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Assessment of epiphytic yeast diversity in rice (*Oryza sativa*) phyllosphere in Thailand by a culture-independent approach

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Abstract The epiphytic yeast diversity in rice phyllosphere in Thailand was investigated by a culture-independent technique based on the RFLP pattern and the sequence of the D1/D2 domain of the large subunit rRNA gene. Forty-four samples of rice leaf were collected randomly from six provinces. The DNA was extracted from leaf washing samples and the D1/D2 domain was amplified using PCR technique. The PCR products were cloned and then screened by colony PCR. Of total 1121 clones, 451 clones (40.2 %) revealed the D1/D2 domain sequences closely related to sequences of yeasts in GenBank, and they were clustered into 45 operational taxonomic units (OTUs)

at 99 % homology. Of total yeast related clones, 329 clones (72.9 %) were identified as nine known yeast species, which consisted of 314 clones (8 OTUs) in the phylum Basidiomycota including *Bullera japonica*, *Pseudozyma antarctica*, *Pseudozyma aphidis*, *Sporobolomyces blumeae*, *Sporobolomyces carnicolor* and *Sporobolomyces oryzicola* and 15 clones (6 OTUs) in the phylum Ascomycota including *Metschnikowia koreensis*, *Meyerozyma guilliermondii* and *Wickerhamomyces anomalus*. The D1/D2 sequences (122 clones) that could not be identified as known yeast species were closest to 3 and 14 species in Ascomycota and Basidiomycota, respectively, some of which may be new yeast species. The most predominant species detected was *P. antarctica* (42.6 %) followed by *B. japonica* (25.9 %) with 63.6 and 22.7 % frequency of occurrence, respectively. The results of OTU richness of each sampling location revealed that climate condition and sampling location could affect epiphytic yeast diversity in rice phyllosphere.

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Rice · rRNA gene

Introduction

Rice is a monocotyledonous plant of the family Poaceae. Rice is one of the most widely produced and consumed foods in the world, making its

cultivation of vital global social and economic importance. In Thailand, rice (*Oryza sativa*) is the main economic crop which is mostly cultivated in north, northeast and central Thailand. During the period 2013–2014 the rice cultivation area was approximately 12.64 million hectares and grain production approximately 38.24 million tons as reported by the Department of Rice, Ministry of Agriculture and Cooperative, Thailand.

Phyllosphere or phylloplane describes the above ground surfaces of the plant as habitat for microorganisms; the phyllosphere is dominated by the leaves. The total surface area available for microbial colonization has been estimated to be about $2\text{--}6 \times 10^8 \text{ km}^2$ (Morris 2001; Lindow and Brandl 2003). Microbial epiphytes including bacteria, yeasts and filamentous fungi can inhabit the phyllosphere by using leaf surface nutrients as carbon sources including carbohydrates, amino acids, organic acids, sugar alcohols and salts (Tukey 1970; Fiala et al. 1990; Weibull et al. 1990; Dik et al. 1991; Leveau and Lindow 2001). In addition, the epiphytes have been reported to produce plant hormones which promote cell wall loosening and release of saccharides from plant cells as substrates (Fry 1989; Brandl and Lindow 1998; Lindow and Brandl 2003). Furthermore, it has been reported that epiphytes may produce biosurfactants which facilitate wettability and further enhance the leaching of substrates (Bunster et al. 1989; Schreiber et al. 2005). The abundance of available nutrients is dependent on leaf age, plant species and growing conditions all of which shape microbial diversity in the phyllosphere. Moreover, environmental factors including radiation, pollution and nitrogen fertilization, plus biotic factors including leaf age and competing microorganisms all affect microbial diversity (Irvine et al. 1978; Jumpponen and Jones 2010; Zimmerman and Vitousek 2012).

The diversity of microorganisms in the environment has been evaluated by culture-dependent methods, however a major obstacle has been unculturable or slow growing strains which may lead to exclusion of important members of the composting microbial community (Amann et al. 1995; Takaku et al. 2006). Therefore recently culture-independent methods have been applied to more accurately elucidate microbial diversity (Su et al. 2012). Over the past 20 years, molecular ecological methods based on the direct amplification and analyses of ribosomal RNA genes have been developed for investigations of microbial

communities in diverse environments such as denaturing gradient gel electrophoresis (DGGE) (Muyzer 1999; Liu et al. 2002), temperature gradient gel electrophoresis (TGGE) (Muyzer 1999), restriction fragment length polymorphisms (RFLP) (Esteve-Zarzoso et al. 1999), terminal restriction fragment length polymorphisms (T-RFLP) (Schutte et al. 2008), random amplified polymorphic DNA (RAPD) (Chiocchetti et al. 1999), amplified fragment length polymorphism (AFLP) (de Barros Lopes et al. 1999), quantitative PCR (qPCR) (Hierro et al. 2006) single-strand-conformation polymorphism (SSCP) (Peters et al. 2000) and capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) (Martins et al. 2014). Meanwhile, alternative molecular techniques such as fluorescence in situ hybridization (FISH) (Moter and Gobel 2000) and micro arrays (Bodrossy and Sessitsch 2004) have successfully been used for the identification of environmental microorganisms. Moreover, the high-throughput sequencing in combination with DNA tagging were utilized efficiently in evaluation of fungal communities (Jumpponen and Jones 2009).

The most numerous microorganisms colonising the phyllosphere are bacteria. However yeast and filamentous fungi are often considered active inhabitants of leaf surfaces (Vorholt 2012). Most studies identifying microorganisms in the phyllosphere have focused on bacteria. In recent years the diversity of yeast communities in the phyllosphere has been studied intensively, however most of which were culture-dependent. Additionally, only a small number of articles have focused on yeasts that colonize the phyllosphere of monocotyledonous plants (Nakase et al. 2001; Limtong and Kaewwichian 2014) and very few of those investigating the diversity of epiphytic yeasts from the phyllosphere were undertaken with samples from Thailand. Therefore, this study is the first research aimed at investigating the diversity of epiphytic yeasts of rice phyllosphere in Thailand by using a culture-independent technique.

Materials and methods

Sample collection

Forty-four samples of rice (*Oryza sativa*) leaf were randomly collected from rice fields in six provinces in Thailand between October 2011 and March 2012

(Table 1). Leaf samples were put in plastic bags, sealed and kept in an ice-box before transferring to the laboratory. The samples were stored at 4 °C until used. DNA was extracted from the leaf washing of the samples within a maximum of 7 days post collection.

DNA extraction of epiphytes

Leaf samples (6 g) were cut and submerged in washing buffer (1X Phosphate buffered saline, Tween 20), sonicated for 7 min using ultrasonic cleaning bath (Bransonic, USA) to dislodge microbes from sample surfaces. The leaf washing was centrifuged at 5000×g for 5 min and the supernatant was discarded. For rapid extraction of DNA from cell pellets the method of Makimura et al. (Makimura et al. 1994) with slight modification was performed. Briefly, the cell pellet was suspended in 100 µl of lysis buffer (100 mM Tris–HCl, pH 7.5, 0.5 % w/v SDS, 30 mM EDTA) and incubated at 95 °C for 15 min. A 100 µl of 2.5 M potassium acetate was added and incubated on ice for 60 min, then centrifuged at 12,000×g for 5 min. The supernatant was transferred to new tube and DNA precipitated with an equal volume of isopropanol, then washed with 70 % ethanol. The precipitated DNA was air dried, resuspended in 30 µl of TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0) and stored at –20 °C.

PCR amplification of D1/D2 domain of large subunit (LSU) rRNA gene

PCR amplification of D1/D2 domain of LSU rRNA gene was performed using the following primers: NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTC AAG ACG G-3')

(Kurtzman and Robnett 1998). The amplification was carried out in 25 µl of the reaction mixture containing 10–100 ng of genomic DNA, 1X PCR buffer (Vivantis, USA), 1.5 mM MgCl₂ (Vivantis, USA), 100 µM of each dNTP (Vivantis, USA), 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Vivantis, USA). The PCR reactions were performed on a Geneamp PCR system 2400 (Perkin Elmer, USA). The reaction conditions were 94 °C for 5 min; 35 cycles of 45 s at 94 °C, 30 s at 52 °C, and 45 s at 72 °C; and a final extension at 72 °C for 10 min. PCR products with expected size of 600 bp were visualized on 1 % agarose gel (w/v) and purified by Nucleic Acid Extraction Kit (Vivantis, USA).

