254^T was isolated from the external surface of sugarcane leaves by an enrichment technique. Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of the strain DMKU-RK254^T was closest to *Candida diddensiae* and *Candida naeodendra* with 1.8% nucleotide substitutions (nine nucleotide substitutions out of 493 nt) and to *Candida kanchanaburiensis* with 2.0% nucleotide substitutions (10 nucleotide substitutions out of 493 nt). The sequences of the ITS region (AB734049) of strain DMKU-RK254^T differed by 6.6% nucleotide substitutions (36 nucleotide substitutions and 13 gaps out of 545 nt) from *C. diddensiae* by 6.6% nucleotide substitutions (39 nucleotide

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substitutions and 30 gaps out of 589 nt) from *C. naeodendra* and by 5.8% nucleotide substitutions (34 nucleotide substitutions and 11 gaps out of 585 nt) from *C. kanchanaburiensis*.

The phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that the strain DMKU-RK254^{T} connects to *C. kanchanaburiensis* and is placed in the same subclade that contains *C. naeodendra*, *C. diddensiae* and the three strains of another two novel species (Figure 13).

Cells of the strain DMKU-RK254^{T} are globose, subglobose to ovoid and proliferated by multilateral budding (Figure 14a). Pseudohyphae but not true hyphae were formed (Figure 14b). Ascospores were not produced on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C.

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene and the ITS region, we concluded that the strains DMKU-RK254^{T} represent a novel *Yamadazyma* species although formation of ascospores was not observed because of the rules under of the International Code of Nomenclature of algae, fungi, and plants (ICN). The nomenclatural rules for fungi were several important changed, the most important of which was probably the adoption of the principle of "one fungus, one name" (Miller *et al*., 2011). The name *Yamadazyma siamensis* sp. nov. (MB801056) is proposed. The type strain is DMKU-RK254^{T} (= BCC 50730^{T} = NBRC 108901^{T} = CBS 12573^{T}).

In practice, *Y. siamensis* sp. nov. can be distinguished from its closest species *C. diddensiae, C. naeodendra* and *C. kanchanaburiensis*, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also some phenotypic characteristics as shown in Table 23.

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Table 23 Phenotypic characteristics that differentiate *Yamadazyma siamensis* sp. nov. from their closest species, *C. didensiae*, *C. naeodendra*, *C.kanchanaburiensis*

Characteristics	Species			
	1	2[a]	3[b]	4[c]
Fermentation				
Galactose	w	+	s	w
Sucrose	-	-/s	-	-
Maltose	-	-/s	-	-
Trehalose	-	s	+	-
Cellobiose	+	n	n	-
Assimilation of carbon/nitrogen compounds				
L-Sorbose	+	v	s	l
Trehalose	+	v	+	+
Soluble starch	-	-	+	-
D-Arabinose	s	v	s	v
L-Rhamnose	+	-	s	l
DL-lactic acid	w	-	-	-
D-glucosamine	+	v	+	s
2-keto-D-gluconic acid	-	v	-	-
D-Glucono-δ-lactone	-	+	-	-
Propane-1,2-diol	-	n	n	+
L-lysine	+	+	+	w
Other growth characteristics				
10% NaCl+5% Glucose	+	+	-	v
0.01%Cycloheximide	w	-	-	n

1, *Yamadazyma siamensis* sp. nov.; 2, *Candida didensiae*; 3, *C. naeodendra*; 4, *C. kanchanaburiensis*.

[a,b] Data from Lachance *et al.* (2011). [c]Data from Nakase *et al.* (2008).

+, positive; -, negative; l, delayed positive; s, slow positive; w, weakly positive; v, variable; n, no data.

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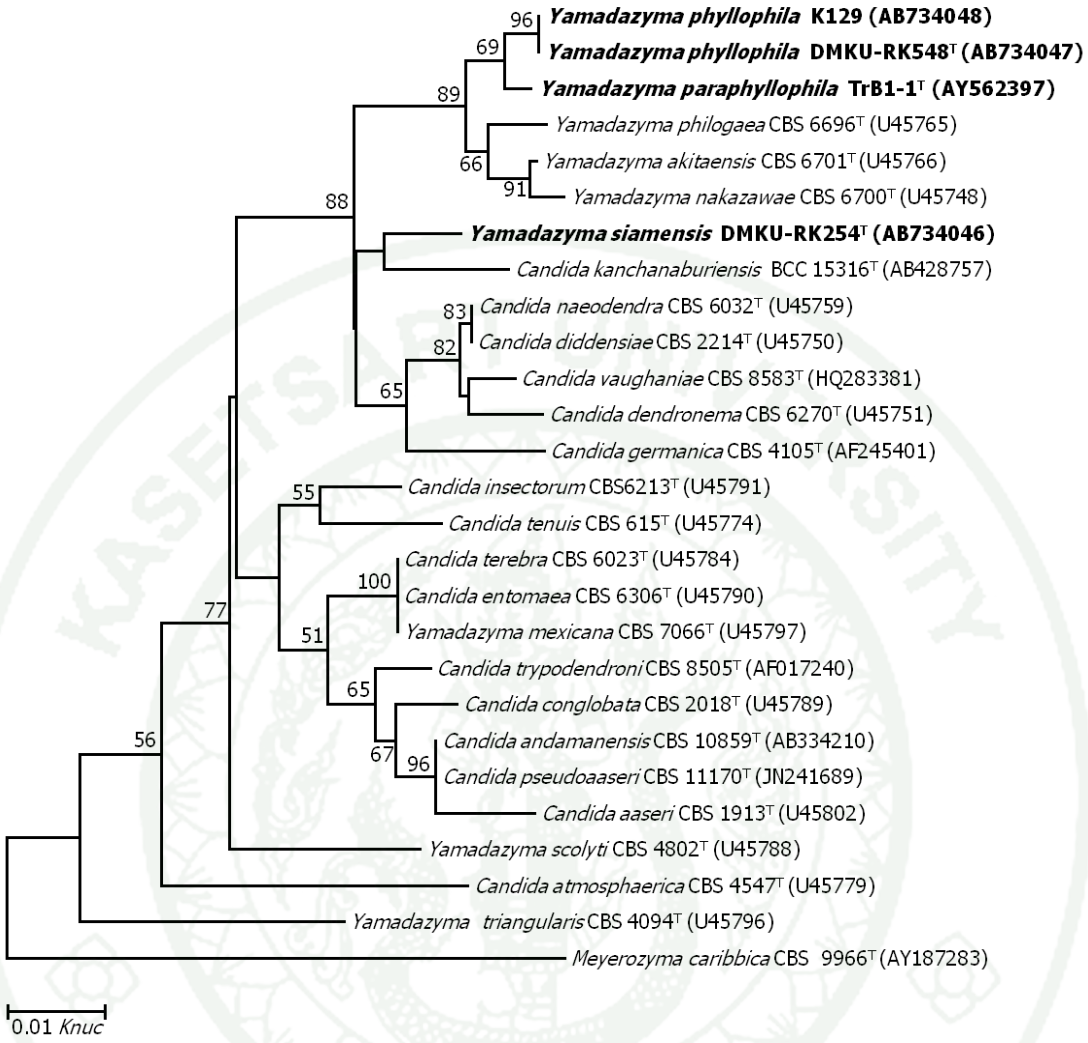


Figure 13 Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Yamadazyma siamensis* sp. nov. (DMKU-RK254^T) and *Yamadazyma phyllophila* sp. nov. (DMKU-RK548^T and K129), with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 4.0. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. The numbers in parentheses are GenBank accession numbers. *Meyerozyma caribbica* was the outgroup in the analysis.

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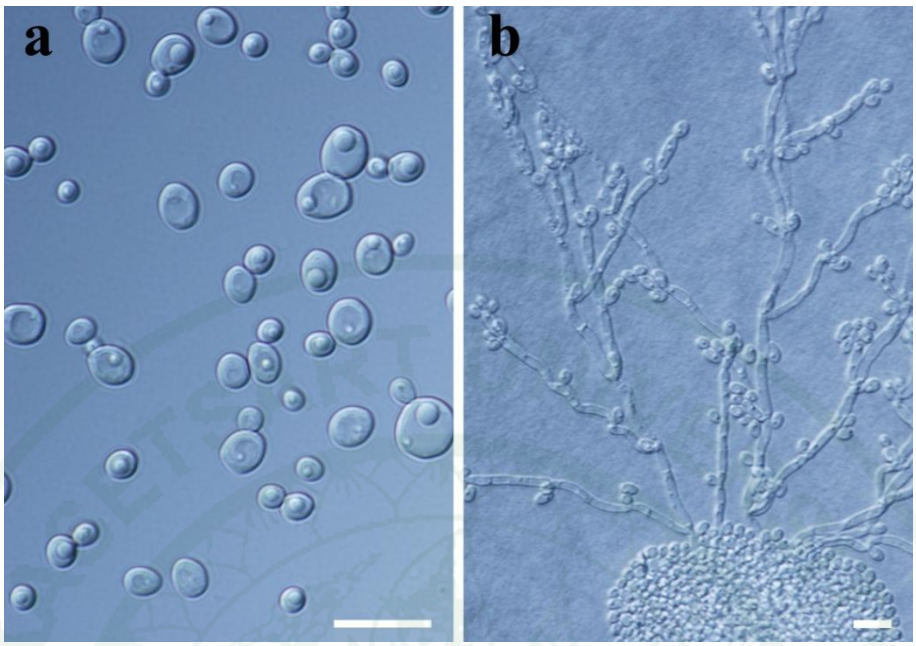


Figure 14 *Yamadazyma siamensis* sp. nov. (DMKU-RK254^{T}) (a) budding cells in YM broth after 3 days at 25°C (b) pseudohyphae formed on PDA after 3 days at 25°C. Scale bar, 10 μm.

Description of *Yamadazyma siamensis* Kaewwichian, Yongmanitchai, Kawasaki, Wang, Yang & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25°C, cells are globose, subglobose to ovoid (2-7 x 3-8 μm) and occur singly, in pairs or in groups (Figure 14a). Budding is multilateral. Growth on YM agar: After 3 days at 25°C, the streak culture is grayish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on potato dextrose agar (PDA) after 3 days at 25°C (Figure 14b). Ascospores were not produced on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C. Fermentation of D-glucose, D-galactose (weak) and cellobiose are positive but negative for sucrose, maltose, lactose, raffinose, trehalose, xylose. D-Glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melezitose, D-xylose, L-arabinose, D-arabinose (slow), D-ribose, L-rhamnose, ethanol, glycerol, ribitol,

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erythritol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, DL-lactic acid (weak), succinic acid, citric acid, arbutin, D-gluconic acid, D-glucosamine, N-acetyl-D-glucosamine, xylitol, arabinitol, ethylamine, L-lysine and cadaverine are assimilated, but, lactose, melibiose, raffinose, inulin, soluble starch, methanol, galactitol, D-glucuronic acid, D-galacturonic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, inositol, hexeadecane, propane-1,2-diol, butane-2,3-diol, D-glucono-δ-lactone, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is positive (weak). Growth on medium containing 50% (w/v) glucose, 60% (w/v) glucose and 10% (w/v) sodium chloride/5% (w/v) glucose are positive, but 16% (w/v) sodium chloride/5% (w/v) glucose are negative. Growth with 0.01% cylcloheximide is positive but 0.1% cylcloheximide is negative. Growth at 25, 30, 35, 37, 40°C is positive, but at 42 and 45°C is negative. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 46.2 mol%. The major ubiquinone is Q-9.

Holotype

DMKU-RK254^{T} is the holotype of *Yamadazyma siamensis* (MB801056). The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Phetchabun province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50730^{T}; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108901^{T} and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 12573^{T}.

Etymology

The species epithet *siamensis* refers to Siam the old name of Thailand, where the strain was isolated.

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3.2.2 *Yamadazyma phyllophila* sp. nov. (DMKU-RK548^T)

The strain DMKU-RK548^T was isolated from the external surface of corn leaves by an enrichment technique. Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of the strain DMKU-RK548^T was closest to *Candida* sp. K129 but differed by three nucleotide substitutions from strain TrB1-1^T. The closest known species to the three strains was *Yamadazyma philogaea*; the two strains (DMKU-RK548^T and K129) differed by 1.1% nucleotide substitutions (six nucleotide substitutions out of 530 nt) while strain TrB1-1^T differed by 1.7% nucleotide substitutions (nine nucleotide substitutions out of 530 nt). The sequences of ITS region of the two strains (DMKU-RK548^T; AB734050 and K129; AB734051) were identical and differed from strain TrB1-1^T (AY559447) by six nucleotide substitutions and one gap out of 582 nt. The two strains (DMKU-RK548^{T}and K129) differed by 5.2% nucleotide substitutions (30 nucleotide substitutions and five gaps out of 582 nt) while strain TrB1-1^T differed by 5.5% nucleotide substitutions (32 nucleotide substitutions and six gaps out of 582 nt) from *Y. philogaea*. Moreover, the sequences of the ACT1 gene and the EF2 gene of the two strains (DMKU-RK548^T; AB761313, AB761316 and K129; AB761312, AB761315) were identical and differed from strain TrB1-1^T (AB761314, AB761317) by 17 nucleotide substitutions out of 523 nt (3.3% nucleotide substitutions) and 36 nucleotide substitutions out of 606 nt (5.9% nucleotide substitutions), respectively. The difference in the ACT1 and EF2 genes of the two strains (DMKU-RK548^T and K129) from strain TrB1-1^T is high enough to justify them as two separate species as report for the ACT1 gene in *Clavispora* spp. by Lachance *et al.* (2003) and for EF2 gene in *Wickerhamomyces* spp. by Imanishi *et al.* (2009).

The phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that strains DMKU-RK548^T and K129 are located in the same position and connect to strain TrB1-1^T. They cluster with *Yamadazyma philogaea*, the closest known species, and *Yamadazyma nakazawae* and *Yamadazyma akitaensis* with high bootstrap support (Figure 13).

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Cells of the strain DMKU-RK548^T and K129 were ovoid to ellipsoid (2-4 x 3-6 μm) and proliferated by multilateral budding (Figure 15a). Pseudohyphae were formed but true hyphae were not formed in slide culture on PDA after 3 days at 25°C (Figure 15b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C.

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene, the ITS region, the ACT1 gene and the EF2 gene, we concluded that the strain DMKU-RK548^T and K129 represent a novel *Yamadazyma* species although formation of ascospores was not observed because of the rules under of the International Code of Nomenclature of algae, fungi, and plants (ICN). The nomenclatural rules for fungi were several important changed, the most important of which was probably the adoption of the principle of "one fungus, one name" (Miller *et al*., 2011). The name *Yamadazyma phyllophila* sp. nov. (MB801057) is assigned. The type strain is DMKU-RK548^T (= BCC 50736^T = NBRC 108906^T = CBS 12572^T).

In practice, *Y. phyllophila* sp. nov. can be distinguished from its closest species *Y. philogaea*, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene, the ITS region, the ACT1 gene and the EF2 gene but also some phenotypic characteristics. *Y. phyllophila* sp. nov. fermented galactose but did not fermented trehalose. Lactose, L-arabinose (weak), L-rhamnose and salicin are assimilated, but L-sorbose, D-arabinose, D-ribose, citric acid, D-glucosamine, 2-keto-D-gluconic acid and hexeadecane are non-assimilated. Growth on medium containing 10% NaCl+5% glucose (weak) is positive while the type strain of *Y. philogaea* fermented galactose (weak) and trehalose. L-arabinose, L-sorbose, D-arabinose, D-ribose, citric acid, D-glucosamine, 2-keto-D-gluconic acid and hexeadecane are assimilated, but lactose, L-rhamnose and salicin are non-assimilated. Growth on medium containing 10% NaCl+5% glucose is positive.

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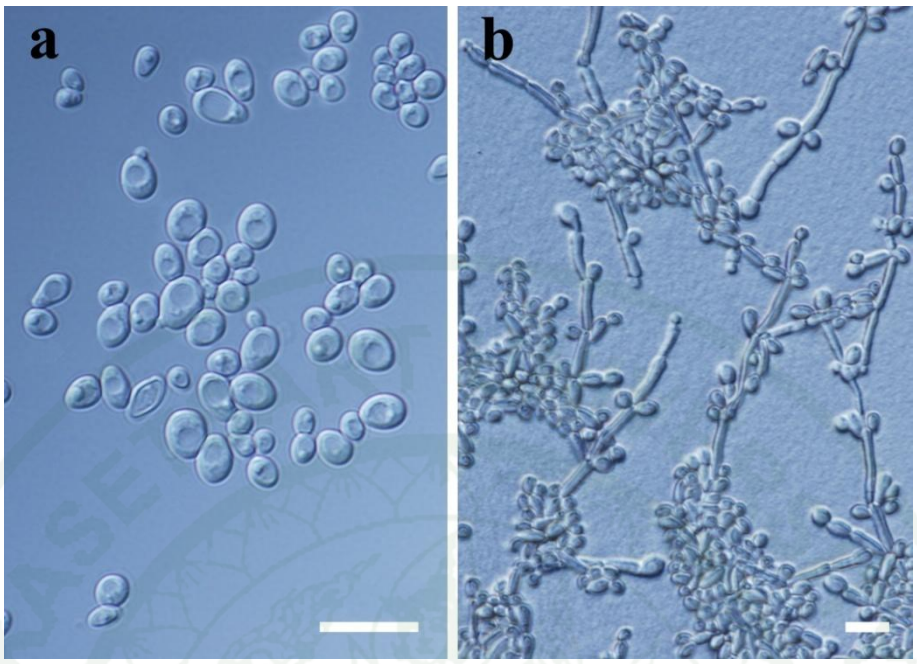


Figure 15 *Yamadazyma phyllophila* sp. nov. (DMKU-RK548^{T}) (a) budding cells in YM broth after 3 days at 25°C (b) pseudohyphae formed on PDA after 3 days at 25°C. Scale bar, 10 μm.

Description of *Yamadazyma phyllophila* Kaewwichian, Yongmanitchai, Kawasaki, Wang, Yang & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25°C, cells are ovoid to ellipsoid (2-4 x 3-6 μm) and occur singly, in pairs or in groups (Figure 15a). Budding is multilateral. Growth on YM agar after 3 days at 25°C, the streak culture is white, smooth and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25°C (Figure 15b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C. Fermentation of D-glucose and D-galactose are positive but negative for sucrose, maltose, lactose, raffinose, trehalose, xylose and cellobiose. D-Glucose, D-galactose, sucrose, maltose, cellobiose, trehalose, lactose, melezitose, D-xylose, L-arabinose

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(weak), L-rhamnose, ethanol, glycerol, erythritol, ribitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, succinic acid, D-gluconic acid, N-acetyl-D-glucosamine, D-glucono-δ-lactone, xylitol, ethylamine, L-lysine and cadaverine are assimilated, but L-sorbose, melibiose, raffinose, inulin, soluble starch, D-arabinose, D-ribose, methanol, galactitol, D-galacturonic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, DL-lactic acid, citric acid, D-glucosamine, arbutin, inositol, hexeadecane, propane-1,2-diol, butane-2,3-diol, D-glucuronic acid, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is negative. Growth on medium containing 50% (w/v) glucose, 60% (w/v) glucose and 10% (w/v) sodium chloride/5% (w/v) glucose are positive, but 16% (w/v) sodium chloride/5% (w/v) glucose is negative. Growth with 0.01% cylcloheximide and 0.1% cylcloheximide is negative. Growth at 25, 30, 35, 37 and 40°C is positive, but at 42 and 45°C is negative. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 38.7 mol%. The major ubiquinone is Q-9.

Holotype

DMKU-RK548^{T} is the holotype of *Yamadazyma phyllophila* sp. nov. (MB801057). The strain was isolated from phylloplane of corn (*Zea mays*) collected from Nakhon Ratchasima province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50736^{T}; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108906^{T} and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 12572^{T}.

Etymology

The species epithet *phyllophila* refers to phylloplane where the three strains of this species were isolated.

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Most of the members of the *Yamadazyma* clade such as *C. michaelii, C. gorgasii, C. lessepsii, C. cerambycidarum,* C. *endomychidarum, C. amphixiae, C. diddensiae, C. naeodendra* and *C. dendronema* exhibit an association with the gut of beetles and other insects (Lachance *et al*., 2011). However, some strains of the recognized species were reported to have been isolated from plants i.e. a strain of *Y. akitaensis* (CBS 6701^T) that was isolated from exudate of a willow, two strains of *Y. mexicana* (CBS 7066^T and CBS 7067) that were isolated from cactus, *C. diospyri* (CBS 9769^T) that was isolated from a kaki fruit and *C. buinensis* (CBS 6796^T) that was isolated from the gelatinous exudate of a tree fern (Kurtzman, 2011d; Lachance *et al*., 2011). In Thailand few *Candida* species in the *Yamadazyma* clade have been isolated i.e. *C. khaothaluensis,* which was isolated from an unidentified tree (Groenwald *et al*., 2011) and *C. kanchanaburiensis,* which was isolated from a fruit body of a unidentified mushroom (*Coprinus* sp.) and a rotted guava fruit (Nakase *et al*., 2008). Until the present, no members of this clade have been reported to have been isolated from the phylloplane. In this study, four strains of the two novel *Yamadazyma* species are proposed, but from limited number of strains derived in this study, we could not conclude that the phylloplane is one of the habitats of yeast in this clade. However, the phylloplane is interesting as the subject for further investigation of yeast in this clade.

3.3 *Wicherhamomyces* clade

3.3.1 *Wicherhamomyces siamensis* sp. nov. (DMKU-RK359^T)

The strain DMKU-RK359^T was isolated from the external surface of sugarcane leaves by an enrichment technique. Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of the strain DMKU-RK359^T was closest to *Wickerhamomyces ciferrii* with 0.7% nucleotide substitutions (four nucleotide substitutions and four gaps out of 571 nt). The sequences of the ITS region of strain DMKU-RK359^T (AB714257) differed by 6% nucleotide substitutions (33 nucleotide substitutions and eight gaps out of 550 nt) from *W. ciferrii.*

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The phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene shows that strain DMKU-RK359^T is closely related with *W. ciferrii*, *W. edaphicus* and *C. silvicultrix* with high bootstrap support and belongs in the *Wickerhamomyces* clade at a position distinct from the other members of the clade (Figure 16).

Cells of the strain DMKU-RK359^{T}are globose, subglobose to ovoid (3-8 x 4-8 μm) and and proliferated by multilateral budding (Figure 17a). Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25°C (Figure 17b). Ascospores were produced on YPD agar and 5% malt extract agar after 7 days at 25°C. Asci were persistent, unconjugated and formed four hat shaped ascospores (Figure 17c). Strain DMKU-RK359^T was found to be homothallic.

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene and the ITS region, we concluded that the strains DMKU-RK359^T represent a novel *Wickerhamomyces*. The name *Wickerhamomyces siamensis* sp. nov. (MB802886) is proposed. The type strain is DMKU-RK359^T (=BCC 50732^T = NBRC 108900^T = CBS 12570^T).

In practice, *W. siamensis* sp. nov. can be distinguished from its closest species *W. ciferrii*, *W. edaphicus* and *C. silvicultrix*, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also some phenotypic characteristics as shown in Table 24.

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Table 24 Phenotypic characteristics that differentiate *Wickerhamomyces siamensis* sp. nov. from its closely related species, *W. ciferrii, W. edaphicus* and *C. silvicultrix*

Characteristics	Species			
	1	2[a]	3[b]	4[c]
Assimilation of carbon/nitrogen compounds				
Melibiose	-	-	+	+
L-Arabinose	-	+/w	w/-	+
D-Arabinose	-	-	-	+
D-Ribose	-	+	nd	+
L-Rhamnose	-	+/w	+	-
Ribitol	-	+	+	+
Citric acid	-	+	+	+
Hexeadecane	-	+	nd	-
D-Glucono-δ-lactone	-	nd	nd	+
Nitrate	-	+	+	-
Nitrite	-	nd	+	-
Ethylamine	+	nd	+	-
Other growth characteristics				
Vitamin-free medium	-	+	+	+
10% NaCl+5% Glucose	-	w	+	+
Growth at 37°C	+	w/-	w	+
Growth at 40°C	+	nd	-	nd

1, *W. siamensis*; 2, *W. ciferrii*; 3, *W. edaphicus*; 4, *C. silvicultrix*

[a] Data from Kurtzman (2011c), [b]Data from Limtong *et al*. (2009), [c]Data from Lachance *et al*. (2011).

+, positive; w, weak positive; -, negative; nd, no data.

