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Four Novel Ustilaginomycetous Anamorphic Yeast Species Isolated as Endophytes from the Medicinal Plant *Hyoscyamus muticus*

[1,2]F.F. Abdel-Motaal, [2]S.A. El-Zayat, [1]Y. Kosaka, [1,2]M.A. El-Sayed, [2]M.S.M. Nassar and [1]Shin-ichi Ito
[1]Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Yamaguchi, 753-8515, Japan
[2]Department of Botany, Faculty of Science, South Valley University, Aswan 81528, Egypt

Abstract: Eleven isolates of basidiomycetous yeast were obtained from *Hyoscyamus muticus* plants as endophytes; they were observed to comprise four *Pseudozyma* species based on morphological and physiological analyses. Molecular taxonomic analysis based on nucleotide sequences of the D1/D2 domain of the large subunit ribosomal RNA gene (D1/D2), internal transcribed spacer region of the rRNA gene (ITS) and mitochondrial rRNA genes (both large and small subunits) revealed that the four isolates represented distinct species and formed a cluster with *Macalpinomyces ericachnes* and *Moesziomyces eriocauli* (Ustilaginaceae). D1/D2 and ITS sequence analyses also indicated that the four isolates were genetically distinct from all known *Pseudozyma* species, suggesting that the isolates belonged to four new species.

Key words: Endophytic yeast, *Pseudozyma*, ustilaginomycetous yeast

INTRODUCTION

Hyoscyamus muticus L. (Egyptian henbane) which belongs to the family Solanaceae is considered an important medicinal plant. The plant is famous for its valuable secondary metabolites of which tropane alkaloids are the majority. The main alkaloids present are scopolamine and hyoscyamine. The effects of these alkaloids include stimulation of the central nervous system and simultaneous depression of the peripheral nerves typical for a parasympathomimetic. Among the medicinal uses are spasmolytic, antiasthmatic, anticholinergic, narcotic and anaesthetic properties (McClatchey *et al*., 2009). Recently, we found that antifungal activity of hyoscyamine and *H. muticus* plants may contain antifungal compounds other than alkaloids (Abdel-Motaal *et al*., 2009a).

Despite that *H. muticus* has a potential antifungal activity, some fungi can colonize this plant as rhizospheric, surface and endophytic fungi (El-Zayat *et al*., 2008). Endophytes are present in all plants and survive without causing any apparent damage or diseases (Petrini *et al*., 1992). Moreover, endophytes play an important role in host protection against pathogens (Azevedo *et al*., 2000). Endophytic fungi in *H. muticus* plant exhibited potential biocontrol agents against some plant pathogenic fungi (Abdel-Motaal *et al*., 2009b).

Endophytic yeast has been isolated from different plants, including roots of banana (Cao *et al*., 2002) and maize (Nassar *et al*., 2005), stems of cottonwood (Xin *et al*., 2009), leaves of tomato (Larran *et al*., 2001), wheat (Larran *et al*., 2002) and rice (Tian *et al*., 2004). Few studies have dealt with the role of endophytic yeasts to plants so far. Some endophytic yeasts isolated from roots of maize are found to be plant-growth promoting organisms through production of auxins (Nassar *et al*., 2005). Similar function of three yeasts occurring in *Populus* trees that are able to produce indole-3-acetic acid has been reported by Xin *et al*. (2009).

Identification of yeast species from morphologically and physiologically characters has been improved by molecular methods in recent years. Nucleotide sequences of domains 1 and 2 (D1/D2) of large-subunit of 26S rRNA have been used as a rapid and effective method to identify species (Fell *et al*., 2000; Kurtzman, 2006). Moreover, the internal transcribed spacer (ITS) regions are useful to distinguish between basidiomycetous yeast species (Scorzetti *et al*., 2002).

Yeasts can be classified into two phylogenetic groups; ascomycetes and basidomycetes (Kurtzman and Robnett, 1998). *Pseudozyma* sp. as anamorphic yeast-like species belonging to the Ustilaginales, have been described morphologically, physiologically and phylogenetically (Begerow *et al*., 2000, 2006; Boekhout,

Corresponding Author: Shin-ichi Ito, Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Yamaguchi 753-8515, Japan
Tel: +81 83 933 5846 Fax: +81 83 933 5820

1995; Boekhout *et al*., 1995). Ustilaginales including smut genera *Lundquistia*, *Melanopsichium*, *Moesziomyces*, *Macalpinomyces*, *Sporiosorium* and *Ustilago* (Stoll *et al*., 2005) contains three clades: *Sporisorium*, *Ustilago* and a basal clade of both *Ustilago* and *Sporisorium* species. Each *Pseudozyma* species belongs to one of these groups. For example, *P. antarctica* is included in *Ustilago* clade (Wei *et al*., 2005), *P. graminicola* is located in *Sporisorium* clade (Golubev *et al*., 2007), while *P. jejuensis* is clustered in the *Ustilgo-Sporisorium* clade (Seo *et al*., 2007).

