Study on epiphytic yeasts in corn phylloplane from Phetchabun province, Thailand by culture-independent approach

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Abstract

This research aimed to investigate the diversity of epiphytic yeasts in corn phylloplane by culture-independent method. Yeast identification based on D1/D2 domain sequence of the large subunit rRNA gene. Eight samples of corn leaf were randomly collected from Phetchabun province, Thailand. Of total 289 clones obtained from clone libraries, 240 clones (83.0%) were related to filamentous fungi and 49 clones (17.0%) were related to yeasts. Yeast related sequences were clustered into 29 operational taxonomic units (OTUs) based on BLAST and restriction analysis. Of this total, 10 OTUs were identified as 7 known yeast species in 3 genera. These included *Bullera derxii*, *Pseudozyma aphidis*, *Pseudozyma hubeiensis*, *Pseudozyma prolifica*, *Pseudozyma rugulosa*, *Sporobolomyces carnicolor* and *Sporobolomyces odoratus*. Other 19 OTUs were unable to identified as yeast species, however they were closely related to the yeast type strains which falling into 3 orders of Basidiomycota consisting of Tremellales, Sporidiobolales and Ustilaginales related to 4 genera including *Bullera*, *Cryptococcus*, *Pseudozyma* and *Sporidiobolus*. Ascomycetous yeast was not found in corn leaf surfaces in this study. The most frequently detected OTU from the clone libraries was identified as *Pseudozyma aphidis* (26.5%, 13 clones) followed by *Pseudozyma hubeiensis* (14.3%, 7 clones). Other species were detected within the clone libraries but showed low relative frequencies (2.04% to 8.16%).

Keywords: corn, epiphytic yeast, PCR, RFLP, large subunit rRNA gene

Introduction

The surface of plant leaf usually refered to the phylloplane or phyllosphere. Plant leaf has been recognized as a source of energy for plant due to the photosynthesis occurs. In addition, it is an important habitat for microorganisms that can use leaf surface nutrients as their carbon sources (Dik et al., 1991; Fiala et al., 1990; Leveau and Lindow, 2001; Tukey, 1970; Weibull et al., 1990). Some epiphytic yeasts have been reported to produce plant growth hormone such as indole-3-acetic acid (IAA) (Limtong and Koowadjanakul, 2012) and gibberellins (GA3) (El-Tarabily and Sivasithamparam, 2006) which promote cell wall loosening and release saccharides from plant cell as substrates (Fry, 1989; Lindow and Brandl, 1998 and 2003).

Although epiphytic yeasts have been isolated from phylloplane of various plant varieties in many countries, but until present there is less information of the diversity of epiphytic yeasts. In Thailand, corn is an important economic crop cultivated mostly in the north. To date, there has no report of yeast diversity in corn. Therefore, this study aimed at investigating the diversity of epiphytic yeasts of corn phylloplane by using a culture-independent approach.

Methodology

Corn leaf samples

Samples of corn leaf were randomly collected from 8 cultivated fields in Phetchabun province, Thailand. Three samples were collected from Chon Dean district (16º11′21″ N 100º51′35″ E); C15PB, C19PB. Five samples were collected from Nong Phai district (15º59′21″ N 101º3′45″ E); C20PB, C77PB, C80PB, C81PB, C88PB and C89PB. The left samples were kept at 4^{o}C until used.

DNA extraction

Corn leaf samples were cut (6 g) and submerged in 40 ml of washing buffer (1X Phosphate buffered saline, Tween-20). The microbes were taken out from leaf surfaces by sonicating for 7 min using ultrasonic cleaning bath (Bransonic, USA). The suspension was centrifuged at 5,000 x g for 5 min and the supernatant was discarded. DNA was extracted from the cell pellet by suspending in 100 µl of lysis buffer (100 mM Tris-HCl, 0.5% w/v SDS, 30 mM EDTA, pH 7.5) and incubated at 95^{o}C for 15 min. Then 100 µl of 2.5 M potassium acetate was added and incubated on ice for 60 min and centrifuged at 12,000 x g for 5 min. The supernatant was transfered to new tube and DNA was precipitated with an equal volume of isopropanol. The precipitated DNA was washed with 70% ethanol and air dried. The DNA was resuspended in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at -20 ^{o}C until used.

