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Molecular Identification and Diversity of Yeasts Associated with *Apis cerana* Foraging on Flowers of *Jatropha integerrima*

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There are only a few reports from tropical countries, and none from Indonesia, on yeasts associated with the Asiatic honeybee, *Apis cerana*. Here we report on yeasts associated with *A. cerana* foraging on flowers of *Jatropha integerrima* in the campus of the Universitas Indonesia, Depok, Indonesia. Yeasts were isolated from guts of 30 individual pollen-collecting bees (PCB) and nectar-collecting bees (NCB), and identified by their internal transcribed spacer (ITS) regions of their rDNA sequences. Based on ITS regions sequence data, 14 representative yeast isolates obtained from *A. cerana* were found to be closely related to *Aureobasidium pullulans*, *Dothioraceae* sp., *Candida* cf. *apicola, C.* cf. *azyma*, *C. cellae, Metschnikowia* sp., *Kodamaea ohmeri* and *Yarrowia lipolytica*. Undescribed yeast of the genus of *Metschnikowia* was also discovered in this study. At present, we assume there is association between *C.* cf. *apicola* and species closely related to *C. cellae* with *A. cerana*. Yeasts species associated with PCB differ from those found in NCB, indicating that PCB and NCB possess different and specific yeasts communities. Some yeasts species isolated from *A. cerana* show a low degree of similarity to their closest related species. Our study sheds light on the detection of several new taxa of yeasts associated with *A. cerana*.

Key words: Apis cerana, yeasts, diversity, ITS regions of rDNA

The association between yeasts and insects has been extensively reported by many scientists over the last three decades (Batra *et al*. 1973; Shandu and Waraich 1985; Lachance *et al*. 1990; Hagler *et al*. 1993; Lachance *et al*. 1998; Lachance *et al*. 2001a; *Teixeira et al*. 2003; Zacchi and Vaughan-Martini 2003; Such and Blackwell 2004; Lachance *et al.* 2005; Such *et al*. 2005; de Vega *et al*. 2009; Unal *et al.* 2009). Insect gut habitats harbour an astonishing diversity of previously undescribed yeasts (Suh *et al*. 2005). Their finding suggests that many unknown yeasts are waiting to be found.

Many scientists working in temperate countries have reported the functional relationships between honeybees and their associated yeasts, but the reports on yeasts associated with honeybees in tropical countries are scarce. Most of honeybee-associated yeasts studied were isolated from *Apis mellifera*, with only one study was reporting on *A. cerana* (Sandhu and Waraich 1985).

Since relatively little is known about the association of yeasts and the honeybee in tropical countries, we investigated yeasts associated with *A. cerana* collected on the campus of the University of Indonesia, Depok, Indonesia. *Apis cerana* or the Asiatic honeybee (or the Eastern honeybee) is distributed widely in Indonesia and is native to most parts of Asia. This local honeybee can be found living in the wild or domesticated. This honeybee is one out of five species of honeybees native to Indonesia (Hadisoesilo 2001).

In this study, we examined yeast diversity from samples isolated from the guts of pollen-collecting bees (PCB) and nectar-collecting bees (NCB) of *A. cerana* foraging on flowers of *J. integerrima* on the campus of the University of Indonesia. Yeast identification was determined based on sequence data of internal transcribed spacer (ITS) regions of ribosomal DNA. Phylogenetic analysis of closely related species is possible by the use of the region spanning the two intergenic transcribed spacers (ITS1 & ITS2) and the 5.8S ribosomal subunit. The ITS region is subdivided into the ITS1 region which separates the conserved 18S and the 5.8S rRNA genes (James *et al.* 1996). The ITS2 region is found between the 5.8S and 28S rRNA genes. The ITS regions are less conserved as a result of fewer evolutionary constraints, and hence they can be used to discriminate between yeast species (Kurtzman 2001). Intergenic transcribed spacers divergence in yeast species is marked, species being clearly separated by at least 1% sequence diversity (Sugita *et al*. 1999; Caligiorne *et al*. 2005).

