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The assessment of epiphytic yeast diversity in sugarcane phyllosphere in Thailand by culture-independent method

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ABSTRACT

The diversity of epiphytic yeasts from sugarcane (*Saccharum officinarum* Linn.) phyllospheres in Thailand was investigated by culture-independent method based on the analysis of the D1/D2 domains of the large subunit rRNA gene sequences. Forty-five samples of sugarcane leaf were collected randomly from ten provinces in Thailand. A total of 1342 clones were obtained from 45 clone libraries. 426 clones (31.7 %) were closely related to yeast strains in the GenBank database, and they were clustered into 31 operational taxonomic units (OTUs) with a similarity threshold of 99 %. All OTU sequences were classified in phylum *Basidiomycota* which were closely related to 11 yeast species in seven genera including *Cryptococcus flavus*, *Hannaella coprosmaensis*, *Rhodotorula taiwanensis*, *Jaminaea angkoreiensis*, *Malassezia restricta*, *Pseudozyma antarctica*, *Pseudozyma aphidis*, *Pseudozyma hubeiensis*, *Pseudozyma prolifica*, *Pseudozyma shanxiensis*, and *Sporobolomyces vermiculatus*. The most predominant yeasts detected belonged to *Ustilaginales* with 89.4 % relative frequency and the prevalent yeast genus was *Pseudozyma*. However, the majority were unable to be identified as known yeast species and these sequences may represent the sequences of new yeast taxa. In addition, The OTU that closely related to *P. prolifica* was commonly detected in sugarcane phyllosphere.

Introduction

Phyllosphere or phylloplane, the aboveground surfaces of the plant, has been recognized as an important habitat for microorganisms. Bacteria, yeasts, and filamentous fungi can inhabit the phyllosphere using nutrients on leaf surfaces as carbon sources such as carbohydrates, amino acids, organic acids, sugar alcohols, and salts (Tukey 1970; Fiala *et al.* 1990;

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Weibull et al. 1990; Dik et al. 1991; Leveau & Lindow 2001). In addition, some epiphytic microorganisms have been reported to produce plant hormones which promote cell wall loosening and release of saccharides from plant cells as their substrates (Fry 1989; Brandl & Lindow 1998; Lindow & Brandl 2003). Furthermore, they may produce biosurfactants which facilitate wettability and further enhance the leaching of substrates (Bunster et al. 1989; Schreiber et al. 2005). The microbial communities of leaf surfaces depend on many factors including the availability of nutrients which is dependent on leaf age, plant species and growing conditions as well as environmental factors such as radiation, pollution and nitrogen fertilization. Moreover, biotic factors including competing microorganisms affect microbial communities of the plant phyllosphere (Irvine et al. 1978; Jumpponen & Jones 2010; Zimmerman & Vitousek 2012).

