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Yeasts associated with *Euploea* butterflies

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Abstract

The yeasts were observed attached to the mouthparts, wings, and forelegs of migratory butterflies in Taiwan. Fifty-eight dominant yeast strains were isolated from 56 *Euploea* butterflies and identified by rDNA ITS and D1/D2 sequencing. The yeasts which associated with *Euploea* butterflies included ascomycetous yeasts, such as *Aureobasidium* sp., *Candida chanthaburiensis*, *C. corydalis*, *Metschnikowia koreensis*, *Metschnikowia* sp., and *Debaryomyces hansenii*; and basidiomycetous yeasts, such as *Cryptococcus rajasthanensis*, *Dirkmeia churashimaensis*, *Filobasidium globisporum*, *Hannaella pagnoccae*, *Papiliotrema flavescens*, *Pseudozyma hubeiensis*, *P. tsukubaensis*, *Pseudozyma* sp., *Rhodotorula mucilaginosa*, *R. subericola*, and *Rhodospordiobolus poonsookiae*. The most common yeast, *Candida corydalis*, associated with 4 migratory *Euploea* species and seemed transmitted through nectar. When the butterflies feed on nectar, bask in the sun on the leaf, sip at the moisture on leaves, in puddles or wet sand and soil, they come into contact with yeasts. This is the first report about the yeast microbiome of migratory butterflies.

Key words – insect-fungus associations – insect microbiome – milkweed butterflies – Saccharomycotina

Introduction

More than 400 species of butterflies have been recorded in Taiwan (Hsu 1999), and including four species of the tribe Danaini (i.e. *Euploea tulliolus koxinga* Fruhstorfer, *E. sylvester swinhoei* Wallace & Moore, *E. mulciber barsine* Fruhstorfer and *E. eunice hobsoni* Butler). Species of *Euploea* overwinter as adults and have migration behaviour as the Monarch butterfly (*Danaus plexippus*) (Chao et al. 2007). From April to September, they are widely distributed in Taiwan. When air temperature decreases in winter, they migrate from north to south and overwinter from October to March in several warm and windless valleys in Lukuea, Taiwu, Laiyi and Dawu (Wang & Emmel 1991). Their adults feed on nectars of flowers, such as *Eupatorium* spp., *Lantana camara* and some other species (Hsu 1999), while the larval feed on milkweeds.

The interaction between pollinators and flowers provides opportunities for transferring pollen grains and dispersing microorganisms. The pollinators such as bees, beetles, flies, hummingbirds, and bats have vectored numerous species of microbes including yeasts when they visited flowers (Brysch-Herzberg 2004, Lachance et al. 2005, Belisle et al. 2014, Pozo et al. 2014). Yeasts

commonly present in nectar and utilize the sugars in the nectar (Herrera et al. 2010, Peay et al. 2011).

Butterflies are important pollinators, but only a small number of studies reported the yeasts associated with butterflies (Harrison et al. 2016, Whitaker et al. 2016). Migratory butterflies provide opportunities for long-distance pollen grains transferring, at the same time, they may harbor and transfer the nectar-specialist yeasts or transient yeasts. The objective of this study was to document the dominant yeast diversity associated with four migratory *Euploea* butterflies.

Materials & Methods

We observed the yeasts on the mouthparts, wings, and forelegs of butterflies under a scanning electron microscope (SEM). Tiny pieces of mouthparts, wings or forelegs from butterflies were fixed with 2.5% glutaraldehyde at 4°C for 12 hrs and then washed 3 times with 0.1M phosphate buffered saline for 15 min. After this fixation process, the specimens were dehydrated in graded ethanol (30%, 50%, 70%, 85%, 95%, 100%, 15 min each), and critical-point dried. Samples were coated with gold and examined with a scanning electron microscope.

The captured butterfly was put into a sterilized ziplock plastic bag with an acid YM plate (Yarrow 1998) for 15 minutes. They stood on the medium and suck it very often, their mouthparts, legs, and wings touched the medium. We released the butterflies and collected the plates. All plates were incubated at room temperature three days and checked daily for yeast growth. There were diverse yeasts in an individual butterfly, a dominant colony type of each sample was selected and purified. Fifty-eight yeast strains which belong to 17 yeast species and 9 genera were isolated from 56 individuals of 4 *Euploea* butterflies (4 *Euploea tulliolus koxinga*, 6 *E. sylvester swinhoei*, 30 *E. mulciber barsine* and 16 *E. eunice hobsoni*). To preserve the isolated yeasts, they were grown on YM agar plates for 3-7 d at 25°C, transferred to broth cultures supplemented with 30% (w/v) glycerol and stored at -80°C. Total DNA from the yeasts was extracted by the CTAB method (Doyle & Doyle 1990). For sequence analyses of the yeasts, the PCR amplification of the rDNA ITS and D1/D2 region was undertaken using the universal primers ITS1/ITS4 (White et al. 1990) and F63/LR3 (Fell et al. 2000), respectively. PCR products were directly sequenced by an ABI PRISM 3730 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). Yeast strains were identified using nucleotide sequences. The nucleotide sequences were compared with sequences deposited in NCBI GenBank by BLAST, with the defaults settings. We assigned names to the sequences according to the following criteria: (1) sequence identity of $\geq 98.0\%$; (2) $\geq 1.0\%$ separation from the next closest species; (3) $\geq 95.0\%$ sequence coverage for the matching sequence; and (4) matching sequence published in a peer-reviewed journal article or submitted by the culture collection centers (CLSI 2008). Additionally, some species which were not able to identify by ITS sequences, such as *Cryptococcus* species, were identified to species level based on LSU sequences following the same criteria.