PCR cloning and screening

The purified PCR products were ligated into pTG19-T cloning vector (Vivantis, USA) performed using manufacturer's recommended protocol, and transformed into *E.coli* DH5α competent cells. The recombinant clones were screened by colony PCR using the primers described above. The reaction mixture was manipulated as described above except that cell suspension (2 µl) was used as template. The reaction conditions were 94 °C for 10 min; 35 cycles of 45 s at 94 °C, 30 s at 52 °C, and 45 s at 72 °C; and a final extension at 72 °C for 10 min. PCR products with expected size of 600 bp were visualized on 1 % agarose gel (w/v).

RFLP analyses

PCR products of the expected size were analyzed using RFLP after enzymatic digestion with the

Table 1 Rice leaf samples collected from six provinces in Thailand

Region	Province	Location	Sampling period	No. of samples	Average atmospheric temperature (°C)	Average rainfall (mm.)
East	Chachoengsao	13°41'15"N, 101°4'13"E	Dec 2011	6	28.1	3.4
Central	Nakhon Nayok	14°12'44"N, 101°12'6"E	Dec 2011	3	25.1	0.8
	Nakhon Pathom	13°49'14"N, 100°3'45"E	Jan 2012	13	27.3	19.9
	Suphanburi	14°28'3"N, 100°7'1"E	Mar 2012	9	30.0	29.9
North-East	Sisaket	15°7'12"N, 104°19'18"E	Oct 2011	10	26.4	182.5
	Surin	14°52'48"N, 103°29'24"E	Oct 2011	3	26.4	182.5

restriction enzymes, *Hae*III, *Hinf*I and *Cfo*I. The restriction fragment patterns were visualized using a 2 % agarose gel (w/v). The RFLP patterns were clustered and the representatives of the PCR products of each pattern from each clone libraries were further purified and sequenced.

Sequencing and phylogenetic analyses

The DNA sequencing was performed by Sanger dideoxy sequencing with four colour fluorescence using NL1 primer and an ABI Prism 3730XL DNA sequencer (Applied Bioscience, USA) at the DNA sequencing services, Bio Basic Inc. (Ontario, Canada). The sequences of the D1/D2 domain were submitted to BLASTn homology search (Altschul et al. 1997). The collection of taxonomically related type sequences and related matches were obtained from GenBank for further analyses. The sequences were aligned with related species using CLUSTALW (Thompson et al. 1994). The GenBank accession numbers of related yeast type strain sequences are AF444760 (*Bullera japonica*), AF075525 (*Cryptococcus cellulolyticus*), AF189834 (*Cryptococcus curvatus*), AB035042 (*Cryptococcus flavescens*), AF075497 (*Cryptococcus flavus*), DQ00317 (*Cryptococcus heimaeyensis*), AF075525 (*Cryptococcus laurentii*), AF189884 (*Hannaella sinensis*), AJ965480 (*Hannaella zae*), AF189977 (*Sporidiobolus pararoseus*), AB279628 (*Sporobolomyces carnicolor*), AF189990 (*Sporobolomyces oryzaicola*), AJ235302 (*Pseudozyma antarctica*), AB089363 (*Pseudozyma aphidis*), DQ008953 (*Pseudozyma hubeiensis*), AF296438 (*Metschnikowia koreensis*), U45709 (*Meyerozyma guilliermondii*) and U74592 (*Wickerhamomyces anomalus*).

The recombinant clones of the D1/D2 domain sequences were clustered into operational taxonomic units (OTUs) at 99 % homology using the furthest neighbour approach of mothur v.1.10 (Schloss et al. 2009). The sequence analysis regarding to the number of the nucleotide substitution compared to the D1/D2 sequences of yeast type strains were also considered (Kurtzman and Robnett 1998; Fell et al. 2000).

A phylogenetic tree was constructed from the evolutionary distance data using Kimura 2-parameter model (Kimura 1980) and the neighbor-joining method (Saitou and Nei 1987) performed with MEGA 6 (Tamura et al. 2013). All positions containing gaps were eliminated. Confidence level of the clades was estimated using bootstrap analysis (1000 replicates). Yeast related

sequence data have been submitted to the GenBank database under accession numbers KJ937682, KJ937684–KJ937698, KJ937700–KJ937728, KJ937730–KJ937750, KJ937752–KJ937781, KM078919–KM078921 and KM999142–KM999219.

Data analyses

Diversity indices were calculated using the Shannon–Wiener index (H') and Shannon equitability (E_H);

$$H' = - \sum_{i=0}^S P_i (\ln P_i)$$

where P_i is the ratio of the number of clones of the i th OTU to the total number of clones.

$$E_H = H' / \ln S$$

where S is the total number of OTUs in the total samples of rice phyllosphere.

Equitability assumes a value between 0 and 1. A value close to 1 means that the sample is diverse, while a value close to 0 means that the sample is not diverse.

Relative frequency (%) was calculated as the number of clones of a particular OTU as a proportion of the total number of clones.

Frequency of occurrence (%) was calculated as the number of samples, where a particular OTU was observed, as a proportion of the total number of samples.

Statistical data analyses

Comparisons of multiple means with standard deviations (SDs) obtained from OTU richness, the number of OTUs of each sample, in association with different provinces were performed by StatPlus v2009 (AnalystSoft Inc., USA), with one-way analysis of variance (ANOVA) and Fisher LSD tests at appropriate significant level of $P = 0.05$ or lower.

Results and discussion

Yeast identification and phylogenetic analyses

Genomic DNA from the cell pellets of leaf washings of 44 samples of rice phyllospheres was successfully

isolated. The amplified PCR products of the D1/D2 domain of LSU rRNA gene were cloned. A total of 1121 clones were obtained from 44 clone libraries. The representative sequences (621 sequences) of each library as a result of restriction analysis were sequenced. Among 1121 clones, 53.3 % (405 sequences, 598 clones) were related to filamentous fungi, 40.2 % (176 sequences, 451 clones) were related to yeasts and 6.4 % (40 sequences, 72 clones) were of unknown origin, the sequences with no or low significant similarity found in database. For the purposes of this study we focused on yeasts present in the rice phyllosphere samples.

Ascomycetous yeasts were identified by analysis of areas of homology for the D1/D2 domain of LSU rRNA gene sequences and those of type strains. Sequences showing greater than 1 % nucleotide substitutions (6 nucleotides) in the ca. 600-nucleotide D1/D2 domain are likely to be of different species and that strains with 0–3 nucleotide differences are either conspecific or sister species (Kurtzman and Robnett 1998). According to the suggestion of Fell et al. (2000) for basidiomycetous yeasts, strains that differ by two or more nucleotides in the D1/D2 region, represent different taxa. Therefore to identify yeasts species present in this study the numbers of nucleotide substitutions within the D1/D2 domain were also considered according to ascomycetous and basidiomycetous yeast identification suggested above.

A total of 176 sequences of 451 clones related to yeasts were classified into 45 OTUs by 99 % similarity cut off. The sequences under the same OTUs that had different number of nucleotide substitutions were distinguished and identified (Table 2). The basidiomycetous yeasts (96 %) were the major components of the clone libraries, in contrast to only 4 % of the ascomycetous yeasts. The total clones were classified into 4 yeast subphylum, three were under phylum Basidiomycota (Agaricomycotina, Pucciniomycotina and Ustilaginomycotina) and one under phylum Ascomycota (Saccharomycotina). The majority of yeast related clones were in subphylum Ustilaginomycotina (60.0 %) followed by Agaricomycotina (33.7 %). The number of clones classified in Saccharomycotina and Pucciniomycotina were only 4.0 and 2.2 %, respectively. As can be seen, some sequences under the same OTUs had different identification results according to the number of nucleotide substitutions (see OTU1, OTU23, OTU31). The sequences that differ by more

than 1 nucleotide substitution in the case of basidiomycetous yeasts and 6 nucleotide substitutions in the ascomycetous yeasts were treated as different species in this study. 329 out of 451 clones (72.9 %) were precisely identified into nine known yeast species in six genera. Of these total, 314 clones (under 8 OTUs) were identified as basidiomycetous yeasts including *B. japonica*, *P. antarctica*, *P. aphidis*, *Sporobolomyces blumeae*, *S. carnicolor* and *S. oryzzicola*. Other 15 clones (under 6 OTUs) were identified as known ascomycetous yeast species including *M. koreensis*, *M. guilliermondii* and *W. anomalus*.