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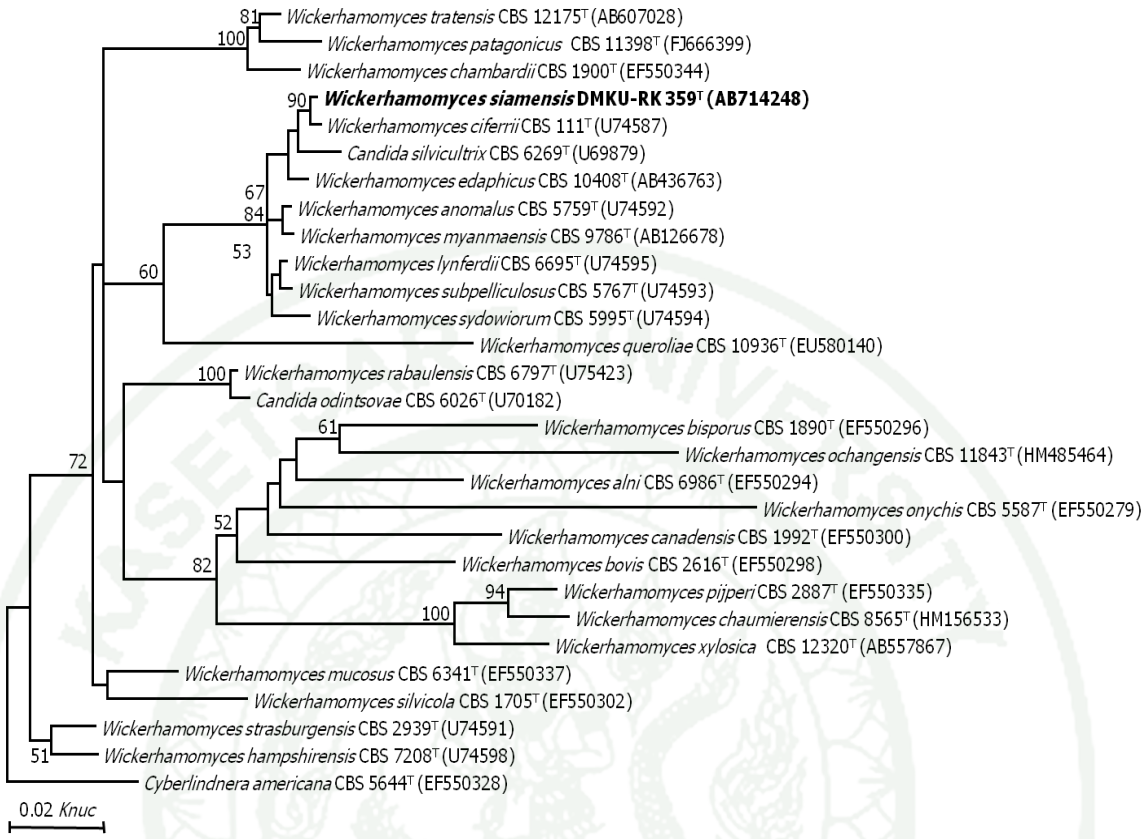


Figure 16 Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Wickerhamomyces siamensis* sp. nov. (DMKU-RK359^{T}), with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA soft-ware version 4.0. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. The numbers in parentheses are GenBank accession numbers. *Cyberlindnera americana* was the outgroup in the analysis.

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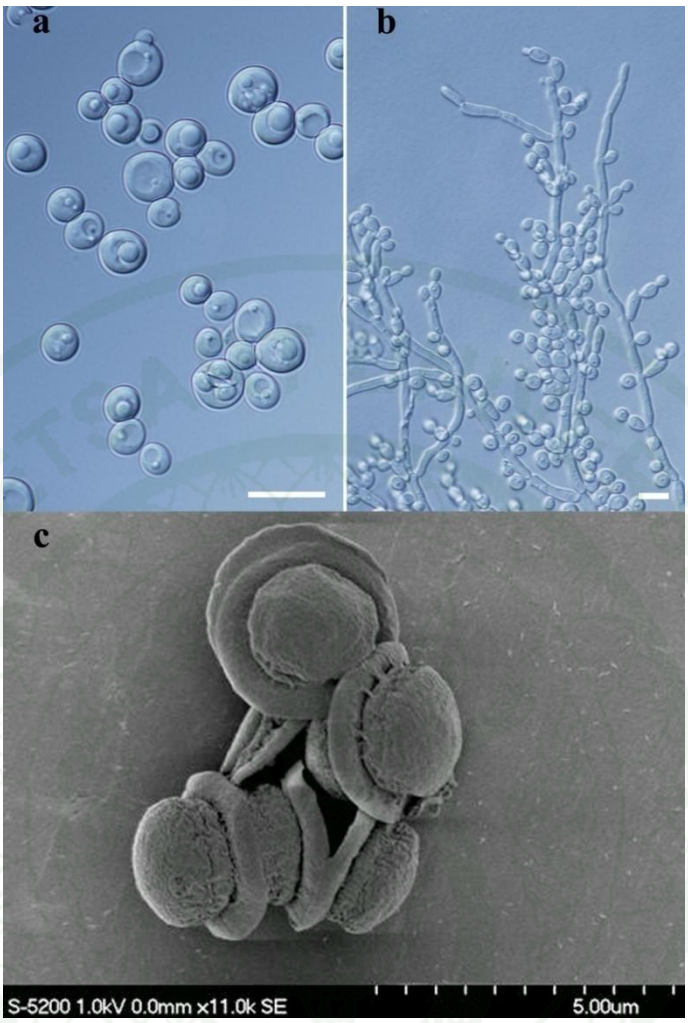


Figure 17 *Wickerhamomyces siamensis* sp. nov. (DMKU-RK359^{T}) (a) budding cells in YM broth after 3 days at 25°C (b) pseudohyphae formed on PDA after 3 days at 25°C. Scale bar, 10 μm. (c) ascospore on 5% malt extract agar after 7 days at 25°C. Scale bar, 5 μm.

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Description of *Wickerhamomyces siamensis* Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25°C, cells are globose, subglobose to ovoid (3-8 x 4-8 μm) and occur singly, in pairs or in groups (Figure 17a). Budding is multilateral. Growth on YM agar: After 3 days at 25°C, the streak culture is grayish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25°C (Figure 17b). One to four hat-shaped ascospores are formed in a persistent ascus on YPD agar and 5% malt extract agar after 7 days at 25°C (Figure 17c). Fermentation of D-glucose, D-galactose, sucrose, maltose (weak) and raffinose (weak) are positive but negative for lactose, trehalose, xylose and cellobiose. D-Glucose, D-galactose (slow), sucrose, maltose, cellobiose (weak), trehalose (weak), raffinose (weak), melezitose (slow), soluble starch (weak), D-xylose (slow), ethanol, glycerol, erythritol (weak), D-mannitol (weak), D-glucitol (weak), α-methyl-D-glucoside (weak), salicin (weak), D-gluconic acid, DL-lactic acid, succinic acid, arbutin (weak), propane-1,2-diol (weak), xylitol (weak) , ethylamine, L-lysine (weak) and cadaverine are assimilated, but L-sorbose, lactose, melibiose, inulin, L-arabinose, D-arabinose, L-rhamnose, D-ribose, N-acetyl-D-glucosamine, methanol, ribitol, galactitol, D-glucuronic acid, D-galacturonic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, citric acid, inositol, D-glucosamine, hexeadecane, butane-2,3-diol, arabinitol, nitrate and nitrite are not assimilated. No growth in vitamin free medium. Growth on medium containing 50% glucose and 60% glucose are positive, but on 10% sodium chloride/5% glucose and 16% sodium chloride/5% glucose are negative. Growth with 0.01% cylcloheximide and 0.1% cylcloheximide are negative. Growth at 25, 30, 35, 37, 40 and 42°C are positive, but at 45°C are negative. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 36.1 mol%. The major ubiquinone is Q-7.

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Holotype

DMKU-RK359^{T} is the holotype of *Wickerhamomyces siamensis*. The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Lopburi province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50732^{T}; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108900^{T} and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12570^{T}.

Etymology

The species epithet "*siamensis*" was chosen for this novel species referring to Siam, the old name of Thailand, where the type strain was isolated.

Member of *Wickerhamomyces* species are often found to associate with plants, however, only a few species were reported to isolate from phylloplane such as *W. edaphicus* which were isolated from *Terminalia catappa* Linn. (Limtong and Koowadjanakul, 2012). In this study from 159 yeast strains obtained from phylloplane of 94 samples of sugarcane leaf only the strain DMKU-RK359^{T} was isolated, therefore, it is clear that the novel species is not common in phylloplane. On the other hand *Meyerozyma caribbica* was found to be the most common yeast species in phylloplane, 36 strains of this species were obtained in this study.

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3.4 *Nakazawaea* clade

3.4.1 *Nakazawaea siamensis* sp. nov. (DMKU-RK467^T)

The strain DMKU-RK467^T was isolated from the external surface of sugarcane leaves by an enrichment technique. Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of the strain DMKU-RK467^T was closest to *Candida wickerhamii* but with 1.9% nucleotide substitutions (11 nucleotide substitutions out of 569 nt). The sequences of the ITS region of strain DMKU-RK467^T (AB793784) differed by 5.2% nucleotide substitutions (35 nucleotide substitutions and ninety nine gaps out of 680 nt) from *C. wickerhamii*.

The phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that the strain DMKU-RK467^T cluster with *C. wickerhamii*, the closest known species, and *Nakazawaea holstii* and *Candida* species in *Nakazawaea* clade namely *C. anatomiae, C. chilensis, C. ernobii, C. heliconiae, C. ishiwadae, C. karawaiewii, C. peltata, C. pomicola, C. populi, C. sinolaborantium* and *C. wyomingensis* (Figure 18).

Cells of the strain DMKU-RK467^T are globose to subglobose (3-5 x 3-5 μm) and proliferated by multilateral budding (Figure 19). Pseudohyphae and true hyphae are not formed in slide culture on PDA. Ascospores were not produced on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C.

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene, we concluded that the strains DMKU-RK467^T represent a novel *Nakazawaea* species although formation of ascospores was not observed because of the rules under of the International Code of Nomenclature of algae, fungi, and plants (ICN). The nomenclatural rules for fungi were several important changed, the most important of which was probably the adoption of the principle of "one fungus, one

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name" (Miller *et al*., 2011). The name *Nakazawaea siamensis* sp. nov. (MB 803008) is proposed. The type strain is DMKU-RK467^{T} (= BCC 50734^{T} = NBRC 108903^{T} = CBS 12569^{T}).

In practice, *N. siamensis* sp. nov. can be distinguished from its closest species, *C. wickerhamii*, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene but also some phenotypic characteristics. *N. siamensis* sp. nov. assimilated sucrose, maltose, melezitose, inulin, soluble starch, erythritol (weak), galactitol (latent), a-methyl-D-glucoside and xylitol but did not assimilated nitrate and nitrite. Growth on medium containing 50% Glucose and 10% NaCl+5% glucose are positive while the type strain of *C. wickerhamii* assimilated nitrate and nitrite but did not assimilated sucrose, maltose, melezitose, inulin, soluble starch, erythritol, galactitol, a-methyl-D-glucoside and xylitol. Growth on medium containing 50% Glucose and 10% NaCl+5% glucose are negative.

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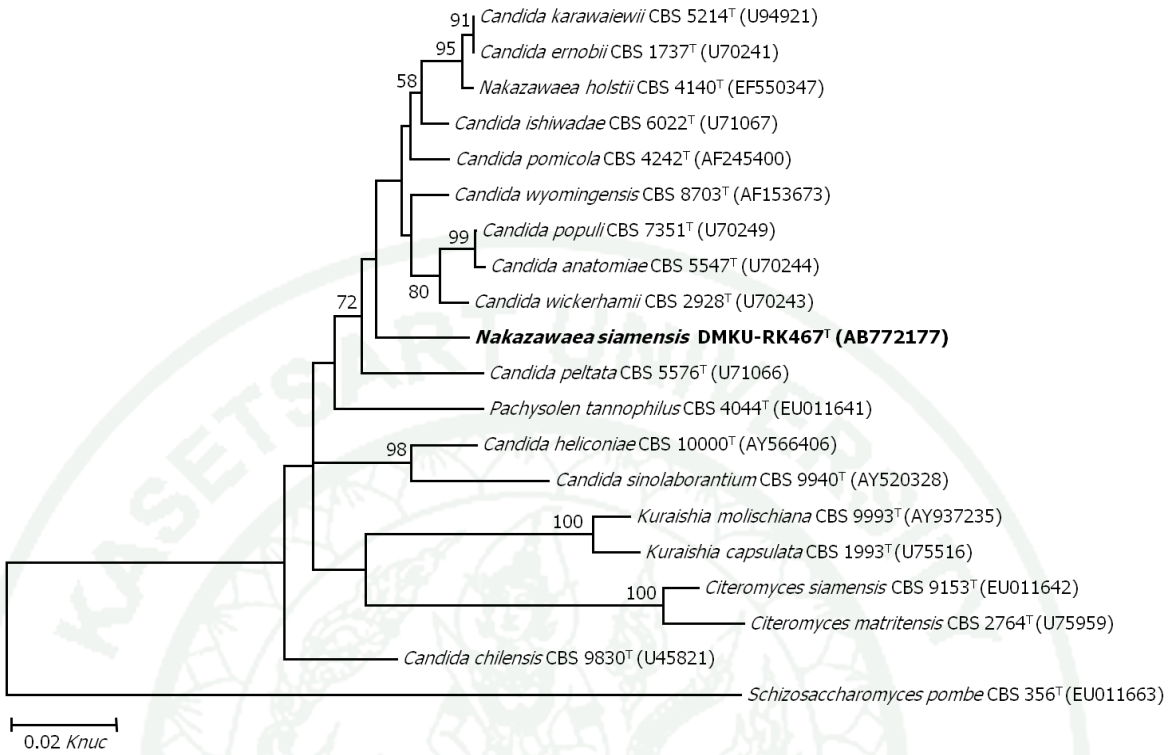


Figure 18 Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Nakazawaea siamensis* sp. nov. (DMKU-RK467^{T}), with respect to closely related species.
The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA soft-ware version 4.0. Numbers indicate percentages of bootstrap sampling, derived from 1000 samples. The numbers in parentheses are GenBank accession numbers. *Schizosaccharomyces pombe* was the outgroup in the analysis.

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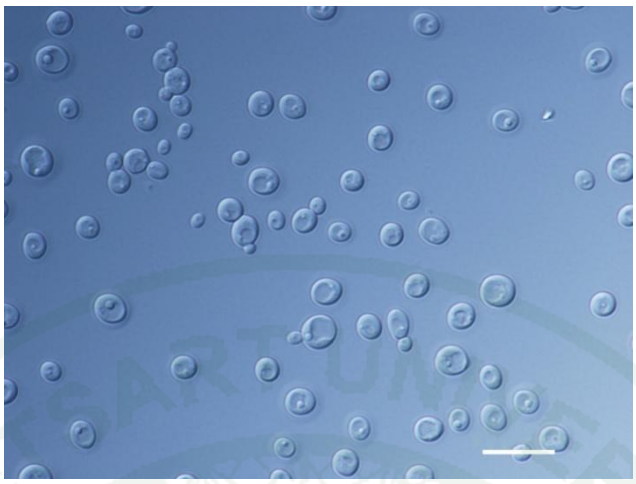


Figure 19 *Nakazawaea siamensis* sp. nov. (DMKU-RK467^{T}) budding cells in YM broth after 3 days at 25°C. Scale bar, 10 μm.

Description of *Nakazawaea siamensis* Kaewwichian, Yongmanitchai, Kawasaki, & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25°C, cells are globose to subglobose (3-5 x 3-5 μm) and occur singly, in pairs or in groups (Figure 19). Budding is multilateral. Growth on YM agar: After 3 days at 25°C, the streak culture is white to cream, smooth, soft and has an entire margin. Pseudohyphae and true hyphae are not formed in slide culture on PDA. Ascospores were not produced on YM agar, YPD agar, 5% malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25°C. Fermentation of D-glucose are positive but negative for D-galactose, sucrose, maltose, raffinose, lactose, trehalose, xylose and cellobiose. D-Glucose, D-galactose (slow), L-sorbose (slow), sucrose, maltose, cellobiose, trehalose, melezitose, soluble starch, inulin, D-xylose, L-arabinose (latent), D-arabinose (slow), L-rhamnose, D-ribose, ethanol, glycerol, erythritol, ribitol, galactitol (latent), D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, D-gluconic acid (slow), succinic acid, citric acid, arbutin, N-acetyl-D-glucosamine, 2-keto-D-gluconic acid (weak), propane-1,2-diol (weak), butane-2,3-diol (weak), D-glucono-δ-lactone, xylitol, D-galacturonic acid, arabinitol, ethylamine, L-lysine (weak) and cadaverine are assimilated but lactose,

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melibiose, raffinose, DL-lactic acid, methanol, D-glucuronic acid, 5-keto-D-gluconic acid, inositol, D-glucosamine, hexeadecane, nitrate and nitrite are not assimilated. No growth in vitamin free medium. Growth on medium containing 50% glucose, 60% glucose and 10% sodium chloride/5% glucose are positive, but on 16% sodium chloride/5% glucose are negative. Growth with 0.01% cylcloheximide and 0.1% cylcloheximide are positive (weak). Growth at 25, 30, 35, 37 and 40°C are positive, but at 42, 45°C are negative. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. The major ubiquinone is Q-7.

Holotype

DMKU-RK467^{T} is the holotype of *Nakazawaea siamensis*. The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Nakhon Ratchasima province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50734^{T}; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108903^{T} and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12569^{T}.

Etymology

The species epithet "*siamensis*" was chosen for this novel species referring to Siam, the old name of Thailand, where the type strain was isolated.

At present there are seven species of the genus *Nakazawaea* and *Candida* in the *Nakazawaea* clade namely *N. holstii, C. chilensis, C. heliconiae, C. populi, C. sinolaborantium, C. wickerhamii* and *C. wyomingensis*. The species in this clade have been isolated from a wide variety of substrates (Kurtzman, 2011b). Many strains of *N. holstii* seems to be primarily associated with bark beetles infesting pine, spruce and fir trees (Wickerham, 1960), but it is also isolated from other substrates, such as cider and apples, insect frass, streams and lichens. *C. chilensis* were collected from species

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of *Tritoma* sp., a basidiocarp-feeding beetle, *C. heliconiae* was isolated from water accumulated in flower bracts of *Heliconia, C. populi* was isolated from sap flux of trembling aspen, *C. sinolaborantium* was isolated from the gut of a handsome fungus beetle and the gut and surface of a larva of a cerambycid beetle, *C. wickerhamii* was isolated from silage and *C. wyomingensis* was isolated from feed in sap fluxes of trees (Kurtzman, 2011b). Therefore, plants and substrates associated with plants seem to be the habitat of yeast species in the *Nakazawaea* clade.

4. Determination of indole-3-acetic acid production by phylloplane yeast

Among the 441 strains of yeast, 148 strains in 40 species, four strains of an undescribed species and two strains of a novel species showed the capability of producing IAA when cultivated in YPD broth supplemented with 0.1 % L-tryptophan (Table 25). *Rhodosporidium fluviale* DMKU-RK253 which was collected from sugarcane leaves produced the highest IAA concentration of 417.8 mg/L. The other 287 strains grew in this medium, but could not produced IAA. Among the strains of the phylum Ascomycota 93 strains in 29 species produced IAA in the range of 2.2-259.0 mg/L. Twelve ascomycete yeast strains produced IAA higher than 100 mg/L. They were *C. michaelii* DMKU-RK301 (259.0 mg/L), *C. sojae* DMKU-RK538 (161.3 mg/L), *C. ruelliae* DMKU-RK434 (131.9 mg/L), *M. caribbica* DMKU-RK203 (131.8 mg/L), *M. guilliermondii* DMKU-RK311 (130.2 mg/L), *C. orthopsilosis* DMKU-RK28 (129.2 mg/L), *C. mesorugisa* DMKU-RK179 (118.0 mg/L), *Y. philogeae* DMKU-RK544 (115.9 mg/L), *K. ohmeri* DMKU-RK552 (110.2 mg/L), *H. opuntiae* DMKU-RK551 (106.9 mg/L), *L. elongisporus* DMKU-RK440 (105.7 mg/L) and *M. caribbica* DMKU-RK6 (104.1 mg/L). Fifty-five strains in 11 species of the phylum Basidiomycota produced IAA in the range of 3.3-417.8 mg/L. Fifteen basidiomycete yeast strains produced IAA higher than 100 mg/L. They were nine strains of *R. fluviale* including DMKU-RK253 (417.8 mg/L), DMKU-RK498 (269.1 mg/L), DMKU-RK422 (259.1 mg/L), DMKU-RK537 (223.0 mg/L), DMKU-RK539 (217.3 mg/L), DMKU-RK545 (198.3 mg/L), DMKU-RK416 (146.0 mg/L), DMKU-RK472 (115.5 mg/L) and DMKU-RK406 (105.4 mg/L); three strains of *R. paludigenum* including DMKU-RK528 (186.2 mg/L), DMKU-RK342 (144.6 mg/L) and DMKU-RK266 (144.0 mg/L); two strains of *R. toruloides* namely DMKU-

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RK334 (153.0 mg/L) and DMKU-RK336 (125.0 mg/L); and *R. taiwanensis* DMKU-RK247 (137.1 mg/L). Investigation of the IAA production capability of yeast from three crops phylloplane in this study revealed that approximately 34.9 % of investigated yeast strains possessed this ability.

All strains of *R. fluviale* (13 strains) and *R. paludigenum* (4 strains) produced IAA while all strain of *C. rhodanensis* (14 strains), *M. koratensis* (8 strains), *C. nivariensis* (6 strains), *T. asahii* (6 strains), *W. edaphicus* (3 strains) and *C. heveanensis* (3 strains) could not product IAA. However, the species which have only one or two strains did not include in these summary. Some strains of some species viz. *C. catenulata, C. glabrata, C. jaroonii, C. orthopsilosis, C. pseudolambica, C. quercitrusa, C. rugosa, C. tropicalis, C. fabianii, D. nepalensis, H. guilliermondii, H. opuntiae, K. ohmeri, M. koreensis, M. caribbica, M. guillermondii, P. kudriavzevii, C. flavescens, C.* aff. *laurentii, C. laurentii, C. rajasthanensis, R. taiwanensis, S. ruineniae* and *S. nylandii* were able to produce IAA while the others were not.

The result indicated IAA production was strain dependent for some species while some strains of some species were able to produce IAA but the others were not. This finding is similar to the result reported by Limtong and Koowadjanakul (2012). They reported that among the species of one genus, both IAA producing species and non-producing species were detected. Moreover, not all strains within the same species had the ability to produce IAA. Therefore, it seems that IAA production capability is strain-dependent in these phylloplane yeasts.In this study, all stains in three species of the genus *Rhodosporidium* namely *R. fluviale, R. paludigenum* and *R. toruloides* produced IAA though we could not conclude that IAA production capability in these species is species dependent but these three species of the genus *Rhodosporidium* are interesting to be screened for potent IAA production.

Variation in IAA biosynthesis among strains of actinomycetes and fungi within the same species has also been reported by the other investigators (Ruanpanun *et al*., 2010; Tsavkelova *et al*., 2006). IAA production by *Rhodosporidium fluviale* DMKU-RK253 (417.8 mg/L) seemed to be higher than the other yeasts such as *C. maltosa* LM114 (314.3 mg/L) (Limtong and Koowadjanakul, 2012), *Lindera* (*Williopsis*) *saturnus* (22.5 mg/L) (Nassar *et al*., 2005); fungi such as *Aspergillus*

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niger (132.7 μg/mL) (Bilkay *et al*., 2010); actinomycetes such as *Streptomyces* sp. CMU-H009 (143.95 μg/mL) (Khamna *et al*., 2010); and bacteria such as *Rubrivivax benzoatilyticus* JA2 (58.1 mg/L) (Mujahid *et al.*, 2001) and *Klebsiellas* sp. SN1 (291 mg/L) (Xinxian *et al*., 2011). However, the concentration of IAA produced by *R. fluviale* DMKU-RK253 needs to be confirmed with more specific method for IAA determination such as high-performance liquid chromatography or gas chromatography-mass spectrometry.