Pseudozyma species are distributed worldwide and are mainly associated with plants (Boekhout and Fell, 1998) and isolated from leaves in China (Wang *et al*., 2006), Russia (Golubev *et al*., 2007) and South Korea (Seo *et al*., 2007) and from flowers in Taiwan (Wei *et al*., 2005; Liou *et al*., 2009). *Pseudozyma* species have also been isolated from human blood samples (Sugita *et al*., 2003).

During a survey of endophytic fungi habited in *H. muticus*, 11 isolates belonging to Basidiomycetes were identified based on morphological, physiological and molecular analyses. In the present study, we determined that the 11 isolates represented four novel species belonging to the genus *Pseudozyma*.

MATERIALS AND METHODS

Yeast isolation: Yeasts were isolated from *H. muticus* plants growing in a green house (temperature, 10-37°C; humidity >50%; light, 10-14 h) at Yamaguchi University, Yamaguchi, Japan. The study was conducted from 19 December, 2006 to 20 November, 2008. Plants were cut into roots, stems and leaves. All plant organs were surface-sterilized in 70% ethanol for 1 min followed by application of 5% sodium hypochlorite solution for 5 min, washed twice with sterilized distilled water and then blotted with sterilized filter papers (Rossman *et al*., 1998). The samples were longitudinally cut into pieces measuring 0.5-1 cm and were placed directly on a sterilized petri dish containing Corn Meal Agar (CMA). Four replicate plates were incubated at 25°C for 2-3 weeks.

Morphological and physiological characteristics: The morphological and physiological characteristics were examined following the methods described by Yarrow (1998).

Molecular phylogenetic analysis: DNA sequences of D1/D2 domains of the large subunit ribosomal RNA (D1/D2), the internal transcribed spacer region of the rRNA gene (ITS) and mitochondrial rRNA genes (large and small subunits both) were amplified by PCR (Arias *et al*., 2002; White *et al*., 1990). Primer pairs used were as follows: NL-1 (5'-GCATATCAATAAGCGGAG GAAAA-3')/NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') forD1/D2,ITS1(5'-TCCGTAGGTGAACCTGCGG-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for ITS, ML7 (5'-GACCCTATGCAGCTTCTACTG-3')/ML8(5'-TTATCCCTAGCGTAACTTTTATC-3') for a large subunit of mitochondrial rRNA (LSU mt-rRNA) and MS1 (5'-CAGCAGTCAAGAATATTAGTCAATG-3')/MS2 (5'-GCGGATTATCGAATTAAATAAC-3') for the small subunit of mitochondrial rRNA (SSU mt-rRNA), respectively. Sequencing reactions were preformed employing the BigDye Terminator v. 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were determined using an AB1 Prism 3100 genetic analyzer (Applied Biosystems). The sequence data were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) at NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) and were aligned with the multiple alignment program CLUSTALX version 2 (Larkin *et al*., 2007). A phylogenetic tree was constructed from the evolutionary distance data with Kimura's two-parameter correction (Kimura, 1980). Phylogenetic analysis was performed using the neighbor-joining method (Saitou and Nei, 1987) with the program Seaview (Galtier *et al*., 1996) and bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

Nucleotide sequences: Accession numbers: The nucleotide sequences determined in this study have been deposited in the DNA Data Bank of Japan (DDBJ) under the following accession numbers: Strain Y2-09 (AB500690, AB385595), Strain Y3-09 (AB500691, AB385596), Strain Y4-09 (AB500692, AB447396) and Strain Y7-09 (AB500693, AB385599) for the ITS and D1/D2 regions, respectively.

RESULTS

The characters of the eleven strains of endophytic yeast, which were isolated from *H. muticus* plant, were studied based on morphological examination of cultures growing in 2% glucose-yeast extract-peptone (GYP), yeast malt extract (YM) and on GYP agar (GYPA), YM agar (YMA), morphology agar (MA), CMA and potato dextrose agar (PDA) and physiological characterization according to Yarrow (1998) and Boekhout and Fell (1998).