The sequences (119 clones) of basidiomycetous yeasts that could not have been identified to species level regarding to the substitution numbers were closest to 14 yeast species. These included *B. japonica*, *C. cellulolyticus*, *C. curvatus*, *C. flavescens*, *C. flavus*, *C. heimaeyensis*, *C. laurentii*, *H. sinensis*, *Hannaela zae*, *P. antarctica*, *P. aphidis*, *P. hubeiensis*, *S. pararoseus* and *S. carnicolor*. For their identification to species level further study such as ITS sequence analysis is needed. Some of these sequences may represent the sequences of new yeast taxa. The sequences (3 clones) of ascomycetous yeasts which differed by more than 6 nucleotide substitutions represented new yeast species closest to *M. koreensis*, *M. guilliermondii* and *W. anomalus*.

The most frequently detected OTU from the clone libraries was OTU23 (47.2 %, 213 clones) closest to *P. antarctica* followed by OTU1 (27.5 %, 124 clones) closest to *B. japonica* and OTU31 (8.2 %, 37 clones) closest to *P. aphidis* (Table 2). Other OTUs were detected within the clone libraries but showed low relative frequencies (0.2–1.8 %). The relative frequencies of the total number of clones from all OTUs identified as *P. antarctica* and *B. japonica* were 42.6 % (192 clones) and 25.9 % (117 clones), respectively.

Phylogenetic trees based on analysis of the D1/D2 domain sequences of subphylum Agaricomycotina (Fig. 1), Pucciniomycotina (Fig. 2), Ustilaginomycotina (Fig. 3) and Saccharomycotina (Fig. 4) were constructed. The phylogenetic tree of OTUs in the subphylum Agaricomycotina illustrated that the sequences with high number in substitutions (differ by 6 nucleotides) such as OTU3, OTU4, OTU5, OTU6, OTU7, OTU8, OTU9, OTU11, OTU12, OTU13, OTU15, OTU16, OTU19 and OTU20 were confirmed of long distance relation to the closest strains. These

Table 2 Yeast identification for 45 OTUs based on BLAST analysis and their frequency of occurrence (%) from rice phyllospheres

OTU	No. of clones	Closest yeast species	Nucleotide substitution (gap) ^a	Identity (%)	Identification	Frequency of occurrence (%)
<i>Phylum Basidiomycota</i>						
<i>Subphylum Agarinomycotina</i>						
1	40	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	0 (0–2)	99.7–100	<i>Bullera japonica</i>	13.6
	77	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	1 (0–3)	99.5–99.8	<i>Bullera japonica</i>	18.2
	3	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	2 (0, 3)	99.1–99.6	Closest to <i>Bullera japonica</i>	6.8
	4	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	3 (1)	99.1–99.3	Closest to <i>Bullera japonica</i>	4.5
2	1	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	4 (0)	99.3	Closest to <i>Bullera japonica</i>	2.3
3	1	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	19 (4)	96.1	Closest to <i>Bullera japonica</i>	2.3
4	1	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	20 (1)	96.4	Closest to <i>Bullera japonica</i>	2.3
5	1	<i>Bullera japonica</i> CBS 2013 ^T (AF444760)	35 (10)	92.0	Closest to <i>Bullera japonica</i>	2.3
6	1	<i>Cryptococcus cellulolyticus</i> CBS 8294 ^T (AF075525)	52 (4)	90.1	Closest to <i>Cryptococcus cellulolyticus</i>	2.3
7	1	<i>Cryptococcus curvatus</i> CBS570 ^T (AF189834)	6 (2)	98.6	Closest to <i>Cryptococcus curvatus</i>	2.3
	1	<i>Cryptococcus curvatus</i> CBS570 ^T (AF189834)	7 (2)	98.5	Closest to <i>Cryptococcus curvatus</i>	2.3
	1	<i>Cryptococcus curvatus</i> CBS570 ^T (AF189834)	8 (1)	98.5	Closest to <i>Cryptococcus curvatus</i>	2.3
8	2	<i>Cryptococcus curvatus</i> CBS570 ^T (AF189834)	6 (1–2)	98.6	Closest to <i>Cryptococcus curvatus</i>	2.3
9	1	<i>Cryptococcus flavescens</i> CBS 942 ^T (AB035042)	3 (1)	99.3	Closest to <i>Cryptococcus flavescens</i>	2.3
10	8	<i>Cryptococcus flavus</i> CBS 331 ^T (AF075497)	3 (0–1)	99.1–99.5	Closest to <i>Cryptococcus flavus</i>	9.1
11	1	<i>Cryptococcus heimaeyensis</i> CBS 8933 ^T (DQ00317)	48 (11)	89.8	Closest to <i>Cryptococcus heimaeyensis</i>	2.3
12	2	<i>Cryptococcus laurentii</i> CBS139 ^T (AF075525)	23 (10)	94.4	Closest to <i>Cryptococcus laurentii</i>	4.5
13	1	<i>Hannaella sinensis</i> CBS7238 ^T (AF189884)	50 (15)	88.6	Closest to <i>Hannaella sinensis</i>	2.3
14	1	<i>Hannaella zeae</i> HB 1207 ^T (AJ965480)	7 (0)	98.8	Closest to <i>Hannaella zeae</i>	2.3
	1	<i>Hannaella zeae</i> HB 1207 ^T (AJ965480)	8 (0)	98.6	Closest to <i>Hannaella zeae</i>	2.3
	1	<i>Hannaella zeae</i> HB 1207 ^T (AJ965480)	9 (1)	98.2	Closest to <i>Hannaella zeae</i>	2.3
15	1	<i>Hannaella zeae</i> HB 1207 ^T (AJ965480)	53 (6)	88.7	Closest to <i>Hannaella zeae</i>	2.3
16	1	<i>Hannaella zeae</i> HB 1207 ^T (AJ965480)	54 (13)	87.7	Closest to <i>Hannaella zeae</i>	2.3

Table 2 continued

OTU	No. of clones	Closest yeast species	Nucleotide substitution (gap) ^a	Identity (%)	Identification	Frequency of occurrence (%)
Subphylum Pucciniomycotina						
17	1	<i>Sporidiobolus pararoseus</i> CBS491 ^T (AF189977)	3 (2)	99.1	Closest to <i>Sporidiobolus pararoseus</i>	2.3
18	3	<i>Sporidiobolus pararoseus</i> CBS491 ^T (AF189977)	5 (0–1)	98.9–99.1	Closest to <i>Sporidiobolus pararoseus</i>	2.3
19	1	<i>Sporobolomyces blumeae</i> CBS 9094 ^T (AB279628)	0 (0)	100	<i>Sporobolomyces blumeae</i>	2.3
20	2	<i>Sporobolomyces carnicolor</i> CBS 4215 ^T (AY070008)	1 (0)	99.8	<i>Sporobolomyces carnicolor</i>	2.3
	1	<i>Sporobolomyces carnicolor</i> CBS 4215 ^T (AY070008)	2 (0)	99.6	Closest to <i>Sporobolomyces carnicolor</i>	2.3
21	1	<i>Sporobolomyces carnicolor</i> CBS 4215 ^T (AY070008)	5 (1)	98.8	Closest to <i>Sporobolomyces carnicolor</i>	2.3
22	1	<i>Sporobolomyces oryzicola</i> CBS7228 ^T (AF189990)	0 (1)	99.7	<i>Sporobolomyces oryzicola</i>	2.3
Subphylum Ustilaginomycotina						
23	117	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	0 (0–2)	99.6–100	<i>Pseudozyma antarctica</i>	54.5
	70	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	1 (0–2)	99.5–99.8	<i>Pseudozyma antarctica</i>	34.1
21	21	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	2 (0–2)	99.3–99.6	Closest to <i>Pseudozyma antarctica</i>	25.0
	5	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	3 (0–3)	98.9–99.3	Closest to <i>Pseudozyma antarctica</i>	6.8
24	1	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	1 (17)	96.7	<i>Pseudozyma antarctica</i>	2.3
25	4	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	1 (2)	99.5	<i>Pseudozyma antarctica</i>	2.3
26	1	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	2 (0)	99.6	Closest to <i>Pseudozyma antarctica</i>	2.3
27	1	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	2 (2)	99.3	Closest to <i>Pseudozyma antarctica</i>	2.3
28	1	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	5 (1)	99.0	Closest to <i>Pseudozyma antarctica</i>	2.3
29	1	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	7 (0)	98.9	Closest to <i>Pseudozyma antarctica</i>	2.3
30	2	<i>Pseudozyma antarctica</i> CBS 214.83 ^T (AJ235302)	7 (1)	98.6	Closest to <i>Pseudozyma antarctica</i>	2.3
31	1	<i>Pseudozyma aphidis</i> JCM 10318 (AB089363)	1 (0)	99.8	<i>Pseudozyma aphidis</i>	2.3
	21	<i>Pseudozyma aphidis</i> JCM 10318 (AB089363)	2 (1)	99.5	Closest to <i>Pseudozyma aphidis</i>	4.5
5	5	<i>Pseudozyma aphidis</i> JCM 10318 (AB089363)	3 (0, 2)	99.1–99.4	Closest to <i>Pseudozyma aphidis</i>	6.8
	10	<i>Pseudozyma aphidis</i> JCM 10318 (AB089363)	4 (1)	99.1	Closest to <i>Pseudozyma aphidis</i>	4.5
32	1	<i>Pseudozyma aphidis</i> JCM 10318 ^T (AB089363)	6 (1)	98.8	Closest to <i>Pseudozyma aphidis</i>	2.3

