Isolation and identification of yeasts associated with dung beetles (*Heliocopris bucephalus* Farbricius) in the north of Thailand.

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Abstract

Dung beetles are feces decomposer which construct brood ball to lay their egg. These insects spend their whole life cycle in lignocellulose-rich habitat. In this study we examined yeasts associated with six dung beetles in the north of Thailand. Ninety six isolates, eleven yeast species were isolated from dung beetles. An undescribed yeast of which relative is *Trichosporon veenhuisii* was predominant and obtained from all samples. Also, we isolated at least four undescribed species of which relatives were *Trichosporon mycotoxinivoran, Trichosporon siamense, Galactomyces geotrichum* and *Schwanniomyces occidentalis*

Keywords: Beetle-symbiosis, *Trichosporon veenhuisii* relative, brood ball.

Introduction

Insects are considered as most diverse group of living organism, more than one million species were recognized (Foottit and Adler 2009). The gut of insects belonging to orders Coleoptera (beetles), Homoptera (aphids), and Hymenoptera (ants) have been reported as rich source of yeast. In addition, various new yeast species have been discovered from insect gut (Suh and Blackwell 2005; Starmer and Lachance 2011). Associated yeast played an important role in providing extra nutritional source, improving nutrition, promoting development and detoxifying toxic compound for the host insect. Furthermore, yeasts were carried by insect to the nutrient rich source (Vega and Dowd 2005). The dung beetle *Heliocopris bucephalus* Farbricius (Cleoptera, Scarabaeidae) played an important role in nutrient cycling by decomposing large herbivore dung. Also, they were secondary seeds disposal which protect seeds from rodent predators by burying them in brood ball (Hanski and Cambefort 1991).Moreover, In some regions including Thailand, Lao, Vietnam, Ecuador, etc. both beetle larvae and pupae serve as human food (Durst et al. 2010). Despite a wide variety of insects, studies reporting yeasts associated with them are limited (Abe et al. 2010; Endoh et al. 2011; Lachance et al. 2006; Urbina et al. 2012). Thus most species of insects including dung beetles are left unexplored in the context of yeast biodiversity. The objective of this work is to expand the knowledge of diversity of yeast associated with dung beetle. Because dung beetles lived in lignocellulose-rich habitat, isolation of new yeasts from the insects may increase opportunities for application to fermentation processes and production of enzymes

Methodology

Collection of sample and yeast isolation

Dung beetles used for yeast isolation were collected from cattle pen at various locations in the north of Thailand. Surface sterilization was done twice by immersing the samples in 95% ethanol for 5 minute, 70% ethanol for 10 minutes, and rinsed with 0.85% NaCl solution. The rinsed liquid was streak on YPD agar (2% glucose, 0.3% yeast extract, 0.3% bactopeptone, 2% agar, adjusted pH 5.0 with HCl) supplemented with 100 ppm chloramphenicol as negative control. After removal of cephalon, elythron, wings and ambulatory legs, insect body was grinded in 0.85% NaCl solution with mortar and pestle. The suspension was inoculated into YPD broth and incubated at 30^{o}C on orbital shaker at 200 rpm for 72 h. The culture was purified by streaking on YPD agar and incubated at 30^{o}C for 48 h. Subsequently, pure cultures were preliminary grouping and distinguished by the observation of colony morphology. Representatives of each colony type were kept on YPD agar slant at 4^{o}C.

DNA analysis

Yeast genomic DNA was extracted with glass bead method as described in Endoh et al. (2011). Representative strains for sequencing of rRNA gene were selected, PCR fingerprinting was performed using a microsatellite primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3'). The reaction mixture included 5 µl of 2x GoTaq Green (Promega), 0.2 µl of primer $(GTG)_5$ (10 pmol/µl), 1 µl of template (2-50 ng/µl) in total volume 10 µl. PCR program was as follows; 5 min of pre-denaturation at 95^{o}C followed by 40 cycles of 45 sec of denaturation at 93^{o}C , 1 min of annealing at 50^{o}C, and 1 min of extension at 72^{o}C, and 6 min for final extension. After that, representative strains were selected for amplification and sequencing of LSU rRNA gene (D1/D2 region) using primers NL1 (forward) (5'-GCATATCAATAAG- CGGAGGAAAAG-3') and NL4 (reverse) (5'-GGTCCGTGTTTCAAGACGG-3'). PCR was performed following the method described in Endoh et al. (2008). PCR products were directly sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Stafford, USA) and analyzed with an auto-sequencer ABI Prism 3100 following the manufacturer's instructions. The data were edited manually using BioEdit v7.2.5 (Hall, 1999). The D1/D2 sequences obtained were compared to those available in the DDBJ/EMBL/GenBank database using BLASTn program (http://www.ncbi.nlm.nih.gov/blast). Strains exhibited 0-3 nucleotide substitutions were likely to be conspecific, whereas strains with 6 or more nucleotide substitutions compared are different (Kurtzman 2011). Phylogenetic tree was constructed by using MEGA 6 (Tamura et al. 2013). Kimura-2-parameter model was used to calculate nucleotide-sequence divergence, where gaps were completely deleted, and bootstrap values were obtained from 1000 replications (Felsenstein, 1985).