Table 25 Indole-3-acetic acid productions in YPD broth supplemented with 1 mg/L L-tryptophan in a test tube after 7 days by shake cultivation at 150 rpm and 30 ± 3 °C

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
Phylum Ascomycota					
Candida akabanensis			DMKU-RK433	0.0	0.0
Candida dendronema			DMKU-RK365	0.0	0.0
Candida catenulata	DMKU-RK53			37.9	3.4
	DMKU-RK81			0.0	0.0
		DMKU-RK524		49.4	5.4
Candida conglobata		DMKU-RK540		54.5	6.4
Candida glabrata	DMKU-RK165			0.0	0.0
		DMKU-RK318		0.0	0.0
		DMKU-RK523		14.9	1.9
Candida jaroonii	DMKU-RK94			0.0	0.0
	DMKU-RK101			42.0	7.2
	DMKU-RK138			0.0	0.0
Candida membranifaciens	DMKU-RK57			0.0	0.0
Candida mesorugisa		DMKU-RK305		61.7	6.4
			DMKU-RK179	118.0	8.2
			DMKU-RK363	24.2	1.6
Candida michaelii			DMKU-RK301	259.0	27.1
			DMKU-RK343	0.0	0.0
Candida nivariensis			DMKU-RK290	0.0	0.0
			DMKU-RK393	0.0	0.0
			DMKU-RK445	0.0	0.0
			DMKU-RK455	0.0	0.0
			DMKU-RK468	0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
			DMKU-RK488	0.0	0.0
Candida orthopsilosis		DMKU-RK322		0.0	0.0
		DMKU-RK324		97.7	15.8
			DMKU-RK28	129.2	10.6
Candida pseudolambica	DMKU-RK48			0.0	0.0
		DMKU-RK11		58.3	10.0
		DMKU-RK505		16.1	3.0
		DMKU-RK515		0.0	0.0
Candida quercitrusa		DMKU-RK1		13.7	3.1
		DMKU-RK4		0.0	0.0
		DMKU-RK5		19.3	3.1
		DMKU-RK504		0.0	0.0
		DMKU-RK516		0.0	0.0
			DMKU-RK14	37.5	4.0
Candida ruelliae	DMKU-RK434			131.9	14.7
Candida rugosa	DMKU-RK164			22.7	1.6
	DMKU-RK483			38.2	4.4
		DMKU-RK407		0.0	0.0
			DMKU-RK299	0.0	0.0
			DMKU-RK389	0.0	0.0
			DMKU-RK495	0.0	0.0
Candida sojae		DMKU-RK538		161.3	20.0
Candida sorboxylosa		DMKU-RK3		0.0	0.0
Candida terebra	DMKU-RK436			7.8	1.1
Candida tropicalis	DMKU-RK34			8.0	0.8
	DMKU-RK36			0.0	0.0
	DMKU-RK37			0.0	0.0
	DMKU-RK41			12.0	0.9
	DMKU-RK43			0.0	0.0
	DMKU-RK45			0.0	0.0
	DMKU-RK60			0.0	0.0
	DMKU-RK62			20.3	1.4
	DMKU-RK104			0.0	0.0
	DMKU-RK121			0.0	0.0
	DMKU-RK123			0.0	0.0
	DMKU-RK125			0.0	0.0
	DMKU-RK126			0.0	0.0
	DMKU-RK131			0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
	DMKU-RK137			0.0	0.0
	DMKU-RK145			0.0	0.0
	DMKU-RK152			0.0	0.0
	DMKU-RK161			0.0	0.0
	DMKU-RK162			0.0	0.0
	DMKU-RK163			0.0	0.0
	DMKU-RK166			0.0	0.0
	DMKU-RK219			0.0	0.0
	DMKU-RK226			0.0	0.0
	DMKU-RK235			30.3	3.0
	DMKU-RK284			0.0	0.0
	DMKU-RK462			0.0	0.0
	DMKU-RK464			0.0	0.0
	DMKU-RK485			0.0	0.0
		DMKU-RK9		34.7	4.1
		DMKU-RK245		29.1	3.6
		DMKU-RK312		0.0	0.0
		DMKU-RK313		0.0	0.0
		DMKU-RK319		0.0	0.0
		DMKU-RK476		0.0	0.0
		DMKU-RK479		0.0	0.0
		DMKU-RK553		64.3	7.0
			DMKU-RK26	65.6	4.9
			DMKU-RK196	95.2	7.0
			DMKU-RK200	0.0	0.0
			DMKU-RK210	67.8	6.0
			DMKU-RK214	39.2	3.8
			DMKU-RK256	49.4	4.4
			DMKU-RK261	0.0	0.0
			DMKU-RK286	28.8	2.4
			DMKU-RK390	0.0	0.0
			DMKU-RK399	0.0	0.0
			DMKU-RK419	0.0	0.0
			DMKU-RK421	0.0	0.0
			DMKU-RK425	2.5	0.2
			DMKU-RK447	0.0	0.0
			DMKU-RK491	0.0	0.0
Candida xylopsoci			DMKU-RK209	0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
Clavispora lusitaniae	DMKU-RK484			0.0	0.0
Cyberlindnera fabianii	DMKU-RK32			0.0	0.0
	DMKU-RK35			0.0	0.0
	DMKU-RK38			0.0	0.0
	DMKU-RK40			0.0	0.0
	DMKU-RK42			0.0	0.0
	DMKU-RK56			0.0	0.0
	DMKU-RK59			0.0	0.0
	DMKU-RK63			0.0	0.0
	DMKU-RK84			0.0	0.0
	DMKU-RK95			0.0	0.0
	DMKU-RK108			0.0	0.0
	DMKU-RK110			0.0	0.0
	DMKU-RK114			0.0	0.0
	DMKU-RK118			7.7	0.7
	DMKU-RK122			0.0	0.0
	DMKU-RK124			11.2	0.8
	DMKU-RK127			0.0	0.0
	DMKU-RK132			0.0	0.0
	DMKU-RK134			0.0	0.0
			DMKU-RK208	6.9	0.8
			DMKU-RK413	0.0	0.0
			DMKU-RK424	0.0	0.0
			DMKU-RK451	0.0	0.0
			DMKU-RK487	0.0	0.0
Cyberlindnera rhodanensis	DMKU-RK144			0.0	0.0
	DMKU-RK151			0.0	0.0
	DMKU-RK463			0.0	0.0
	DMKU-RK481			0.0	0.0
	DMKU-RK482			0.0	0.0
		DMKU-RK529		0.0	0.0
			DMKU-RK349	0.0	0.0
			DMKU-RK428	0.0	0.0
			DMKU-RK452	0.0	0.0
			DMKU-RK466	0.0	0.0
			DMKU-RK489	0.0	0.0
			DMKU-RK492	0.0	0.0
			DMKU-RK493	0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
			DMKU-RK501	0.0	0.0
Debaryomyces nepalensis	DMKU-RK71			0.0	0.0
	DMKU-RK78			0.0	0.0
	DMKU-RK142			0.0	0.0
	DMKU-RK153			0.0	0.0
	DMKU-RK156			0.0	0.0
	DMKU-RK158			0.0	0.0
	DMKU-RK280			0.0	0.0
			DMKU-RK176	47.6	4.8
Geotrichum phurueaensis		DMKU-RK310		0.0	0.0
Hanseniaspora guilliermondii		DMKU-RK525		5.8	1.1
		DMKU-RK549		65.9	13.5
			DMKU-RK497	0.0	0.0
Hanseniaspora opuntiae		DMKU-RK327		0.0	0.0
		DMKU-RK520		2.2	1.0
		DMKU-RK521		7.5	2.1
		DMKU-RK541		78.3	17.5
		DMKU-RK543		50.3	11.4
		DMKU-RK546		0.0	0.0
		DMKU-RK551		106.9	24.4
		DMKU-RK555		81.6	17.9
Hanseniaspora thailandica		DMKU-RK532		18.8	7.3
		DMKU-RK534		22.3	6.3
Kluyveromyces marxianus		DMKU-RK371		0.0	0.0
			DMKU-RK458	0.0	0.0
Kodamaea ohmeri	DMKU-RK67			53.8	7.2
	DMKU-RK79			0.0	0.0
		DMKU-RK308		0.0	0.0
		DMKU-RK317		0.0	0.0
		DMKU-RK328		0.0	0.0
		DMKU-RK477		0.0	0.0
		DMKU-RK533		13.6	1.8
		DMKU-RK536		13.6	1.7
		DMKU-RK550		91.1	11.0
		DMKU-RK552		110.2	13.2
		DMKU-RK554		98.2	13.6
		DMKU-RK556		66.3	7.8

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
Lachancea thermotolerans	DMKU-RK275			0.0	0.0
			DMKU-RK361	0.0	0.0
Lodderomyces elongisporus			DMKU-RK440	105.7	8.4
Metschnikowia koreensis	DMKU-RK61			0.0	0.0
	DMKU-RK70			0.0	0.0
	DMKU-RK91			0.0	0.0
			DMKU-RK66	58.5	6.1
			DMKU-RK382	0.0	0.0
			DMKU-RK431	0.0	0.0
Meyerozyma caribbica	DMKU-RK237			18.3	2.3
	DMKU-RK240			0.0	0.0
	DMKU-RK273			0.0	0.0
	DMKU-RK279			0.0	0.0
	DMKU-RK281			0.0	0.0
	DMKU-RK282			0.0	0.0
	DMKU-RK461			0.0	0.0
		DMKU-RK6		104.1	13.9
		DMKU-RK8		48.6	7.4
		DMKU-RK12		34.5	5.8
		DMKU-RK268		0.0	0.0
		DMKU-RK331		0.0	0.0
		DMKU-RK333		0.0	0.0
		DMKU-RK369		0.0	0.0
		DMKU-RK378		0.0	0.0
		DMKU-RK510		12.9	1.3
		DMKU-RK514		0.0	0.0
			DMKU-RK27	68.9	5.8
			DMKU-RK173	67.9	5.4
			DMKU-RK183	75.9	8.4
			DMKU-RK184	97.2	11.3
			DMKU-RK186	0.0	0.0
			DMKU-RK187	82.3	7.7
			DMKU-RK190	64.8	6.1
			DMKU-RK199	39.2	4.6
			DMKU-RK201	0.0	0.0
			DMKU-RK203	131.8	16.6
			DMKU-RK212	48.7	5.5
			DMKU-RK257	29.7	3.2

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
			DMKU-RK259	0.0	0.0
			DMKU-RK287	72.9	7.5
			DMKU-RK288	0.0	0.0
			DMKU-RK295	50.6	5.7
			DMKU-RK303	0.0	0.0
			DMKU-RK340	0.0	0.0
			DMKU-RK344	5.7	0.6
			DMKU-RK346	0.0	0.0
			DMKU-RK347	24.2	10.5
			DMKU-RK351	0.0	0.0
			DMKU-RK352	0.0	0.0
			DMKU-RK366	0.0	0.0
			DMKU-RK385	0.0	0.0
			DMKU-RK391	5.9	0.6
			DMKU-RK398	0.0	0.0
			DMKU-RK401	36.4	4.8
			DMKU-RK409	0.0	0.0
			DMKU-RK410	15.1	1.6
			DMKU-RK415	0.0	0.0
			DMKU-RK417	8.5	1.1
			DMKU-RK426	7.5	1.1
			DMKU-RK448	0.0	0.0
			DMKU-RK496	0.0	0.0
			DMKU-RK499	6.4	0.7
Meyerozyma guilliermondii	DMKU-RK149			0.0	0.0
	DMKU-RK216			0.0	0.0
	DMKU-RK221			0.0	0.0
	DMKU-RK228			48.0	5.9
		DMKU-RK311		130.2	23.5
		DMKU-RK375		10.3	1.1
		DMKU-RK526		28.4	3.7
Millerozyma acaciae		DMKU-RK379		0.0	0.0
Millerozyma koratensis	DMKU-RK238			0.0	0.0
	DMKU-RK241			0.0	0.0
	DMKU-RK272			0.0	0.0
		DMKU-RK265		0.0	0.0
			DMKU-RK297	0.0	0.0
			DMKU-RK412	0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
			DMKU-RK418	0.0	0.0
			DMKU-RK442	0.0	0.0
Pichia galeiformis		DMKU-RK473		0.0	0.0
		DMKU-RK542		62.4	7.4
Pichia kluyveri		DMKU-RK531		10.1	1.8
Pichia kudriavzevii	DMKU-RK105			0.0	0.0
	DMKU-RK133			0.0	0.0
	DMKU-RK135			0.0	0.0
	DMKU-RK140			0.0	0.0
	DMKU-RK283			0.0	0.0
	DMKU-RK460			0.0	0.0
		DMKU-RK304		0.0	0.0
		DMKU-RK320		0.0	0.0
		DMKU-RK474		0.0	0.0
		DMKU-RK475		0.0	0.0
		DMKU-RK480		0.0	0.0
		DMKU-RK522		0.0	0.0
		DMKU-RK530		14.1	2.0
		DMKU-RK535		0.0	0.0
			DMKU-RK202	24.2	2.4
			DMKU-RK394	0.0	0.0
			DMKU-RK490	0.0	0.0
Torulaspora delbrueckii			DMKU-RK193	69.6	6.1
Torulaspora globosa		DMKU-RK547		0.0	0.0
Wickerhamomyces ciferrii	DMKU-RK30			0.0	0.0
	DMKU-RK136			0.0	0.0
Wickerhamomyces edaphicus		DMKU-RK326		0.0	0.0
		DMKU-RK512		0.0	0.0
			DMKU-RK411	0.0	0.0
Yamadazyma mexicanum	DMKU-RK465			0.0	0.0
Yamadazyma philogaea		DMKU-RK544		115.9	12.7
Yarrowia lipolytica	DMKU-RK82			0.0	0.0
Phylum Basidiomycota					
Cryptococcus flavescens	DMKU-RK99			0.0	0.0
	DMKU-RK129			0.0	0.0
	DMKU-RK141			0.0	0.0
		DMKU-RK10		10.0	2.5

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
		DMKU-RK13		5.4	1.4
		DMKU-RK18		12.8	4.7
		DMKU-RK23		0.0	0.0
		DMKU-RK381		0.0	0.0
			DMKU-RK197	17.5	3.0
			DMKU-RK204	76.4	10.3
Cryptococcus heveanensis		DMKU-RK519		0.0	0.0
			DMKU-RK293	0.0	0.0
			DMKU-RK302	0.0	0.0
Cryptococcus aff. *laurentii*	DMKU-RK169			0.0	0.0
	DMKU-RK171			0.0	0.0
	DMKU-RK230			0.0	0.0
		DMKU-RK243		18.8	5.6
		DMKU-RK373		0.0	0.0
Cryptococcus laurentii	DMKU-RK76			0.0	0.0
		DMKU-RK267		0.0	0.0
		DMKU-RK335		0.0	0.0
		DMKU-RK337		0.0	0.0
		DMKU-RK402		56.6	8.5
		DMKU-RK405		0.0	0.0
		DMKU-RK503		0.0	0.0
		DMKU-RK513		0.0	0.0
			DMKU-RK192	50.0	4.1
			DMKU-RK248	0.0	0.0
			DMKU-RK345	0.0	0.0
			DMKU-RK350	0.0	0.0
			DMKU-RK355	0.0	0.0
			DMKU-RK357	31.5	13.7
			DMKU-RK449	0.0	0.0
			DMKU-RK459	0.0	0.0
			DMKU-RK469	0.0	0.0
Cryptococcus rajasthanensis	DMKU-RK54			0.0	0.0
	DMKU-RK167			0.0	0.0
	DMKU-RK224			0.0	0.0
			DMKU-RK175	57.7	6.2
			DMKU-RK255	0.0	0.0
Hannaella aff. *coprosmaensis*			DMKU-RK250	0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
Pseudozyma antarctica	DMKU-RK112			0.0	0.0
Pseudozyma aphidis	DMKU-RK86			12.4	1.4
Rhodosporidium fluviale		DMKU-RK306		26.0	4.8
		DMKU-RK380		13.2	1.8
		DMKU-RK404		37.1	5.1
		DMKU-RK406		105.4	15.4
		DMKU-RK472		115.5	16.1
		DMKU-RK537		223.0	40.7
		DMKU-RK539		217.3	25.9
		DMKU-RK545		198.3	27.5
			DMKU-RK253	417.8	66.8
			DMKU-RK362	10.4	3.7
			DMKU-RK416	146.0	17.9
			DMKU-RK422	259.1	28.0
			DMKU-RK498	269.1	37.4
Rhodosporidium paludigenum		DMKU-RK266		144.0	27.9
		DMKU-RK509		56.8	6.5
		DMKU-RK528		186.2	22.0
			DMKU-RK342	144.6	14.4
Rhodosporidium toruloides		DMKU-RK334		153.0	21.7
		DMKU-RK336		125.0	19.8
Rhodotorula mucilaginosa			DMKU-RK294	0.0	0.0
Rhodotorula sesimbrana			DMKU-RK494	0.0	0.0
Rhodotorula taiwanensis	DMKU-RK33			0.0	0.0
	DMKU-RK44			0.0	0.0
	DMKU-RK47			0.0	0.0
	DMKU-RK49			0.0	0.0
	DMKU-RK52			0.0	0.0
	DMKU-RK58			0.0	0.0
	DMKU-RK64			3.6	0.4
	DMKU-RK73			18.1	2.1
	DMKU-RK75			0.0	0.0
	DMKU-RK80			0.0	0.0
	DMKU-RK85			6.1	0.7
	DMKU-RK90			0.0	0.0
	DMKU-RK92			0.0	0.0
	DMKU-RK98			19.1	2.8
	DMKU-RK100			0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
	DMKU-RK103			0.0	0.0
	DMKU-RK106			0.0	0.0
	DMKU-RK107			0.0	0.0
	DMKU-RK109			0.0	0.0
	DMKU-RK113			14.7	14.7
	DMKU-RK117			0.0	0.0
	DMKU-RK128			0.0	0.0
	DMKU-RK146			0.0	0.0
	DMKU-RK148			0.0	0.0
	DMKU-RK150			0.0	0.0
	DMKU-RK155			0.0	0.0
	DMKU-RK157			0.0	0.0
	DMKU-RK168			0.0	0.0
	DMKU-RK172			0.0	0.0
	DMKU-RK215			0.0	0.0
	DMKU-RK227			0.0	0.0
	DMKU-RK229			0.0	0.0
	DMKU-RK231			0.0	0.0
	DMKU-RK274			0.0	0.0
	DMKU-RK435			0.0	0.0
	DMKU-RK437			0.0	0.0
		DMKU-RK242		90.4	17.0
		DMKU-RK264		0.0	0.0
		DMKU-RK315		0.0	0.0
		DMKU-RK321		9.6	1.8
		DMKU-RK323		0.0	0.0
		DMKU-RK325		36.4	7.3
		DMKU-RK330		0.0	0.0
		DMKU-RK332		0.0	0.0
		DMKU-RK338		10.5	1.9
		DMKU-RK372		11.5	1.7
			DMKU-RK65	53.5	5.2
			DMKU-RK174	0.0	0.0
			DMKU-RK180	52.1	6.7
			DMKU-RK181	50.0	5.7
			DMKU-RK182	61.8	7.7
			DMKU-RK185	65.2	8.0
			DMKU-RK188	0.0	0.0

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
			DMKU-RK189	42.5	10.0
			DMKU-RK191	79.4	7.4
			DMKU-RK211	16.7	2.2
			DMKU-RK247	137.1	19.0
			DMKU-RK262	0.0	0.0
			DMKU-RK292	14.4	2.0
			DMKU-RK294	0.0	0.0
			DMKU-RK300	0.0	0.0
			DMKU-RK427	0.0	0.0
			DMKU-RK443	0.0	0.0
			DMKU-RK454	0.0	0.0
Sporidiobolus pararoseus		DMKU-RK511		0.0	0.0
Sporidiobolus ruineniae		DMKU-RK368		0.0	0.0
			DMKU-RK383	21.5	3.6
			DMKU-RK392	11.0	1.4
Sporobolomyces blumeae	DMKU-RK31			0.0	0.0
	DMKU-RK39			0.0	0.0
Sporobolomyces carnicolor			DMKU-RK249	0.0	0.0
Sporobolomyces nylandii		DMKU-RK263		3.3	0.8
		DMKU-RK307		0.0	0.0
			DMKU-RK207	10.6	1.6
			DMKU-RK360	91.2	13.3
			DMKU-RK400	0.0	0.0
Trichosporon asahii	DMKU-RK51			0.0	0.0
	DMKU-RK69			0.0	0.0
	DMKU-RK72			0.0	0.0
	DMKU-RK77			0.0	0.0
	DMKU-RK102			0.0	0.0
	DMKU-RK130			0.0	0.0
Undescribed species					
Similar to *Candida* sp. 05-7-186T		DMKU-RK7		1.9	0.3
		DMKU-RK21		1.6	0.2
			DMKU-RK206	0.0	0.0
			DMKU-RK470	0.0	0.0
Similar to *Candida* sp. BG01-8-20-001B-C1			DMKU-RK15	106.7	11.8

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Table 25 (Continued)

Species	Strain from rice leaves	Strain form corn leaves	Strain form sugarcane leaves	IAA (mg/L)	IAA (mg/g dry cell weight)
Similar to			DMKU-RK354	0.0	0.0
Rhodosporidium			DMKU-RK356	0.0	0.0
sp. APSS 849			DMKU-RK384	8.7	1.0
New species					
Metschnikowia lopburiensis	DMKU-RK277			0.0	0.0
Metschnikowia saccharicola			DMKU-RK16	0.5	0.1
			DMKU-RK24	0.0	0.0
			DMKU-RK198	14.4	2.2
			DMKU-RK500	0.0	0.0
Nakazawaea siamensis			DMKU-RK467	0.0	0.0
Wickerhamomyces siamensis			DMKU-RK359	0.0	0.0
Yamadazyma phyllophila		DMKU-RK548		0.0	0.0
Yamadazyma siamensis			DMKU-RK254	0.0	0.0

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5. Determination of biological control activity against plant pathogenic fungi of phylloplane yeasts

To determine the potential to be biological control yeasts, the antagonism activity of 441 yeast strains, which were isolated from leaves samples, against eight plant pathogenic fungi were tested. The plant pathogenic fungi used in this study were *Fusarium moniliforme*, the causal agent of bakanae diseases of rice; *Helminthosporium oryzae*, the causal agent of brown spot diseases of rice; *Rhizoctonia solani*, the causal agent of sheath blight diseases of rice; *Bipolaris maydis*, the causal agent of southern leaf blight of corn; *Bipolaris zeicola*, the causal agent of northern leaf spot of corn; *Exserohilum turcicum*, the causal agent of northern leaf blight of corn; *Fusarium moniliforme*, the causal agents of stem rot of corn and *Macrophomina phaseolina*, the causal agents of stem rot of corn. Each pathogenic fungus and yeast were grown side by side on PDA and incubated at 25°C for 7 days. The result showed that only one strain namely *Torulaspora globosa* DMKU-RK547 showed antagonistic activity againt three pathogenic fungi of rice.

The efficacy of *T. globosa* (DMKU-RK547) against *F. moniliforme, R. solani* and *H. oryzae* on PDA incubated at 25°C for 7 days were showed in Figure 20, 21 and 22, respectively. The percentage of inhibition of fungal growth by *T. globosa* (DMKU-RK547) against *F. moniliforme, R. solani* and *H. oryzae* were 28.72%, 29.38% and 11.76%, respectively (Table 26).

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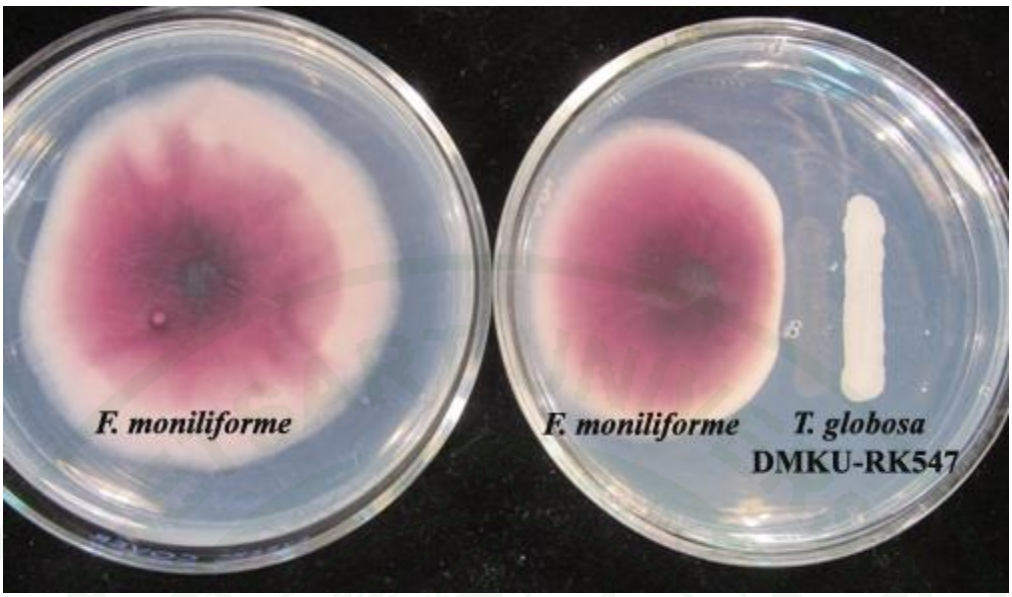


Figure 20 Antagonistic interaction between *Fusarium moniliforme* and *Torulaspora globosa* DMKU-RK547. *F. moniliforme* (control) and dual cultures were grown on PDA at 25°C for 7 days.