Amplification of D1/D2 domain of LSU rRNA gene

The total genomic DNA extracted from corn phylloplane was used as DNA template for the amplification of the D1/D2 domain of LSU rRNA gene by polymerase chain reaction (PCR) 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 reaction mixture containing 50 ng of genomic DNA, 1X PCR buffer (Vivantis, USA), 1.5 mM $MgCl_2$ (Vivantis, USA), 200 µM of each dNTP (Vivantis, USA), 20 pmol of each primer and 1 U of *Taq* DNA polymerase (Vivantis, USA). The reaction was performed in a thermal cycler programmed as followed: initial DNA denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 52 °C for 30 s and extension at 72°C for 45 s and final extension at 72°C for 10 min. Determination of amplified DNA was performed by electrophoresis using 0.8% (w/v) agarose gel in 1X TAE buffer (Tris-acetate buffer, EDTA, pH 8.0), then stained with ethidium bromide and visualized under UV illuminator. The amplified DNA was purified with Nucleic acid Extraction kit (Vivantis, USA).

PCR cloning, screening and restriction analysis

The purified PCR products with correct size (approximately 600 bp) were ligated into pTG19-T cloning vector (Vivantis, USA) performed using manufacturer's protocol and transformed into *E.coli* DH5α competent cells using method of Sambrook et al. (1989). The recombinant clones were screened by colony PCR using cell suspension (2 µl) as template with the same PCR condition described above. PCR products with the correct size of the DNA insert were further restriction analyzed by digesting with the restriction enzymes, *Hae*III, *Hin*fI and *Cfo*I, then separated in 2% (w/v) agarose gel electrophoresis in 1X TAE buffer. The patterns of restriction fragment length polymorphism (RFLP) were grouped in operational taxonomic units (OTUs). The inserted DNA of D1/D2 from the representative transformants of each OTUs were subjected to sequence.

Sequence analysis

The sequences of D1/D2 region were submitted to BLASTN homology search and aligned with the sequences of related species retrieved from GenBank using CLUSTALW 1.6 multiple alignment program. The phylogenetic were constructed using MEGA version 6.0 (Tamura et al., 2013) by Kimura 2-parameter model and neighbor-joining method. Bootstrap analysis was performed 1,000 random replications.

Results

Among 289 clones, 83.0% (240 clones) were closely related to filamentous fungi (data not shown) and 17.0% (49 clones) were closely related to yeasts (Table 1). Yeast related sequences were clustered into 26 OTUs by RFLP patterns and 3 sub-OTUs were distinguished as the difference of the number of nucleotide substitutions (see OTU4, OTU18 and OTU26). The OTU sequences were identified as yeast species based on BLAST analysis and the number of nucleotide substitutions within the D1/D2 domain was also considered.

As can be seen, in Table 1 yeasts presented in most corn samples apart from sample C89PB. The basidiomycetous yeasts were commonly found in this study. Among 29 OTUs shown, 10 OTUs were identified as 7 known yeast species within 3 genera with the number of nucleotide substitutions ranging from 0 to 2 according to the basidiomycetous yeast

Table 1 Epiphytic yeasts in corn phylloplane samples identified based on D1/D2 domain of LSU rRNA gene by restriction and BLAST analysis