Table 2 continued

OTU	No. of clones	Closest yeast species	Nucleotide substitution (gap) ^a	Identity (%)	Identification	Frequency of occurrence (%)
33	4	<i>Pseudozyma aphidis</i> JCM 10318 ^T (AB089363)	41 (5)	91.8	Closest to <i>Pseudozyma aphidis</i>	2.3
34	3	<i>Pseudozyma aphidis</i> JCM 10318 ^T (AB089363)	44 (7)	91.0	Closest to <i>Pseudozyma aphidis</i>	2.3
35	1	<i>Pseudozyma aphidis</i> JCM 10318 ^T (AB089363)	45 (3)	91.4	Closest to <i>Pseudozyma aphidis</i>	2.3
36	1	<i>Pseudozyma hubeiensis</i> CBS 10077 ^T (DQ008953)	4 (1)	99.2	Closest to <i>Pseudozyma hubeiensis</i>	2.3
<i>Phylum Ascomycota</i>						
Subphylum Saccharomycotina						
37	1	<i>Metschnikowia koreensis</i> KCTC 7998T (AF296438)	1 (2)	99.4	<i>Metschnikowia koreensis</i>	2.3
38	1	<i>Metschnikowia koreensis</i> KCTC 7998T (AF296438)	1 (1)	99.6	<i>Metschnikowia koreensis</i>	2.3
39	1	<i>Metschnikowia koreensis</i> KCTC 7998T (AF296438)	13 (2)	97.0	Closest to <i>Metschnikowia koreensis</i>	2.3
40	3	<i>Meyerozyma guilliermondii</i> NRRL Y-2075T (U45709)	2 (1)	99.5	<i>Meyerozyma guilliermondii</i>	2.3
	1	<i>Meyerozyma guilliermondii</i> NRRL Y-2075T (U45709)	3 (1)	99.3	<i>Meyerozyma guilliermondii</i>	2.3
41	1	<i>Meyerozyma guilliermondii</i> NRRL Y-2075T (U45709)	17 (4)	95.9	Closest to <i>Meyerozyma guilliermondii</i>	2.3
42	1	<i>Wickerhamomyces anomalus</i> NRRL Y-366T (U74592)	0 (1)	99.8	<i>Wickerhamomyces anomalus</i>	2.3
	1	<i>Wickerhamomyces anomalus</i> NRRL Y-366T (U74592)	1 (1)	99.6	<i>Wickerhamomyces anomalus</i>	2.3
	3	<i>Wickerhamomyces anomalus</i> NRRL Y-366T (U74592)	2 (1)	99.5	<i>Wickerhamomyces anomalus</i>	4.5
43	3	<i>Wickerhamomyces anomalus</i> NRRL Y-366T (U74592)	3 (1)	99.3	<i>Wickerhamomyces anomalus</i>	2.3
44	1	<i>Wickerhamomyces anomalus</i> NRRL Y-366T (U74592)	3 (2)	99.1	<i>Wickerhamomyces anomalus</i>	2.3
45	1	<i>Wickerhamomyces anomalus</i> NRRL Y-366T (U74592)	20 (6)	95.3	Closest to <i>Wickerhamomyces anomalus</i>	2.3

^a Number of nucleotides that does not match with the closest yeast sequence subtract with number of gaps

sequences may represent the sequences of new yeast taxa. Other OTUs with lower numbers of substitutions such as OTU1, OTU2, OTU9 and OTU10 with 2-4 substitutions were closely related to the type strains seen in the phylogenetic tree however OTUs could not have been identified by only one region in DNA. The similar interpretation could be described for the phylogenetic results in subphylum Pucciniomycotina (Fig. 2), Ustilaginomycotina (Fig. 3) and Saccharomycotina (Fig. 4).

Frequency of occurrence and diversity of epiphytic yeasts

The frequency of occurrence of yeast OTUs within the 44 rice samples (Table 2) showed that OTU23, which was identified as *P. antarctica*, was commonly found in rice samples followed by OTU1 identified as *B. japonica*. Other OTUs occurred but at lower frequencies of occurrence. Of the yeast species detected *P. antarctica* and *B. japonica* were the most common

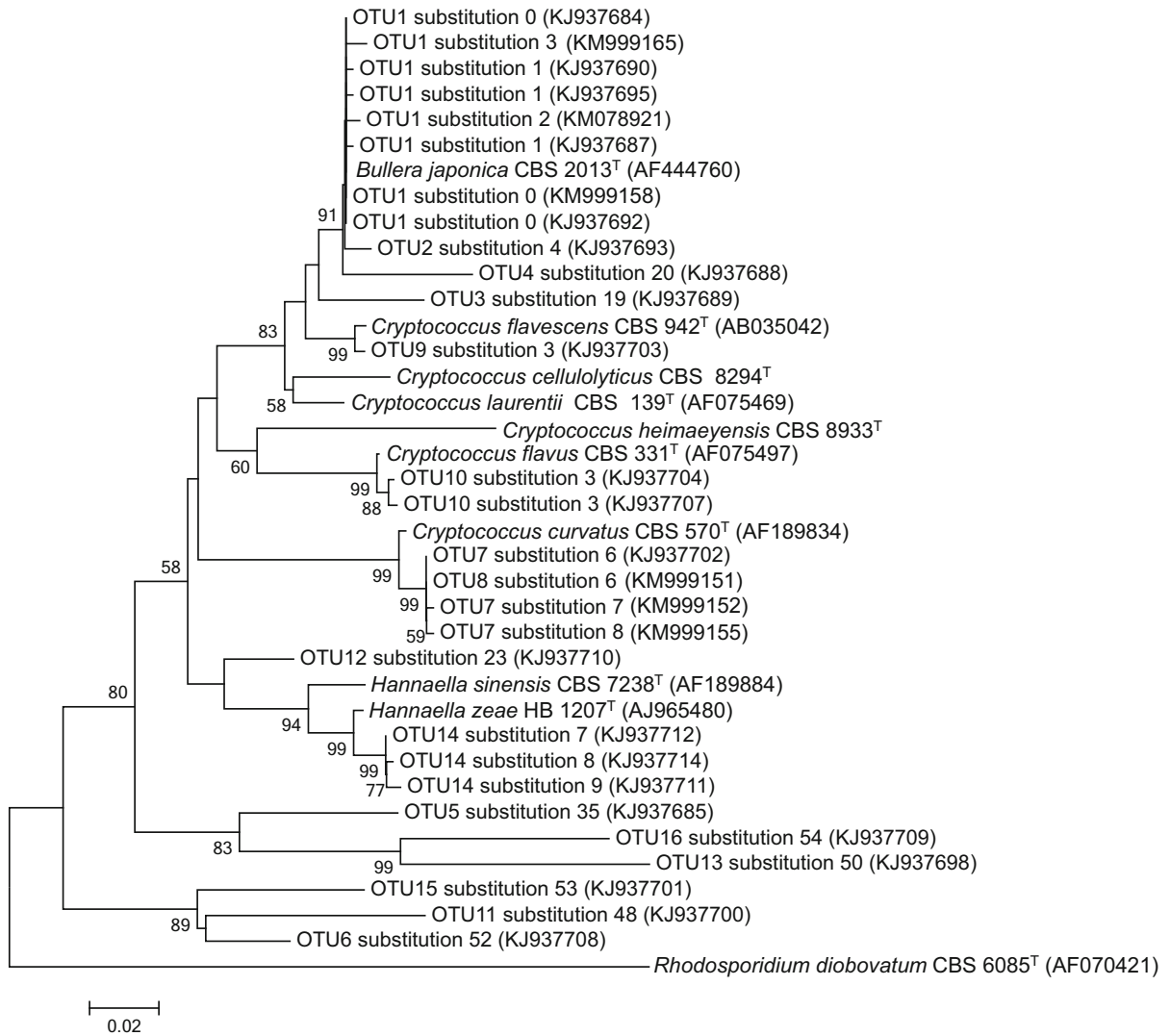


Fig. 1 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Agarinomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model.

Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The scale bar corresponds to a genetic distance of 0.02 substitutions per position

with 63.6 % (28 samples) and 22.7 % (10 samples) frequencies of occurrence, respectively. Other species were less frequently detected with less than 10 % frequency of occurrence.

The average OTU richness values ranged from 1 to 3 over 6 provinces (Fig. 5). This figure showed that there were significant differences between the values of OTU richness for samples collected in different provinces. The OTU richness of the samples collected in Chachoeangsao (CC, 1.00 ± 0.89) was lowest and was significantly different from those of Surin (SR,

3.67 ± 1.00 , $P = 0.007$), Nakhon Nayok (NN, 3.00 ± 1.00 , $P = 0.039$) and Nakhon Pathom (NP, 3.08 ± 1.71 , $P = 0.003$) except Suphanburi (SP, 2.11 ± 1.05 , $P = 0.89$) and Sisaket (SK, 1.5 ± 1.08 , $P = 0.312$) which showed no significant difference. In addition, those of the samples collected in Sisaket was also significantly different from those of Surin ($P = 0.030$) and Nakhon Pathom ($P = 0.018$). These results revealed that the OTU richness of the rice samples collected in Chachoeangsao and Sisaket provinces were significantly lower than other provinces. These could

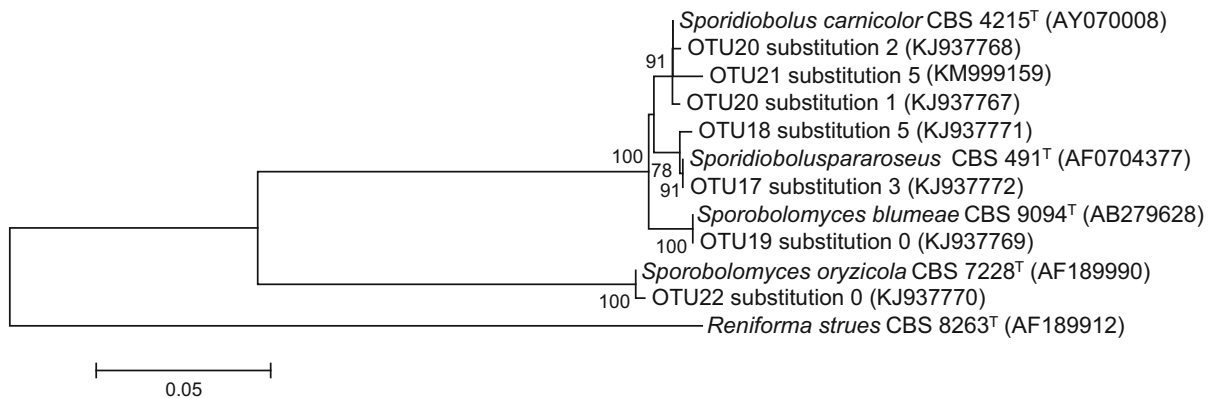


Fig. 2 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Pucciniomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model.

Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The *scale bar* corresponds to a genetic distance of 0.05 substitutions per position

suggest that the richness of yeast strains varied by sampling locations. However, the inter-provincial variation was not the focus of this study.

The diversity index and equitability of yeast evaluated in this study were calculated based on the identification results according to nucleotide substitution numbers. Therefore the sequences that could not be identified at the species level were distinguished as different genotypes in those particular OTUs. Thus, we could separate these sequences into 57 genotypes. The Shannon–Weiner index and equitability of yeast genotypes detected in rice samples were 2.191 and 0.542, respectively, as shown in Table 3.

Discussion

This is the first study that we are aware of that has used a culture-independent molecular approach to determine the diversity of epiphytic yeasts in rice (*Oryza sativa*) phyllosphere in Thailand. The basidiomycetous yeasts were the major components of the clone libraries in our study. These results for rice are in accordance with a previous study in Moscow by Glushakova and Chernov (2010), which reported that the number of basidiomycetous yeasts were greater than those of ascomycetous yeasts on the phyllosphere of plants. This study was performed using a culture-dependent technique and yeasts were identified based on morphological, physiological characteristics and the analysis of the rDNA ITS1-5.8S-ITS2 and D1/D2 nucleotide sequences.

Additionally, the basidiomycetous yeasts have been reported as the dominant yeasts in previous studies of other plants and with alternative methodologies. For instance, the study of yeast communities associated with sugarcane in Rio de Janeiro, Brazil showed that the prevalent species were basidiomycetous yeasts including *C. laurentii*, *Cryptococcus albidus* and *Rhodotorula mucilaginosa* (de Azeredo et al. 1998). It has been postulated that the yeast species most commonly found on monocotyledonous plants are from the phylum Basidiomycota (Spencer and Spencer 1997). The ballistoconidium-fall isolation method was used to isolate ballistoconidium-forming yeasts from dead leaves and stems of rice (*Oryza sativa* L.) in Japan. The isolated yeasts belonged to the genera *Bullera*, *Sporobolomyces* and *Tilletopsis* (Nakase and Suzuki 1985). The common ballistosporous yeasts isolated from rice leaf and other plants collected in the Asia-Pacific region were the genera *Bensingtonia*, *Bullera*, *Kockovaella*, *Sporidiobolus* *Sporobolomyces*, *Tilletiopsis* and *Udeniomyces* (Nakase 2000). A year later Nakase et al. (2001) also reported that the basidiomycetous yeasts isolated from plant phyllosphere collected in Thailand by using the ballistoconidium-fall isolation method, were identified as 21 species in six genera including *Bensingtonia*, *Bullera*, *Kockovaella*, *Sporidiobolus*, *Sporobolomyces* and *Tilletiopsis*. In the present study we also detected the common ballistosporous yeasts in the genera *Bullera* and *Sporobolomyces* by using culture-independent method from rice phyllosphere in Thailand.