Y2-09: Growth on different media was as follows: the cells were ellipsoidal, elongated or cylindrical to fusiform with 2-5 oil droplets, in variable sizes (Fig. 1a-c) in GYP, GYPA

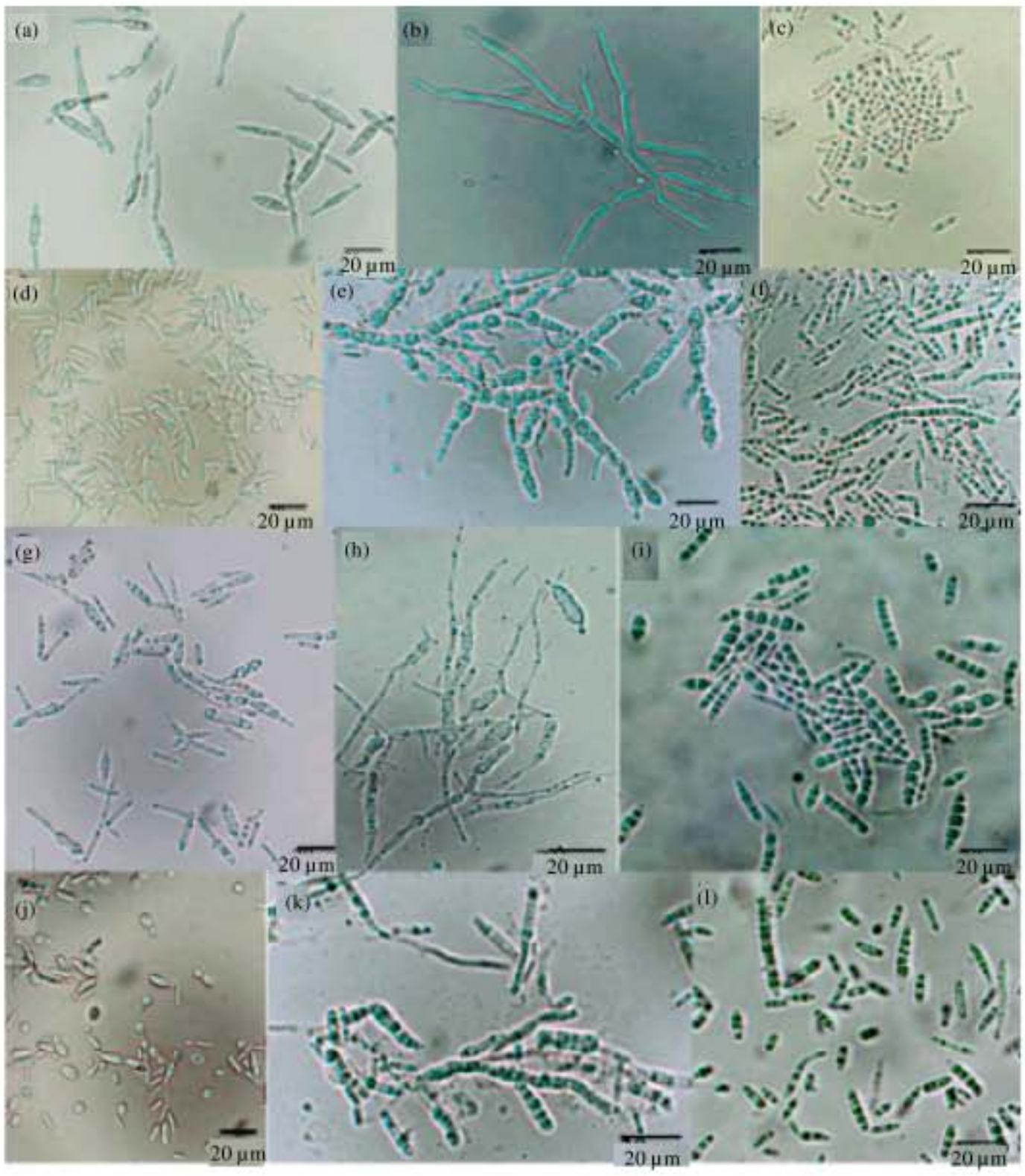

Fig. 1: Light microscopy of vegetative cells of *Pseudozyma* isolates, Y2-09, Y3-09, Y4-07 and Y7-09, incubated at 25°C. (a, d, g, j) Initial growth of vegetative yeast-like cells with polar budding in glucose-yeast extract-peptone broth at 3 days, (b, e, h, k) hyphae and chain of blastoconidia on yeast morphology agar (YMA) at 5 days and (c, f, i, l) cells contain storage material visible as prominent globules on YMA at 5 days

and PDA they measured 3.0-13.0×1.0-3.0 µm, while in YM and on YMA, they measured 6.0-26.0×1.2-2.4 µm. In the Dalmau plate culture on CMA and MA, cells measured 2.0-14.2×0.8-3.0 µm with the formation of pseudohyphae that measured 0.6-2.4 µm in diameter. Conidiogenesis was largely polar with bipolar conidiogenesis being common and acropetal, fusiform and lanceolate blastoconidia were formed in stigmata-like outgrowths arising near the hyphae septa. The streak culture was varied: color, creamy to light brown; texture, smooth to wrinkled; semi-glistening; netted, entire to lobbed margins. In GYP, after one-month incubation, a sediment and ring were present. A study of its physiological characteristics, as shown in Table 1, demonstrated that cellobiose, lactose and melibiose were assimilated; ribitol, galactitol and citric acid were weakly utilized, while salicin could not be assimilated. Growth at 37°C and in vitamin-free media was positive.

