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Pseudozyma antarctica in Taiwan: a description based on morphological, physiological and molecular characteristics

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Abstract. The genus *Pseudozyma* is an anamorph that belongs to the Ustilaginales. *Pseudozyma* species are unusual yeast-like fungi and are most frequently isolated from plant materials. The *Pseudozyma* strain, BCRC 33871, was isolated from the flower of *Albizia julibrissin* in Taiwan. BCRC 33871 was identified as *P. antarctica* and described based on morphological, physiological, and molecular data. This discovery marked the first finding of this species in Taiwan. To ensure correct identification, the intraspecific variations of *P. antartica* were illustrated based on physiological characteristics and phylogenetic analysis of ITS1 and ITS2 rDNA sequences. Additionally, distinguishing characteristics of *P. antartica* and other related species were discussed.

Keywords: Morphology; New Record; Physiological characteristics; *Pseudozyma*; rDNA sequencing; Taiwan.

Introduction

Ustilaginales are economically important fungi. *Ustilago* is the largest genus, and most of its species parasitize monocotyledonous hosts. A number of anamorphs of Ustilaginales have been classified in diverse genera, for example *Candida, Pseudozyma, Sporobolomyces, Sterigmatomyces, Stephanoascus,* and *Trichosporon* (Boekhout et al., 1998). These species have been reclassified into *Pseudozyma* Bandoni emend. Boekhout by morphological, physiological, biochemical, and molecular data (Boekhout, 1995). Phylogenetic analysis inferred from partial sequences of the 26S rDNA indicated that *Pseudozyma* species and Ustilaginales parasitizing grasses form a monophyletic group. Furthermore, the type species of *Pseudozyma*, *P. prolifica*, is most closely related to *Ustilago maydis*. Accordingly, *Pseudozyma* species are anamorphs of Ustilaginales that parasitize grasses (Boekhout, 1995; Boekhout et al., 1995; Begerow et al., 2000).

Pseudozyma species are unusual yeast-like fungi and are most frequently isolated from plant materials, such as leaves, flowers, and fruits (Boekhout and Fell, 1998; Trindade et al., 2002). Seven species are listed in the genus *Pseudozyma* by Boekhout and Fell (1998): *P. antarctica, P. aphidis, P. flocculosa, P. fusiformata, P. prolifica, P. rugulosa*, and *P. tsukubaensis*. Recently, Sugita et al. (2003) isolated *Pseudozyma* strains from patient blood in Thailand and named two new species, *P. parantarctica* and *P. thailandica*.

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A project involving the screening of osmophilic yeast-like fungi in Taiwan isolated one species of *Pseudozyma* which was identified as *P. antarctica*. This discovery marked the first finding of this species in Taiwan. Assessing identity and diversity is extremely crucial in identifying of *P. antarctica* for industrial procedures. This investigation aimed to (i) describe the local strain, and (ii) analyze the intraspecific and interspecific variation of *P. antarctica* using a polyphasic approach.

Materials and Methods

Isolation of BCRC 33871

The *Pseudozyma* isolate, No. 176, was isolated from a flower of *Albizia julibrissin* in Taiwan using a slight modification of the method developed by Hajny et al. (1964). The collected samples were inoculated into 20-ml flasks containing 5 ml of a medium comprising 40% glucose and 1% yeast extract and were incubated at 30°C under shaking for six days. The cultures were streaked on plate medium containing 20% glucose, 1% yeast extract, and 2% agar. Pure cultures were established by picking and transferring individual colonies to the same medium. The culture was deposited as BCRC 33871 in the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute.

Morphological, Physiological and Biochemical Characteristics

The morphological and physiological characteristics were examined using the methods described by Yarrow (1998). Moreover, BCRC 33871 was compared with the type

strains of *P. antarctica*, BCRC 33858, and published descriptions of related species (Boekhout and Fell, 1998; Sugita et al., 2003).

Preparation of Genomic DNA

The culture was inoculated into YM broth (DIFCO 0712) and harvested after incubation at 20°C for ten days. Additionally, DNA for PCR was extracted using the Chelex method (Chen, 1998).