Results and discussion

Yeast isolation and identification

Ninety six yeast strains were obtained from 6 samples of dung beetle which collected from the north of Thailand. All of yeast strains were categorized by PCR fingerprints. Among the 98 yeast isolates, we distinguished 12 PCR fingerprint types (fig.1). Twenty six representative strains showeddifferent PCR fingerprint patterns, all of which were employed for sequencing of D1/D2 region of LSU rRNA gene. As the result of identity search of the region, twelve strains were likely to be known species including *Candida glabrata*, *Candida*

tropicalis, *Candida stellimalicola*, *Candida quercitrusa*, *Candida orthopsilosis* and *Trichosporon cataneum*. Fourteen strains were indicated to be undescribed yeasts of which relative species were *Trichosporon veenhuisii*, *Trichosporon mycotoxinivoran*, *Trichosporon siamense*, *Galactomyces geotrichum* and *Schwanniomyces occidentalis*(Table 1).The predominant yeast (74 out of 96 strains) was a relative of *Trichosporon veenhuisii*, all of which showed 95% similarity with 16 nucleotide substitutions.

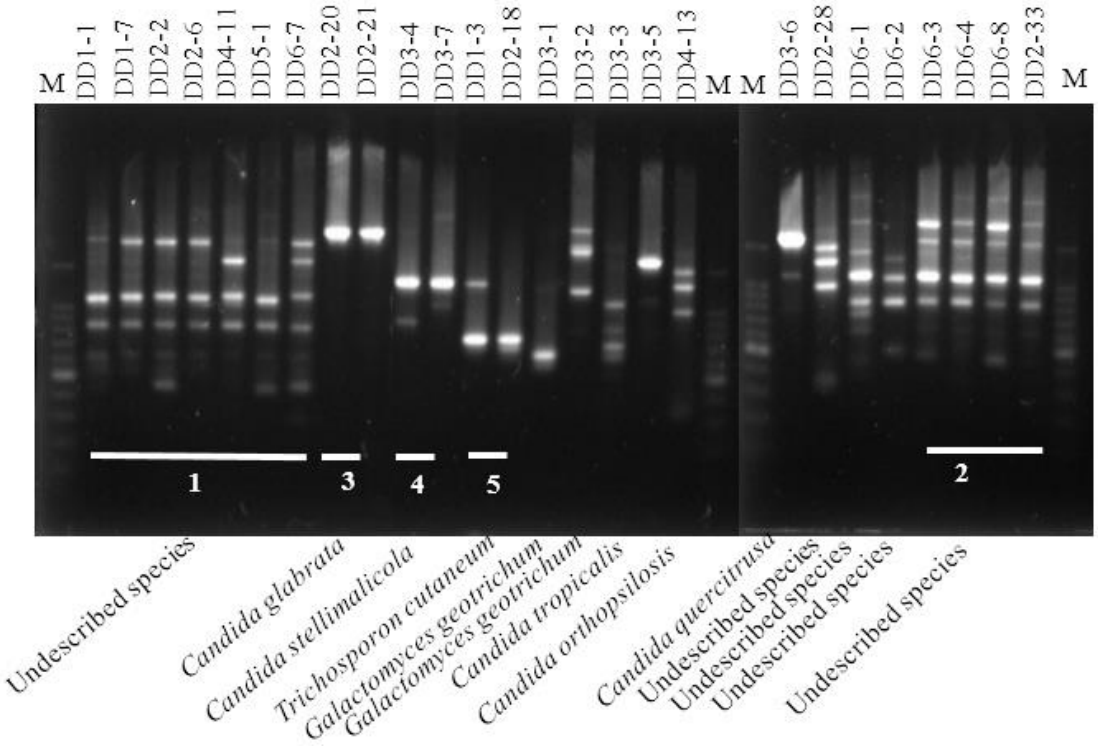

Figure 1: Comparison of PCR fingerprints of the representative strains. Lane M, 100 bp DNA ladder**.**

Table 1: Isolation and identification of the representative strains based on LSU rRNA gene (D1/D2 region) sequence.