Y3-09: Growth on different media was as follows: cells were ellipsoidal, elongate, fusiform, or cylindrical in shape; single, in pairs, or in chains; in variable sizes; and contained many oil droplets (Fig. 1d-f). In GYP, GYPA and PDA, cells measured 2.8-17.0×1.0-4.0 µm; on YMA, 9.0-44.0×1.6-5.0 µm; in Dalmau plate culture on CMA and MA, 3.0-22.0×0.8-4.0 µm with formation of pseudohyphae measuring 1.0-4.0 µm in diameter. Budding was polar with monopolar budding being common. The aerial mycelium

Table 1: Physiological characterization of four new *Pseudozyma* isolates and other *Pseudozyma* species

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Fermentation of carbon compounds	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Assimilation of carbon compounds																	
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	LW	V	L	L
Galactose	+	+	+	+	+	+	L	+	+	+	L	-	L	LW	V	L	L
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	LW	V	L	L
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	LW	V	L	L
Cellobiose	+	+	+	+	+	+	+	L	L	+	+	L	L	LW	V	L	W
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	LW	V	L	L
Lactose	+	+	-	+	+	-	+	+	-	+	+	-	-	LW	V	L	L
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	LW	V	L	L
Salicin	-	+	+	-	+	W	-	L	L	+	-	V	+	-	V	L	W
Ribitol	W	+	+	-	+	+	L	L	L	L	-	L	L	+	V	-	L
L-Rhamnose	+	+	+	-	+	-	+	+	+	V	-	L	-	-	V	-	-
Melibiose	+	+	+	-	+	+	-	+	L	-	-	+	L	LW	V	-	L
Soluble starch	+	+	+	+	+	+	+	+	+	+	+	-	L	LW	V	-	L
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-
D-xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	L	L
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	L	L
D-Mannitol	+	W	+	W	+	+	+	+	+	+	-	+	+	+	V	L	L
D-Glucitol	+	W	+	+	+	+	+	+	+	+	-	+	+	+	V	+	L
Insitol	+	+	+	+	+	+	+	+	+	+	+	+	+	-	W	-	L
Galactitol	W	W	-	+	-	-	-	-	-	-	-	-	-	-	V	-	-
Succinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	-	L
Citric acid	W	+	+	+	W	LW	-	+	+	+	+	L	+	-	V	L	L
Assimilation of nitrogen compounds																	
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrite	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vitamin free medium	+	+	-	W	W	-	+	-	W	-	+	+	+	+	+	+	W
Growth at 37°C	-	+	+	+	+	+	-	+	+	V	-	-	-	+	+	+	-
Production of starch-like substances	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Growth on 50% (w/w) glucose-yeast extract agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

1: Y2-09, 2: Y3-09, 3: Y4-09, 4: Y7-09, 5: *P. parantactica DMST 15422*[a], 6: *P. thailandica DMST 15423*[a], 7: *P. prolifica CBS 319.87*[b], 8: *P. aphidis CBS 517.83*[b], 9: *P. rugulosa CBS 179.88*[b], 10: *P. antarctica CBS 214.83*[b], 11: *P. tsukubaensis CBS 422.96*[b], 12: *P. fusiformata CBS 6951*[b], 13: *P. flocculosa CBS 167.88*[b], 14: *P. hubeiensis WS 6.4*[c], 15: *P. shanxiensis SH 64*[c], 16: *P. jejuensis OL71*[d], 17: *P. graminicola L120*[e]. [a]Sugita *et al.* (2003), [b]Boekhout and Fell (1998), [c]Wang *et al.* (2006), [d]Seo *et al.* (2007), [e]Golubev *et al.* (2007), +: Positive; L: Latent; W: Weak; LW: Latent and weakly positive; V: Variable, -: Negative

consisted of ramifying and acropetal chains of fusiform blastoconidia. The streak culture was light brown to red brown in color, which changed to dark brown with long incubation; smooth to rough; rugose; and with lobbed margins. In GYP, after one-month incubation, a sediment and ring were present. Physiological characteristics are shown in Table 1. Most of carbon sources were assimilated except inuline was not assimilated while D-Mannitol, D-Glucitol and galactitol were weakly assimilated. Both nitrate and nitrite were utilized. Growth at 37°C and in vitamin free media were positive.