PCR Amplification and Direct DNA Sequencing of rDNA

The internal transcribed spacer (ITS) regions of the rRNA gene were defined using primers ITS5 (5´-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5´-TCCTCCGCTTATTGATATGC) (White et al., 1990). The reaction was performed in a GeneAmp PCR system 9700 (Applied Biosystems) using 30 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min with an initial denaturation at 94°C for 5 min before a cycling and a final extension at 72°C for 7 min. Sequencing reactions were performed employing the ABI PRISM™ BigDye™ Terminator, v 3.0 Ready Reaction Cycle Sequencing Kit as directed by the manufacturer. Finally, the PCR products were sequenced using the ABI PRISM™ 3700 DNA analyzer.

Molecular Phylogenetic Analysis

The BCRC 33871 sequence was compared with those of *Pseudozyma antarctica* and other related species (Table 1). Sequences were aligned using CLUSTAL W (Thompson et al., 1994). Additionally, the alignment of all sequences was visually checked and optimized, and alignment gaps were treated as an additional character state. A phylogenetic analysis was conducted, using a neighbor joining program from the package PHYLIP 3.5C (Felsenstein, 1993). The parsimony program implemented in PHYLIP was used to compare the tree topologies. For neighbor-joining analysis, the distances between sequences were calculated using the Maximum Likelihood model (Kishino and Hasegawa, 1989). Finally, a bootstrap analysis was performed with 1,000 replications. Trees were viewed using TreeView (Page, 1996).

Results and Discussion

Morphological and Physiological Description of BCRC 33871

Pseudozyma antarctica (S. Goto, Sugiyama & Iizuka) Boekhout, 1995, J. Gen. Appl. icrobiol. 41: 359-366. (Figure 1)

=*Sporobolomyces antarcticus* S. Goto, Sugiyama & Iizuka, 1969, Mycologia 61: 759.

Growth on 5% malt extract agar: After five days at 20°C, cells are cylindrical to fusiform, with variable size, 5.0-8.1 × 1.8-2.4 μm. Conidiogenesis is polar on short denticles and has sympodial proliferation. Hyphae are abundant, 1.5-2.4 μm width and with sterigmata on which fusiform blastoconidia are formed. Colonies are dimorphic, smooth to somewhat irregularly furrowed, pale cream-white, and with the margin fringed.

Slide culture on 5% malt extract agar: After five days at 20°C, cells are cylindrical to fusiform, with variable size, 3.6-16.9 × 1.5-3.1 μm. Conidiogenesis is polar on short denticles and with sympodial proliferation. Hyphae are abundant, 1.5-2.2 μm wide and with sterigmata on which fusiform blastoconidia are formed.

Habitat. flower of *Albizia julibrissin.*

Specimen examined. **TAIWAN.** Chanhua, Teinwei, April 17, 1998. (BCRC 33871= isolation no. 176)

Fermentation of carbon compounds: see Table 2

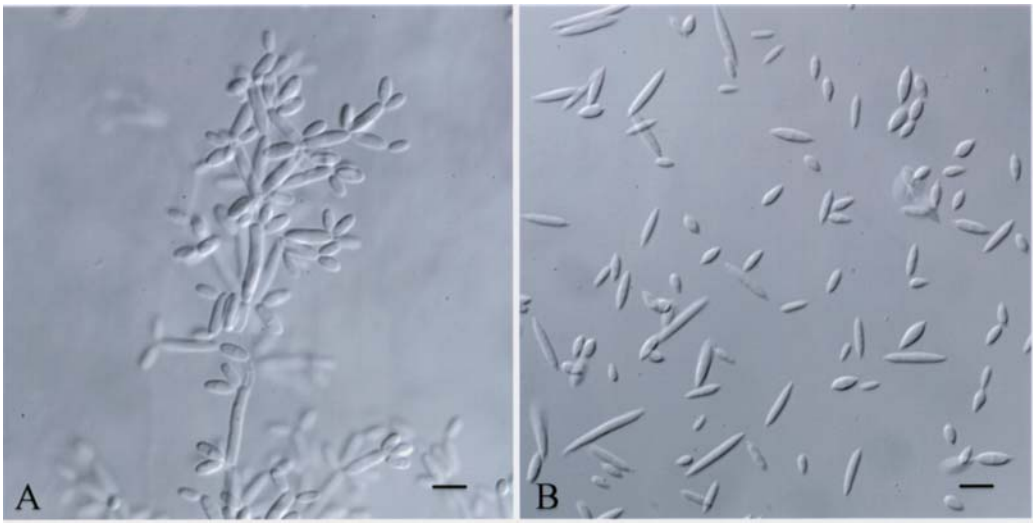

Figure 1. *Pseudozyma antarctica* BCRC 33871 growth on 5% malt extract agar at 20°C for 5 days. A, hyphae and chains of blastoconidia; B, blastoconidia, scale bar = 5 μm.