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MATERIALS AND METHODS

Collection of Honeybees. Sampling of 30 honeybees from the Universitas Indonesia (UI), Depok, campus was conducted between January and April 2009. Collections of *A. cerana* were made repeatedly at different localities on the UI campus. Thirty adult worker bees of *A. cerana* collecting pollen (PCB) and nectar (NCB) were captured when foraging on flowers of *J. integerrima*. The bees were collected in sterile polyethylene bags (each individual adult worker honeybee was placed in a separate plastic bag), brought to the laboratory, and processed immediately. The bees were kept in the freezer after collection to make them inactive and easy to handle. The bees were kept alive until dissection.

Yeast Isolation and Culture. Bees are surface disinfected by submerging in 5.25% (v/v) NaOCl solution for 1 min to disinfect the surface. The NaOCl wash was followed by a 0.7% (w/v) NaCl rinse and the rinse-liquid was then plated on yeast extract agar 50% glucose (YAG 50%) supplemented with chloramphenicol as a negative control. Sterile forceps were used to dissect each bee under a stereo microscope. The bee gut was removed aseptically and transferred to mortar containing 1 mL sterile MilliQ water. Gut segments were crushed in the sterile with MilliQ water using a pestle. The suspension then was transferred to an Erlenmeyer flask containing 99 mL sterile water and vortexed for 1 min to homogenize the suspension. The suspension was filtered using Whatman filter paper and then the filter paper was transferred to YAG 50% plate. The filtrate was again filtered on a millipore membrane (pore size 0.45 µm) using a vacuum pump. The millipore membrane was transferred to YAG 50% plate. Plates were incubated at room temperature (26-28°C), and after three days all single colonies were picked up using sterile toothpicks and put into colony libraries. The representative colonies of each morphological type were purified at least two times on yeast malt extract Agar (YMA) plates supplemented with chloramphenicol (100 mg L^{-1}) and maintained on potato dextrose agar (PDA) slants. The cultures from this study were deposited in the University of Indonesia Culture Collection (UICC).

Amplification of ITS Regions. A cell suspension with 1 loopful of yeast cells in 250 µL of sterile MilliQ water was boiled for 20 min, and a 9 µL aliquot of supernatant from centrifugation for 15 min at 16 200 x g was used directly for the polymerase chain reaction (PCR) to amplify the ITS regions of rDNA (about 300-900 bp). The PuRe*Taq*™ Ready-To-Go™ PCR beads [GE Healthcare] was used for PCR reaction with the total volume of 25 µL. The primer sets ITS5-ITS4 were used for amplifying 5.8S rDNA and internal transcribed spacer (ITS) sequences (White *et al*. 1990) using the polymerase chain reaction (PCR). PCR products were purified using a DNA purification kit QIAquick PCR columns (Qiagen). The purified PCR products were used as templates for sequencing with an ABI PRISM™ BigDye Terminator Cycle sequencing kit, version 3.1 (PE Applied Biosystems, Foster City, CA). The cycle sequencing products were purified using QIAquick Spin Column (Qiagen). The complete sequence of 5.8S rDNA, including ITS regions of the rDNA, were sequenced with the primers ITS4 and ITS5 using an ABI PRISM 310 automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

DNA Sequence Analysis. The ITS sequences data were sent to online international DNA database for homology search by the Basic Local Algorithm Search Tool (BLAST) (Altschul *et al.* 1997). BLAST searches were used to identify new isolates. DNA sequences were aligned with other sequences obtained from the GenBank database using the multialignment program Clustal X (Thompson *et al.* 1994). The phylogenetic tree was constructed by Neighbour-Joining method using Kimura two parameters (Saitou and Nei 1987). Bootstrap values were obtained from 1 000 replications (Felsenstein 1985).

RESULTS

Identification of Yeasts from *Apis cerana*. We selected 14 out of 35 yeast isolates from the gut of PCB and NCB of *A. cerana* as representative isolates for identification. The representative yeast isolates were selected after two-times screening, based on their morphological type, and are presented in Table 1. Yeast isolates with the same morphotype were omitted from this table.