Y4-09: Cells were ovoid, elongate, cylindrical, or fusiform in shape; single, in pairs, or in chains (Fig. 1g-i); with 2-8 oil droplets; with variable sizes on different media, i.e., 2.4-14.0×1.0-3.2 μm on GYPA and PDA, respectively. The pseudohyphae diameter on Dalmau plate culture on CMA and MA was 1.0-2.9 μm and the vegetative cell size was 2.4-14.0×0.4-2.2 μm. Conidiogenesis was polar with presence of stigmata on which fusiform and cylindrical blastoconidia were formed. In YM and on YMA, cell size ranged between 6.0-28×2.0-4.0 μm. The streak culture was light brown to dark brown in color, smooth in texture, rugose and with lobbed margins. In GYP, after one-month incubation, a sediment and ring were present. The dissimilarity between this strain and other *Pseudozyma* species is explained in Table 1. Strain Y4-09 unable to assimilate lactose, inuline and galactitol while the remaining carbon sources and both nitrogen sources (nitrate and nitrite) were well utilized and growth at 37°C was positive while growth in vitamin free media was negative.

Y7-09: Cells were ovoid, fusiform, or cylindrical in shape; single or in pairs; with variable sizes, i.e., 1.2-18.4×1.0-4.0 μm in GYP, GYPA and PDA and 5.6-34.4×1.0-5.0 μm in YM and YMA. In Dalmau plate culture on CMA and MA, the cell size was 0.4-8.0×0.2-0.6 μm with formation of pseudohyphae with a diameter of 1.0-3.6 μm. Budding was polar with formation