Table 1. Strains used in this study.

Species / Strain no.	Source	GenBank accession numbers
P. antarctica (S. Goto, Sugiyama & Iizuka) Boekhout		
JCM 10317^T (CBS 214.83)	Lake sediment, Antarctica	AB089358[a]
CBS 516.83	Unpolished rice, Japan	AF294698[b]
M9935	Thailand	AB089376[a]
M9954	Blood, man, Thailand	AB089374[a]
BCRC 33871	Flower of *Albizia julibrissin*, Taiwan	AY641557
P. aphidis (Henninger & Windisch) Boekhout		
JCM 10318^T (CBS 517.83)	Secretion of Aphididae, Germany	AB089362[a]
Pseudozyma flocculosa (Traquair, Shaw & Jarvis) Boekhout & Traquair		
JCM10321^T (CBS 167.88)	Leaf of clover infected with mildew, Canada	AB089364[a]
Pseudozyma fusiformata (Buhagiar) Boekhout		
JCM3931^T (CBS 423.96)	Cauliflower, Great Britain	AB089366[a]
Pseudozyma parantarctica Sugita, Takashima, Mekha & Poonwan		
JCM 15422^T	Blood, woman, Thailand	AB089356[a]
Pseudozyma prolifica Bandoni		
CBS 319.87^T	Litter of *Scirpus microcarpus*, Canada	AF294700[b]
Pseudozyma rugulosa (Traquair, Shaw & Jarvis) Boekhout & Traquair		
JCM 10323^T (CBS 170.88)	Leaf of maize infected with molds, Canada	AB089370[a]
Pseudozyma thailandica Sugita, Takashima, Mekha & Poonwan		
JCM 11753^T	Blood, woman, Thailand	AB089354[a]
Pseudozyma tsukubaensis (Onishi) Boekhout		
JCM10324^T (CBS 422.96)	Flower, Japan	AB089372[a]
Sporisorium aegypticum (Fischer von Waldheim) Vánky		
Specimen[b]	*Schismus arabicus*	AY344970[b]
Sporisorium cruentum (Kuhn) Vánky		
Specimen[b]	*Sorghum halepense*	AY344974[b]
Sporisorium scitamineum (Syd.) M. Piepenbr., M. Stoll & Oberw.		
Specimen[b]	*Saccharum* sp.	AY345007[b]
Sporisorium sorghi Ehrenb. ex Link		
Specimen[c]	*Sorghum bicolor*	AF038828[c]
Ustilago crameri Korn		
Specimen[b]	*Setaria italica*	AY344999[b]
Ustilago maydis (De Candolle) Corda		
Specimen[c]	*Zea mays*	AF038826[c]
Specimen[b]	*Zea mays*	AY345004[b]

[T]Type strain; [a]Sugita et al. (2003); [b]Stoll et al. (2003); [c]Roux et al. (1998).

BCRC, Bioresource Collection and Research Center, Food Industry Research & Devolopment Institute, Hsinchu, Taiwan, ROC; CBS, Centraalbureau voor Schimmelcultures; Utrecht, The Netherlands; JCM, Japan Collection of Microorganisms; RIKEN, Saitama, Japan; M, Meiji Pharmaceutical University, Tokyo, Japan.

Assimilation of carbon compounds and nitrogen compounds: see Table 2.

rDNA Sequence Analysis

The phylogenetic tree presented here indicated a close relationship between *Pseudozyma* and *Ustilago*. This result was in accordance with Boekhout (1995) and Begerow et al. (2000). On the other hand, one *Sporisorium* species, *S. aegypticum*, was assigned to the *Ustilago-Pseudozyma* clade. However, Stoll et al. (2003) illustrated the basal position of *S. aegypticum* to *Ustilago*.

In our phylogram, BCRC 33871 was clustered in the clade of *Pseudozyma antartica* (Figure 2). The sequences of ITS regions of BCRC 33871 were identical to the type strain of *P. antarctica*, JCM 10317^T (CBS 214.83), and patients' blood isolates, M9935 & M9954. BCRC 33871 and *P. antarctica* CBS 516.83, isolated from unpolished rice in Japan, displayed 99.8% similarity. Sugita et al. (1999) found that conspecific strains have a less than 1% nucleotide difference in their ITS regions, after comparing the nuclear DNA-DNA hybridization. The overall ITS sequence similarity between strains of *P. antarctica* was more than 99%.