Molecular identification of the representative yeast isolates based on their internal transcribed spacers (ITS) regions of ribosomal DNA sequences data showed that they were closely related to *Aureobasidium pullulans*, *Candida* cf. *apicola, Candida* cf. *azyma*, *C. cellae, Dothioraceae* sp., *Kodamaea ohmeri*, *Metschnikowia* sp. and *Yarrowia lipolytica*. The homology of the sequences of some isolates show a low degree of similarity to their closest related species (homology ≤98%) (Table 1). Three isolates have 99% homology of their sequences to their closed related species, *i.e*. C*andida cf. apicola, Candida cf. azyma and Y. lipolitica*.

The *Aureobasidium* closely-related isolates were found in both PCB and NCB (Table 1). On the other hand, C*andida* cf. *apicola, Candida.* cf. *azyma, Y. lipolytica, K. ohmeri* and *Metschnikowia* sp. closely-related isolates were found only in PCB, while *C. cellae and Dothioraceae* sp. were only isolated from NCB. The f*requency of occurrence* of yeast isolates from PCB and NCB is presented in Table 2. *Candida cf. apicola* has the highest frequency of occurrence in PCB (33%), followed by *K. ohmeri* closely related isolates with frequency of occurrence of 22%. In NCB, the highest

Table 1 Identification of yeast isolates from pollen- and nectar-collecting bees of *Apis cerana* foraging on *Jatropha integerrima*

Isolate codes	Closely related species (% homology)	Bee type
P 1 M (A)	*Candida* cf. *apicola* (98%)	PCB
P 1 M (B)	*C.* cf. *apicola* (99%)	PCB
P 1 M (C)	*Kodamaea ohmeri* (98%)	PCB
P 1 M (D)	*C.* cf. *azyma* (99%)	PCB
P 3 M (2)	*Aureobasidium pullulans* (96%)	NCB
P 3 M (3)	*C. cellae* (91%)	NCB
P 3 M (4)	*C. cellae* (92%)	NCB
P 3 M (5)	*C. cellae* (89%)	NCB
B 2 M (A)	*Dothioraceae* (98%)	NCB
B 5 M	*Metschnikowia* sp. (87%)	PCB
KM 1 M	*C.* cf. *apicola* (98%)	PCB
KM 2 M (A)	*Yarrowia lipolitica* (99%)	PCB
KM 3 M (B)	*A. pullulans* (98%)	PCB
IK 2 M (A)	*K. ohmeri* (97%)	PCB

PCB = pollen-collecting bee; NCB = nectar-collecting bee.

Table 2 The f*requency of occurrence* of yeast isolates from pollen- and nectar-collecting bees of *Apis cerana* foraging on *Jatropha integerrima*

Closely-related Species	Frequency of occurrence (%) in	
	PCB	NCB
Aureobasidium pullulans	11	20
Candida cf. *apicola*	33	-
C. cf. *azyma*	11	-
C. cellae	-	60
Dothioraceae	-	20
Kodamaea ohmeri	22	-
Metschnikowia sp.	11	-
Yarrowia lipolitica	11	-

PCB = pollen-collecting bee; NCB = nectar-collecting bee.