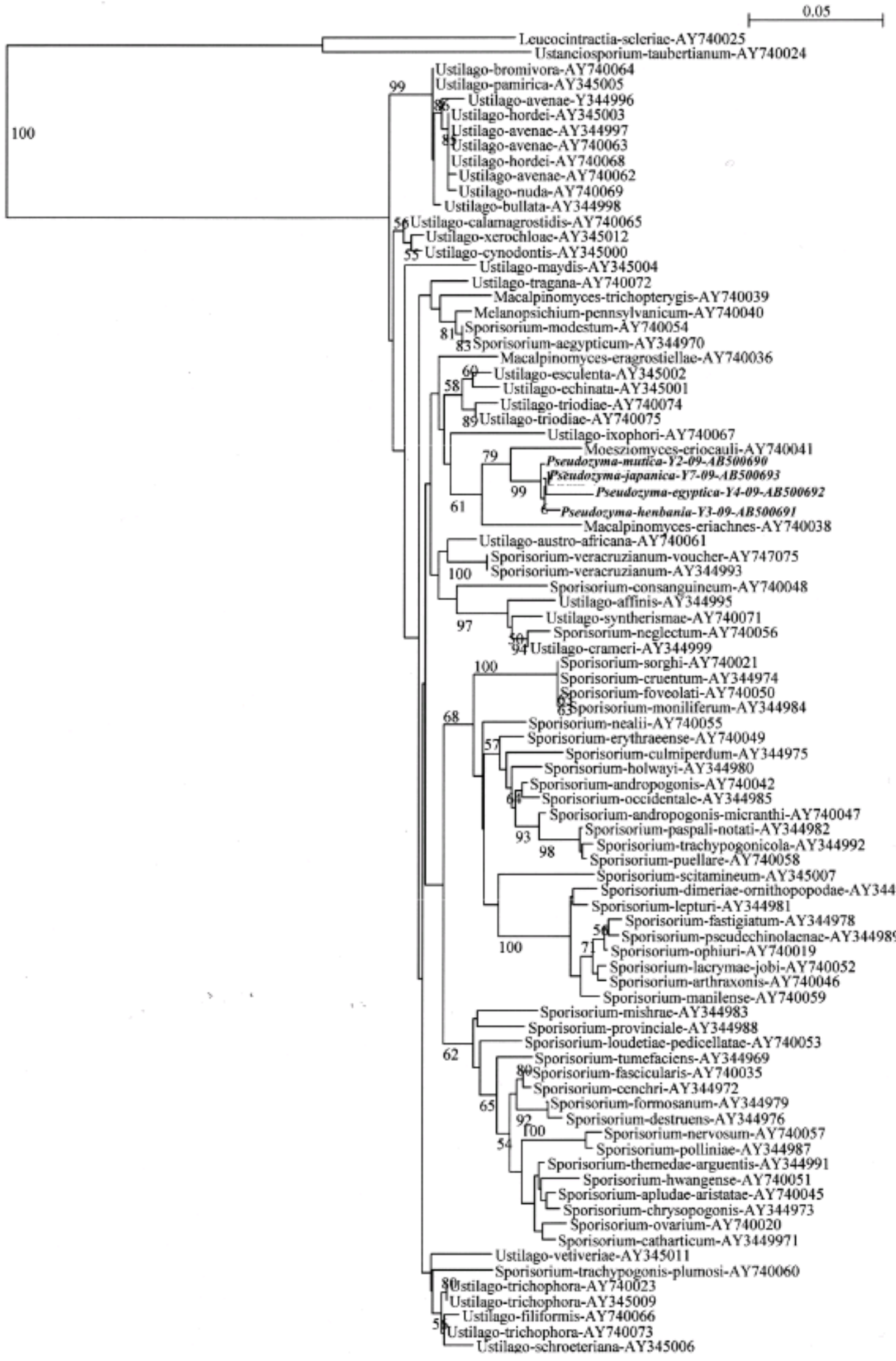

Fig. 2: A phylogenetic tree of basidiomycetous yeasts generated by the neighbor-joining algorithm based on the distance calculated by Kimura's two-parameter model from the sequences of ITS regions of the four new *Pseudozyma* species. The percentage numbers at the nodes indicate the levels of bootstrap support (> 50%) for branch points based on 1000 bootstrap replicates. *Leucocintractia scleriae* AY740025 and *Ustanciosporium taubertianum* AY 740024 were used as outgroups. Reference sequences were retrieved from GenBank under the accession numbers indicated

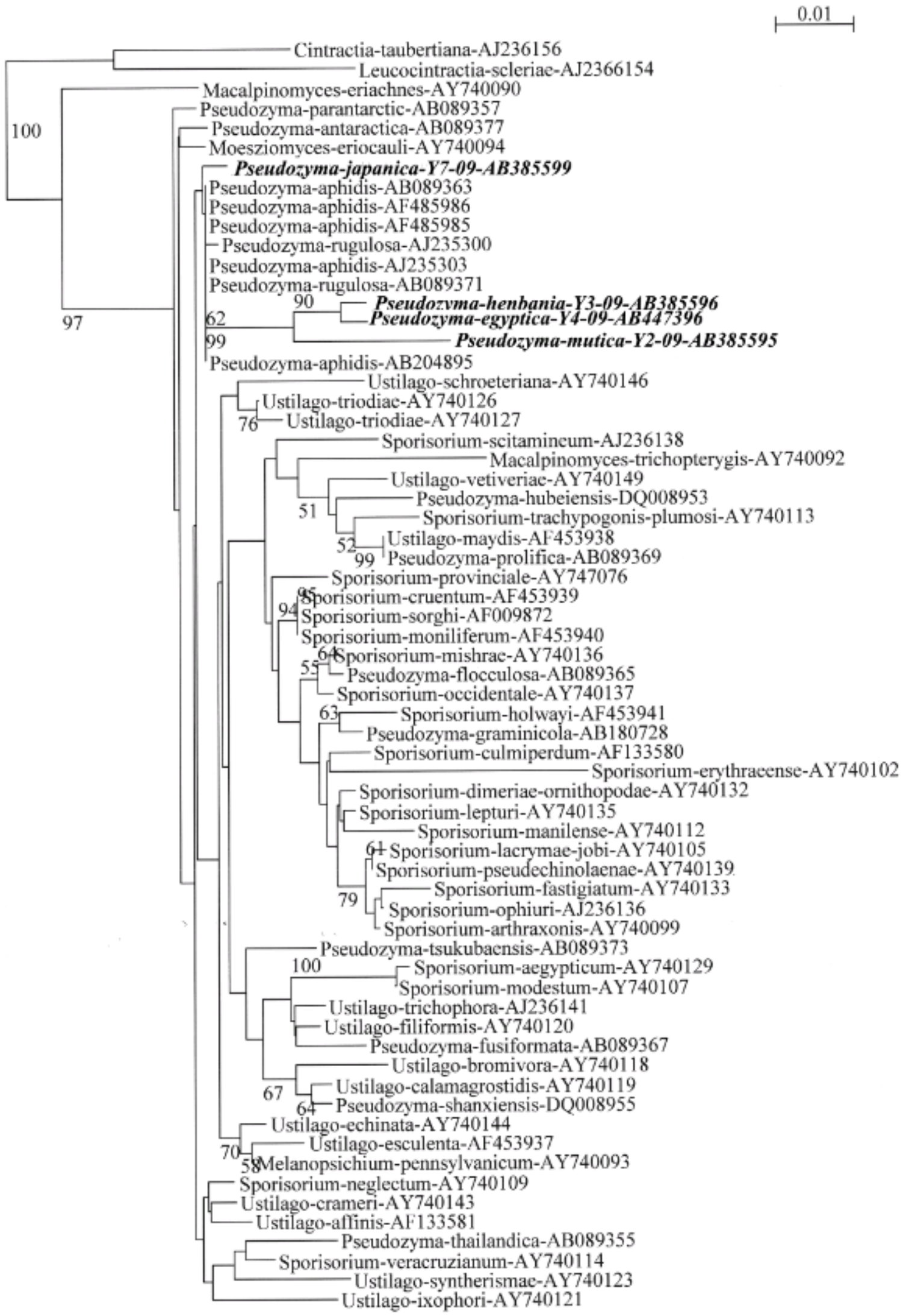

Fig. 3: Phylogenetic relationship of the four new *Pseudozyma* species and related taxa, based on a sequence analysis of the D1/D2 domain of the 26S rRNA gene. Clustering was performed with the neighbor-joining method using Kimura's two-parameter model. The percentage numbers at the nodes indicate the levels of bootstrap support (> 50%) for branch points based on 1000 bootstrap replicates. *Leucocintractia scleriae* AJ236154 and *Cintractia taubertiana* AJ236156 were used as outgroups. Reference sequences were retrieved from GenBank under the accession numbers indicated