Results

SEM pictures show that there were typical globose or ellipsoid yeast cells attached on the mouthparts, wings, and forelegs of butterflies which collected in the field (Fig. 1). In Fig. 1, the budding yeast cells on the mouthpart and foreleg indicated that they could grow there. According to their ITS and D1/D2 sequences, the dominant yeasts which associated *Euploea* butterflies included *Aureobasidium* sp., *Candida chanthaburiensis*, *C. corydalis*, *Cryptococcus rajasthanensis*, *Debaryomyces hansenii*, *Dirkmeia churashimaensis*, *Filobasidium globisporum*, *Hannaella pagnoccae*, *Metschnikowia koreensis*, *Metschnikowia* sp., *Papiliotrema flavescens*, *Pseudozyma hubeiensis*, *P. tsukubaensis*, *Pseudozyma* sp., *Rhodotorula mucilaginosa*, *R. subericola*, and *Rhodospordiobolus poonsookiae* (Table 1). *Candida corydalis* was isolated most frequently and associated with four *Euploea* species. There were 3, 4, 16 and 12 isolates of *C. corydalis* isolated as dominant from 3 *Euploea tulliolus koxinga*, 4 *E. sylvester swinhoei*, 12 *E. mulciber barsine* and 11 *E. eunice hobsoni*, respectively. *Dirkmeia churashimaensis* and *P. hubeiensis* both associated with an *E. mulciber barsine* and an *E. eunice hobsoni*. The other 14 species were found as dominant

yeasts only on individual butterflies. Among 18 yeast species, *Candida corydalis*, *Cryptococcus rajasthanensis*, *Dirkmeia churashimaensis*, *Pseudozyma hubeuensis*, *Pseudozyma tsukubaensis* and *Rhodospordiobolus poonsookiae* were new records to Taiwan.

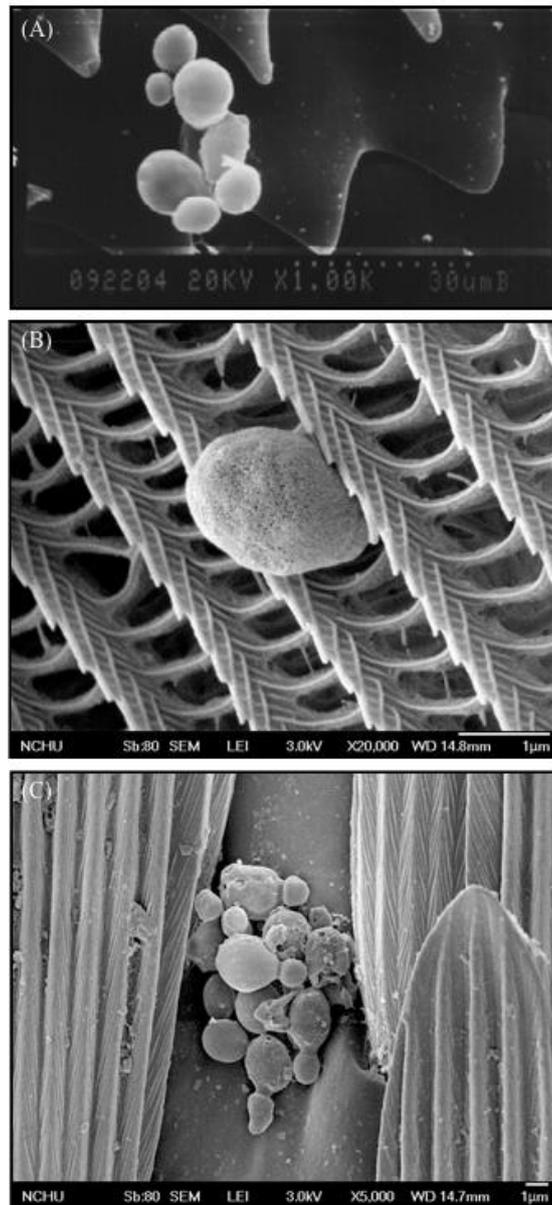


Figure 1 – The SEM pictures show that there were yeasts on the mouthparts (A) wings. (B) and (C) forelegs of butterflies which collected in the field.