Isolation No.	%identities	Nucleotide substitution	Top hit of similarity search
DD1-1	95%	16	*Trichosporon veenhuisii* (JN939494)
DD1-7	95%	16	*Trichosporon veenhuisii* (JN939494)
DD2-2	94%	16	*Trichosporon veenhuisii* (JN939494)
DD2-6	94%	16	*Trichosporon veenhuisii* (JN939494)
DD4-11	94%	17	*Trichosporon veenhuisii* (JN939494)
DD5-1	94%	17	*Trichosporon veenhuisii* (JN939494)
DD6-7	94%	18	*Trichosporon veenhuisii* (JN939494)
DD2-20	100%	0	*Candida glabrata* (FN393990)
DD2-21	100%	0	*Candida glabrata* (FN393990)
DD3-4	100%	0	*Candida stellimalicola* (FM180552)
DD3-7	100%	0	*Candida stellimalicola* (FM180552)
DD1-3	99%	5	*Trichosporon cutaneum* (AJ749837)
DD2-18	99%	4	*Trichosporon cutaneum* (AJ749837)
DD3-1	99%	1	*Galactomyces geotrichum* (JN938931)
DD3-2	99%	1	*Galactomyces geotrichum* (JN938931)
DD3-3	99%	1	*Candida tropicalis* (FJ455116)
DD3-5	99%	3	*Candida tropicalis* (FJ455116)
DD4-13	99%	1	*Candida orthopsilosis* (FJ432622)
DD3-6	99%	3	*Candida quercitrusa* (JN544056)
DD2-28	99%	6	*Trichosporon siamense* (AB164370)
DD6-1	92%	19	*Trichosporon veenhuisii* (JN939494)
DD6-2	91%	27	*Schwanniomyces occidentalis* (KC006471)
DD6-3	95%	8	*Trichosporon dulcitum* (GQ222351)
DD6-4	95%	8	*Trichosporon mycotoxinivorans* (JX111953)
DD6-8	94%	10	*Trichosporon mycotoxinivorans* (AB557756)
DD2-33	94%	16	*Trichosporon veenhuisii* (JN939494)

Discussion

We grouped the yeasts based on PCR fingerprints generated by oligonucleotide primer $(GTG)_5$ and on the similarity search of D1/D2 region of the LSU rRNA gene. The result showed that among PCR fingerprint group 1 and 2 even though PCR fingerprint patterns were similar, the strains may have 1-2 nucleotide substitutions of the region (fig.1). In contrast, Wuczkowski and Prillinger (2004) reported that PCR fingerprint pattern among yeasts isolated from soil were different even though they showed 100% identity with each other. Thus, only one oligonucleotide primer may be not enough for perfect categorization. The gut of wood borer beetle and fungus feeding insects have been reported as a reservoir of novel yeast. Indeed, more than a hundred undescribed yeast species were isolated from insect gut (Suh et al. 2004). In this study most of yeast isolates were member of the genera *Trichosporon* and *Candida*, while a few were *Geotrichum* and *Schwanniomyces*. Related yeast species have been isolated from another source for instance buffalo dung (Middelhoven et al. 2000), termite (Molna´r et al. 2004), and insect frass (Nakase et al. 2006). Moreover, 84% of yeast isolates obtained in this study were undescribed species belonging to the genus *Trichosporon*. *Trichosporon* species are well known because of their clinical importance. The dominant yeast of dung beetles was relative species of *Trichosoron veenhuisii* of which isolation source was buffalo feces. Further studies are needed to disclose why and how *Trichosporon* species serves as a symbiont of dung beetles. It is a next challenge to reveal the enzymatic characteristics of the yeasts, and how to provide the host beetle with benefit in the lignocellulose-rich habitat.

Conclusion

The gut of dung beetle may be a promising source of novel yeast. Many of yeast are still not known. Therefore future study is required to expand the knowledge about yeast diversity in dung beetle gut.

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