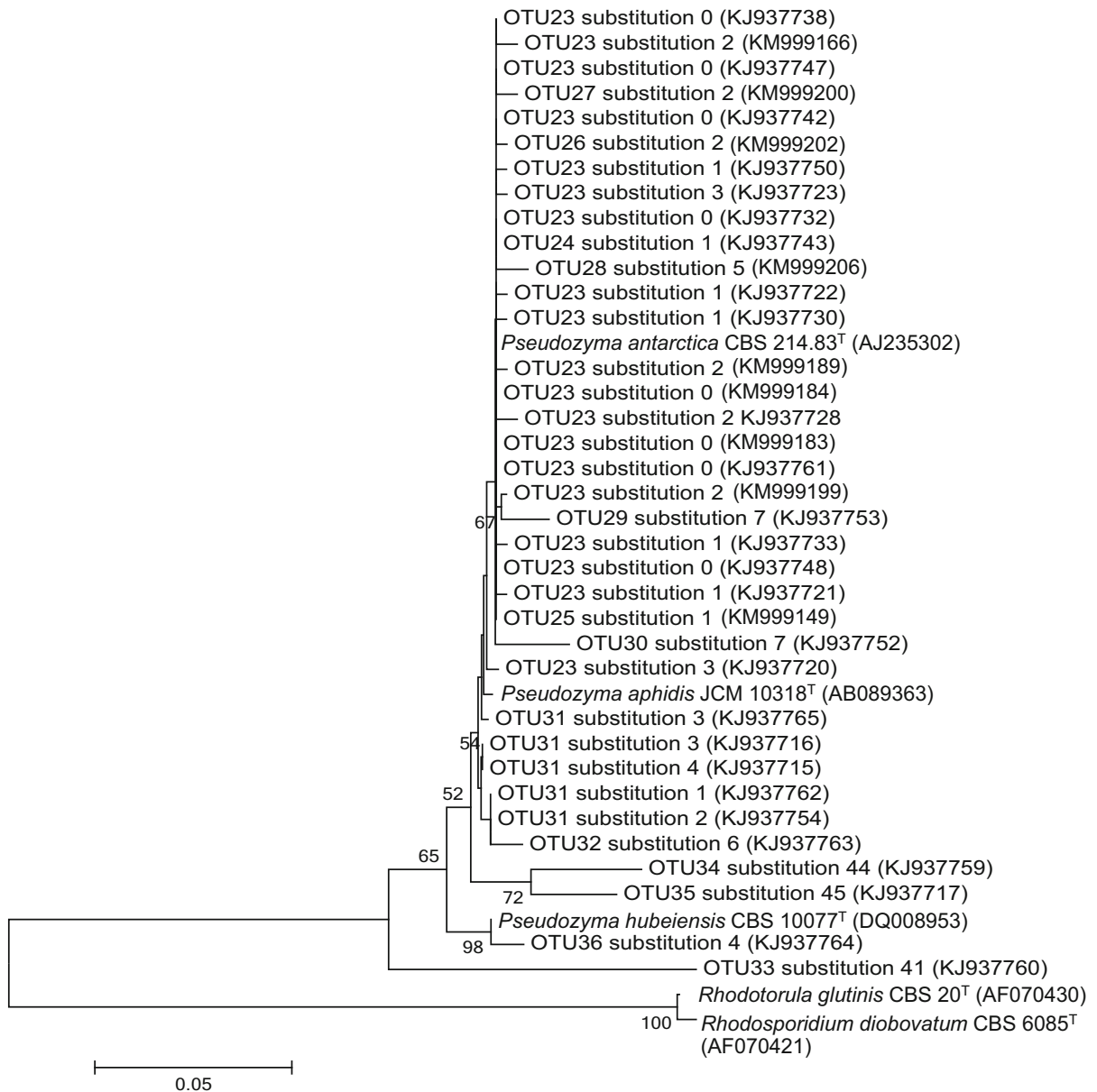


Fig. 3 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Ustilaginomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model.

Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The scale bar corresponds to a genetic distance of 0.05 substitutions per position

Although the most common phyllosphere yeasts are basidiomycete yeast species various ascomycete yeast species have also been isolated from the phyllosphere such as *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Kazachstania barnettii*, *Metschnikowia lophuriensis*, *Metschnikowia pulcherrima*, *Metschnikowia 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. 2007; Glushakova and Chernov 2010; Landell et al. 2010; Koowadjanakul et al. 2011; Kaewwichian et al. 2012). The ascomycetous yeasts were mostly detected in phyllosphere when using the

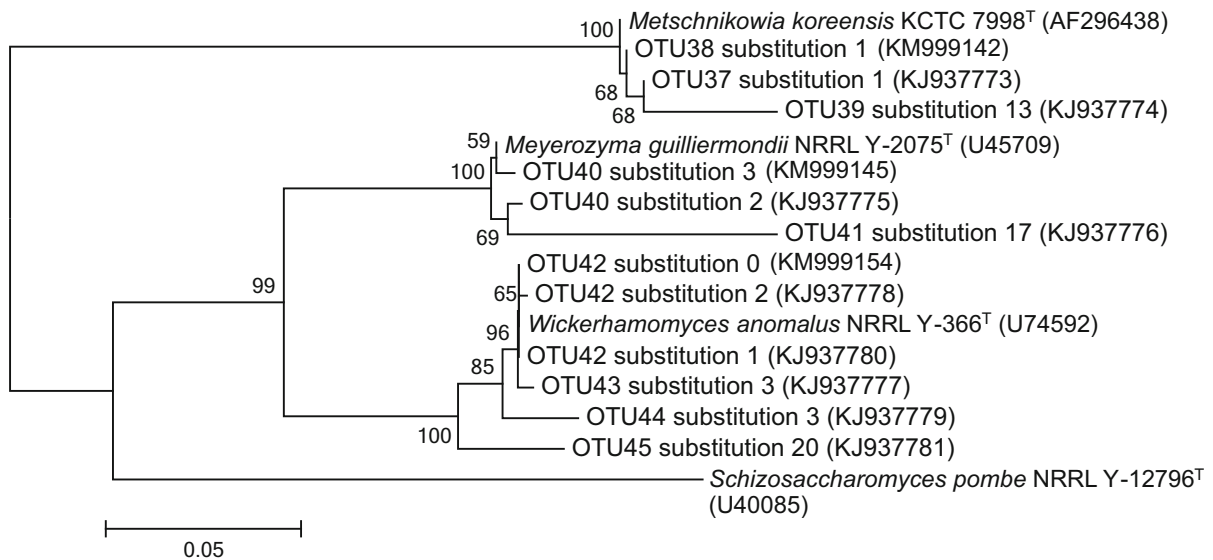


Fig. 4 Phylogenetic tree calculated from neighbor-joining of OTU sequences in subphylum Saccharomycotina (D1/D2 domain, LSU rRNA gene) and reference sequences retrieved from the GenBank database using Kimura 2-parameter model.

Numbers on branches are the bootstrap percentage (>50 %) from 1000 random replications. The scale bar corresponds to a genetic distance of 0.05 substitutions per position

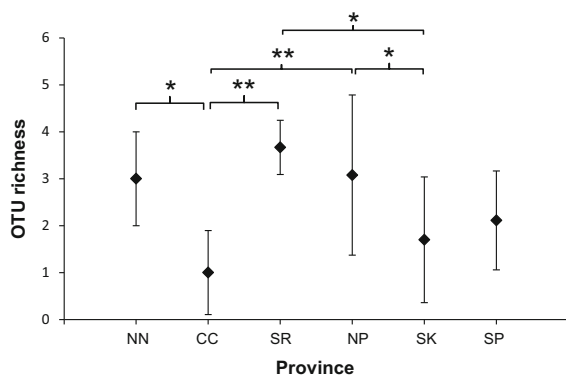


Fig. 5 OTU richness in the sub-samples depending on province; Nakhon Nayok (NN), Chachoengsao (CC), Surin (SR), Naphon Pathom (NP), Sisaket (SK) and Suphanburi (SP). Bars are confidence interval and middle points are the respective mean. Significant differences are indicated by * $P \leq 0.05$ and ** $P \leq 0.01$

culture-dependent approach based on the enrichment isolation technique and the D1/D2 domain sequence analysis (Limtong and Koowadjanakul 2012; Limtong and Kaewwichian 2014; Limtong et al. 2014). Only 7 related yeast species detected in this study were also found in rice phyllospheres when using the enrichment isolation technique (Limtong and Kaewwichian 2014).

Table 3 Diversity indices of yeast genotypes presenting in 44 rice phyllosphere samples

Diversity indices	Values
Total number of clones	451
Total number of genotypes (S)	53
Average population size	8.51
Shannon–Weiner index (H')	1.996
Equitability index (E_H)	0.5028

These consisted of *C. flavecens*, *C. laurentii*, *Metschnikowia koreensis*, *P. antarctica*, *P. aphidis*, *M. guilliermondii* and *S. blumae*. Furthermore, the dominant species detected in our study were present in relatively low frequencies (*P. antarctica*) and was not found (*B. japonica*) when using enrichment culture. Also of note is that the yeast species showing a relatively high frequency of isolation using the enrichment isolation technique did not appear in this study. These included *Rhodotorula taiwanensis*, *Candida tropicalis* and *Cyberlindnera fabianii* (Limtong and Kaewwichian 2014). These results indicated that different yeasts were obtained from different techniques of investigations. The enrichment technique is likely to support the growth of small populations of

ascomycetous yeasts leading to a greater representation of these yeast species in the results. The species that were not found by enrichment technique but sequences of which were obtained in this study were related to that of *B. japonica*, *C. curvatus*, *C. flavus*, *H. zaeae*, *P. hubeiensis*, *S. pararoseus*, *S. carnicolor*, *S. oryzicola* and *W. anomalus*. This likely indicates that the culture techniques used during enrichment do not support growth of these populations and these maybe under-represented in the results.