of blastoconidia (Fig. 1j-l). The streak culture was yellow creamy to dull brown in color, smooth to somewhat furrowed in texture, with entire to lobbed margins. In GYP, after one-month incubation, a sediment and ring were present. Physiological characteristics as shown in Table 1, strain Y7-09 assimilated cellobiose, lactose and galactitol, weakly assimilated d-mannitol, while salicin, ribitol, l-rhamnose and melibiose were not assimilated. Growth in vitamin-free media was weak. Best growth was at 37°C.

Molecular identification of yeast isolates: The phylogenetic analysis indicated that the four strains were clustered in Ustilaginales clade in the D1/D2 sequence tree and more related to the genus *Macalpinomyces eriachnes* and *Moesziomyces eriocauli* out of 81 members belonging to five genera of ustilaginales based on D1/D2 and ITS regions using *Leucocintractia scleriae* and *Ustanciosporium taubertianum* as outgroups (Fig. 2). Analysis of D1/D2, ITS, SSU mt-rRNA and LSU mt-rRNA regions suggested that the strains are most closely related to the genus *Pseudozyma*. Moreover, mismatching in D1/D2 (2-6%), ITS (1-5%), SSU mt-rRNA (3-5%) and LSU mt-rRNA (1-2%) between the four strains Y2-09, Y3-09, Y4-09 and Y7-09 indicated that the four strains are different from each other. Neighbor-joining analysis based on D1/D2 sequences clustered the four isolates in the clade including *Pseudozyma aphidis*, *P. rugulosa*, *P. antractica* and *P. parantarctica* (Fig. 3).

DISCUSSION

Phenotypic characteristics: Eleven strains (Y1-11) were isolated from *H. muticus* plants (root and stem) as endophytic yeasts on CMA and were determined to belong to Basidiomycetes based on the morphological characterization as observed when grown in GYP, YM and on GYPA, YMA, MA, CMA and PDA as well as the physiological characterization according to Yarrow (1998) and Boekhout and Fell (1998). The 11 isolates were later proved to comprise four different putative species. Thus, four representative strains were chosen from the 11 isolates and named as Y2-09, Y3-09, Y4-09 and Y7-09.

On agar media, the isolates Y2-09, Y3-09, Y4-09 and Y7-09 formed yeast-like colonies fringed by pseudohyphae without clamp-connections at the margin and produced blastoconidia by budding on short stigmata-like stalks. Hyphae occur with the cytoplasm retracted in the cells. Sexual structures were not observed. Ballistoconidia and arthroconidia were not recognized (Fig. 1). The physiological characteristics of the four isolates revealed the absence of both fermentation and production of starch-like compounds, while the urease test was positive and myo-inositol was assimilated (Table 1). According to the morphological and physiological profiles, we identified the isolates as those belonging to the genus *Pseudozyma* as defined by Boekhout (1995) and Boekhout and Fell (1998). Comparison of the profiles with those of the *Pseudozyma* species reported in recent studies (Sugita *et al*., 2003; Wang *et al*., 2006; Seo *et al*., 2007; Golubev *et al*., 2007) also supported the identification. On the other hand, the closest species to the isolates were *P. aphidis* and *P. rugulosa* with primary differences in the assimilation of some carbon sources and growth in vitamin-free media at 37°C.

Comparison of physiological characteristics of Y2-09 with the most related species, *P. aphidis*, which shows late assimilation of cellobiose, salicin and ribitol, no assimilation of galactitol and no growth in vitamin-free media, but able to grow at 37°C, suggests that Y2-09 is different species from *P. aphidis*. Physiological characteristics of Y2-09 were also different from those of another related species, *P. rugulosa*, which shows the late assimilation of celliobiose, salicin, ribitol and melibiose, no assimilation of lactose and galactitol, but assimilates citric acid, grows at 37°C and weakly grows in vitamin-free media, suggesting that Y2-09 is different from *P. rugulosa*.

There were certain differences between Y3-09 and other *Pseudozyma* species. The results indicates that Y3-09 is differentiated from the maximally related species *P. aphidis* and *P. rugulosa*, because it was able to assimilate cellobiose, lactose, salicin, ribitol and melibiose but weakly assimilated d-mannitol, d-glucitol and galactitol and grew well in vitamin-free media.