Discussion

In this study, *Candida* and *Metschnikowia* were the most common yeasts associated with *Euploea* butterflies. They were found in highly abundance in flower nectar (Lachance et al. 2001, Brysch-Herzberg 2004, Lachance 2006, Herrera et al. 2009, Pozo et al. 2011, Belisle et al. 2012, Pozo et al. 2011) and considered as specialized nectar-dwelling yeasts (Brysch-Herzberg 2004, Lachance 2006, Herrera et al. 2010). Most of them grow fast and are able to tolerate an environment of high osmotic concentration (Lachance et al. 2001, Alvarez-Pérez & Herrera 2013, Mittelbach et al. 2015, Mittelbach et al. 2016). *Candida* was the most common yeasts associated with pollinating bees (Sandhu & Waraich 1985) and *Drosophila* (Chandler et al. 2012).

Candida corydali was isolated most frequently from the 4 *Euploea* butterfly species and nectar of their nectariferous plants *Eupatorium tashiroi* Hayata and *Bidens pilosa* (our unpublished data). These butterflies might get the yeasts by sucking the nectar and cleaning the mouthpart by the front pair of legs, the yeasts harbored on the butterfly. The budding yeast cells on the mouthpart and foreleg of *Euploea* butterflies were observed by SEM demonstrated that they grew there, which indicated some yeasts may inhabit on butterfly as an ecological niche. These 4 migratory *Euploea* butterfly species could be the carriers to disperse yeasts.

In this study, basidiomycetes yeasts *Cryptococcus*, *Pseudozyma*, *Rhodotorula*, and *Rhodospordiobolus* were also isolated as dominant yeast. They are primary phyllosphere colonizers (Allen et al. 2006, Last & Price 1969, Fonseca & Inácio 2006, Mittelbach et al. 2015) or come from soil (Fonseca & Inácio, 2006, Yurkov et al. 2012). The butterflies may carry these transient basidiomycetous yeasts by sucking up water from leaf surface or staying at the phylloplane for foraging and basking (Mittelbach et al. 2016). They were rarely isolated in the flower nectar and have been hardly associated with nectar-foraging insects (Lachance et al. 2001, Brysch-Herzberg 2004, Pozo et al. 2011, Mittelbach et al. 2016). A next-generation sequencing study show that *Malassezia restricta*, *M. globose* and *Rhodospordium diobovatum* associated with *Lycaeides melissa* adults developed from larval in the laboratory (Harrison et al. 2016). These three species belong to basidiomycetous yeasts, they persisted on the larval and leaves of larval food plant (Harrison et al. 2016). Their results indicated that butterfly can obtain basidiomycetous yeasts from phylloplane.

Our results demonstrated at least 17 dominant yeast species harbored on the mouthparts, wings, and forelegs of 4 migratory *Euploea* butterflies, including nectariferous ascomycetous yeasts and phyllosphere basidiomycetous yeasts. When the butterflies feed on nectar, bask in the sun on the leaf, sip at the moisture on the leaf, in puddles or wet sand and soil, they come into contact with yeasts. There are high diversity yeasts in nectar, litter, leaf surfaces, fruits, plant tissues (Fonseca & Inácio 2006) and soil (Yurkov et al. 2012). To our knowledge, this is the first report about the yeast microbiome of migratory butterflies.

Table 1 Yeasts associated with *Euploea* butterflies.

	Yeasts associated with <i>Euploea</i> butterflies				Total
	<i>E. tulliolus koxinga</i>	<i>E. sylvester swinhoei</i>	<i>E. mulciber barsine</i>	<i>E. eunice hobsoni</i>	
<i>Aureobasidium</i> sp.	1	0	0	0	1
<i>Candida chanthaburiensis</i>	0	1	0	0	1
<i>Candida corydali</i>	3	4	16	12	35
<i>Cryptococcus rajasthanensis</i>	0	0	1	0	1
<i>Debaryomyces hansenii</i>	0	0	1	0	1
<i>Dirkmeia churashimaensis</i>	0	0	1	1	2
<i>Filobasidium globisporum</i>	0	0	1	0	1
<i>Hannaella pagnoccae</i>	0	0	2	0	2
<i>Metschnikowia koreensis</i>	0	0	2	0	2
<i>Metschnikowia</i> sp.	0	0	1	0	1
<i>Papiliotrema flavescens</i>	0	0	1	0	1
<i>Pseudozyma hubeiensis</i>	0	0	4	1	5
<i>Pseudozyma</i> sp.	0	0	0	1	1
<i>Pseudozyma tsukubaensis</i>	0	0	1	0	1
<i>Rhodotorula mucilaginoso</i>	0	1	0	0	1
<i>Rhodotorula subericola</i>	0	0	0	1	1
<i>Rhodospordiobolus poonsookiae</i>	0	0	1	0	1
Numbers of yeast strains	4	6	32	16	58
Numbers of yeast species	2	3	12	5	17

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