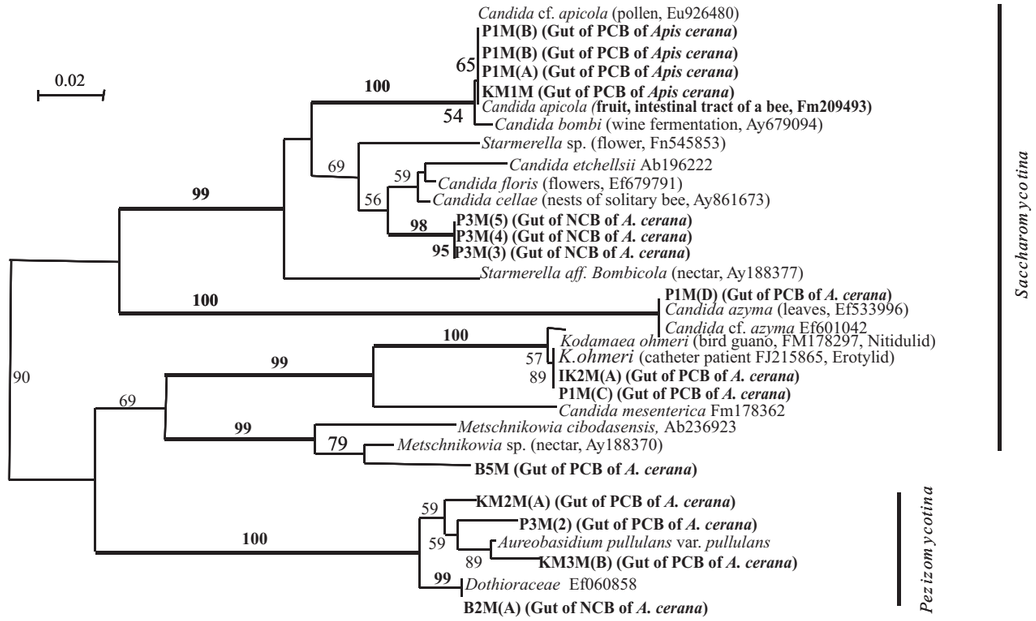

Fig 1 Unrooted phylogenetic tree of yeast isolates from *Apis cerana* foraging on *Jatropha integerrima* and their closely related species and substrates where found. Yeasts from *A. cerana* were grouped into the sub-phyla *Saccharomycotina* and *Pezizomycotina* of the phylum *Ascomycota*. Yeasts isolated from this study are indicated by bold. The tree was constructed by Neighbour-Joining method based on ITS region sequence data.

frequency of occurrence belongs to C. cellae closely related isolates (60%).

Phylogenetic Placement. The phylogenetic placement of yeast isolates within the phylum of *Ascomycota* and their relationship to neighboring species is presented in Fig 1. This tree shows that all yeasts from *A. cerana* were grouped into the sub-phyla *Saccharomycotina* and *Pezizomycotina* of the phylum *Ascomycota.* Some species of yeasts from *A. cerana* isolated in this study were clustered with other yeasts associated with bees or other insects obtained from the GenBank database. Other species from *A. cerana* were closely related to yeasts associated with flower, nectar, leaves or other substrates.

DISCUSSION

All yeasts isolated from the gut of *A. cerana* found in this study are phylogenetically closely related to yeasts species within the phylum *Ascomycota*. The genera of *Aureobasidium* and *Dothioraceae* belong to the class *Euascomycetes* of the sub-phylum *Pezizomycotina*. Other species, i.e. *Candida* cf. *apicola, C.* cf. *azyma*, *C. cellae, K. ohmeri*, *Metschnikowia* sp. and *Y. lipolytica* belong to the class *Hemiascomycetes* of the sub-phylum *Saccharomycotina*. Lachance *et al*. (2001b) mentioned that ascomycetous yeasts from the order *Saccharomycetales* are the dominant yeasts in beetle-flower-yeast system. Brysch-Hetzberg (2004) also found that similar ascomycete species predominated in both flowers and bees. The *Ascomycota* were able to tolerate high sugar concentrations while *Basidiomycota* were not.