Table 2. Comparison of physiological characteristics of BCRC 33871 with type and reference strains of *Pseudozyma antarctica* and related species.

	P. antarctica BCRC 33871	*P. antarctica* BCRC33858[T]	*P. antarctica*[a]	*P. aphidis*[a]	*P. rugulosa*[a]	*P. parantarctic*[b]
Fermentation of carbon compounds:						
Carbon compounds	–	–	–	–	–	–
Assimilation of carbon compounds:						
Glucose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Sorbose	+	+	+	S	+	W
Glucosamine	+	W	+	+	+	+
Ribose	+	+	+	+	+	+
Xylose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+
D-Arabinose	W	W	+	+	+	+
Rhamnose	+	–	V	+	+	+
Sucrose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Threhalose	+	+	+	+	+	+
α-Methyl-D-glucoside	+	+	+	+	+	+
Cellobiose	+	+	+	S	S	+
Salicin	W	W	+	S	S	+
Arbutin	W	W	+	+	+	+
Melibiose	+	–	–	+	S	+
Lactose	+	+	+	+	–	+
Raffinose	+	+	+	+	+	+
Melezitose	+	+	+	+	+	+
Inulin	–	–	–	–	–	–
Starch	+	+	+	+	+	+
Glycerol	+	+	+	S	+	+
Erythritol	S	+	+	+	S	L
Ribitol	W	L	L	S	S	+
Xylitol	+	+	n	n	n	L
Arabinitol	S	+	n	n	n	–
Glucitol	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Galactitol	–	–	–	–	–	–
myo-inositol	+	+	+	+	+	+
Glucono-1.5-lactone	+	+	n	n	n	+
2-keto-gluconate	+	+	+	+	+	+
Gluconate	S	S	+	S	+	+
Glucuronate	+	+	+	+	+	+
Galacturonate	+	+	n	n	n	+
Lactate	W	W	+	+	+	+
Succinate	+	+	+	+	+	+
Citrate	W	W	+	+	+	W
Methanol	–	–	–	–	W	–
Ethanol	+	+	+	–	+	–
Propane	+	+	n	n	n	+
Butane	–	–	n	n	n	–
Quinate	+	+	n	n	n	n
Saccharate	–	–	–	+	+	+
Galactonate	–	–	n	n	n	n
Assimilation of nitrogen compounds:						
Nitrate	+	+	+	+	+	+
Nitrite	+	+	+	+	+	+
Ethylamine	+	+	n	n	n	–
Lysine	+	+	n	n	n	+
Cadaverine	+	+	n	n	n	+
Creatine	–	–	n	n	n	n
Creatinine	–	–	n	n	n	n
Glucosamine	+	+	+	+	+	N
Imidazole	–	–	n	n	n	N
Other characteristics:						
w/o Vitamins	–	–	–	–	–	W
0.1% cycloheximide	–	–	n	n	n	n
0.01% cycloheximide	–	–	n	n	n	n
Growth at 30°C	+	+	+	+	+	+
Growth at 37°C	+	–	V	+	+	+

[T]Type strain; [a]Boekhout and Fell (1998); [b]Sugita et al. (2003). Carbon compounds: glucose, galactose, maltose, methyl-D-glucoside, sucrose, trehalose, melibiose, lactose, cellobiose, melezitose, raffinose, inuline, D-xylose; +, positive; L, delayed positive (latent); S, slow positive; V, variable; W, weakly positive; –, negative; n, not determined.