Some of the yeast species found in this study had also been detected previously in various plant species in Thailand, including *C. flavescens*, *M. koreensis*, *M. guilliermondii*, *S. blumeae* and *S. carnicolor* (Nakase 2000; Limtong and Kaewwichian 2014; Limtong et al. 2014). *Pseudozyma* was the most prevalent yeast genus in the rice phyllospheres of this study and this is not unexpected as the *Pseudozyma* genus is known generally inhabit numerous plant phyllospheres (Allen et al. 2004; Seo et al. 2007; Kitamoto et al. 2011; Limtong and Kaewwichian 2014; Yoshida et al. 2014). In the present study, *P. antarctica* was detected in relatively high frequency and was a common species in the rice phyllosphere samples. Some strains of this yeast species have been reported to provide beneficial effects to the host plant, such as microbiological control (Clement-Mathieu et al. 2008). There have been reported that *P. antarctica* secreted mannosylerythritol lipids (MELs) on plant surfaces that could be expected to play a significant role in fungal morphological development and propagation on plant surfaces (Morita et al. 2010; Yoshida et al. 2014). Therefore, these epiphytic yeast species detected in this study could be used as promising plant pathogenic biocontrol agents. In recent years, the enzyme production ability of this species which allows for the breakdown of biodegradable plastics has been reported (Kitamoto et al. 2011; Shinozaki et al. 2013a, 2013b). Other predominant yeasts in this study were in the genus *Bullera*, which have also been commonly found in other plant phyllopheres (Nakase 2000; Sampaio et al. 2004; Inácio et al. 2005; Fungsin et al. 2006). *B. japonica* another predominant species in this study has also been found to be so in other plant phyllospheres (Sampaio et al. 2004). Other epiphytic yeasts that have been reported of their capability to promote plant growth by producing plant growth hormone, indole-3-acetic acid (IAA) were also found in this study such as *P. aphidis* and *C. flavus* (Sun et al.

2014). Therefore these yeasts could be useful for further development in rice production.

It should be noted that a few yeasts species found in this study have been reported of their pathogenic risk in human i.e. *P. antarctica* has been isolated from the blood of a patient with a spontaneous pneumothorax in Thailand (Sugita et al. 2003). This should be aware of as this yeast species was predominantly found in rice phyllosphere in Thailand. *M. guilliermondii*, Risk Group 2 microorganism, was also detected in this study (San Millan et al. 1997; Pfaller et al. 2006). An appearance of pathogenic yeasts in rice fields may be due to the fact that human beings are involved in growing rice in Thailand and these microorganisms may be brought by farmers. In addition, many animals can also be sources of pathogenic microorganism contamination in the field.

Since the diversity of epiphytic yeasts in the rice phyllosphere has received little attention, only few novel yeast species isolated from the rice phyllosphere in Thailand have been proposed so far. These included *M. saccharicola* and *M. lobburiensis* (Kaewwichian et al. 2012). In our study, there were several OTUs that could not have been identified into a species level on the basis of the D1/D2 domain of the LSU rRNA gene sequence analysis with strongly supporting phylogenetic analysis, and some of these were suggested to be the sequences of new yeast taxa.

This study was not intended to investigate the relation between sampling locations and yeast communities. However, we primarily found significant differences in the OTU richness between some provinces. The significantly lowest OTU richness was shown in Chachoensao province. This may be a result of the minimal level of rainfall (3.4 mm) during the sampling period in comparison with the rainfall level in other locations. This result is accordance with the report of Swinfield et al. (2012) which indicated that dry climates and when rainfall is less frequent could reduce fungal diversity. Nevertheless, the rainfall level was lowest (0.8 mm) in Nakhon Nayok province but this factor did not affect the OTU richness. There may be other factors that have an effect on yeast diversity. There have been reported that environmental factors such as seasonal and climatic conditions also geography could affect microbial biodiversity (Lachance et al. 2003; Glushakova and Chernov 2010; Jumpponen and Jones 2010; Gayevskiy and Goddard 2012; Voriskova et al. 2014).

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References

- Allen TW, Quayyum HA, Burpee LL, Buck JW (2004) Effect of foliar disease on the epiphytic yeast communities of creeping bentgrass and tall fescue. *Can J Microbiol* 50:853–860
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59:143–169
- Bodrossy L, Sessitsch A (2004) Oligonucleotide microarrays in microbial diagnostics. *Curr Opin Microbiol* 7:245–254
- Brandl MT, Lindow SE (1998) Contribution of indole-3-acetic acid production to the epiphytic fitness of *Erwinia herbicola*. *Appl Environ Microbiol* 64:3256–3263
- Bunster L, Fokkema NJ, Schippers B (1989) Effect of surface-active *Pseudomonas* spp. on leaf wettability. *Appl Environ Microbiol* 55:1340–1345
- Chiocchetti A, Ghignone S, Minuto A, Gullino ML, Garibaldi A, Migheli Q (1999) Identification of *Fusarium oxysporum* f. sp. *basilici* isolated from soil, basil seed, and plants by RAPD analysis. *Plant Dis* 83:576–581
- Clement-Mathieu G, Chain F, Marchand G, Belanger RR (2008) Leaf and powdery mildew colonization by glycolipid-producing *Pseudozyma* species. *Fungal Ecol* 1:69–77
- de Azeredo L, Gomes E, Mendonca-Hagler L, Hagler A (1998) Yeast communities associated with sugarcane in Campos, Rio de Janeiro, Brazil. *Int Microbiol* 1:205–208
- de Barros Lopes M, Rainieri S, Henschke PA, Langridge P (1999) AFLP fingerprinting for analysis of yeast genetic variation. *Int J Syst Bacteriol* 49:915–924
- Dik AJ, Fokkema NJ, Van Pelt JA (1991) Consumption of aphid honeydew, a wheat yield reduction factor, by phyllosphere yeasts under field conditions. *Neth J Plant Pathol* 97:209–232
- Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* 49:329–337
- Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* 50:1351–1371
- Fiala V, Glad C, Martin M, Jolivet E, Derridj S (1990) Occurrence of soluble carbohydrates on the phylloplane of maize (*Zea mays* L.): variations in relation to leaf heterogeneity and position on the plant. *New Phytol* 115:609–615
- Fry SC (1989) Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. *Physiol Plant* 75:532–536
- Fungsin B, Takashima M, Sugita T, Artjariyasripong S, Potacharoen W, Tanticharoen M, Nakase T (2006) *Bullera koratensis* sp. nov. and *Bullera lagerstroemiae* sp. nov., two new ballistoconidium-forming yeast species in the Trichosporonales clade isolated from plant leaves in Thailand. *J Gen Appl Microbiol* 52:73–81
- Gayevskiy V, Goddard MR (2012) Geographic delineations of yeast communities and populations associated with vines and wines in New Zealand. *ISME J* 6:1281–1290
- Glushakova AM, Chernov IY (2010) Seasonal dynamics of the structure of epiphytic yeast communities. *Microbiology* 79:830–839
- Glushakova AM, Yurkov AM, Chernov IY (2007) Massive isolation of anamorphous ascomycete yeasts *Candida oleophila* from plant phyllosphere. *Microbiology* 76:799–803
- Hierro N, Esteve-Zarzoso B, Gonzalez A, Mas A, Guillamon JM (2006) Real-time quantitative PCR (QPCR) and reverse transcription-qPCR for detection and enumeration of total yeasts in wine. *Appl Environ Microbiol* 72:7148–7155
- Inácio J, Portugal L, Spencer-Martins I, Fonseca Á (2005) Phylloplane yeasts from Portugal: seven novel anamorphic species in the Tremellales lineage of the Hymenomycetes (Basidiomycota) producing orange-coloured colonies. *FEMS Yeast Res* 5:1167–1183
- Irvine JA, Dix NJ, Warren RC (1978) Inhibitory substances in acer platanoides leaves: seasonal activity and effects on growth of phylloplane fungi. *Trans Br Mycol Soc* 70:363–371
- Jumpponen A, Jones KL (2009) Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytol* 184:438–448
- Jumpponen A, Jones KL (2010) Seasonally dynamic fungal communities in the *Quercus macrocarpa* phyllosphere differ between urban and nonurban environments. *New Phytol* 186:496–513
- Kaewwichian R, Yongmanitchai W, Kawasaki H, Limtong S (2012) *Metschnikowia saccharicola* sp. nov. and *Metschnikowia lopburiensis* sp. nov., two novel yeast species isolated from phylloplane in Thailand. *Anton Leeuw* 102:743–751
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kitamoto H, Shinozaki Y, X-h Cao et al (2011) Phyllosphere yeasts rapidly break down biodegradable plastics. *AMB Express* 1:44
- Koowadjanakul N, Jindamorakot S, Yongmanitchai W, Limtong S (2011) *Ogataea phyllophila* sp. nov., *Candida chumphonensis* sp. nov. and *Candida matranensis* sp. nov., three methylotrophic yeast species from phylloplane in Thailand. *Anton Leeuw* 100:207–217
- Kurtzman C, Robnett C (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Anton Leeuw* 73:331–371
- Lachance M-A, Bowles JM, Starmer WT (2003) Geography and niche occupancy as determinants of yeast biodiversity: the yeast–insect–morning glory ecosystem of Kípuka Puuulu, Hawai'i. *FEMS Yeast Res* 4:105–111
- Landell MF, Billodre R, Ramos JP, Leoncini O, Vainstein MH, Valente P (2010) *Candida aechmeae* sp. nov. and *Candida*