Yeasts have been isolated frequently from the gut or surface of insects that feed on a variety of materials, including basidiomycete fruiting bodies, woody substrates, ephemeral flowers and nectar exudates (Suh and Blackwell 2004; Nguyen *et al.* 2007; Nakase *et al.* 2009). Some yeast species found in this study have been reportedly isolated from bees or other insects associated flowers. Lachance *et al*. (2001b) collected *A. pullulans* and *C. azyma* from bees. They found *A. pullulans* and *C. azyma* were among the most frequently isolated yeasts from the flower-beetle-yeast system. *Aureobasidium* sp. was also isolated from honeybee *A. mellifera* (Johnson *et al*. 2005). Rosa *et al*. (2003) found *A. pullulans* and *K. ohmeri* from stingless bee species, and strains identified as members of the *C. apicola* complex from a stingless bee *Melipona quadrifasciata*. Stratford *et al*. (2002) suggested strongly that *C. apicola* was one of several yeast species that is primarily associated with the Aculeates (bees and wasps). In their study carried out in Atlantic rain forest of Brazil, Pimentel *et al*. (2005) isolated *C. cellae* from pollen-nectar provisions of a solitary bee *Centris tarsata*. The yeast genus *Metschnikowia* is the predominant genus in both bees and flowers. Most species of *Metschnikowia* were found in the nectar or corolla of flowers or in decaying fruit or plant tissue; they are transmitted to new niches by insects, such as bees and drosophilids (Lachance *et al.* 2001a). According to Hagler *et al*. (1993), the genus *Metschnikowia* isolated from terrestrial habitats is typically associated with flowers or fruits and insects.

The present study shows that the yeast species isolated from *A. cerana* differ from yeast species previously reported

by Shandu and Waraich (1985). The difference might be due to a distinct origin of *A. cerana*, geographically and taxonomically at the sub-species level, and/or their visited flowers. Our study revealed that a specific community of yeasts could be found in the gut of *A. cerana* native to Indonesia and foraging mainly on the flowers of *J. integerrima*. Manson *et al*. (2007) mentioned that the composition of the yeast community is highly dependent on the types of insects involved. In his study, Brysch-Hetzberg (2004) concluded that the attractiveness of plants to the flower-visiting insects appears to have had a greater impact on the abundance and frequency of yeasts in the nectar of different plant species.

Our study also found different yeast communities to be present in PCB and NCB. The only species common to both PCB and NCB was *Aureobasidium* closely-related species. The study shows the occurrence of specific yeast species in PCB and NCB, suggesting the specificity of yeasts in PCB and NCB. Based on the frequency of occurrence of *C. apicola* (33%) in PCB and C. cellae closely related isolates (60%) in NCB, we assume there is an association between both yeast species with A. cerana. This finding is supported by Rosa *et al*. (2003) who showed that *Candida cellae* belongs to the *Starmerella* clade, a group of several related species that are generally associated with bees. Stratford *et al*. (2002) also speculated that insects may form the normal environment for *C. apicola*. However, we need to strengthen our hypothesis by thoroughly isolating and identifying yeasts from more PCB and NCB. Some yeasts isolated from *A. cerana* found in this study showed a low degree of similarity to their closest related species (homology ≤98%). In this study, yeast identification at species level was based on the species guideline from the work of Sugita *et al*. (1999). The isolate was assigned to a species if the sequence revealed a homology of ≥99% to a reference sequence. Our study sheds light on the existence of several new taxa of yeasts associated with *A. cerana*. We need to carriedcarry out further studies to identify them.

Some of the yeasts species found in this study are consistent with the previous studies on bee-flower-associated yeasts conducted by other workers. Lachance *et al*. (2001a) and Stratford *et al*. (2002) believe that the relatedness of bee-associated yeast species indicate the functional relationship between yeasts and bees as supported by phylogenetic evidence. Suh *et al*. (2005) found host specialization among some of the associated insects and yeasts indicated a significant interaction between the two group organisms.

Gilliam (1997) reported that bees use yeasts to process pollen before it is suitable as a food. Fermentation has been the suspected means of transforming pollen into bee bread. Insects, principally nitidulid beetles and drosophilid flies, are vector of a highly specific yeast community that may serve as food for the larvae of the insects (Lachance *et al.* 2001b; Lachance and Bowles, 2002). *Aureobasidium* sp. was known to be antagonist to *A*. growth. *Ascosphaera apis is* the causative agent of the brood disease chalkbrood in *A. mellifera* larvae (Johnson *et al*. 2005). At present, yeasts are implicated in bee nutrition, but there may be additional roles of yeasts in the bee life history.

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