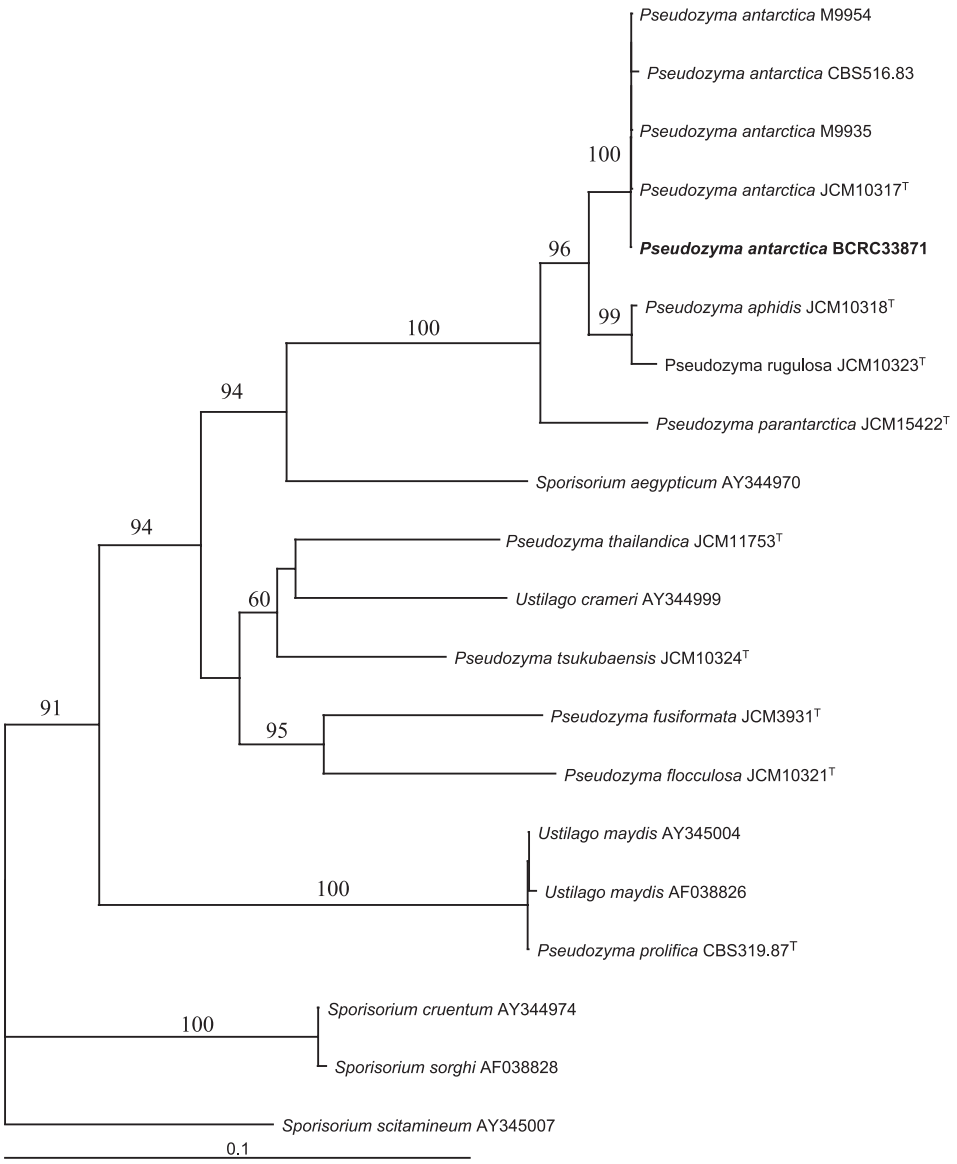

Figure 2. Neighbor-joining tree of strains of *Pseudozyma antarctica* and related species inferred from 587 nucleotides from ITS1 and ITS2 rDNA. Topology was rooted with *Sporisorium scitamineum*. Numbers given on branches indicate the confidence level from a 1,000-replicate bootstrap sampling. (Frequencies below 50% are not indicated.)

On the other hand, the ITS sequences displayed a less than 97% similarity between *P. antarctica* and known *Pseudozyma* species.

The type strain of *P. antarctica* was isolated from lake sediment in Antarctica and was initially classified in the genus *Sporobolomyces* based on morphological and physiological properties. However, morphologically it differs from other *Sporobolomyces* species by lacking ballistospores (Goto et al., 1969; Boekhout, 1995). Recent studies have suggested a closer relationship to the genus *Pseudozyma* (Boekhout, 1995; Begerow et al., 2000). More recently, the strains of *P. antarctica* were isolated from unpolished new crop rice in Japan (Boekhout and Fell, 1998), various fruits in Brazil (Trindade et al., 2002), blood from a patient in Thailand (Sugita et al., 2003), and the *Albizia julibrissin* flower in Taiwan. These strains from Antarctica, Japan, Thailand, and Taiwan display highly similar physiological characteristics (Table 2) and sequences in the ITS regions of their rDNA (Figure 2). Therefore, based on the morphological, physiological, and molecular data, BCRC 33871 was identified as *P. antarctica*.