- vrieseae* sp. nov., novel yeast species isolated from the phylloplane of bromeliads in Southern Brazil. *Int J Syst Evol Microbiol* 60:244–248
- Leveau JHJ, Lindow SE (2001) Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proc Natl Acad Sci USA* 98:3446–3453
- Limtong S, Kaewwichian R (2014) The diversity of culturable yeasts in the phylloplane of rice in Thailand. *Ann Microbiol*. doi:10.1007/s13213-014-0905-0
- Limtong S, Koowadjanakul N (2012) Yeasts from phylloplane and their capability to produce indole-3-acetic acid. *World J Microbiol Biotechnol* 28:3323–3335
- Limtong S, Kaewwichian R, Yongmanitchai W, Kawasaki H (2014) Diversity of culturable yeasts in phylloplane of sugarcane in Thailand and their capability to produce indole-3-acetic acid. *World J Microbiol Biotechnol* 30:1785–1796
- Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. *Appl Environ Microbiol* 69:1875–1883
- Liu W-T, Huang C-L, Jiang Yong H, Song L, Say Leong O, Wun Jun N (2002) Denaturing gradient gel electrophoresis polymorphism for rapid 16S rDNA clone screening and microbial diversity study. *J Biosci Bioeng* 93:101–103
- Makimura K, Murayama SY, Yamaguchi H (1994) Detection of a wide range of medically important fungi by the polymerase chain reaction. *J Med Microbiol* 40:358–364
- Martins G, Vallance J, Mercier A, Albertin W, Stamatopoulos P, Rey P, Lonvaud A, Masneuf-Pomarede I (2014) Influence of the farming system on the epiphytic yeasts and yeast-like fungi colonizing grape berries during the ripening process. *Int J Food Microbiol* 177:21–28
- Morita T, Ito E, Kitamoto HK, Takegawa K, Fukuoka T, Imura T, Kitamoto D (2010) Identification of the gene *PaEMT1* for biosynthesis of mannosylerythritol lipids in the basidiomycetous yeast *Pseudozyma antarctica*. *Yeast* 27: 905–917
- Morris CE (2001) Phyllosphere. In: Encyclopedia of life sciences. Nature Publishing Group, London. doi:10.1038/npg.els.0000400
- Moter A, Gobel UB (2000) Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J Microbiol Methods* 41:85–112
- Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* 2:317–322
- Nakase T (2000) Expanding world of ballistosporous yeasts: distribution in the phyllosphere, systematics and phylogeny. *J Gen Appl Microbiol* 46:189–216
- Nakase T, Suzuki M (1985) Ballistospore-forming yeasts found on the surface of the Japanese rice plant, *Oryza sativa* L. *J Gen Appl Microbiol* 31:457–474
- Nakase T, Takashima M, Itoh M, Fungsin B, Potacharoen W, Athasampunna P, Komagata K (2001) Ballistoconidium-forming yeast found in the phyllosphere of Thailand. *Microbiol Cult Collect* 17:23–35
- Peters S, Koschinsky S, Schwieger F, Tebbe CC (2000) Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation polymorphism-based genetic profiles of small-subunit rRNA genes. *Appl Environ Microbiol* 66:930–936
- Pfalter MA, Diekema DJ, Mendez M, Kibbler C, Erzsebet P, Chang SC, Gibbs DL, Newell VA, The Global Antifungal Surveillance Group (2006) *Candida guilliermondii*, an opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. *J Clin Microbiol* 44:3551–3556
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sampaio JP, Inacio J, Al Fonseca, Gadanho M, Spencer-Martins I, Scorzetti G, Fell JW (2004) *Auriculibuller fuscus* gen. nov., sp. nov. and *Bullera japonica* sp. nov., novel taxa in the Tremellales. *Int J Syst Evol Microbiol* 54:987–993
- San Millan RM, Wu L-C, Salkin IF, Lehmann PF (1997) Clinical isolates of *Candida guilliermondii* include *Candida fermentati*. *Int J Syst Bacteriol* 47:385–393
- Schloss PD, Westcott SL, Ryabin T et al (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537–7541
- Schreiber L, Krimm U, Knoll D, Sayed M, Auling G, Kropenstedt RM (2005) Plant–microbe interactions: identification of epiphytic bacteria and their ability to alter leaf surface permeability. *New Phytol* 166:589–594
- Schutte UE, Abdo Z, Bent S, Shyu C, Williams C, Pierson J, Forney L (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Appl Microbiol Biotechnol* 80:365–380
- Seo HS, Um HJ, Min J, Rhee SK, Cho TJ, Kim YH, Lee J (2007) *Pseudozyma jejuensis* sp. nov., a novel cutinolytic ustilaginomycetous yeast species that is able to degrade plastic waste. *FEMS Yeast Res* 7:1035–1045
- Shinozaki Y, Kikkawa Y, Sato S et al (2013a) Enzymatic degradation of polyester films by a cutinase-like enzyme from *Pseudozyma antarctica*: surface plasmon resonance and atomic force microscopy study. *Appl Microbiol Biotechnol* 97:8591–8598
- Shinozaki Y, Morita T, X-h Cao et al (2013b) Biodegradable plastic-degrading enzyme from *Pseudozyma antarctica*: cloning, sequencing, and characterization. *Appl Microbiol Biotechnol* 97:2951–2959
- Slavikova E, Vadkertiova R, Vranova D (2007) Yeasts colonizing the leaf surfaces. *J Basic Microbiol* 47:344–350
- Spencer JFT, Spencer DM (1997) Ecology: where yeasts live. In: Spencer JFT, Spencer DM (eds) Yeasts in natural and artificial habitats. Springer, New York, pp 33–58
- Su C, Lei L, Duan Y, Zhang K-Q, Yang J (2012) Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. *Appl Microbiol Biotechnol* 93:993–1003
- Sugita T, Takashima M, Poonwan N, Mekha N, Malaithao K, Thungmuthasawat B, Prasarn S, Luangsook P, Kudo T (2003) The first isolation of ustilaginomycetous anamorphic yeasts, *Pseudozyma* species, from patients' blood and a description of two new species: *P. parantarctica* and *P. thailandica*. *Microbiol Immunol* 47:183–190
- Sun P-F, Fang W-T, Shin L-Y, Wei J-Y, Fu S-F, Chou J-Y (2014) Indole-3-acetic acid-producing yeasts in the phyllosphere of the carnivorous plant *Drosera indica* L. *PLoS One*. doi:10.1371/journal.pone.0111496
- Swinfield T, Lewis OT, Bagchi R, Freckleton RP (2012) Consequences of changing rainfall for fungal pathogen-

- induced mortality in tropical tree seedlings. *Ecol Evol* 2:1408–1413
- Takaku H, Kodaira S, Kimoto A, Nashimoto M, Takagi M (2006) Microbial communities in the garbage composting with rice hull as an amendment revealed by culture-dependent and -independent approaches. *J Biosci Bioeng* 101:42–50
- Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Tukey HB (1970) The leaching of substances from plants. *Annu Rev Plant Physiol* 21:305–324
- Vorholt JA (2012) Microbial life in the phyllosphere. *Nat Rev Microbiol* 10:828–840
- Voriskova J, Brabcova V, Cajthaml T, Baldrian P (2014) Seasonal dynamics of fungal communities in a temperate oak forest soil. *New Phytol* 201:269–278
- Weibull J, Ronquist F, Brishammar S (1990) Free amino acid composition of leaf exudates and phloem sap: a comparative study in oats and barley. *Plant Physiol* 92:222–226
- Yoshida S, Morita T, Shinozaki Y et al (2014) Mannosylerythritol lipids secreted by phyllosphere yeast *Pseudozyma antarctica* is associated with its filamentous growth and propagation on plant surfaces. *Appl Microbiol Biotechnol* 98:6419–6429
- Zimmerman NB, Vitousek PM (2012) Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. *Proc Natl Acad Sci USA* 109:13022–13027