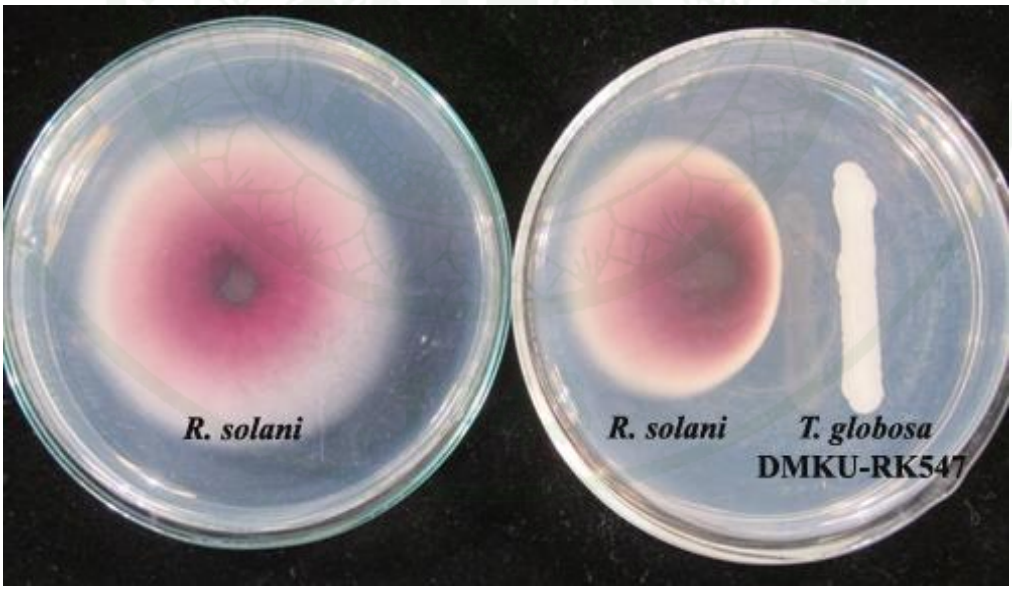


Figure 21 Antagonistic interaction between *Rhizoctonia solani* and *Torulaspora globosa* DMKU-RK547. *R. solani* (control) and dual cultures were grown on PDA at 25°C for 7 days.

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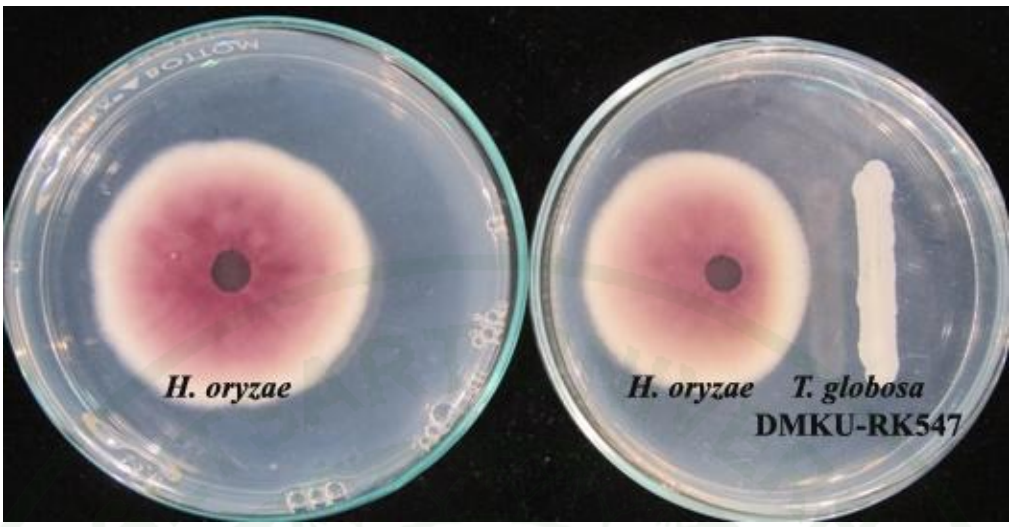


Figure 22 Antagonistic interaction between *Helminthosporium oryzae* and *Torulaspora globosa* DMKU-RK547. *H. oryzae* (control) and dual cultures were grown on PDA at 25°C for 7 days.

Table 26 Diameter of fungal colony grew on PDA medium incubated at 25°C for 7 days and percentage of inhibition of fungal growth by *Torulaspora globosa* DMKU-RK547 against three pathogenic fungi of rice

	Diameter of fungal colony (cm)[a]	% Inhibition[b]
F. moniliforme (Control)	6.50±0.10	-
DMKU-RK547 + *F. moniliforme*	4.63±0.06	28.72
R. solani (Control)	5.90±0.10	-
DMKU-RK547+ *R. solani*	4.17±0.06	29.38
H. oryzae (Control)	5.10±0.10	-
DMKU-RK547+ *H. oryzae*	4.50±0.10	11.76

[a] Diameter of fungal colony grew on PDA medium incubated at 25°C for 7 days was averaged from triplicate. "±" Standard deviation.

[b] % Inhibition = (Diameter of control fungal colony - Diameter of fungal colony grown with yeast) x 100/ Diameter of control fungal colony

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The low number of antagonist isolated from the environment was not a surprise, since many other works reported similar results such as Chanchaichaovivat *et al.* (2007) demonstrated the primary screening of 225 yeast cultures isolated from 11 species of fruits and vegetables by their abilities to resist fungal invasion on plate culture technique resulted in only 54 positive isolates. Secondary screening of these isolates for biocontrol efficacy against *Colletotrichum capsici* growth using the well test showed that only four yeast strains which were identified to be *Pichia guilliermondii*, *Candida musae*, *Pichia kudriavzevii* and *Candida quercitrusa,* were effective in inhibition of *C. capsici* growth. These four yeast strains were tested to confirm their potential in controlling chilli anthracnose caused by *C. capsici*. The result showed that *P. guilliermondii* had the highest biocontrol efficacy, both in vivo and in vitro. Wang *et al.* (2009) screened plant epiphytic yeasts to be used as the biocontrol agents of bacterial fruit blotch (BFB), *Acidovorax avenae* subsp. *citrulli*. Results showed that 24 out of 463 yeast strains isolated from leaves or flowers of plants collected from three provinces in China were antibiotic against *Acidovorax avenae* subsp. *citrulli* on agar medium. Among these eight antagonistic yeast strains including strain 0732-1, which was isolated from watermelon grown in Xinjiang, formed inhibition zones larger than 18 mm in diameter. Spray application of strain 0732-1 was effective in reducing incidence and severity of disease caused by *Acidovorax avenae* subsp. *citrulli* on leaves of hami melon. Based on morphological and physiological characteristics and analysis of the DNA sequence of the internal transcribed spacer region of rRNA gene, the yeast strain 0732-1 was identified as *Pichia anomala*. Rosa *et al.* (2011) reported that the antagonism tests with 317 yeast strains against *Colletotrichum graminicola* and *Colletotrichum sublineolum.* The result showed that only eight isolates (2.5%) were able to inhibit the fungus growth, with six strains belonging to the species *Torulaspora globosa* and two strains were identified to be *Candida intermedia.*

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6. Evaluation of mechanisms for controlling of plant pathogenic fungi of the selected biological control yeast

6.1 Evaluation of antagonism on solid medium

The three pathogenic fungi of rice and yeast were grown side by side on PDA. After incubated at 25°C for 7 days, mycelial samples were removed from different parts of the PDA and studied microscopically at 400X magnification. The result revealed that mycelia of three pathogenic fungi of rice located two positions, close to the yeast cell and far away from the yeast cell, appeared normal (Figure 23, 24 and 25). The result suggested that *T. globosa* DMKU-RK547 did not cause of lethal hyphal deformities whereas Rosa *et al.* (2011) reported that *T. globosa* strain 1S112 inhibited *C. sublineolum* growth in PDA. Deformities of the *C. sublineolum* hyphae were observed when mycelial samples were removed from different parts of the PDA. The hyphae located near the yeast cell appeared wilted, folded and coiled. The median portion of the mycelium growth, which was located between the hyphae near yeast cell and the hyphae far away from yeast cell, showed vesicle formation or granulation along the hyphae and the hyphae far away from the yeast cell appeared normal. This result demonstrated that *T. globosa* strain 1S112 acted on the mycelium but causing non-lethal hyphal deformities, which could affect the hyphal penetration into the host tissues.

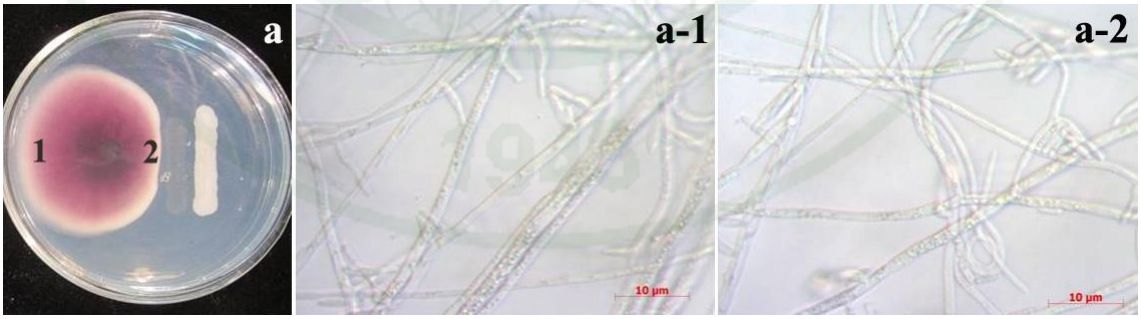


Figure 23 Antagonism by the *Torulaspora globosa* DMKU-RK547 against *Fusarium moniliforme* (a) *F. moniliforme* and the strain DMKU-RK547 inoculated on PDA (1 far away from the yeast cell; 2 close to the yeast cell); (a-1) and (a-2): hyphal morphology seen under optical microscopy from samples taken from different parts in the PDA.

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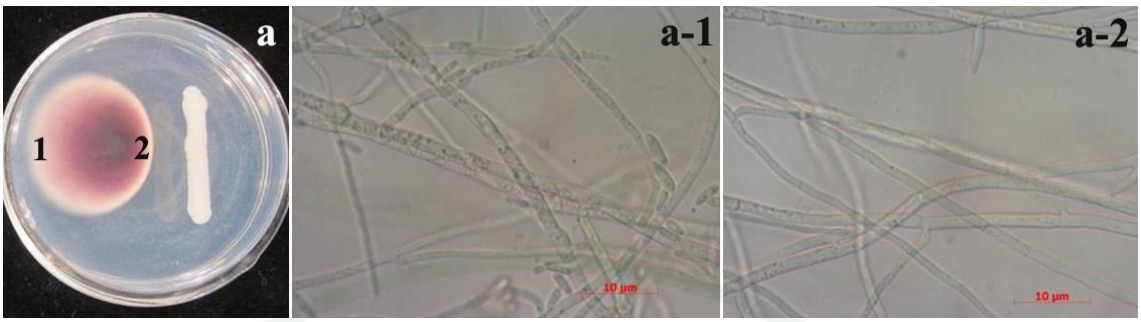


Figure 24 Antagonism by the *Torulaspora globosa* DMKU-RK547 against *Rhizoctonia solani* (a) *R. solani* and the strain DMKU-RK547 inoculated on PDA (1 far away from the yeast cell; 2 close to the yeast cell); (a-1) and (a-2): hyphal morphology seen under optical microscopy from samples taken from different parts in the PDA.

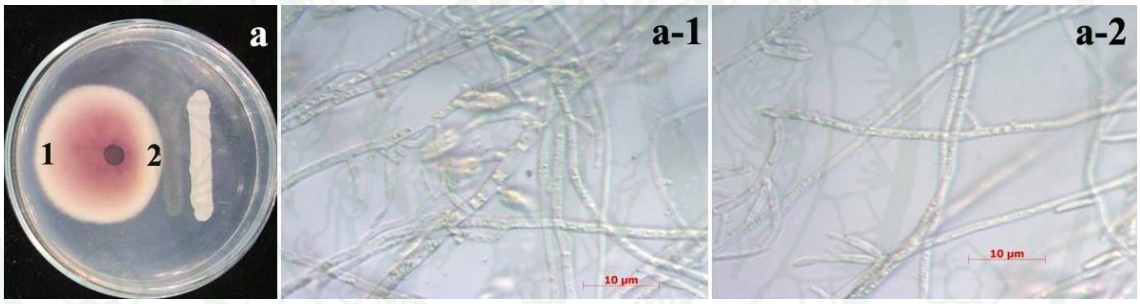


Figure 25 Antagonism by the *Torulaspora globosa* DMKU-RK547 against *Helminthosporium oryzae* (a) *H. oryzae* and the strain DMKU-RK547 inoculated on PDA (1 far away from the yeast cell; 2 close to the yeast cell); (a-1) and (a-2): hyphal morphology seen under optical microscopy from samples taken from different parts in the PDA.

6.2 Production of antifungal volatile compounds

The three pathogenic fungi of rice and yeast were grown side by side on PDA that has partition in the middle. After incubated at 25°C for 7 days, the mycelial growth of three pathogenic fungi of rice were difference between without inoculation and with inoculation with yeast on PDA with a central division that prevented any contact between the fungal and the yeast. The mycelium growth of three pathogenic

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fungi of rice, which was inoculated with yeast were reduce and their fungal colony in dual cultivation was smaller than control (without inoculation with yeast). This indicated *T. globosa* DMKU-RK547 produce the volatile compounds that inhibite the mycelial growth of *F. moniliforme*, *R. solani* and *H. oryzae* (Figure 26, 27 and 28). This result was similar to the previously studies such as Wiater *et al.* (2012) reported that *Candida melibiosica* produced the volatile compounds to against *Botrytis cinerea, Alternaria alternata* and *A. radicina*, pathogenic fungi of carrot roots. Chaurasia *et al.* (2005) studied the efficient antagonistic strain of *Bacillus subtilis* and found that it was the cause of structural deformities in six pathogenic fungi (*Alternaria alternata*, *Cladosporium oxysporum*, *Fusarium oxysporum*, *Paecilomyces lilacinus*, *Paecilomyces variotii*, and *Pythium afertile*) in vitro culture conditions. This effect was attributed to the production of diffusible and volatile antifungal compounds. The bacterial strain successfully restricted the growth of all test fungi in dual cultures, and induced morphological abnormalities such as mycelial and conidial deviations. However, Rosa *et al.* (2010) reported that *T. globosa* strain 1S112 did not produce any volatile compound. The mycelia grown in the absence of yeast and with the inoculated yeast were not different.

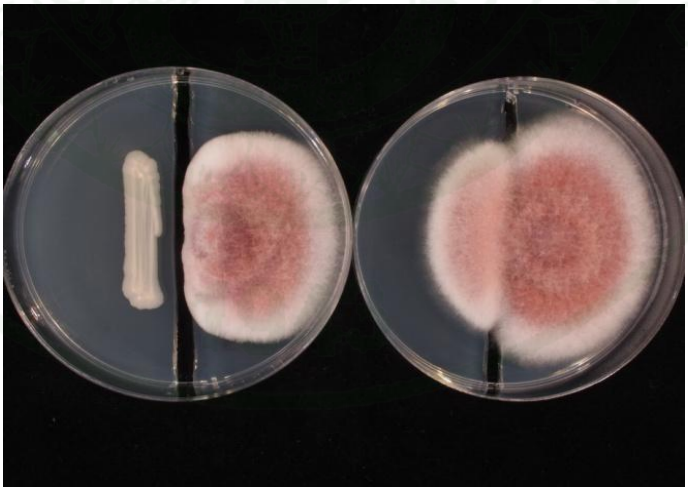


Figure 26 Dual cultures of *Torulaspora globosa* DMKU-RK547 and *Fusarium moniliforme* and sole *F. moniliforme* (control) on PDA with central division at 25°C for 7 days for testing the production of antifungal volatile compounds.

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Figure 27 Dual cultures of *Torulaspora globosa* DMKU-RK547 and *Rhizoctonia solani* and sole *R. solani* (control) on PDA with central division at 25°C for 7 days for testing the production of antifungal volatile compounds.

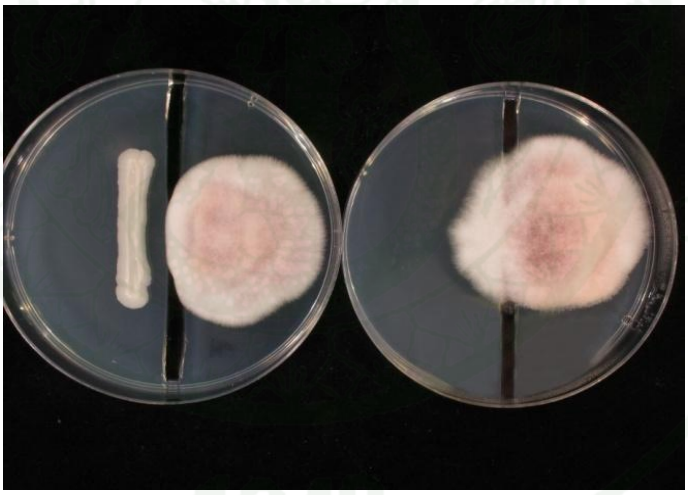


Figure 28 Dual cultures of *Torulaspora globosa* DMKU-RK547 and *Helminthosporium oryzae* and sole *H. oryzae* (control) on PDA with central division at 25°C for 7 days for testing the production of antifungal volatile compounds.

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6.3 Siderophores production

The result of determination of siderophores production by *T. globosa* DMKU-RK547 revealed that this yeast strain grew and changed the color of the CAS-blue medium (Figure 29) suggested that it produced siderophore whereas Rosa *et al.* (2010) reported that *T. globosa* strain 1S112 did not produce siderophore compounds.

The siderophore production was a mechanism involved with the competition for nutrients because the molecules of this substance were capable of stealing the iron (III) ions into the medium, making them unavailable to other microorganisms (Calvente *et al.*, 1999). This mechanism of action may involve in *T. globosa* DMKU-RK547 for using control three rice fungal pathogen.

Moreover, yeast species were able to produce siderophore compounds that inhibit fungal growth such as *Rhodotorula glutinis* strains produced siderophores that improved the biological control of blue rot caused by *Penicillium expansum* in postharvested apples (Calvente *et al.*, 1999). The extracts containing siderophore alone or combined with antagonist microorganisms would be an interesting alternative for control of postharvest diseases including the grey mold pathogen causing by *B. cinerea* (Calvente *et al.*, 2001).

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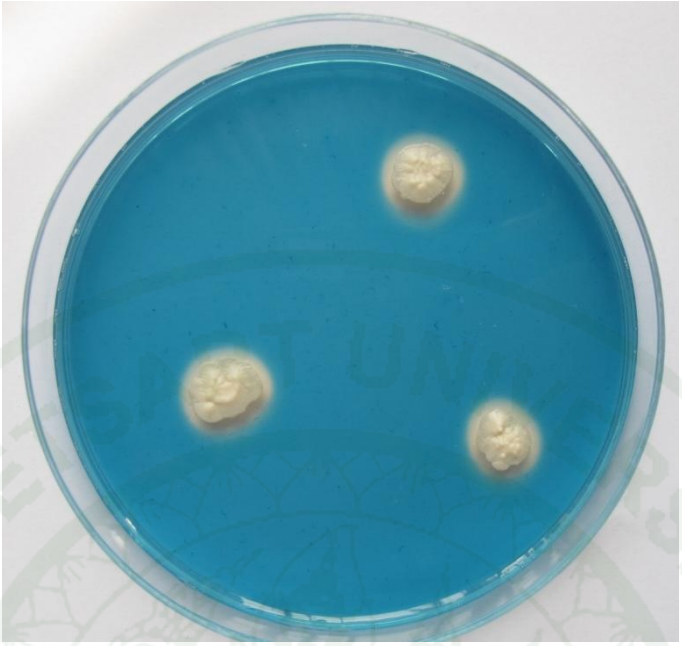


Figure 29 *Torulaspora globosa* DMKU-RK547 showing the color changes around colonies on CAS-blue medium.

6.4 Detection of killer activity

T. globosa DMKU-RK547 was characterized for the production of killer toxin. *Rhodotorula sloffiae* CBS 7095, which was the sensitive strains, was spread over the buffered YPD-methylene blue agar. After drying, *T. globosa* DMKU-RK547 was point inoculated and incubated at 30°C for 5 days. The result showed that on the buffered YPD-methylene blue agar no blue zone surrounding the inhibition halo was not observed indicated that *T. globosa* DMKU-RK547 has no killer activity against *R. sloffiae* CBS 7095. Rosa *et al.* (2011) reported that *T. globosa* strain 1S112 exhibited killer activity against two sensitive yeast strains, *Saccharomyces cerevisiae* NCYC1006 and *Torulopsis glabrata* ATCC 15126, and the killer toxin production was one mechanisms utilized by this yeast strain in the biocontrol. Walker *et al.* (1995) reported the potential ability of killer yeasts to inhibit pathogenic fungi. These killer yeasts were shown to strongly inhibit the vegetative growth of *Heterobasidion annosum*, *Rhizoctonia solani*, *Fusarium equiseti*, and a range of plant pathogenic fungi. Weiler and Schimitt (2003) showed the significant control of the fungus

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Fusarium oxysporum by the yeast *Zygosaccharomyces bailii*. Santos and Marquina (2004) reported that *Pichia membranifaciens* controlled the causal agent of grey mold disease in grapevines (*B. cinerea*) by producing a killer toxin.

6.5 Production of hydrolytic enzymes (β-glucanase and chitinase)

To evaluate β-1,3-glucanase activity of *T. globosa* DMKU-RK547, the colorimetric quantification of glucose (reducing sugars) released from laminarin and chitinase activity of *T. globosa* DMKU-RK547 by estimating the release of N-acetylglucosamine (NAG) from the substrate colloidal chitin was used. The result revealed that *T. globosa* DMKU-RK547 could not produce both β-glucanase and chitinase. This finding is similar to the result reported by Rosa *et al.* (2010) for the incapacity of *T. globosa* 1S112 to produce hydrolytic enzymes, which was one of the modes of action of biocontrol agent.

Some yeast species possess the capacity to produce hydrolytic enzymes, such as β-glucanases and chitinases, that destroy other fungal cell walls (Saligkarias *et al*., 2002). Saravanakumar *et al.* (2009) showed the ability of *Metschnikowia pulcherrima* MACH1 to secrete chitinase in liquid media containing apple juice and the enzyme could be involved in the postharvest biological control of *B. cinerea*. Chanchaichaovivat *et al*. (2008) reported that the secretion of hydrolytic enzymes of *P. guilliermondii* strain R13 was one of the modes of action to suppress chilli anthracnose cause by *C. capsici.* Zhang *et al.* (2010) suggested that the production of β-1,3-glucanases and chitinase were important modes of action of yeast like fungus, *A. pullulans* PL5, to antagonize postharvest pathogens of fruits.

6.6 Effect of nutrient concentration on yeast-fungal pathogen interaction

The effect of nutrient concentration on the interaction of *T. globosa* DMKU-RK547 with *F. moniliforme, R. solani* and *H. oryzae* was studied by using dual cultivation on PDA with different concentrations (39; normal concentration of PDA,

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19.5, 9.7, and 3.9 g/l) and incubated at 25°C for 7 days. The results revealed that *T. globosa* DMKU-RK547 dual cultured with *F. moniliforme, R. solani* and *H. oryzae* on PDA at 39 g/l showed the highest % inhibition of fungal growth by 24.47, 25.51 and 10.87%, respectively, which were significantly different with the other PDA concentrations. When dual cultured with *F. moniliforme* on PDA at 19.5 g/l, *T. globosa* DMKU-RK547 reduced the fungal growth by 13.21% which was significantly different when dual cultivation on PDA at 39.0, 9.7 and 3.9 g/l concentration. The dual cultured with *R. solani* on PDA at 19.5 g/l, *T. globosa* DMKU-RK547 reduced the fungal growth by 19.79 % while it did not inhibite the fungal growth on PDA at 9.7 g/l and 3.9 g/l. When the dual cultured with *H. oryzae* on PDA (19.5, 9.7, and 3.9 g/l), *T. globosa* DMKU-RK547 reduced the fungal growth by 4.55, 2.09 and 1.02%, respectively (Table 27). Therefore, the nutrient concentrations had significant on fungal growth inhibition by *T. globosa* DMKU-RK547.

In the study of Zhang *et al.* (2010), they reported that dual cultured with the pathogens on culture medium with lower nutrient concentrations, the ability of *A. pullulans* PL5 to inhibit the fungal pathogens growth was greatly improved. Their result supported that the competition for nutrients is the role in the activity of *A. pullulans* PL5 against postharvest pathogens.

Our result indicated that nutrient concentration played the role on the biocontrol activities of *T. globosa* DMKU-RK547 to against the three rice pathogens.

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Table 27 Effect of nutrient concentration on the inhibition of growth of *Fusarium moniliforme*, *Rhizoctonia solani* and *Helminthosporium oryzae* by *Torulaspora globosa* DMKU-RK547 by using different concentrations of PDA

Culture medium (PDA) (g/l)	% Inhibition[b]		
	Fusarium moniliforme	*Rhizoctonia solani*	*Helminthosporium oryzae*
39.0[a]	24.47a	25.51a	10.87a
19.5	13.21b	19.79b	4.55b
9.7	4.72c	10.91c	2.09b
3.9	0.00c	8.00c	1.02b

[a] 39 g/l is normal concentration of PDA

[b] % Inhibition = (Diameter of control fungal colony - Diameter of fungal colony grown with yeast) x 100/ Diameter of control fungal colony

Values followed by the same letter in the same column are not significantly different at $p \leq 0.05$ (Duncan's Multiple Range Test, SPSS).