Sample no. (positive clone)	Yeast OTUs/ Total OTUs	OTU No.	D1/D2 size (bp)	RFLP			Closest relative species (No. of clone)	Accession no.	%Identity	Nucleotide substitution	
				*Hae*III	*Hinf*I	*Cfo*I				No.	%
C15PB (45)	6/21	1	588	524,64	401,187	588	*Cryptococcus luteolus* CBS 943^T (1)	AF075482.1	98.8(581/588)	7	1.23
		2	592	364,123,48,39,18	222,184,138,48	592	*Pseudozyma aphidis* JCM 10318^T (2)	AB089363.1	89.1(507/569)	45	7.91
		3	560	513,47	275,184,101	514,46	*Sporobolomyces carnicolor* CBS 4215^T (1)	AY070008.1	99.6(559/561)	1	0.18
		4-1	561	387,174	376,185	561	*Sporobolomyces odoratus* IGC 5694^T (3)	AF387125.1	99.8(554/555)	1	0.18
		4-2	562	387,175	377,185	562	*Sporobolomyces odoratus* IGC 5694^T (1)	AF387125.1	99.6(553/555)	2	0.36
		5	560	384,176	275,184,101	560	*Sporidiobolus pararoseus* CBS 491^T (1)	AF189977.1	99.1(556/561)	4	0.71
		6	558	384,174	275,184,99	512,46	*Sporidiobolus pararoseus* CBS 491^T (1)	AF189977.1	98.7(552/559)	6	1.07
C19PB (45)	2/9	7	582	521,61	385,187	582	*Bullera derxii* CBS 7225^T (1)	AF189857.1	100.0(582/582)	0	0.00
		8	593	363,123,48,39,20	224,185,184	436,157	*Pseudozyma antarctica* CBS 214.83^T (1)	AJ235302.1	98.3(563/573)	8	1.40
C20PB (41)	1/17	9	582	270,248,64	398,82,54,48	318,183,81	*Bullera sinensis* CBS7238^T (1)	AF189884.1	89.3(526/589)	52	8.83
C77PB (32)	10/14	10	563	501,62	399,164	495,68	*Bullera coprosmaensis* CBS 8284^T (1)	AF363660.1	93.6(513/548)	34	6.20
		11	578	516,62	399,179	578	*Cryptococcus zeae* HB 1207^T (1)	AJ965480.1	96.8(541/559)	15	2.68
		12	590	411,123,39,17	221,185,184	433,157	*Pseudozyma aphidis* JCM 10318^T (1)	AB089363.1	98.4(553/562)	7	1.25
		13	583	352,123,48,39,21	225,183,146,29	583	*Pseudozyma hubeiensis* CBS 10077^T (1)	DQ008953.1	90.0(448/498)	37	7.43
		14	594	411,123,39,21	225,185,184	437,112,45	*Pseudozyma hubeiensis* CBS 10077^T (1)	DQ008953.1	99.7(593/595)	1	0.17
		15	594	414,123,39,18	222,188,184	434,160	*Pseudozyma hubeiensis* CBS 10077^T (1)	DQ008953.1	96.3(573/595)	19	3.19
		16	589	407,123,39,20	224,184,181	436,112,41	*Pseudozyma hubeiensis* CBS 10077^T (1)	DQ008953.1	98.3(580/590)	8	1.36
		17	583	347,123,48,39,20,6	224,184,175	436,147	*Pseudozyma rugulosa* JCM 10323^T (1)	AB089371.1	97.7(507/519)	11	2.12
		18-1	567	337,123,48,39,20	224,184,159	436,131	*Pseudozyma rugulosa* JCM 10323^T (1)	AB089371.1	99.6(532/534)	2	0.37
		18-2	607	349,123,48,48,39	252,184,171	464,143	*Pseudozyma rugulosa* JCM 10323^T (1)	AB089371.1	99.2(521/525)	3	0.57
		19	562	333,123,48,39,19	223,184,155	435,127	*Pseudozyma rugulosa* JCM 10323^T (1)	AB089371.1	98.7(523/530)	7	1.32
C80PB (17)	2/4	20	592	412,123,39,18	222,186,184	434,112,46	*Pseudozyma hubeiensis* CBS 10077^T (2)	DQ008953.1	99.8(592/593)	0	0.00
		21	593	411,123,39,20	224,185,184	436,157	*Pseudozyma prolifica* JCM 10319^T (1)	AB089369.1	97.5(549/563)	10	1.78
C81PB (37)	5/11	22	593	363,123,48,39,20	224,185,184	436,157	*Pseudozyma aphidis* JCM 10318^T (1)	AB089363.1	98.9(556/562)	4	0.71
		23	597	365,163,48,21	339,151,75,32	376,159,62	*Pseudozyma aphidis* JCM 10318^T (1)	AB089363.1	89.3(499/559)	47	8.41
		20	595	412,123,39,21	225,186,184	437,112,46	*Pseudozyma hubeiensis* CBS 10077^T (4)	DQ008953.1	99.8(595/596)	0	0.00
		24	591	411,123,39,18	222,185,184	434,157	*Pseudozyma prolifica* JCM 10319^T (1)	AB089369.1	99.6(560/562)	0	0.00
		25	591	256,135,123,39,18	222,185,184	434,157	*Pseudozyma prolifica* JCM 10319^T (1)	AB089369.1	99.3(558/562)	2	0.36
C88PB (26)	1/2	26-1	591	364,123,48,39,17	221,186,184	433,158	*Pseudozyma aphidis* JCM 10318^T (13)	AB089363.1	99.5(559/562)	2	0.35
		26-2	590	360,123,48,39,20	224,184,182	436,154	*Pseudozyma aphidis* JCM 10318^T (1)	AB089363.1	99.1(552/557)	3	0.54
C89PB (46)	0/1						ND				