Sugarcane is a monocotyledonous plant of the family *Poaceae*. It is hypothesized that sugarcane originated in New Guinea (Daniels et al. 1996). To date, sugarcane is grown in more than 70 countries, mainly in tropical areas. In Thailand, sugarcane (*Saccharum officinarum* Linn.) is the second most important economic crop. The Office of Cane and Sugar Board under the jurisdiction of the Thailand Ministry of Industry reported in 2012–2013 approximately 1.52 million hectares was used for sugarcane cultivation with a production yield of 107.44 million tons of sugar. In 2013 the Food and Agriculture Organization of the United Nations reported Thailand was the fourth largest sugarcane producer. However, destructive plant diseases caused by pathogenic fungi are a major problem in the sugarcane cultivation industry. It has been previously reported that leaf surfaces are colonized by members of several genera of saprophytic yeasts and that these provide a natural barrier to protect against plant pathogens (Avis & Bélanger 2002; De Costa et al. 2006; Pusey et al. 2009). Therefore, the study of yeast communities associated with this crop and their antagonistic effects on pathogens may facilitate the application of novel and effective biocontrol approaches. In addition, yeast diversity data is fundamental to the comprehensive study of microbiological functions within nature. In recent years the diversity of yeast and fungal communities within the phyllosphere has been studied intensively. However, only a small number of these studies have focused on yeast communities associated with sugarcane (de Azeredo et al. 1998; Nakase et al. 2001; Limtong et al. 2014). De Azeredo et al. (1998) studied the yeast communities associated with sugarcane in Campos, Rio de Janeiro, Brazil by culture dependent method and reported that basidiomycetous yeasts were prevalent on leaf (79.6 %). The prominent yeast species isolated from leaf washings were in phylum *Basidiomycota* including *Cryptococcus albidus*, *C. laurentii*, and *Rhodotorula mucilaginosa*. Whereas *Debaryomyces hansenii* was the prevalent ascomycetous yeast species isolated from sugarcane leaves in Brazil. However using enrichment isolation technique the ascomycetous yeasts (69 %) were found to be more prevalent than basidiomycetous yeasts (31 %) in sugarcane phyllospheres in Thailand (Limtong et al. 2014). The predominant species was *Meyerozyma caribbica* followed by *Rhodotorula taiwanensis* and *Candida tropicalis*, respectively. Nakase et al. (2001) reported that yeasts isolated from phyllospheres of various plant species including sugarcane collected in Thailand by using the ballistoconidium-fall isolation method, were identified as 21 species within the following six genera *Bensingtonia*, *Bullera*, *Kockovaella*, *Sporidiobolus*, *Sporobolomyces*, and *Tilletiopsis*. These differing results appear to be due to the use of different isolation techniques. However, other factors that may affect yeast diversity and cannot be ruled out. It has been reported that environmental factors such as seasonal fluctuations and climatic conditions are likely to have an effect on microbial biodiversity (Glushakova and Chernov, 2010; Jumpponen & Jones 2010; Kachalkin & Yurkov 2012; Voriskova et al. 2014) as well as geography (Maksimova et al. 2009; Yurkov et al. 2015). Additionally, Finkel et al. (2011) demonstrated that microbial phyllosphere communities between plant species show significant similarity within the same locale, whereas trees of the same species and growing in different climatic regions are host to distinct microbial communities.

Since the 1980s, molecular based methods based on the direct amplification and analyses of ribosomal RNA genes have been developed for investigation of microbial diversity in diverse environments. These methods overcome the problems of culture-dependent methods as it is estimated that 99 % of microorganisms presented in nature are not typically able to be cultivated (Amann et al. 1995; Takaku et al. 2006). For yeast identification the rRNA gene has been preferred for molecular taxonomy over other genes because of (1) its universality, which allows comparisons to virtually any organism; (2) the presence of multiple gene copy numbers which evolve in concert; and (3) the belief that it is ancestrally homologous, having originated from a single evolutionary event (Sogin et al. 1986), allowing systematic study of yeast relationships. The nucleotide sequence of the D1/D2 domain of the large subunit (LSU) rRNA gene has been widely used to identify the majority of yeast species in phylums *Ascomycota* and *Basidiomycota* by direct sequence comparison, since the D1/D2 domain nucleotide sequences of nearly all known yeast species are available within the GenBank database (Kurtzman & Robnett 1998; Fell et al. 2000). The aim of this study was therefore to investigate the diversity of epiphytic yeasts of sugarcane phyllosphere using a culture-independent method based on the D1/D2 domain of the large subunit rRNA gene using a PCR based molecular technique.

Materials and methods

Sample collection

Forty-five samples of sugarcane (*S. officinarum* Linn.) leaf were randomly collected from sugarcane plantations in ten provinces in Thailand between January 2012 and April 2012 (Table 1). Leaf samples were stored in plastic bags, sealed and kept refrigerated on ice before transfer to the laboratory. The samples were stored at 4 °C until analysis. DNA was extracted from leaf washings of the samples within 7 d of collection.

DNA extraction of microbial epiphytes

Leaf samples (6 g) were cut and submerged in washing buffer (1× Phosphate buffered saline, Tween 20), sonicated for 7 min