6.7 Effect of yeast cell suspension, culture filtrate and autoclaved yeast cell suspension on pathogic fungal growth

The effect of yeast cell suspension, culture filtrate and autoclaved yeast cell suspension of *T. globosa* DMKU-RK547 with *F. moniliforme, R. solani* and *H. oryzae* were studied on PDA. Aliquots of each suspension were pipetted into each side of the dish and inoculatd with a 5 mm diameter disk of fungal mycelium on another side and incubated at 25°C for 7 days. The results revealed that cell suspension of *T. globosa* DMKU-RK547 showed the highest inhibition on the growth of *F. moniliforme, R. solani* and *H. oryzae* by 31.35, 25.00 and 14.17%, respectively. On the other hand, the culture filtrates and the autoclaved yeast cell suspension of *T. globosa* DMKU-RK547 showed no significant difference of inhibition on the growth of the three pathogens at $p \leq 0.05$ by Duncan's multiple range test. This result

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revealed that living cells of *T. globosa* DMKU-RK547 are necessary for its biocontrol activity. This finding is similar to the result reported by Spadaro *et al.* (2002) who studied the dual cultivation on a synthetic medium of *Metschnikowia pulcherrima* with *Botrytis cinerea*. Their result showed that *B. cinerea* spore germination (10^5 spore/ ml) was completely inhibited by the presence of 10^8 cells of the *M. pulcherrima*, while the culture filtrate and autoclaved suspension were not able to reduce spore germination. Zhang *et al.* (2010) also reported the autoclaved yeast cell and culture filtrate of the antagonist had no effect on the pathogens spore germination or germ tube elongation, suggesting that production of antibiotics were not involved in the modes of action of *A. pullulans* PL5 against the pathogens.

Table 28 Effect of yeast cell suspension, culture filtrate and autoclaved yeast cell suspension of *Torulaspora globosa* DMKU-RK547 on the fungal growth inhibition

Treatment	% Inhibition*
Fusarium moniliforme (Control)	0.00a
Cell suspension + *Fusarium moniliforme*	31.35b
Culture filtrate + *Fusarium moniliforme*	2.09a
Autoclaved yeast cell suspension + *Fusarium moniliforme*	5.08a
Rhizoctonia solani (Control)	0.00a
Cell suspension + *Rhizoctonia solani*	25.00b
Culture filtrate + *Rhizoctonia solani*	0.00a
Autoclaved yeast cell suspension + *Rhizoctonia solani*	0.00a
Helminthosporium oryzae (Control)	0.00a
Cell suspension + *Helminthosporium oryzae*	14.17b
Culture filtrate + *Helminthosporium oryzae*	3.34a
Autoclaved yeast cell suspension + *Helminthosporium oryzae*	5.42a

*% Inhibition = (Diameter of control fungal colony - Diameter of fungal colony grown with yeast) x 100/ Diameter of control fungal colony

Values followed by the same letter in the same column (in each pathogenic fungi) are not significantly different at $p \leq 0.05$ (Duncan's Multiple Range Test, SPSS).

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CONCLUSIONS

1. Yeast isolation:

A total of 244 leaf samples including 85 samples of rice leaves were collected from 14 districts in seven provinces (Ang Thong, Lop Buri, Nakhon Ratchasima, Nakhon Sawan, Phra Nakhon Si Ayutthaya, Pathum Thani and Suphan Buri), 65 samples of corn leaves were collected from 8 districts in four provinces (Lop Buri, Nakhon Ratchasima, Nakhon Sawan and Phetchabun) and 94 samples of sugarcane leaves were collected from 15 districts in seven provinces (Ang Thong, Lop Buri, Nakhon Ratchasima, Nakhon Sawan, Phra Nakhon Si Ayutthaya, Suphan Buri and Uthai Thani) of Thailand. All 441 yeast strains were isolated by the enrichment technique in yeast extract-malt extract broth supplemented with 0.025% sodium propionate and 0.02% chloramphenicol including 156 yeast strains from rice leaves, 126 yeast strains from corn leaves and 159 yeast strains from sugarcane leaves.

2. Yeast identification:

All yeast strains were identified on the basis of the D1/D2 region of the large subunit rRNA gene sequences analysis and phylogeny. One hundred and fifty six yeast strains from rice leaves were identified as 32 known species in 18 genera (155 strains) belonging to Ascomycota i.e. *Candida, Clavispora, Cyberlindnera, Debaryomyces, Kodamaea, Lachancea, Metschnikowia, Meyerozyma, Millerozyma, Pichia, Wickerhamomyces, Yamadazyma* and *Yarrowia,* and Basidiomycota i.e. *Cryptococcus, Pseudozyma, Rhodotorula, Sporobolomyces* and *Trichosporon* and a new species in *Metschnikowia* clade (1 strain). One hundred and twenty six yeast strains from corn leaves were identified as 39 known species in 17 genera (123 strains) belonging to Ascomycota i.e. *Candida, Cyberlindnera, Geotrichum, Hanseniaspora, Kluyveromyces, Kodamaea, Meyerozyma, Millerozyma, Pichia, Torulaspora, Wickerhamomyces* and *Yamadazyma,* and Basidiomycota i.e. *Cryptococcus, Rhodosporidium, Rhodotorula, Sporobolomyces* and *Sporidiobolus,*

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similar to *Candida* sp. 05-7-186T which was undescribed species (2 strains) and a new species in *Yamadazyma* clade (1 strain). One hundred and fifty nine yeast strains from sugarcane leaves were identified to be 36 known species in 19 genera (146 strains) belonging to Ascomycota i.e. *Candida, Cyberlindnera, Debaryomyces, Hanseniaspora, Kluyveromyces, Lachancea, Lodderomyces, Metschnikowia, Meyerozyma, Millerozyma, Pichia, Torulaspora* and *Wickerhamomyces*, and Basidiomycota i.e. *Hannaella, Cryptococcus, Rhodosporidium, Rhodotorula, Sporidiobolus* and *Sporobolomyces*, similar to *Candida* sp. BG01-8-20-001B-C1 (1 strains), similar to *Candida* sp. 05-7-186T (2 strains), similar to *Rhodosporidium* sp. APSS 849 (3 strains) and four new species in *Metschnikowia* clade (4 strains), *Yamadazyma* clade (1 strain), *Wickerhamomyces* clade (1 strain) and *Nakazawaea* clade (1 strains).

3. Yeast biodiversity on phylloplane:

The dominant yeast species from rice phylloplane was *Rhodotorula taiwanensis*, although only 36 strains of this species were isolated from 36 samples, which was calculated to be a frequency of isolation as 23.0%, the dominant yeast species from corn phylloplane was *Kodamaea ohmeri*, *Meyerozyma caribbica* and *Rhodotorula taiwanensis,* although only 10 strains of each species were isolated from 30 samples, represented a 7.9% frequency of isolation of each species and the dominant yeast species from sugarcane phylloplane was *Meyerozyma caribbica*, although only 36 strains of this species were isolated from 36 samples, represented a 22.6% frequency of isolation. Individual leaf sample was generally accommodated by two yeast species (56.5, 60.0 and 48.9% of total sample of rice, corn and sugarcane phylloplane, respectively) while one species was presented in 30.5, 23.1 and 41.5%, three species was presented in 11.8, 16.9 and 8.5% of total sample of rice, corn and sugarcane phylloplane, respectively, and only two samples (1.2 and 1.1% of total sample of rice and sugarcane phylloplane, respectively) and no sample of corn leaf contained up to four species.

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4. Description of new yeast species:

On the basis of polyplasic taxonomy four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) from phylloplane of sugarcane were described as *Metschnikowia saccharicola* sp. nov., strain DMKU-RK277^T from phylloplane of rice was described as *Metschnikowia lopburiensis* sp. nov., strain DMKU-RK254^T from phylloplane of sugarcane was described as *Yamadazyma siamensis* sp. nov., strain DMKU-RK548^T from phylloplane of corn was described as *Yamadazyma phyllophila* sp. nov., strain DMKU-RK359^T from phylloplane of sugarcane was described as *Wicherhamomyces siamensis* sp. nov. and strain DMKU-RK467^T from phylloplane of sugarcane was described as *Nakazawaea siamensis* sp. nov.

5. Indole-3-acetic acid producing capacity of phylloplane yeasts:

Among the 441 strains of yeast, 148 strains in 40 species, four strains of an undescribed species and two strains of a novel species showed the capability to produce IAA cultivated in yeast extract peptone dextrose broth supplemented with 0.1%
L-tryptophan. *Rhodosporidium fluviale* DMKU-RK253 produced the highest IAA concentration of 417.8 mg/L. Among the strains of the phylum Ascomycota 93 strains in 29 species produced IAA in the range of 2.2-259.0 mg/L. Twelve ascomycete yeast strains produced IAA higher than 100 mg/L. They were *C. michaelii* DMKU-RK301 (259.0 mg/L), *C. sojae* DMKU-RK538 (161.3 mg/L), *C. ruelliae* DMKU-RK434 (131.9 mg/L), *M. caribbica* DMKU-RK203 (131.8 mg/L), *M. guilliermondii* DMKU-RK311 (130.2 mg/L), *C. orthopsilosis* DMKU-RK28 (129.2 mg/L), *C. mesorugisa* DMKU-RK179 (118.0 mg/L), *Y. philogeae* DMKU-RK544 (115.9 mg/L), *K. ohmeri* DMKU-RK552 (110.2 mg/L), *H. opuntiae* DMKU-RK551 (106.9 mg/L), *L. elongisporus* DMKU-RK440 (105.7 mg/L) and *M. caribbica* DMKU-RK6 (104.1 mg/L). Fifty-five strains in 11 species of the phylum Basidiomycota produced IAA in the range of 3.3-417.8 mg/L. Fifteen basidiomycete yeast strains produced IAA higher than 100 mg/L. They were nine strains of *R. fluviale* including DMKU-RK253 (417.8 mg/L), DMKU-RK498 (269.1 mg/L), DMKU-RK422 (259.1 mg/L), DMKU-RK537

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(223.0 mg/L), DMKU-RK539 (217.3 mg/L), DMKU-RK545 (198.3 mg/L), DMKU-RK416 (146 mg/L), DMKU-RK472 (115.5 mg/L) and DMKU-RK406 (105.4 mg/L); three strains of *R. paludigenum* including DMKU-RK528 (186.2 mg/L), DMKU-RK342 (144.6 mg/L) and DMKU-RK266 (144.0 mg/L); two strains of *R. toruloides* namely DMKU-RK334 (153.0 mg/L) and DMKU-RK336 (125.0 mg/L); and *R. taiwanensis* DMKU-RK247 (137.1 mg/L). Investigation of the IAA production capability of yeast from the three crops phylloplane in this study revealed that approximately 34.9 % of investigated yeast strains possessed this ability.

6. Biological control capacity of phylloplane yeast:

All 441 yeast strains were determined for their biological control activity against plant pathogenic fungi. The result showed that only one strain namely *Torulaspora globosa* DMKU-RK547 significantly against *F. moniliforme, R. solani* and *H. oryzae*, which were pathogenic fungi of rice, but did not cause of lethal hyphal deformities. The percentage of inhibition of fungal growth by *T. globosa* (DMKU-RK547) against *F. moniliforme, R. solani* and *H. oryzae* were 28.72%, 29.38% and 11.76%, respectively. *T. globosa* DMKU-RK547 produce the volatile and siderophore compounds but did not produce killer toxin and hydrolytic enzymes. Therefore, its biocontrol activity seem to be based on production of volatile and siderophore compounds. The nutrient concentrations had significant effect on fungal growth inhibition by *T. globosa* DMKU-RK547. It dual cultured with *F. moniliforme, R. solani* and *H. oryzae* on PDA at the concentration of 39 g/l showed the highest inhibition of fungal growth by 24.47, 25.51 and 10.87%, respectively. The cell suspension of *T. globosa* DMKU-RK547 showed the highest inhibition on the growth of *F. moniliforme, R. solani* and *H. oryzae* by 31.35, 25.00 and 14.17%, respectively. On the other hand, the culture filtrates and the autoclaved yeast cell suspension of *T. globosa* DMKU-RK547 showed no significant difference of inhibition on the growth of the three pathogens at $p \leq 0.05$ by Duncan's multiple range test. This result revealed that living cells of *T. globosa* DMKU-RK547 are necessary for its biocontrol activity.

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APPENDICES

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Appendix A

Media

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MEDIA

1. Acetate–GSH agar

Potassium acetate	15.0 g
Glucose	0.2 g
Yeast extract	0.1 g
Glutathione	10.0 mM
Agar	15.0g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15 min

2. Acid formation from glucose

Glucose	50.0 g
Calcium carbonate	5.0 g
Yeast extract	5.0 g
Agar	20.0 g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15min

Tubes was cooled to around 45°C and agitated gently to resuspend the chalk and then slanted or pored to plate.

3. Carbon assimilation medium

Ten fold of stock carbon solution (0.2 ml) was added to 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

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4. Christensen's urea agar

1) 20 % urea

Urea	20.0 g
Reverse osmosis water	100 ml

Sterilized by filtration and stored in a freezer at -20°C until use

2) Basal medium

Glucose	1.0 g
Peptone	1.0 g
NaCl	2.0 g
KH_2PO_4	2.0 g
Agar	20.0 g
Phenol red	0.012 g
Reverse osmosis water	1000 ml

Adjust pH = 6.8

Sterilized at 121°C for 15min

Amount of 0.5 ml of 20 % urea was added to 16 x 150 mm test tube containing 4.5 ml sterilized basal medium.

5. Cycloheximide resistant

1) Basal medium

Cycloheximide	1 g in acetone 2.5 ml
Bacto Yeast Nitrogen Base (Difco)	6.7 g
Reverse osmosis water	100 ml

Sterilized by filtration

2) Active medium

Amount of 0.2 ml of 10 fold basal medium was added to 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

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6. Fermentation test medium

1) Basal medium

Yeast extract	4.5 g
Peptone	7.5 g
Bromothymol blue	small amount
Reverse osmosis water	1000 ml

Fermentation basal medium (2 ml) was distributed into the cotton plugged test tubes (13x100 mm), insert Durham tube and sterilize at 121°C for 15 min. After cool down, add concentrate sugar, which was sterilized by membrane filtration to make the final concentration at 2% sugar (except 4% raffinose).

7. 5% malt extract agar

Malt extract	50 g
Agar	15 g
Reverse osmosis water	1000 ml

Sterilized at 115°C for 15 min

8. Nitrogen agar plate:

Ninety ml of 1.67 % agar solution was sterilized at 121°C for 15 min and left for cool down to 50-55°C, then 10 ml of stock nitrogen solution (10X) was added, mixed well and poured into the petri dishes.

9. Osmotic medium

1) 50% glucose medium

Glucose	50.0 g
Yeast extract	1.0 g
Agar	1.5 g

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Reverse osmosis water	100 ml
Sterilized at 110°C for 10 min	

2) 60% glucose medium

Glucose	60.0 g
Yeast extract	1.0 g
Agar	1.5 g
Reverse osmosis water	100 ml
Sterilized at 110°C for 10 min	

3) 5% glucose with 10% sodium choloride medium

NaCl	10.0 g
Glucose	5.0 g
Bacto Yeast Nitrogen Base (Difco)	0.67 g
Agar	20.0 g
Reverse osmosis water	100 ml
Sterilized at 121°C for 15min	

4) 5% glucose with 16% sodium choloride medium

NaCl	16.0 g
Glucose	5.0 g
Bacto Yeast Nitrogen Base (Difco)	0.67 g
Agar	20.0 g
Reverse osmosis water	100 ml
Sterilized at 121°C for 15min	

10. Stock carbon solution (10X)

Yeast Nitrogen Base (Difco)	6.7 g
Carbon compound	5.0 g

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Reverse osmosis water 100 ml

Sterilized by filtration and stored in a freezer at -20°C until use

11. Stock nitrogen solution (10X)

Bacto Yeast Carbon Base (Difco) 11.7 g

Nitrogen compound* x g

Reverse osmosis water 100 ml

Sterilized by filtration and stored in a freezer at -20°C until use

* Nitrogen compound: (NH4)2SO4 (0.5 g) or KNO3 (0.78 g) or NaNO2 (0.26 g) or ethylamine-HCl (0.64 g) or L-lysine-HCl (0.56 g) or cadaverine (0.68 g)

12. Vitamin free medium

Glucose 10.0 g

Vitamin-free cassamino acids (Difco) 5.0 g

$KH_2PO_4.7H_2O$ 1 g

$MgSO_4$ 0.5 g

NaCl 0.1 g

$CaCl_2$ 0.1 g

Reverse osmosis water 1000 ml

Adjust pH = 5.5

Sterilized at 121°C for 15min

13. Yeast Carbon Base (YCB) medium:

1) Stock YCB solution (10X)

Bacto Yeast Carbon Base (Difco) 11.7 g

Reverse osmosis water 100 ml

Sterilized by filtration and stored in a freezer at -20°C until use

2) Ten fold of stock YCB solution (0.2 ml) was added to 13x100 mm test tube containing 1.8 ml sterilized distilled water

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14. Yeast Nitrogen Base (YNB) medium

1) Stock YNB solution (10X)

Bacto Yeast Nitrogen Base (Difco)	6.7 g
Reverse osmosis water	100 ml

Sterilized by filtration and stored in a freezer at -20°C until use

2) Ten fold of stock YNB solution (0.2 ml) was added into 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

15. Yeast extract malt extract (YM) agar

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15 min

16. Yeast extract malt extract (YM) broth

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15 min

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17. Yeast extract malt extract (YM) agar plates supplemented with chloramphinical (200 μg/l) and sodium propionate (0.2%)

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Chloramphenical	200 μg
Sodium propionate	2.0 g
Agar	15 g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15 min

18. Yeast extract malt extract (YM) broth supplemented with chloramphinical (200 μg/l) and sodium propionate (0.2%)

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Chloramphenical	200 μg
Sodium propionate	2.0 g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15 min

19. Yeast extract malt extract (YM) supplemented with 10% glycerol

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Glycerol	100 ml

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Reverse osmosis water	900 ml

Sterilized at 121°C for 15 min

20. Yeast Peptone Dextrose (YPD) broth

Yeast extract	10.0 g
Peptone	10.0 g
Glucose	20.0 g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15 min

21. Yeast Peptone Dextrose (YPD) with 0.1% L-Tryptophan

Yeast extract	10.0 g
Peptone	10.0 g
Glucose	20.0 g
L-Tryptophan	1.0 g
Reverse osmosis water	1000 ml

Sterilized at 121°C for 15 min

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Appendix B

Reagents

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REAGENTS

1. Bromthymol blue stock solution

50 mg of bromthymol blue was dissolved in 75 ml of distilled water and stored in a freezer at 4°C until use.

2. Diazonium blue B reagent (DBB reagent)

Diazonium blue B salt	0.15 g
0.25 M Tris hydroxymethyl aminomethane (pH 7.0)	10 ml

The freshly prepared reagent is kept in an ice bath, and used before it turns dark yellow, which is within about 30 min.

3. Loading dye

Bromophenol blue	0.25 g
Glycerol	30.0 ml
Reverse osmosis water	100 ml

4. Lugol's solution

Iodine	1.0 g
Potassium iodide	2.0 g
Reverse osmosis water	300 ml

5. Salkowski reagent

Ferric-3-chloride	1.2 g
Absolute sulfuric acid	79.0 ml
Reverse osmosis water	21.0 ml

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6. Stock ethidium bromide

Ethidium bromide	1.0 g
Reverse osmosis water	100 ml

7. 1 kb DNA ladder (DNA marker)

DNA ladder	1.0 µl
6X loading dye	1.0 µl
Sterilized reverse osmosis water	4.0 µl

8. 2.5 mM dNTP

dATP	25.0 µl
dCTP	25.0 µl
dGTP	25.0 µl
dTTP	25.0 µl
Sterilized reverse osmosis water	900 µl

9. 10X TBE buffer

Tris base	10.8 g
Boric acid	5.5 g
0.5 mM EDTA (pH 8.0)	4.0 ml
Reverse osmosis water	100 ml
Sterilized at 121°C for 15min	

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Appendix C

Publications

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ORIGINAL PAPER

Metschnikowia saccharicola sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand

Rungluk Kaewwichian · Wichien Yongmanitchai · Hiroko Kawasaki · Savitree Limtong

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Abstract Five strains representing two novel anamorphic yeast species were isolated from the external surface of sugarcane leaves (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) and rice leaf (DMKU-RK277^T) by an enrichment technique. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, the sequence analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene and the internal transcribed spacer region, the five strains were assigned to be two novel species of the *Metschnikowia,* although the formation of ascospores was not observed. Four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) where the sequences of the D1/D2 domain of the LSU rRNA gene were identical and differed by 4.1 % nucleotide substitutions (20 nucleotide substitutions and four gaps out of 490 nt) from the type strain of *Candida succicola*, their closest known species in terms of pairwise sequence similarity, represent a single novel species, for which the name *Metschnikowia saccharicola* sp. nov. is proposed. The type strain is DMKU-RK500^T (= BCC 50735^T = NBRC 108904^T = CBS 12575^T). Strain DMKU-RK277^T was closest to *Candida hainanensis* but with 4.0 % nucleotide substitutions (18 nucleotide substitutions and three gaps out of 449 nt) in the D1/D2 domain of the LSU rRNA gene was assigned to be *Metschnikowia lopburiensis* sp. nov. (type strain DMKU-RK277^T = BCC 50731^T = NBRC 108902^T = CBS 12574^T)

Keywords *Metschnikowia saccharicola* sp. nov. · *Metschnikowia lopburiensis* sp. nov. · Phylloplane yeast · Ascomycetous yeast · New yeast species · Thailand

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Introduction

Many yeast species of the genus *Metschnikowia* and a few related *Candida* species are often found associated with a variety of substrates including insects and plants, especially beetles and flowers (Lachance et al. 2011, 2005; Manson et al. 2007; Nguyen et al. 2006). More recently, yeast species of the genus *Candida* in *Metschnikowia* clade were reported to have been collected from flowers, tree sap, gum produced by trees and exudates of *Ficus racemosa* Linn.; these species included *Candida hainanensis*, *Candida heveicola*, *Candida pseudoflosculorum*, *Candida robnettiae*, *Candida eppingiae, Candida danieliae* and *Candida succicola* (Groenewald et al. 2011; Nakase et al. 2011; Wang et al. 2008).

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The external surface of plant leaves is usually referred to as the phylloplane or phyllosphere (Fonseca and Inacio 2006; Phaff and Starmer 1987). Phylloplane from various regions of the world are found to be colonized by members of both basidiomycetous and ascomycetous yeasts (Fonseca and Inacio 2006; Glushakova et al. 2007; Landell et al. 2010; Nakase et al. 2001; Slavikova et al. 2009). Although most common phylloplane yeasts are reported to be basidiomycete species (Fonseca and Inacio 2006; Nakase et al. 2001) such as the genera *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo et al. 1998; Glushakova and Chernov 2010; Slavikova et al. 2009), various ascomycetous yeast species have also been detected on the phylloplane i.e. *Debaryomyces hansenii*, *Hanseniaspora uvarum, Kazachstania barnetii*, *Metschnikowia pulcherrima*, *Pichia membranifaciens*, *Saccharomyces cerevisiae* and various species of *Candida* including *Candida aechmeae*, *Candida olephila*, *Candida chumphonensis, Candida mattranensis* and *Candida vrieseae* (Glushakova et al. 2007; Glushakova and Chernov 2010; Koowadjanakul et al. 2011; Landell et al. 2010; Slavikova et al. 2009).

During an investigation of yeasts on the external surface of sugarcane leaves and rice leaves in Thailand, various known yeast species were found and five strains, including four strains from phylloplane of sugarcane and one strain from phylloplane of rice, were found to represent two novel *Metschnikowia* species. In this paper, the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) are described as *Metschnikowia saccharicola* sp. nov. and strain DMKU-RK277^T is described as *Metschnikowia lopburiensis* sp. nov.

Materials and methods

Sample collection and yeast isolation

Green and undamaged leaves of rice (85 samples) and sugarcane (95 samples) were respectively collected from rice and sugarcane fields in central and northeastern Thailand. Yeasts were isolated from surface of plant leaves by an enrichment technique using acidified yeast extract malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong et al. 2007). Three grams of cut leaves were aseptically placed in 250 ml Erlenmeyer flask, containing 50 ml of acidified YM broth and incubated on a rotary shaker at room temperature (27 ± 3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature until yeast colonies appeared. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

A total of 154 strains were isolated from rice leaf samples and 166 strains were from sugarcane leaf samples. Four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were obtained from four leaf samples and strain DMKU-RK277^T was from a rice leaf sample (Table 1).