ND = yeast not detected

identification by Fell et al., (2000). These included *Bullera derxii* (OTU7), *Pseudozyma aphidis* (OTU26-1), *Pseudozyma hubeiensis* (OTU14 and OTU20), *Pseudozyma prolifica* (OTU24 and OTU25), *Pseudozyma rugulosa* (OTU18-1), *Sporobolomyces carnicolor* (OTU3) and *Sporobolomyces odoratus* (OTU4-1 and OTU4-2). The most frequently detected OTU from the clone libraries was identified as *Pseudozyma aphidis* (26.5%, 13 clones) followed by *Pseudozyma hubeiensis* (14.3%, 7 clones). Other species were detected within the clone libraries but showed low relative frequencies (2.04% to 8.16%). In addition, most samples had different OTUs.

There were 19 OTUs that had more than three nucleotide substitutions were closely related to 10 yeast species including *Bullera coprosmaensis* (OTU10), *Bullera sinensis* (OTU9), *Cryptococcus luteolus* (OTU1), *Cryptococcus zeae* (OTU11), *Pseudozyma antarctica* (OTU8), *Pseudozyma aphidis* (OTU2, OTU12, OTU22, OTU23 and OTU26-2), *Pseudozyma hubeiensis* (OTU13, OTU15 and OTU16), *Pseudozyma prolifica* (OTU21), *Pseudozyma rugulosa* (OTU17, OTU18-2 and OTU19) and *Sporidiobolus pararoseus* (OTU5 and OTU6). These OTU sequences that were unable to be identified as particular yeast species were confirmed to have long distances from their closest yeast species by phylogenetic analysis (Figure 1). These sequences may represent the sequences of new yeast taxa. The majority of these OTUs were in order Ustilaginales.

The phylogenetic tree (Figure 1.) illustrated that the epiphytic yeasts in corn phylloplane were classified into 3 orders of Basidiomycota consisting of Tremellales, Sporidiobolales and Ustilaginales supported with 100% of bootstrap values. Ascomycetous yeast was not found in corn leaf surfaces in this study.

Discussion

The epiphytic yeasts detected in corn phylloplane in this study were identified as basidiomycetous yeasts. This results corresponded to the previous study by Glushakova and Chernov (2010) which reported that the basidiomycetous yeasts were predominate on plant leaves. In addition, the basidiomycetous yeasts have been reported as the common yeasts in monocotyledonous plants (Spencer et al., 1997; de De Azerezo et al., 1998). However, the ascomycetous yeasts were mostly detected when using the enrichment technique (Limtong and Koowadjanakul, 2012). In our study, *Pseudozyma* was the most common genus found in corn phylloplane which had been reported that it could produce biocontrol agent such as fungicide (Buxdorf et al., 2013). *Pseudozyma aphidis* and *Pseudozyma hubeiensis* were the common yeast species detected in our study. In previous study, *Pseudozyma aphidis* has been reported as biocontrol agent of plant pathogen (Buxdorf et al., 2013). *Pseudozyma hubeiensis* has the ability to produce a hydrophilic Mannosylerythritol lipids (MELs), which is one of the most promising biosurfactants known because of their multifunctionality and biocompatibility (Konishi et al., 2008). *Cryptococcus* was also detected in this study and it has also been reported as a biocontrol agent producer (Cadez et al., 2010).