Based on the phylogenetic analysis inferred from partial sequences of the 18S rDNA (Avis et al., 2001) and D1/D2 26S rDNA sequences (Sugita et al., 2003), *P. antarctica* is closely related to *P. aphidis* and *P. rugulosa*. *Pseudozyma aphidis* was proposed to be conspecific with *P. antarctica* (Kurtzman, 1990). However, based on DNA-DNA hybridization, partial 26S rDNA sequences and physiological differences, *P. aphidis* was considered a separate species (Boekhout and Fell, 1998). Furthermore, analysis

Table 3. Key characters of *P. antarctica* and related species.

	Assimilation of						
	Galactose	L-Rhamnose	Salicin	Lactose	Melibiose	Ethanol	Saccharate
P. antarctica	+	V	+	+	V	+	–
P. aphidis	+	+	+	+	+	–	+
P. rugulosa	+	+	+	–	S	+	+
P. parantarctica	+	+	+	+	+	+	+
P. flocculosa	+	–	+	–	+	–	–
P. fusiformata	–	–	V	–	V	+	–
P. tskubaensis	+	–	–	+	–	+	–
P. thailandica	+	–	+	–	+	+	–
P. prolifica	+	+	–	+	–	+	–
Ustilago maydis	–	+	+	+	–	+	–

+, positive; S, slow positive; V, variable; –, negative.

of the ITS regions supported the conclusion of Boekhout and Fell (1998) as did the findings of Sugita et al. (2003).

Comparison of the Physiological Characteristics of P. antarctica and Related Species

Table 3 lists the physiological characteristics of *P. antarctica* and related species. Carbohydrates are not fermented in the genus *Pseudozyma* (Boekhout and Fell, 1998). Therefore, assimilation tests and other growth characteristics are important for the identification of *Pseudozyma* species. BCRC 33871 differs from *P. antarctica* JCM 10317^T (CBS 214.83) because it grows at 37°C and assimilates melibiose and rhamnose. For *P. antarctica*, growth at 37°C and assimilation of rhamnose are variable, based on the description of the type strain and on the isolate from Japan (Boekhout and Fell, 1998). Melibiose is not assimilated, based on the description of the type strain and other isolates from Thailand and Japan. However, the strain found in Taiwan, BCRC 33871, can assimilate melibiose, and therefore the character of melibiose assimilation in *P. antarctica* is variable.

Moreover, *P. antarctica* displays similar physiological characteristics in the assimilation to *P. aphidis* and *P. rugulosa*. However, *P. antarctica* differs from *P. aphidis* in assimilation of ethanol and saccharate, and differs from *P. rugulosa* in assimilation of lactose and saccharate. In comparing physiological characteristics, the assimilation of lactose, ethanol, and saccharate are key to circumscribing *P. antarctica* and closely related species.

Pseudozyma antarctica is a recently characterized yeast-like fungus capable of bioconverting n-alkalines into glycolipid biosurfactants (Kitamoto et al., 2001). It is also capable of β-glucosidase activity and mycocin production (Trindade et al., 2002). When selecting fungi for a specific function, it is essential to accurately classify and identify the isolates. In conclusion, this investigation indicates that *P. antarctica* can be genetically and physiologically differentiated from other closely related species. On the other hand, the conidial structures of *Pseudozyma* species exhibit poor differentiation and have a highly variable shape and size. Therefore, application of ITS rDNA sequence analysis is useful for the identification of *Pseudozyma* species.

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台灣 *Pseudozyma antarctica* 之形態、生理及分子特性之探討

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Pseudozyma 屬為黑穗菌科無性世代之類酵母真菌，大部分的種分離自植物組織，在台灣自合歡花分離到 *Pseudozyma* 分離株，已寄存於食品工業發展研究所生物資源保存及研究中心，編號為 BCRC 33871，依據形態特徵、生理生化特性及 ITS1-5.8S-ITS2 rDNA 序列分析結果，鑑定種名為 *P. antarctica*，為台灣新紀錄種。此外本文利用生理生化特性及 rDNA 序列分析資料探討 *P. antarctica* 之種內及近似種間的差異，並討論用以區分 *P. antarctica* 與近似種之關鍵性特徵。

關鍵詞： *Pseudozyma*；rDNA序列；台灣；新紀錄種。