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region were determined from PCR products amplified from genomic DNA. Methods for DNA extraction and amplification of the D1/D2 domain of the LSU rRNA gene were described previously (Limtong et al. 2007). The ITS region was amplified with primers, ITS1 and ITS4, following the method of White et al. (1990). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen, Germany). The purified products were submitted to Macrogen Inc. (Korea) for sequencing with primers, NL1 (5′ GCATATCAATAAGCGGAGGA AAAG 3′) and NL4 (5′ GGTCCGTGTTTCAAGACG G 3′), for the D1/D2 domain of the LSU rRNA gene, and with primers, ITS1 (5′ TCCGTAGGTGAACCT GCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGAT ATGC 3′), for the ITS region. The sequences were compared pairwise using a BLAST search (Altschul et al. 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al. 1997). A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura 1980), using the neighbor joining method (Saitou and Nei 1987) by MEGA software version 4.0 (Tamura et al.

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Table 1 Strains of *M. saccharicola* sp. nov. and *M. lopburiensis* sp. nov. isolated from surface of plant leaves used in this study

Strain	Accession number			GenBank accession number		Sample for isolation		
	BCC	NBRC	CBS	D1/D2	ITS	Leaves	Locality	Date
M. saccharicola sp. nov.								
DMKU-RK16	50727	–	–	AB697752	AB697757	Sugarcane 1 (*S. officinarum*)	Sikhio district, Nakhon Ratchasima Province, Thailand	28 Sep. 2009
DMKU-RK24	50728	–	–	AB697753	AB697758	Sugarcane 2 (*S. officinarum*)	Sikhio district, Nakhon Ratchasima Province, Thailand	28 Sep. 2009
DMKU-RK198	5072	–	–	AB697754	AB697759	Sugarcane 3 (*S. officinarum*)	Dan Chang district, Suphan Buri Province, Thailand	22 Mar. 2010
DMKU-RK500^T	50735^T	108904^T	12575^T	AB697755	AB697760	Sugarcane 4 (*Saccharum officinarum*)	Khon Buri district, Nakhon Ratchasima Province, Thailand	6 May 2010
M. lopburiensis sp. nov.								
DMKU-RK277^T	50731^T	108902^T	12574^T	AB697756	AB697761	Rice (*Oryza sativa*)	Ban Mi district, Lop Buri Province, Thailand	5 May 2010

BCC BIOTEC Culture Collection, *NBRC* NITE Biological Resources Center, *CBS* Centraalbureau voor Schimmelcultures

2007). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Felsenstein 1985).

Examination of taxonomic characteristics

The strains were characterized morphologically, biochemically, and physiologically according to the standard methods described by Yarrow (1998). Mycelium formation was investigated on potato dextrose agar (PDA) in slide culture at 25 °C for up to 7 days. Ascospores formation was investigated individually or in pair on 5 % malt extract agar, McClary's acetate agar, Gorodkowa agar, corn meal agar, V8 agar and yeast carbon base supplemented with 0.01 % ammonium sulfate (YCBAS) agar at 15 and 25 °C for up to 4 weeks. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula following the method of Nakase and Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml of yeast extract peptone dextrose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % dextrose) on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada and Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al. 2007). The DNA base composition was determined by HPLC as described by Tamaoka and Komagata (1984) using the DNA-GC kit (Yamasa Shoyu Co., Japan).

Results and discussion

Novel species delineation and identification

Analysis of the D1/D2 domain of the LSU rRNA gene sequence revealed that the sequences of the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were identical and differed by only two nucleotide substitutions from the two strains of undescribed *Candida* species namely strains HN1-1 and HN1-2(a), however, the strains are not available for further comparison. The sequences of these strains showed 4.1 % nucleotide substitutions

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(20 nucleotide substitutions and four gaps out of 490 nt) from the type strain of *C. succicola*, their closest known species, in terms of pairwise sequence similarity. The nucleotide sequences of the ITS regions of the four strains were identical and differed by 12.6 % nucleotide substitutions (37 nucleotide substitutions and one gap out of 294 nt) from *C. succicola*. Strain DMKU-RK277^T was closest to *C. hainanensis* but with 4.0 % nucleotide substitutions (18 nucleotide substitutions and three gaps out of 449 nt) in the D1/D2 domain of the LSU rRNA gene. The sequences of ITS region of strain DMKU-RK277^T and *C. hainanensis* differed by 31.9 % nucleotide substitutions (145 nucleotide substitutions and 51 gaps out of 455 nt).

The phylogenetic tree base on the sequences of the D1/D2 domains of the LSU rRNA gene further demonstrated that all strains were in *Metschnikowia* clade. Four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were in the same position and connected to the two undescribed *Candida* species (strains HN1-1 and HN1-2(a)) that closest to them. They connected to *C. succicola*, the closest known species, and form a subclade with *Metschnikowia lunata* though the bootstrap was low (Fig. 1). Strain DMKU-RK277^T connected to *C. hainanensis*, it closest relative in term of pairwise sequence similarity, with 100 % bootstrap support and was near the subclade containing *Metschnikowia pulcherrima*.

Cells of the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) were globose, subglobose and ovoid (Fig. 2a) and the strain DMKU-RK277^T was ovoid to ellipsoid (Fig. 3). Cells of the five strains proliferated by multilateral budding. Pseudohyphae but not true hyphae were formed by the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) (Fig. 2b). Neither pseudohyphae and true hyphae were formed by the strain DMKU-RK277^T. Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C.

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 domain of the LSU rRNA gene and the ITS region, we concluded that the five strains represent two novel *Metschnikowia* species. The name *M. saccharicola* sp. nov. (MB564869) is proposed for the four strains (DMKU-RK16, DMKU-RK24, DMKU-RK198 and DMKU-RK500^T) and the name *M. lopburiensis* sp. nov. (MB564870) is assigned for the strain DMKU-RK277^T.

Fig. 1 Phylogenetic tree based on the sequences of the D1/D2 domain of the LSU rRNA gene, showing positions of *M. saccharicola* sp. nov. (DMKU-RK500^T) and *M. lopburiensis* sp. nov. (DMKU-RK277^T), with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 4.0. *Numbers* indicate percentages of bootstrap sampling, derived from 1,000 samples. The numbers in *parentheses* are GenBank accession numbers. *Schizosaccharomyces pombe* was the outgroup in the analysis

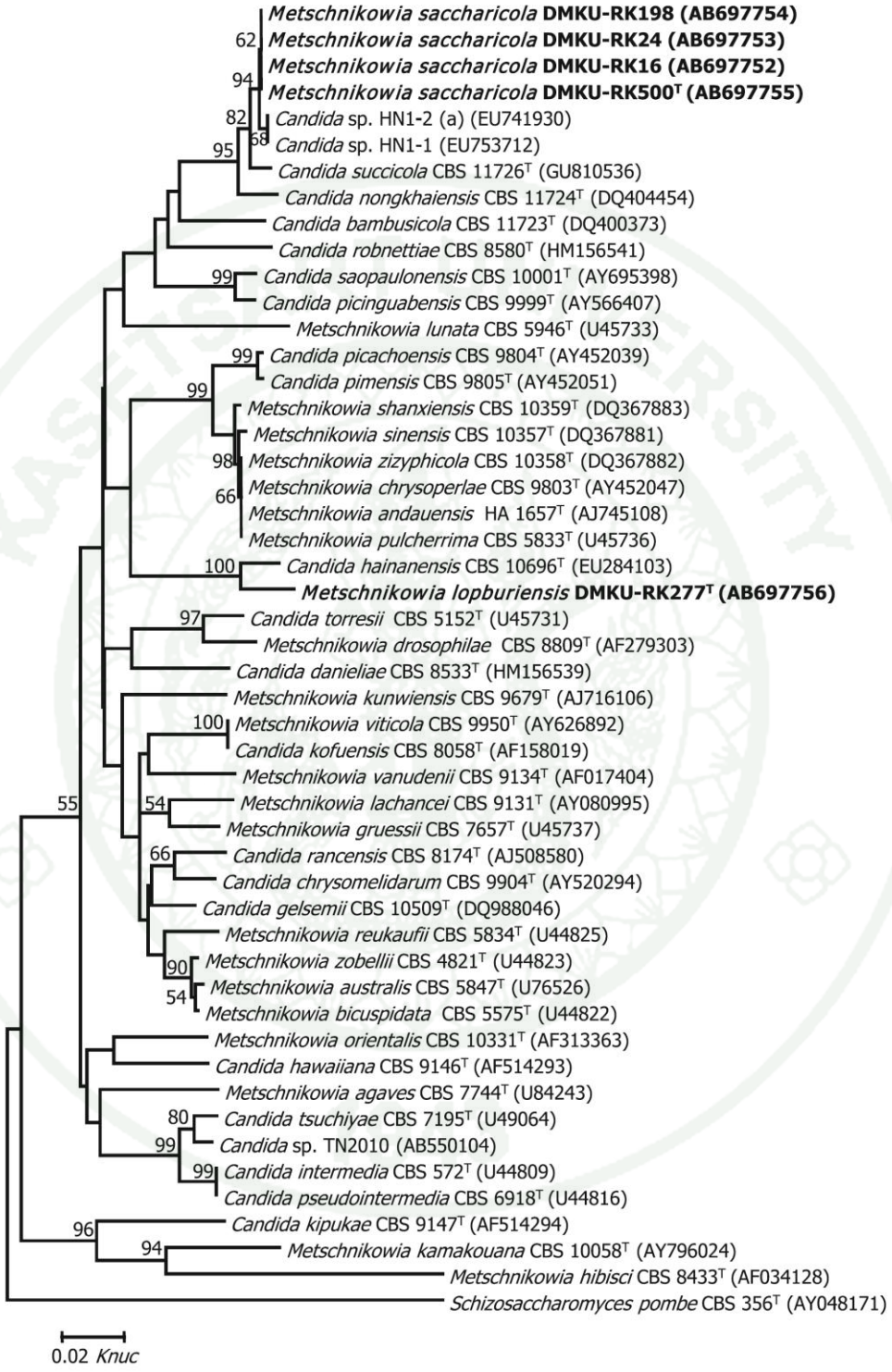
In practice, *M. saccharicola* sp. nov. and *M. lopburiensis* sp. nov. can be distinguished from their closest species *C. succicola* and *C. hainanensis*, respectively, not only on the basis of the sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region but also some phenotypic characteristics as shown in Table 2.

Most of the members of the *Metschnikowia* clade such as *C. nongkhaiensis, C. bambusicola, C. saopaulonensis, C. picinguabensis, C. tsuchiyae, C. intermedia* and *C. pseudointermedia* exhibit an association with insect frass, flowers and mosses (Lachance et al. 2011, Nakase et al. 2011, Ruivo et al. 2006). In the most recent report, the novel *Candida* species in *Metschnikowia* clade, namely *C. succicola,* the species closest to *M. saccharicola* sp. nov., was isolated from exudate of a tree in Thailand (Nakase et al. 2011). *C. hainanensis,* the species closest to *M. lopburiensis* sp. nov., and *C. heveicola* were isolated from flowers and tree sap collected, respectively from tropical forest in Yunnan and Hainan, China (Wang et al. 2008). *C. danieliae*, *C. pseudoflosculorum*, *C. robnettiae* and *C. eppingiae* were reported to isolate from gum produced by trees, and flowers in Thailand and Guyana (Groenewald et al. 2011). Therefore, plants seem to be the habitat of numerous yeast species in the *Metschnikowia* clade.

Description of *M. saccharicola* Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25 °C, cells are globose, subglobose to ovoid (4–7 × 5–8 µm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. Growth on YM

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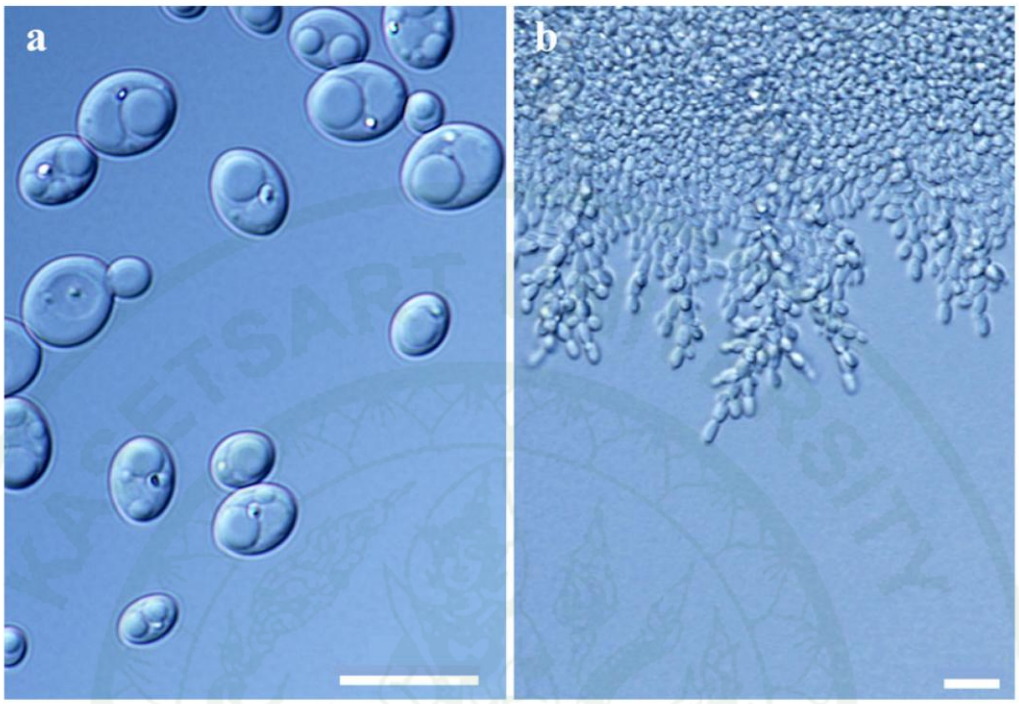


Fig. 2 *Metschnikowia saccharicola* sp. nov. (DMKU-RK500^T) **a** budding cells in YM broth after 3 days at 25 °C and **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar* 10 μm

agar: After 3 days at 25 °C, the streak culture is grayish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on potato dextrose agar (PDA) after 3 days at 25 °C (Fig. 2b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. Fermentation of D-glucose and trehalose are positive but negative for D-galactose, sucrose, maltose, lactose, raffinose, xylose and cellobiose. D-glucose, L-sorbose, sucrose, maltose, trehalose, melezitose, D-xylose, ethanol, ribitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, 2-keto-D-gluconic acid, D-glucono-δ-lactone, succinic acid, citric acid, xylitol, ethylamine, L-lysine and cadaverine are assimilated, but D-galactose, cellobiose, lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, D-arabinose, L-rhamnose, D-ribose, N-acetyl-D-glucosamine, methanol, glycerol, erythritol, galactitol, salicin, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, 5-keto-D-gluconic acid, DL-lactic acid, inositol, D-glucosamine, arbutin, hexeadecane, propane-1,2-diol, butane-2,3-diol, arabinitol, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is positive (weak). Growth on medium containing 50 % (w/v) glucose and 60 % (w/v) glucose are positive, but 10 % (w/v) sodium chloride/5 % (w/v) glucose and 16 % (w/v) sodium chloride/5 % (w/v) glucose are negative. Growth with 0.01 % cylcloheximide is positive but 0.1 % cylcloheximide is negative. Growth at 25, 30 and 35 °C are positive, but at 37, 40, 42 and 45 °C are negative. Acid formation is present. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 41.7 mol %. The major ubiquinone is Q-9.

Holotype

DMKU-RK500^T is the holotype of *M. saccharicola* (MB564869). The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Nakhon Ratchasima province, Thailand. The living culture from type was deposited at the BIOTEC

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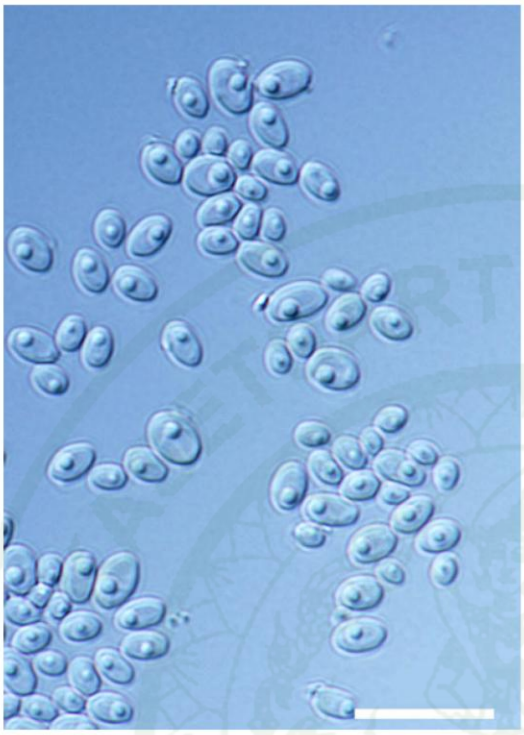


Fig. 3 *Metschnikowia lopburiensis* sp. nov. (DMKU-RK277^T) budding cells in YM broth after 3 days at 25 °C. *Scale bar* 10 µm

Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50735^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108904^T and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12575^T.

Etymology

The species epithet "*saccharicola*" was chosen because this species was isolated from phylloplane of sugarcane, *S. officinarum* collected in Thailand.

Description of *M. lopburiensis* Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov

Growth in yeast extract malt extract (YM) broth: After 3 days at 25 °C, cells are ovoid to ellipsoid (2–3 × 3–5 µm) and occur singly, in pairs or in groups (Fig. 3). Budding is multilateral. Growth on YM agar after 3 days at 25 °C, the streak culture is cream, smooth soft to butyrous and has an entire margin. Pseudohyphae and true hyphae are not formed in slide culture on PDA. Ascospores were not produced on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. Fermentation of D-glucose and trehalose (latent) are positive but negative for D-galactose, sucrose, maltose, lactose, raffinose, xylose and cellobiose. D-glucose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melezitose, D-xylose, D-arabinose (weak), *N*-acetyl-D-glucosamine, ethanol, glycerol (latent), ribitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, D-gluconic acid, D-glucuronic acid, 2-keto-D-gluconic acid, D-glucono-δ-lactone, succinic acid, citric acid, D-glucosamine, arbutin, xylitol, ethylamine, L-lysine and cadaverine are assimilated, but D-galactose, lactose, melibiose, raffinose, inulin, soluble starch, L-arabinose, L-rhamnose, D-ribose, methanol, erythritol, galactitol, D-galacturonic acid, 5-keto-D-gluconic acid, DL-lactic acid, inositol, hexeadecane, propane-1,2-diol, butane-2,3-diol, arabinitol, nitrate and nitrite are non-assimilated. Growth in vitamin free medium is positive (weak). Growth on medium containing 50 % (w/v) glucose, 60 % (w/v) glucose and 10 % (w/v) sodium chloride/5 % (w/v) glucose (weak) are positive, but 16 % (w/v) sodium chloride/5 % (w/v) glucose is negative. Growth with 0.01 % cylcloheximide is positive but 0.1 % cylcloheximide is negative. Growth at 25, 30, 35 and 37 °C are positive, but at 40, 42 and 45 °C are negative. Acid formation is absent. Starch like compounds are not produced. Diazonium blue B color and urease reactions are negative. DNA G+C content is 51.0 mol %. The major ubiquinone is Q-9.

Holotype

DMKU-RK277^T is the holotype of *M. lopburiensis* (MB564870). The strain was isolated from phylloplane of rice (*Oryza sativa*) collected from Lop Buri province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50731^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of

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Table 2 Phenotypic characteristics that differentiate *M. saccharicola* sp. nov. and *M. lopburiensis* sp. nov. from their closest species, *C. succicola* and *C. hainanensis*, respectively

Characteristics	Species			
	M. saccharicola	*C. succicola*[a]	*M. lopburiensis*	*C. hainanensis*[b]
Fermentation				
Sucrose	−	+	−	−
Maltose	−	+	−	−
Assimilation of carbon compounds				
D-Galactose	−	l	−	+
D-Arabinose	−	−	w	−
Glycerol	−	v	l	+
Ribitol	+	l	+	+
D-Glucono-δ-lactone	+	l	+	n
Succinic acid	+	+	+	l
Hexeadecane	−	+	−	−
Arabinitol	−	l	−	n
Other growth characteristics				
Vitamin-free medium	w	w	w	+
Growth at 37 °C	−	+	+	+
Acid formation from glucose	w	−	−	n

+ positive, − negative, *l* delayed positive, *w* weak, *v* variable, *n* no data

[a] Data from Nakase et al. (2011)

[b] Data from Wang et al. (2008)

Technology and Evaluation, Chiba, Japan, as NBRC 108902^T and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 12574^T.

Etymology

The species epithet *lopburiensis* refers to Lop Buri province, Thailand, where the type strain was isolated.

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ORIGINAL PAPER

Yamadazyma siamensis sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov., three novel yeast species isolated from phylloplane in Thailand and Taiwan

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Abstract Four strains representing three novel anamorphic yeast species were isolated from the external surface of sugarcane leaves (DMKU-RK254^T), corn leaves (DMKU-RK548^T), bean leaves (K129) in Thailand and hengchun pencilwood leaves (TrB1-1^T) in Taiwan. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, the sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene, the internal transcribed spacer (ITS) region, the actin gene (ACT1) and the elongation factor 2 gene (EF2), the four strains were determined to represent novel *Yamadazyma* species although formation of ascospores was not observed. Strain DMKU-RK254^T was determined to be related to *Candida diddensiae*, *Candida naeodendra* and *Candida kanchanaburiensis* but with 1.8, 1.8 and 2.0 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene, respectively. It was assigned to *Yamadazyma siamensis* sp. nov. (type strain DMKU-RK254^T = BCC 50730^T = NBRC 108901^T = CBS 12573^T). The sequences of the D1/D2 region of the LSU rRNA gene, the ITS region, ACT1 gene and EF2 gene of two strains (DMKU-RK548^T and K129) were identical but differed from that of strain TrB1-1^T by 0.6, 1.0, 3.3 and 5.9 % nucleotide substitutions, respectively. Therefore, the two strains (DMKU-RK548^T and K129) and strain TrB1-1^T were assigned to be two separate species. The closest species in terms of pairwise sequences similarity of the D1/D2 region to the two novel species was *Yamadazyma philogaea* but with 1.1–1.7 % nucleotide substitutions. The two strains (DMKU-RK548^T and K129) were assigned to *Yamadazyma phyllophila* sp. nov. (type strain DMKU-RK548^T = BCC 50736^T = NBRC 108906^T = CBS 12572^T) and the strain TrB1-1^T was named *Yamadazyma paraphyllophila* sp. nov. (type strain TrB1-1^T = BCRC 23030^T = CCTCC AY 204005^T = CBS 9928^T).

Keywords *Yamadazyma siamensis* sp. nov. · *Yamadazyma phyllophila* sp. nov. · *Yamadazyma paraphyllophila* sp. nov. · Phylloplane yeast · Ascomycete yeast · New yeast species · Thailand · Taiwan

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Introduction

The genus *Yamadazyma* was described by Billon-Grand (1989) to accommodate species that had previously been assigned to the genus *Pichia* and that formed CoQ-9 as their major ubiquinone and formed hat-shaped ascospores (Kurtzman and Robnett 1998). Analysis of the D1/D2 region of the large subunit (LSU) rRNA gene by Kurtzman and Robnett (1998) showed that species assigned to the genus *Yamadazyma* are placed in several clade. Later some of the species that had been assigned to this genus by Billon-Grand (1989) were transferred to the newly described genera *Babjeviella*, *Meyerozyma*, *Millerozyma* and *Priceomyces* based on phylogenetic analysis of the D1/D2 region of the LSU rRNA gene and the nearly complete small subunit rRNA gene (Kurtzman and Suzuki 2010). Then after the genus *Yamadazyma* became a well-support clade (Kurtzman and Suzuki 2010) and a generally accepted genus in the Family Debaryomycetaceae of Order Saccharomycetales (Kurtzman 2011) in The Yeast, a taxonomic study 5th edition *Y. philogaea*, the type species, as well as *Y. akitaensis*, *Y. mexicana*, *Y. nakazawae*, *Y. scolyti*, *Y. triangularis* and 23 *Candida* species are placed in the *Yamadazyma* clade (Kurtzman 2011; Lachance et al. 2011). More recently, a few novel *Candida* species in this clade have been described i.e. *Candida kanchanaburiensis*, which was isolated from the fruit body of a mushroom and rotted fruit of guava collected in the western Thailand (Nakase et al. 2008), *C. khaothaluensis*, which was isolated from unidentified tree in Thailand, *C. vaughaniae* and *C. tallmaniae*, which were isolated from flowers of unidentified plants in French Guiana (Groenewald et al. 2011), and *C. oceani*, which was obtained from an unidentified deep-sea coral, water samples and the stomach of a marine fish on the Mid-Atlantic Ridge (Burgaud et al. 2011).