The variability in the assimilation of certain carbon sources was recognized between Y4-09 and the maximally related species *P. aphidis* and *P. rugulosa*. Y4-09 assimilated cellobiose, salicin, ribitol and melibiose but not lactose; further, growth in vitamin-free media was negative. *P. aphidis* assimilates lactose with late assimilation of cellobiose, salicin and ribitol. *P. rugulosa* shows late assimilation of cellobiose, salicin, ribitol and melibiose and grows weakly in vitamin-free media.

There were clear differences between strain Y7-09 and related *Pseudozyma* species, especially in the assimilation of carbon sources. Strain Y7-09 assimilated cellobiose, lactose and galactitol, weakly assimilated d-mannitol, while salicin, ribitol, l-rhamnose and melibiose were not assimilated. Growth in vitamin-free media was weak. *P. aphidis* is able to assimilate l-rhamnose, melibiose and d-mannitol and shows late assimilation of cellobiose, salicin and ribitol, while it is unable to assimilate galactitol and can grow in vitamin-free media. *P. rugulosa* is able to

Table 2: Sequences similarity analysis based on D1/D2 and ITS regions using GenBank database (DBBJ) comparing with the four novel strains and most closest *Pseudozyma* species (*P. aphidis* and *P. rugulosa*)

	Pseudozyma aphidis		*Pseudozyma rugulosa*	
Strains name	ITS sequences similarity (%)	D1/D2 sequences similarity (%)	ITS sequences similarity (%)	D1/D2 sequences similarity (%)
Y2-09	86-99	95-97	96-97	95-98
Y3-09	95-99	96-98	91-98	96-98
Y4-09	95-99	99-100	95-98	98-99
Y7-09	99	97-98	97-98	97-98

assimilate l-rhamnose and d-mannitol with late assimilation of cellobiose, salicin, ribitol and melibiose, while lactose and galactitol are not assimilated.

Physiological characterization data of the four isolates, Y2-09, Y3-09, Y4-09 and Y7-09, obtained in the present study strongly suggest that they are different species from *P. aphidis* and *P. rugulosa*.

Sequence analysis: Nucleotide sequences of D1/D2, ITS, SSU mt-rRNA and LSU mt-rRNA were determined and compared between Y2-09, Y3-09, Y4-09 and Y7-09. Mismatches in D1/D2, ITS, SSU mt-rRNA and LSU mt-rRNA between the four isolates were 2-6, 1-5, 3-5 and 1-2%, respectively. Kurtzman and Robnett (1998) have reported that yeast strains showing nucleotide substitutions >1% in D1/D2 are generally different species. Therefore, the four isolates, Y2-09, Y3-09, Y4-09 and Y7-09, are likely to be different species from each other and from *P. aphidis* and *P. rugulosa*.

The phylogenetic relationships obtained through the comparison of D1/D2 and ITS sequences of the four isolates with the sequences of 81 members of five Ustilaginales genera (Stoll *et al*., 2005) revealed that the four isolates were located in a cluster with *Macalpinomyces ericachnes* and *Moesziomyces eriocauli*; this illustrated that the four isolates belong to Ustilaginales (Fig. 2). *Macalpinomyces ericachnes* showed 1-3% mismatching bases in the D1/D2 and 10-13% in the ITS when compared to those of the four isolates. *Moesziomyces eriocauli* showed 1-2% mismatching bases and 4-11% in D1/D2 and ITS, respectively, when compared to those of the four isolates. These results indicated that the four isolates are different species from *Moesziomyces eriocauli* and *Moesziomyces eriocauli*.

The phylogenetic relationships of the four isolates with *Pseudozyma* species and related teleomorphs of the Ustilaginales were further analyzed based on the sequences of D1/D2 sequences using *Leucocintractia scleriae* and *Cintractia taubertiana* as outgroups (Fig. 3). Neighbor-joining analysis clustered the four isolates in the clade including *Pseudozyma aphidis*, *P. rugulosa*, *P. antractica* and *P. parantarctica.* On the other hand, sequence similarity analysis of D1/D2 and ITS regions using the GenBank database (DBBJ) indicated that the four isolates were maximally close to *P. aphidis* and *P. rugulosa* (Table 2).

Present data suggest that the four isolates may be novel species. This hypothesis is supported by Sugita *et al*. (2003) in which similarities in both D1/D2 and ITS regions were used to identify *P. pantractica* and *P. thailandica* as new species. We propose the names *P. mutica* Abdel-Motaal, El-Zayat, Kosaka, El-Sayed, Nassar and Ito sp. nov. for Y2-09, *P. henbania* Abdel-Motaal, El-Zayat, Kosaka, El-Sayed, Nassar and Ito sp. nov. for Y3-09, *P. egyptica* Abdel-Motaal, El-Zayat, Kosaka, El-Sayed, Nassar and Ito sp. nov. for Y4-09 and *P. japanica* Abdel-Motaal, El-Zayat, Kosaka, El-Sayed, Nassar and Ito sp. nov. for Y7-09, respectively.

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