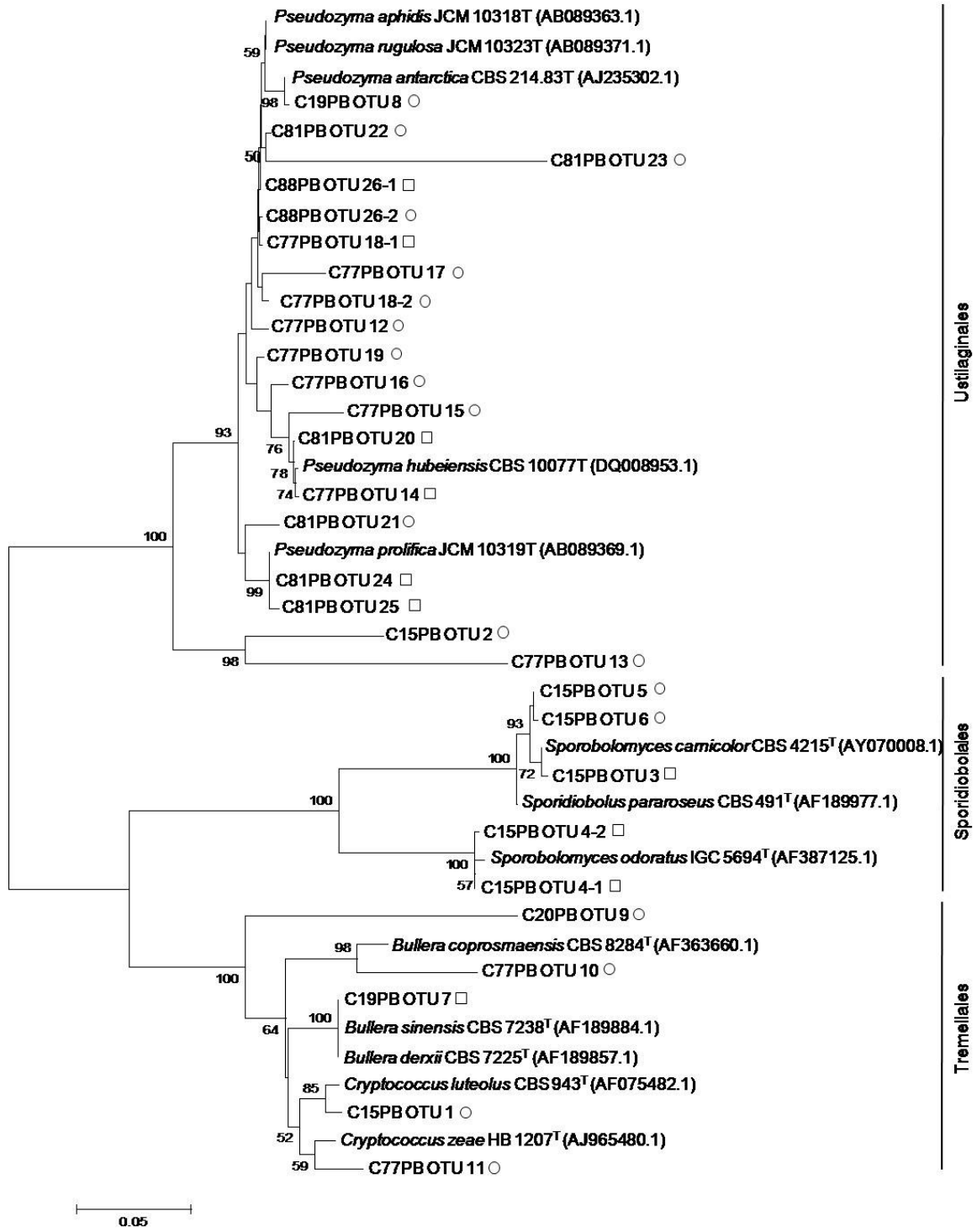

Figure 1: Phylogenetic tree constructed between the type strains of the related species from GenBank and the representative D1/D2 sequences of 29 OTUs. The scale bar corresponded to a genetic distance of 0.05 substitution per position. Numbers on branches are the bootstrap percentage (>50%) from 1,000 random replications. Blank square (□) represent the sequences identified as same species with the particular type strains. Blank circles (○) represent the sequences closely related to the yeast type strains.

Conclusion

Corn phylloplane samples were collected from Phetchabun province, Thailand. Most of recombinant clones were identified as filamentous fungi. Only 17.0% of clones were related to yeast which were in phylum Basidiomycota and classified into 3 groups with phylogenetic analysis. *Pseudozyma* was the most common genus detected in most samples. The sequences of new yeast taxa were also detected in this study.

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