The external surface of plant leaves is usually referred to as the phylloplane, or phyllosphere (Fonseca and Inacio 2006; Phaff and Starmer 1987). The phylloplane has been found to be colonized by members of both basidiomycete and ascomycete yeasts (Fonseca and Inacio 2006; Glushakova et al. 2007; Landell et al. 2010; Nakase et al. 2001; Slavikova et al. 2009). Although most common phylloplane yeasts are yeast species in basidiomycete genera such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo et al. 1998; Fonseca and Inacio 2006; Glushakova and Chernov 2010; Nakase et al. 2001; Slavikova et al. 2009), various ascomycete yeast species have also been detected on the phylloplane i.e. *Debaryomyces hansenii*, *Hanseniaspora uvarum, Kazachstania barnetii*, *Metschnikowia lopburiensis, Metschnikowia pulcherrima*, *Metschnikowia saccharicola, Pichia membranifaciens*, *Saccharomyces cerevisiae* and various *Candida* species including *Candida aechmeae*, *Candida olephila*, *Candida chumphonensis, Candida mattranensis* and *Candida vrieseae* (Glushakova et al. 2007; Glushakova and Chernov 2010; Kaewwichian et al. 2012; Koowadjanakul et al. 2011; Landell et al. 2010; Slavikova et al. 2009).

During an investigation of yeasts on the external leaf surfaces of plants in Thailand and Taiwan, four strains representing three novel species of *Yamadazyma* were isolated from the phylloplane of sugarcane, corn, bean and hengchun pencilwood. In this paper, one strain (DMKU-RK254^T) is described as *Yamadazyma siamensis* sp. nov., two strains (DMKU-RK548^{T}and K129) are described as *Yamadazyma phyllophila* sp. nov. and one strain (TrB1-1^T) is described as *Yamadazyma paraphyllophila* sp. nov.

Materials and methods

Yeast isolation

Strain DMKU-RK254^T, DMKU-RK548^T and K129 were isolated from leaf surfaces of sugarcane, corn and bean collected in Thailand (Table 1) by an enrichment technique using acidified yeast extract malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong et al. 2007). 3 g of cut leaves were aseptically placed in 250 ml Erlenmeyer flask, containing 50 ml of acidified YM broth and incubated on a rotary shaker at room temperature (27 ± 3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature until yeast colonies appeared. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Strain TrB1-1^T

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Table 1 Strains of *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov. isolated from surface of plant leaves used in this study

Strain	Accession number	GenBank accession number				Sample for isolation		
		D1/D2	ITS	ACT1	EF2	Leaves	ocality	Date
Y. siamensis sp. nov.								
DMKU-RK254^T	BCC 50730^T NBRC 108901^T CBS 12573^T	AB734046	AB734049	–	–	Sugarcane (*Saccharum officinarum*)	Bueng Sam Phan district, Phetchabun province, Thailand	23 Mar 2010
Y. phyllophila sp. nov.								
DMKU-RK548^T	BCC 50736^T NBRC 108906^T CBS 12572^T	AB734047	AB734050	AB761313	AB761316	Corn (*Zea mays*)	Pak Chong district, Nakhon Ratchasima province, Thailand	6 May 2010
K129	–	AB734048	AB734051	AB761312	AB761315	Bean (*Phaseolus vulgaris*)	Kanjanaburi province, Thailand	15 Nov 2009
Y. paraphyllophila sp. nov.								
TrB1-1^T	BCRC 23030^T CCTCC AY 204005^T CBS 9928^T	AY562397	AY559447	AB761314	AB761317	Hengchun pencilwood (*Dysoxylum hongkongense*)	NanJen-Shan Natural Reserve on Pindung, Taiwan	2004

BCC BIOTEC Culture Collection, *NBRC* NITE Biological Resources Center, *CBS* Centraalbureau voor Schimmelcultures, *BCRC* Bioresource Collection and Research Center, *CCTCC* China Center for Type Culture Collection

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was isolated from hengchun pencilwood leaves collected in Taiwan (Table 1) according to the procedures described by Rosa et al. (1995) with slight modification. Leaves were collected in clean polyethylene plastic bags, transported to the laboratory and washed with 30–40 ml of sterile distilled water, vortexed for 30 min, an aliquot of 0.1 ml was spread on acidified YM agar supplemented with 0.034 % chloramphenicol adjusted to pH 3.7 by 1 N HCl and incubated at 26 °C. After 2 days, yeast colonies were picked and purified. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

DNA sequencing and phylogenetic analysis

The sequences of the D1/D2 region of the LSU rRNA gene, the internal transcribed spacer (ITS) region, the actin gene (ACT1) and the elongation factor 2 gene (EF2) were determined from PCR products amplified from genomic DNA. The D1/D2 region of the LSU rRNA gene, the ITS region, the ACT1 gene and EF2 gene were amplified and sequenced with primers, NL1 and NL4, ITS1 and ITS4 or ITS5 and ITS4, ACT1 and ACT2, and EFIIF1 and EFIIR2 (Table 2). Methods for DNA extraction and amplification of the D1/D2 region of the LSU rRNA gene, ITS region, and ACT1 gene and EF2 gene of strain DMKU-RK548^T, K129 and TrB1-1^T were as described previously (Limtong et al. 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen, Germany). The purified products were submitted to Macrogen Inc. (Korea) for sequencing. The DNA was extracted for the amplification of the D1/D2 region of the LSU rRNA gene and the ITS region of strain TrB1-1^T by using a variation of a CTAB method (Doyle and Doyle 1990). Dichloromethane was substituted for chloroform in the extraction procedures. Yeast cells were suspended in 500 µl of CTAB extraction buffer [1 ± 4 M NaCl, 100 mM Tris–HCl, pH 8 ± 0, 20 mM EDTA, 2 % w/v PVP-40, 20 mM cetyl trimethyl ammonium bromide (CTAB), and 0 ± 5 % v/v 2-mercaptoethanol] in an eppendorf and placed in a dry bath at 95 °C for 30 min and then in a water bath at 60 °C for 30 min with occasional gentle mixing. An equal volume of dichloromethane:isoamyl alcohol (24:1) was added, mixed by gentle inversion and centrifuged at 1,500×*g* for 4 min at 20 °C. The upper aqueous phase was recovered and DNA was precipitated by the addition of 0.6 volume of isopropanol. The tube was inverted gently and then centrifuged at 460×*g* for 2 min. The DNA pellet was rinsed with 500 µl wash buffer (76 % ethanol and 10 mM ammonium acetate) for 2 min, and rinsed again with wash buffer. The tube was spun at 1,500×*g* for 10 min and the supernatant was decanted. The tube was inverted and air-dried for 10–30 min and then the pellet was resuspended in 20 µl SDW at 37 °C for 30 min. DNA was quantified with a fluorimeter, according to the manufacturer's instructions, and stored at −20 °C. The PCR product was sequenced according to the manufacturer's instructions (ABI prism 3100 Genetic Analyzer, PE Applied Biosystems, USA). The sequences were compared pairwise using a BLAST search (Altschul et al. 1997) and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson et al. 1997).

Table 2 Primers used for PCR amplification and sequencing

Region/gene	Primer	Sequence	Reference
D1/D2	NL1 (forward)	5′ GCATATCAATAAGCGGAGGAAAAG 3′	Kurtzman and Robnett (1998)
	NL4 (reverse)	5′ GGTCCGTGTTTCAAGACGG 3′	
ITS	ITS1 (forward)	5′ TCCGTAGGTGAACCTGCGG 3′	White et al. (1990)
	ITS5 (forward)	5′ GGAAGTAAAAGTCGTAACAACG 3′	
	ITS4 (reverse)	5′ TCCTCCGCTTATTGATATGC 3′	
ACT1	ACT1 (forward)	5′ TACCCAATTGAACACGGTAT 3′	Diezmann et al. (2004)
	ACT2 (reverse)	5′ TCTGAATCTTTCGTTACCAAT 3′	
EF2	EFIIF1 (forward)	5′ AAGTCTCCAAACAAAGCATAAC 3′	Diezmann et al. (2004)
	EFIIR2 (reverse)	5′ GGGAAAGCTTGACCACCAGTAGC 3′	

ITS5 (forward primer) was used for strain TrB1-1

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A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura 1980), using the neighbor joining method (Saitou and Nei 1987) by MEGA software version 4.0 (Tamura et al. 2007). Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) (Felsenstein 1985).

Examination of taxonomic characteristics

The strains were characterized morphologically, biochemically, and physiologically according to the standard methods described by Yarrow (1998). Mycelium formation was investigated on potato dextrose agar (PDA) in slide culture at 25 °C for up to 7 days. Ascospores formation was investigated individually or in pairs on 5 % malt extract agar, McClary's acetate agar, Gorodkowa agar, corn meal agar, V8 agar and yeast carbon base supplemented with 0.01 % ammonium sulfate (YCBAS) agar at 15 and 25 °C for up to 4 weeks. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula following the method of Nakase and Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500 ml Erlenmeyer flask containing 250 ml of yeast extract peptone dextrose (YPD) broth (1 % yeast extract, 2 % peptone and 2 % dextrose) on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada and Kondo (1973) and Kuraishi et al. (1985). Isoprenologues were identified by HPLC as described previously (Limtong et al. 2007). The DNA base composition was determined by HPLC as described by Tamaoka and Komagata (1984) using the DNA-GC kit (Yamasa Shoyu Co., Japan).

Results and discussion

Novel species delineation and identification

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of strain DMKU-RK254^T was closely related to *Candida diddensiae* and *Candida naeodendra* with 1.8 % nucleotide substitutions (9 nucleotide substitutions out of 493 nt) and to *Candida kanchanaburiensis* with 2.0 % nucleotide substitutions (10 nucleotide substitutions out of 493 nt). The sequences of the ITS region of strain DMKU-RK254^T differed by 6.6 % nucleotide substitutions (36 nucleotide substitutions and 13 gaps out of 545 nt) from *C. diddensiae* by 6.6 % nucleotide substitutions (39 nucleotide substitutions and 30 gaps out of 589 nt) from *C. naeodendra* and by 5.8 % nucleotide substitutions (34 nucleotide substitutions and 11 gaps out of 585 nt) from *C. kanchanaburiensis*.

The D1/D2 region of the LSU rRNA gene sequences of the two strains (DMKU-RK548^T and K129) were identical and differed by three nucleotide substitutions from strain TrB1-1^T. The closest known species to the three strains was *Yamadazyma philogaea*; the two strains (DMKU-RK548^T and K129) differed by 1.1 % nucleotide substitutions (6 nucleotide substitutions out of 530 nt) while strain TrB1-1^T differed by 1.7 % nucleotide substitutions (9 nucleotide substitutions out of 530 nt). The sequences of ITS region of the two strains (DMKU-RK548^T and K129) were identical and differed from strain TrB1-1^T by six nucleotide substitutions and one gap out of 582 nt. The two strains (DMKU-RK548^{T}and K129) differed by 5.2 % nucleotide substitutions (30 nucleotide substitutions and five gaps out of 582 nt) while strain TrB1-1^T differed by 5.5 % nucleotide substitutions (32 nucleotide substitutions and 6 gaps out of 582 nt) from *Y. philogaea*. Moreover, the sequences of the ACT1 gene and the EF2 gene of the two strains (DMKU-RK548^T and K129) were identical and differed from strain TrB1-1^T by 17 nucleotide substitutions out of 523 nt (3.3 % nucleotide substitutions) and 36 nucleotide substitutions out of 606 nt (5.9 % nucleotide substitutions), respectively. The difference in the ACT1 and EF2 genes of the two strains (DMKU-RK548^T and K129) from strain TrB1-1^T is high enough to justify them as two separate species as report for the ACT1 gene in *Clavispora* spp. by Lachance et al. (2003) and for EF2 gene in *Wickerhamomyces* spp. by Imanishi et al. (2009).

The phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene further demonstrated that the four strains of the three novel species are in *Yamadazyma* clade. Strain DMKU-RK254^T connects to *Candida kanchanaburiensis* and is placed in the same subclade that contains *C. naeodendra*, *C. diddensiae* and the three strains of another two novel species (Fig. 1). Strains DMKU-RK548^T and

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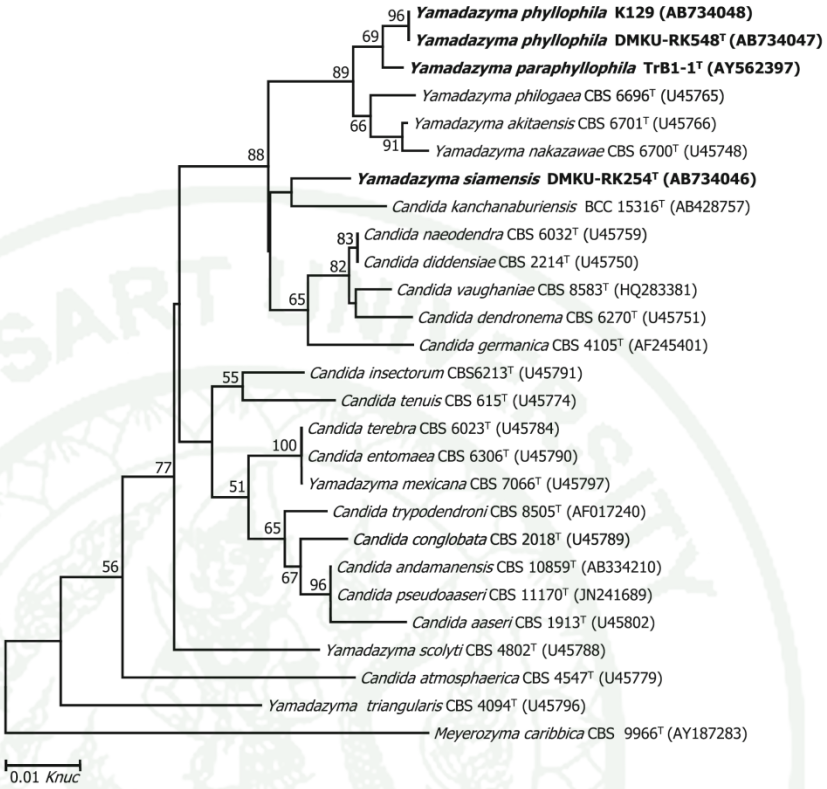


Fig. 1 Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of *Yamadazyma siamensis* sp. nov. (DMKU-RK254^T), *Yamadazyma phyllophila* sp. nov. (DMKU-RK548^T and K129) and *Yamadazyma paraphyllophila* sp. nov. (TrB1-1^T), with respect to closely related species. The phylogenetic tree was constructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbor joining method by MEGA software version 4.0. Numbers indicate percentages of bootstrap sampling, derived from 1,000 samples. The *numbers* in *parentheses* are GenBank accession numbers. *Meyerozyma caribbica* was the outgroup in the analysis

K129 are located in the same position and connect to strain TrB1-1^T. They cluster with *Yamadazyma philogaea*, the closest known species, and *Yamadazyma nakazawae* and *Yamadazyma akitaensis* with high bootstrap support (Fig. 1).

On the basis of morphological, biochemical, physiological, chemotaxonomic characteristics, and the sequence analysis of the D1/D2 region of the LSU rRNA gene, the ITS region, the ACT1 gene and the EF2 gene, we concluded that the four strains represent three novel *Yamadazyma* species although formation of ascospores was not observed. The name *Yamadazyma siamensis* sp. nov. (MB801056) is proposed for the strain DMKU-RK254^T, the name *Yamadazyma phyllophila* sp. nov. (MB801057) is assigned for the two strains (DMKU-RK548^{T}and K129) and the name *Yamadazyma paraphyllophila* sp. nov. (MB802274) is proposed for the strain TrB1-1^T.

In practice, *Yamadazyma siamensis* sp. nov., *Y. phyllophila* sp. nov. and *Y. paraphyllophila* sp. nov. can be distinguished from their close related species *C. diddensiae*, *C. naeodendra* and *C. kanchanaburiensis*, and *Y. philogaea*, respectively, not only on the basis of the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region but also several phenotypic characteristics as shown in Table 3. *Y. phyllophila* sp. nov. can be distinguished from *Y. paraphyllophila* sp. nov. only by the different in genotypic characteristics while the phenotypic characteristics were the same.

Most of the members of the *Yamadazyma* clade such as *C. michaelii*, *C. gorgasii*, *C. lessepsii*, *C. cerambycidarum*, C. *endomychidarum*, *C. amphixiae*, *C. diddensiae*, *C. naeodendra* and *C. dendronema* exhibit an association with the gut of beetles and other insects (Lachance et al. 2011). However, some strains of the recognized species were reported to have been isolated from plants i.e. a strain of *Yamadazyma akitaensis* (CBS 6701^T) that was isolated from exudate of a willow, two strains of *Yamadazyma mexicana* (CBS 7066^T and CBS 7067) that were isolated from cactus, *Candida diospyri* (CBS 9769^T) that was isolated from a kaki fruit and *Candida buinensis*

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Table 3 Phenotypic characteristics that differentiate *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp. nov. and *Yamadazyma paraphyllophila* sp. nov. from their closest species, *Candida diddensiae*, *C. naeodendra*, *C. kanchanaburiensis* and *Yamadazyma philogaea*, respectively

Characteristics	Species						
	1	2[a]	3[b]	4[c]	5	6	7[d]
Fermentation							
Glucose	+	+	+	w	+	+	+
Galactose	w	+	s	w	+	+	w
Sucrose	−	v	−	−	−	−	−
Maltose	−	v	−	−	−	−	v
Lactose	−	−	−	n	−	−	−
Raffinose	−	−	−	n	−	−	−
Trehalose	−	+	+	−	−	−	+
Cellobiose	+	n	n	−	−	−	n
Xylose	−	n	n	−	−	−	n
Assimilation of carbon/nitrogen compounds							
D-Glucose	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+
L-Sorbose	+	v	s	l	−	−	+
Sucrose	+	+	−	+	+	+	+
Maltose	+	+	−	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Trehalose	+	v	+	+	+	+	+
Lactose	−	−	−	−	+	+	−
Melibiose	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−
Melezitose	+	+	+	+	+	+	+
Inulin	−	−	−	−	−	−	−
Soluble starch	−	−	+	−	−	−	−
D-Xylose	+	+	+	+	+	+	+
L-Arabinose	+	v	+	+	w	w	+
D-Arabinose	s	v	s	v	−	−	+
D-Ribose	+	v	+	v	−	−	+
L-Rhamnose	+	−	s	l	+	+	−
Ethanol	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+
Erythritol	+	+	+	+	+	+	+
Ribitol	+	+	+	+	+	+	+
Galactitol	−	−	−	−	−	−	−
D-Mannitol	+	+	+	+	+	+	+
D-Glucitol	+	+	+	+	+	+	+
α-Methyl-D-glucoside	+	v	+	+	+	+	+
Salicin	+	v	+	+	+	+	−
DL-Lactic acid	w	−	−	−	−	−	−
Succinic acid	+	v	+	+	+	+	+

Table 3 continued

Characteristics	Species						
	1	2[a]	3[b]	4[c]	5	6	7[d]
Citric acid	+	v	+	+	−	−	+
Inositol	−	−	−	−	−	−	−
Arbutin	+	n	n	+	−	−	n
D-Gluconic acid	+	+	+	+	+	+	+
D-Glucosamine	+	v	+	s	−	−	+
N-Acetyl-D-glucosamine	+	+	+	+	+	+	+
2-Keto-D-gluconic acid	−	v	−	−	−	−	+
5-Keto-D-gluconic acid	−	n	n	−	−	−	−
Hexeadecane	−	w	+	−	−	−	+
Methanol	−	−	−	−	−	−	−
Propane-1,2-diol	−	n	n	l	−	−	n
Butane-2,3-diol	−	n	n	−	−	−	n
D-Glucono-δ-lactone	−	+	−	−	w	w	n
Xylitol	+	v	+	+	+	+	n
D-Glucuronic acid	−	−	−	−	−	−	n
D-Galacturonic acid	−	n	n	−	−	−	n
Arabinitol	+	n	n	+	−	−	n
Nitrate	−	−	−	−	−	−	−
Nitrite	−	−	−	−	−	−	n
Ethylamine	+	+	+	+	+	+	n
L-Lysine	+	+	+	w	+	+	+
Cadaverine	+	+	+	+	+	+	n
Other growth characteristics							
Vitamin free medium	w	−	−	n	−	−	−
50 % Glucose	+	v	+	n	+	+	n
60 % Glucose	+	n	n	n	+	+	n
10 % NaCl + 5 % glucose	+	+	−	v	w	w	+
16 % NaCl + 5 % glucose	−	n	n	n	−	−	n
0.01 % Cycloheximide	w	−	−	n	−	−	−
0.1 % Cycloheximide	−	−	−	−	−	−	n
Growth at 25 °C	+	+	+	+	+	+	+
Growth at 30 °C	+	+	+	+	+	+	+
Growth at 35 °C	+	+	+	+	+	+	+
Growth at 37 °C	+	+	+	+	+	+	w
Growth at 40 °C	+	n	n	n	+	+	n
Growth at 42 °C	−	n	n	n	−	−	n
Growth at 45 °C	−	n	n	n	−	−	n
Starch production	−	−	−	−	−	−	−

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Table 3 continued

Characteristics	Species						
	1	2[a]	3[b]	4[c]	5	6	7[d]
Diazonium blue B	–	–	–	n	–	–	–
Urease	–	n	n	n	–	–	n
Major ubiquinone	Q9	Q9	Q9	Q9	Q9	Q9	Q9

+, positive; −, negative; l, latent positive; s, slow positive; w, weakly positive; v, variable; n, no data

1 Yamadazyma siamensis sp. nov., *2 Candida diddensiae*, *3 C. naeodendra*, *4 C. kanchanaburiensis*, *5 Y. phyllophila* sp. nov., *6 Y. paraphyllophila* sp. nov., *7 Y. philogaea*

[a,b] Data from Lachance et al. (2011)

[c] Data from Nakase et al. (2008)

[d] Data from Kurtzman (2011)

(CBS 6796^T) that was isolated from the gelatinous exudate of a tree fern (Kurtzman 2011; Lachance et al. 2011). In Thailand few *Candida* species in the *Yamadazyma* clade have been isolated i.e. *Candida khaothaluensis*, which was isolated from an unidentified tree (Groenewald et al. 2011) and *Candida kanchanaburiensis*, which was isolated from a fruit body of a unidentified mushroom (*Coprinus* sp.) and a rotted guava fruit (Nakase et al. 2008). Until the present, no members of this clade have been reported to have been isolated from the phylloplane. In this study, four strains of the two novel *Yamadazyma* species are proposed, but from limited number of strains derived in this study, we could not conclude that the phylloplane is one of the habitats of yeast in this clade. However, the phylloplane is interesting as the subject for further investigation of yeast in this clade.

Description of *Yamadazyma siamensis* Kaewwichian, Yongmanitchai, Kawasaki, Wang, Yang & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: After 3 days at 25 °C, cells are globose, subglobose to ovoid (2–7 × 3–8 µm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. Growth on YM agar: after 3 days at 25 °C, the streak culture is grayish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 2b). Ascospores were not produced on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks

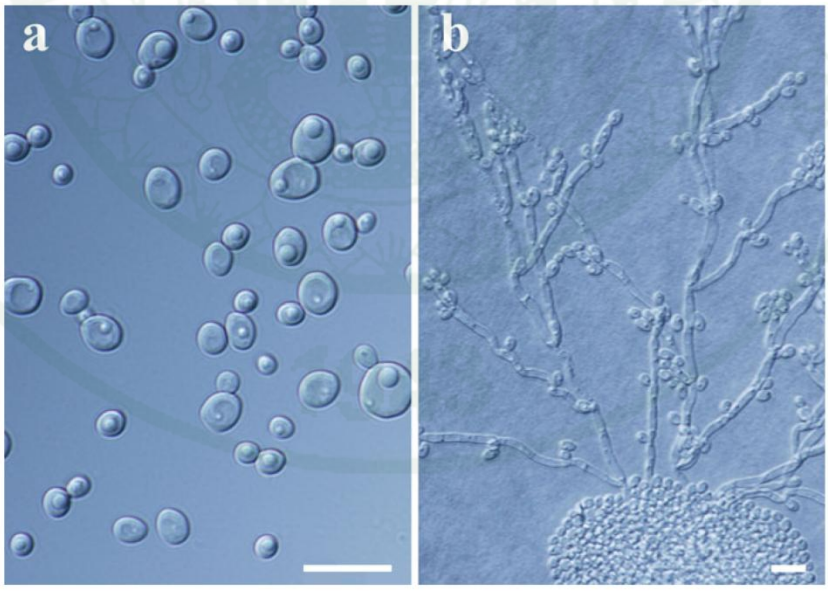


Fig. 2 *Yamadazyma siamensis* sp. nov. (DMKU-RK254^T) **a** budding cells in YM broth after 3 days at 25 °C **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar*, 10 µm

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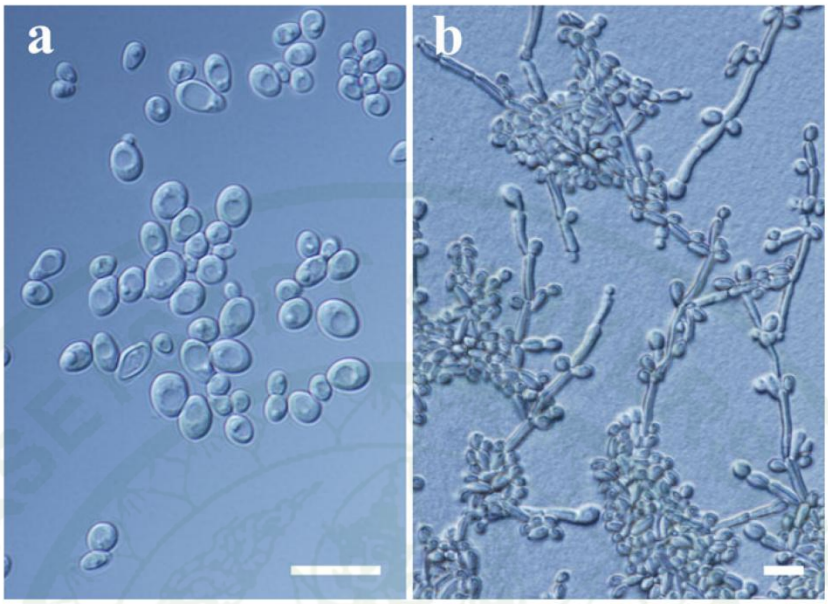


Fig. 3 *Yamadazyma phyllophila* sp. nov. (DMKU-RK548^T) **a** budding cells in YM broth after 3 days at 25 °C **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar*, 10 μm

at 15 and 25 °C. DNA G+C content is 46.2 mol %. The other phenotypic characteristics of the species are shown in Table 3.

Holotype

DMKU-RK254^T is the holotype of *Yamadazyma siamensis* (MB801056). The strain was isolated from phylloplane of sugarcane (*Saccharum officinarum*) collected from Phetchabun province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50730^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108901^T and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 12573^T.

Etymology

The species epithet *siamensis* refers to Siam the old name of Thailand, where the strain was isolated.

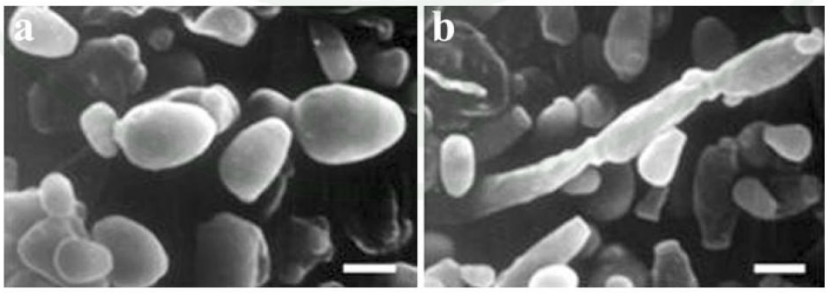


Fig. 4 *Yamadazyma paraphyllophila* sp. nov. (TrB1-1^T) **a** budding cells in YM broth after 3 days at 25 °C **b** pseudohyphae formed on PDA after 3 days at 25 °C. *Scale bar*, 1 μm.

Scanning electron microscopy was prepared according to the procedures described by Lee et al. (1994)

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Description of *Yamadazyma phyllophila* Kaewwichian, Yongmanitchai, Kawasaki, Wang, Yang & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: after 3 days at 25 °C, cells are ovoid to ellipsoid (2–4 × 3–6 µm) and occur singly, in pairs or in groups (Fig. 3a). Budding is multilateral. Growth on YM agar after 3 days at 25 °C, the streak culture is white, smooth and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 3b). Ascospores were not produced by any of the strains either individually or when paired on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. DNA G+C content is 38.7 mol %. The other phenotypic characteristics of the species are shown in Table 3.

Holotype

DMKU-RK548^T is the holotype of *Yamadazyma phyllophila* sp. nov. (MB801057). The strain was isolated from phylloplane of corn (*Zea mays*) collected from Nakhon Ratchasima province, Thailand. The living culture from type was deposited at the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 50736^T; NITE Biological Resources Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108906^T and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 12572^T.

Etymology

The species epithet *phyllophila* refers to phylloplane where the three strains of this species were isolated.

Description of *Yamadazyma paraphyllophila* Wang, Yang, Kaewwichian, Yongmanitchai, Kawasaki & Limtong sp. nov.

Growth in yeast extract malt extract (YM) broth: after 3 days at 25 °C, cells are ovoid to ellipsoid (2–3 × 3–5 µm) and occur singly, in pairs or in groups (Fig. 4a). Budding is multilateral. Growth on YM agar after 3 days at 25 °C, the streak culture is white, smooth and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 4b). Ascospores were not produced on YM agar, YPD agar, 5 % malt extract agar, McClary's acetate agar, corn meal agar, Gorodkowa agar, V8 agar and YCBAS agar after 4 weeks at 15 and 25 °C. The other phenotypic characteristics of the species are shown in Table 3.

Holotype

TrB1-1^T is the holotype of *Yamadazyma paraphyllophila* sp. nov. (MB802274). The strain was isolated from phylloplane of hengchun pencilwood (*Dysoxylum hongkongense*) collected from NanJen-Shan Natural Reserve on Pindung, Taiwan. The living culture from type was deposited at the Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan R.O.C., as BCRC 23030^T; China Center for Type Culture Collection, Wuhan, Hubei, China, as CCTCC AY 204005^T and CBS-KNAW Fungal Biodiversity Center (CBS), Utrecht, The Netherlands as CBS 9928^T.

Etymology

The species epithet *paraphyllophila* indicating affinity with *phyllophila* species.

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Wickerhamomyces siamensis sp. nov., a novel yeast species isolated from the phylloplane in Thailand

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Strain DMKU-RK359^T, representing a novel yeast species, was isolated from the external surface of a sugar-cane leaf collected in Thailand. On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics, sequence analysis of the D1/D2 region of the large-subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region, strain DMKU-RK359^T was assigned to a novel *Wickerhamomyces* species. The novel species was closest to *Wickerhamomyces ciferrii*, but differed from it by 0.7 % nucleotide substitutions in the D1/D2 region of the LSU rRNA gene and 6 % nucleotide substitutions in the ITS region. The name *Wickerhamomyces siamensis* sp. nov. is proposed (type strain DMKU-RK359^T =BCC 50732^T =NBRC 108900^T =CBS 12570^T).

The genus *Wickerhamomyces* was proposed to accommodate 17 species that were transferred from the genera *Pichia*, *Williopsis* and *Hansenula* as a result of phylogenetic analysis of nucleotide divergence in the genes coding for the large-subunit (LSU) and small-subunit rRNAs and for elongation factor 1α (Kurtzman *et al.*, 2008; Kurtzman, 2011). The number of *Wickerhamomyces* species was subsequently increased by the discovery of *Wickerhamomyces edaphicus* (Limtong *et al.*, 2009), *W. queroliae* (Rosa *et al.*, 2009), *W. patagonicus* (de García *et al.*, 2010), *W. chaumierensis* (Groenewald *et al.*, 2011), *W. ochangensis* (Shin *et al.*, 2011), *W. tratensis* (Nakase *et al.*, 2012) and *W. xylosica* (Limtong *et al.*, 2012).

The phylloplane, the external surface of the plant leaf, has been recognized as an important habitat for epiphytic micro-organisms (Phaff & Starmer, 1987; Fonseca & Inácio, 2006). Both basidiomycete and ascomycete yeasts have been found to be phylloplane colonizers (Nakase *et al.*, 2001; Fonseca & Inácio, 2006; Glushakova *et al.*, 2007; Slavikova *et al.*, 2009; Landell *et al.* 2010). Although most common phylloplane yeasts are members of basidiomycete genera such as *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon* (de Azeredo *et al.*, 1998; Nakase *et al.*, 2001; Fonseca & Inácio, 2006; Slavikova *et al.*, 2009; Glushakova & Chernov, 2010), various ascomycete yeast species have also been reported, e.g. *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Kazachstania barnetii*, *Metschnikowia lopburiensis*, *M. pulcherrima*, *M. saccharicola*, *Pichia membranifaciens*, *Saccharomyces cerevisiae* and various *Candida* species including *Candida aechmeae*, *C. olephila*, *C. chumphonensis*, *C. mattranensis* and *C. vrieseae* (Glushakova *et al.*, 2007; Slavikova *et al.*, 2009; Glushakova & Chernov, 2010; Landell *et al.*, 2010; Kaewwichian *et al.*, 2012; Koowadjanakul *et al.*, 2011).

During an investigation of yeasts on the external leaf surfaces of sugar cane in Thailand, strain DMKU-RK359^T, representing a novel species of *Wickerhamomyces*, was obtained. In this paper, it is described as the type strain of *Wickerhamomyces siamensis* sp. nov.

One hundred and sixty-six yeast strains were isolated from the external surfaces of 95 samples of sugar cane (*Saccharum officinarum* L.) leaf collected in Thailand. Strain DMKU-RK359^T was isolated from a sample collected from Nong Muang district, Lop Buri Province, on 5 May 2010, by an enrichment technique using acidified yeast extract-malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose) supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol (Limtong *et al.*, 2007). Cut leaves (3 g) were placed aseptically in a 250-ml Erlenmeyer flask containing 50 ml acidified YM broth and incubated on a rotary shaker at room temperature (27±3 °C) for 3 days. The enrichment culture was then spread on YM agar supplemented with 0.025 % sodium propionate and 0.02 % chloramphenicol and incubated at room temperature (27±3 °C) until yeast colonies appeared. Yeast colonies of different morphologies were picked and

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 region of the LSU rRNA gene and the ITS region of strain DMKU-RK359^T are AB714248 and AB714257, respectively.

The Mycobank number for *Wickerhamomyces siamensis* is MB802886.

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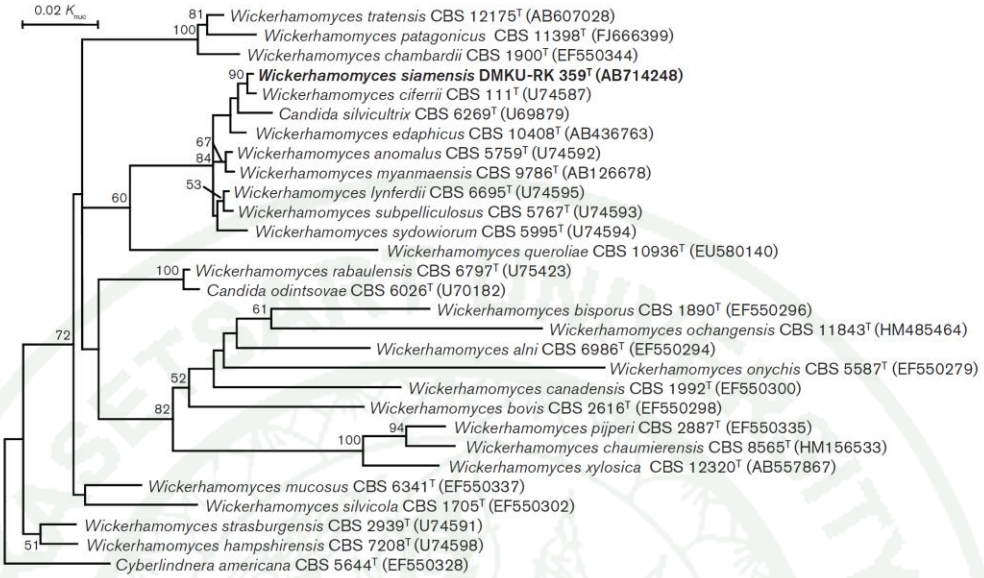


Fig. 1. Phylogenetic tree based on sequences of the D1/D2 region of the LSU rRNA gene, showing the position of *W. siamensis* sp. nov. DMKU-RK359^T with respect to closely related species. The phylogenetic tree was reconstructed from evolutionary distance data with Kimura's two-parameter correction, using the neighbour-joining method with MEGA version 5.0. Numbers at nodes indicate percentages of bootstrap sampling, derived from 1000 samples. *Cyberlindnera americana* CBS 5644^T was the outgroup. Bar, 0.02 K_{nuc}.

purified by cross-streaking on YM agar. Purified yeast strains were suspended in YM broth supplemented with 10 % glycerol and maintained at −80 °C.

The sequences of the D1/D2 region of the LSU rRNA gene and the internal transcribed spacer (ITS) region were determined from PCR products amplified from genomic DNA, using primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1 and ITS4 (White *et al.*, 1990), respectively, for sequencing and amplification. Methods of DNA extraction and amplification were as described previously (Limtong *et al.*, 2007). The PCR products were checked by agarose gel electrophoresis and purified by using the QIAquick purification kit (Qiagen) and the purified products were submitted to Macrogen Inc. (Seoul, Republic of Korea) for sequencing. The sequences were compared pairwise using a BLAST search (Altschul *et al.*, 1997) and were aligned with the sequences of related strains retrieved from GenBank using the multiple alignment program CLUSTAL_X version 1.81 (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980) using the neighbour-joining method (Saitou & Nei, 1987) and MEGA software version 5.0 (Tamura *et al.*, 2011). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

Strain DMKU-RK359^T was characterized morphologically, biochemically and physiologically according to standard methods described by Yarrow (1998). Mycelium formation was investigated on potato dextrose agar (PDA; 20 % potato infusion, 2 % glucose and 1.5 % agar) in slide culture at 25 °C for up to 7 days. Ascospore formation was investigated on YPD agar (1 % yeast extract, 2 % peptone, 2 % glucose and 1.5 % agar) and 5 % malt extract agar (5 % malt extract and 1.5 % agar) at 25 °C. Carbon assimilation tests were conducted in liquid medium according to the method described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid medium with starved inocula following the method of Nakase & Suzuki (1986). Growth at various temperatures was determined by cultivation in YM broth. Ubiquinones were extracted from cells cultivated in a 500-ml Erlenmeyer flask containing 250 ml YPD broth on a rotary shaker at 28 °C for 24–48 h and purified according to the method described by Yamada & Kondo (1973) and Kuraishi *et al.* (1985). Isoprenologues were identified by HPLC as described previously (Limtong *et al.*, 2007). The DNA base composition was determined by HPLC as described by Tamaoka & Komagata (1984) using the DNA-GC kit (Yamasa Shoyu).

Morphological examination of ascospores under the scanning electron microscope was performed as follows. Strain DMKU-RK359^T was grown on YPD agar at 25 °C

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Table 1. Phenotypic characteristics that differentiate *Wickerhamomyces siamensis* sp. nov. DMKU-RK359^T from closely related species

Species/strains: 1, *W. siamensis* sp. nov. DMKU-RK359^T; 2, *W. ciferrii* (data from Kurtzman, 2011); 3, *W. edaphicus* (Limtong *et al.*, 2009); 4, *C. silvicultrix* (Lachance *et al.*, 2011). +, Positive; W, weakly positive; −, negative; ND, no data available.

Characteristic	1	2	3	4
Assimilation of:				
Melibiose	−	−	+	+
L-Arabinose	−	+/W	W/−	+
D-Arabinose	−	−	−	+
D-Ribose	−	+	ND	+
L-Rhamnose	−	+/W	+	−
Ribitol	−	+	+	+
Citric acid	−	+	+	+
Hexadecane	−	+	ND	−
D-Glucono-δ-lactone	−	ND	ND	+
Nitrate	−	+	+	−
Nitrite	−	ND	+	−
Ethylamine	+	ND	+	−
Growth in/at:				
Vitamin-free medium	−	+	+	+
10 % NaCl/5 % glucose	−	W	+	+
37 °C	+	W/−	W	+
40 °C	+	ND	−	ND

for 3 days or until ascospores were produced. An ascospore suspension was prepared in 1 ml normal saline and then centrifuged. An aliquot (0.5 ml) of 0.1 M phosphate buffer, pH 7.0, was added to the cell suspension and the cells were harvested by centrifugation. Ascospores were fixed with 0.5 ml of 3.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at room temperature (27 ± 3 °C) for 1 h and then centrifuged and washed three times with phosphate buffer. An aliquot (0.5 ml) of 2 % osmium tetroxide in 0.1 M phosphate buffer was added and left at room temperature for 1 h and then the suspension was washed three times with phosphate buffer. Fixed ascospores were washed stepwise with 0.5 ml aliquots of increasing ethanol concentrations (30, 50, 70, 80, 90 and 95 %) for 30 min and two 30-min washes in 100 % ethanol. Fixed ascospores were dried with 0.5 ml ethanol/isoamyl alcohol (2 : 1) for 30 min, centrifuged and then repeatedly dried with ethanol/isoamyl alcohol (1 : 1 and 1 : 2). After centrifugation, isoamyl alcohol was added, left for 30 min and then the suspension was centrifuged; this step was repeated. Ascospores were moved to a critical-point dryer and then coated with platinum. Finally, ascospores were observed by scanning electron microscopy (Hitachi S-5200).

Determination of whether strain DMKU-RK359^T was homothallic or heterothallic was performed by the heat-treatment method described by Wickerham & Burton (1954). A loopful of cells from a YM agar plate (24–48 h, 25 °C) was suspended in 2 ml YM broth in a 16-mm test tube and the test tube was placed in a water bath at 62 °C. After 1, 2, 3, 4 and 5 min, loopfuls of the cell suspension were taken and spread on YM agar. The plates were examined after incubation to determine the time taken to kill the vegetative cells. A suspension of a sporulated culture of strain DMKU-RK359^T (3 days, 25 °C on YPD agar) was then prepared in YM broth. The suspension was heated at 62 °C for 7 min and spread on YM agar. Vegetative cells were killed, so that the colonies arising on plates were derived only from surviving ascospores. Twelve to twenty colonies were checked for sporulation. If all or nearly all colonies show good sporulation, the strain is homothallic; otherwise, the culture is heterothallic.

Novel species delineation and identification

Analysis of the D1/D2 region of the LSU rRNA gene sequence revealed that the sequence of strain DMKU-RK359^T was closest to that of *W. ciferrii*, with 0.7 % nucleotide substitutions (four nucleotide substitutions and four gaps out of 571 nt). The sequence of the ITS region of strain DMKU-RK359^T was also analysed; it differed by 6 % nucleotide substitutions (33 substitutions and eight gaps out of 550 nt) from that of *W. ciferrii*.

The phylogenetic tree based on sequences of the D1/D2 domain of the LSU rRNA gene shows that strain DMKU-RK359^T is closely related to *W. ciferrii*, *W. edaphicus* and *Candida silvicultrix* with high bootstrap support and belongs to the *Wickerhamomyces* clade at a position distinct from the other members of the clade (Fig. 1).

Ascospores were produced by strain DMKU-RK359^T on YPD agar and 5 % malt extract agar after 7 days at 25 °C. Asci were persistent and unconjugated and formed four hat-shaped ascospores. Strain DMKU-RK359^T was found to be homothallic.

On the basis of morphological, biochemical, physiological and chemotaxonomic characteristics and sequence analysis of the D1/D2 domain of the LSU rRNA gene and ITS region, we conclude that strain DMKU-RK359^T represents a novel *Wickerhamomyces* species, for which the name *Wickerhamomyces siamensis* sp. nov. is proposed. *W. siamensis* sp. nov. is distinguished from *W. ciferrii*, *W. edaphicus* and *C. silvicultrix*, the most closely related described species, by the phenotypic characteristics shown in Table 1.

Member of *Wickerhamomyces* species are often found associated with plants; however, only a few species have been isolated from the phylloplane, such as *W. edaphicus*, which was isolated from *Terminalia catappa* L. (Limtong & Koowadjanakul, 2012). In this study, of 166 yeast strains obtained from the phylloplane of 95 samples of sugar-cane leaf, strain DMKU-RK359^T was the only strain of *W. siamensis* sp. nov. that was isolated; therefore, it is clear that the novel species is not common in the phylloplane. *Meyerozyma guilliermondii* was found to be the most

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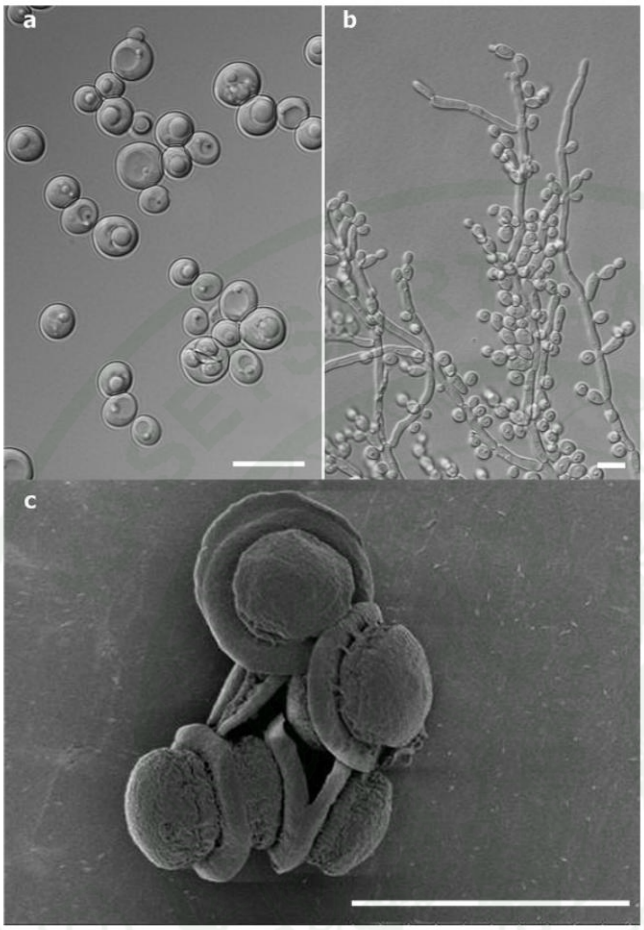


Fig. 2. Morphological analysis of *W. siamensis* sp. nov. DMKU-RK359^T. (a) Budding cells and an ascus with hat-shaped ascospores in YM broth after 3 days at 25 °C. (b) Pseudohyphae formed on PDA after 3 days at 25 °C. (c) Scanning electron micrograph of hat-shaped ascospores produced on 5 % malt extract agar after 7 days at 25 °C. Bars, 10 μm (a, b) and 5 μm (c).

common yeast species in the sugar-cane phylloplane; 25 strains of this species were obtained.

Description of *Wickerhamomyces siamensis* Kaewwichian, Kawasaki & Limtong sp. nov.

Wickerhamomyces siamensis (si.a.men′sis. N.L. fem. adj. *siamensis* of or belonging to Siam, the old name of Thailand, where the type strain was isolated).

Growth in YM broth: after 3 days at 25 °C, cells are globose, subglobose to ovoid (3–8 × 4–8 μm) and occur singly, in pairs or in groups (Fig. 2a). Budding is multilateral. Growth on YM agar: after 3 days at 25 °C, the streak culture is greyish white, smooth, soft to butyrous and has an entire margin. Pseudohyphae are formed but true hyphae are not formed in slide culture on PDA after 3 days at 25 °C (Fig. 2b). Ascospores are produced on YPD agar and 5 % malt extract agar after 7 days at 25 °C. Asci are persistent, unconjugated and formed four hat-shaped ascospores (Fig. 2c). D-Glucose, D-galactose, sucrose, maltose (weak) and raffinose (weak) are fermented but lactose, trehalose, xylose and cellobiose are not fermented. Compounds assimilated include D-glucose, D-galactose (slow), sucrose, maltose, cellobiose (weak), trehalose (weak), raffinose (weak), melezitose (slow), soluble starch (weak), D-xylose (slow), ethanol, glycerol, erythritol (weak), D-mannitol (weak), D-glucitol (weak), methyl α-D-glucoside (weak), salicin (weak), D-gluconic acid, DL-lactic acid, succinic acid, arbutin (weak), propane-1,2-diol (weak), xylitol (weak), ethylamine, L-lysine (weak) and cadaverine. Compounds that are not assimilated include L-sorbose, lactose, melibiose, inulin, L- and D-arabinose, L-rhamnose, D-ribose, *N*-acetyl-D-glucosamine, methanol, ribitol, galactitol, D-glucuronic acid, D-galacturonic acid, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, D-glucono-δ-lactone, citric acid, inositol, D-glucosamine, hexadecane, butane-2,3-diol, arabitol, nitrate and nitrite. No growth in vitamin-free medium. Growth occurs on medium containing 50 and 60 % glucose, but not on 10 % NaCl/5 %

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glucose or 16 % NaCl/5 % glucose. No growth with 0.01 or 0.1 % cycloheximide. Growth occurs at 25, 30, 35, 37, 40 and 42 °C, but not at 45 °C. Starch-like compounds are not produced. Diazonium blue B colour and urease reactions are negative. The major ubiquinone is Q-7. The DNA G+C content of the type strain is 46.2 mol%.

The holotype is DMKU-RK359^T, isolated from the phylloplane of sugar cane (*Saccharum officinarum* L.) collected from Lop Buri province, Thailand. Living cultures from the type have been deposited at the BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand, as BCC 50732^T, the NITE Biological Resources Center, Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as NBRC 108900^T, and the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, as CBS 12570^T. The Mycobank registration number is MB802886.

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