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FULL PAPER

Endospore formation in *Hanseniaspora pseudoguilliermondii*: a key characteristics of the species

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Abstract In the course of a study on yeast diversity in Japan and Thailand, we isolated two yeast strains with bipolar budding patterns. Physiological and phylogenetic analysis suggested that these two strains were identical to *Hanseniaspora pseudoguilliermondii*. However, these strains produced hat-shaped ascospores and endospores, the latter of which was an unknown characteristic of the species. Endospores were produced on yeast extract–malt extract (YM) plates, though ascospores were produced on cornmeal agar of *H. pseudoguilliermondii* cultures. Endospores were formed in a twin-cell structure composed of a mother cell and a daughter cell, which did not separate after budding. Unlike the cell wall of the endospores, that of ascospore was stained with a chitin-specific stain. This was a feature distinguishing endospores and ascospores. Cell morphology of *H. pseudoguilliermondii* was compared with other species of the genus by observing their type strains. Other *Hanseniaspora* species did not show endospore formation under the same condition in which *H. pseudoguilliermondii* did. Therefore, the formation of endospores was considered to be a species-delimiting character of *H. pseudoguilliermondii*.

Keywords Bipolar budding yeast · Cell morphology · Phenotypic characteristics

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Introduction

In order to understand yeast diversity in Asia, we have been studying taxonomy of microorganisms isolated in Thailand and Japan. Among the yeasts isolated from the natural environment in Thailand, we found that 79 undescribed ascomycetous species might be included in those isolates. Some of them were already proposed as new species, for example, *Candida easanensis* Jindam., Thuy & Nakase, *C. pattaniensis* Jindam., Duy & Nakase, and *C. nakhonratchasimensis* Jindam. & Nakase (Jindamorakot et al. 2004), *Pichia nongkratonensis* Nakase & Jindam. (Nakase et al. 2005), *C. jaroonii* Imanishi, Jindam., Nakagiri, Limtong & Nakase and *C. songkhlaensis* Imanishi, Jindam., Nakagiri, Limtong & Nakase (Imanishi et al. 2008). Among the other isolates, two strains of bipolar budding yeast were found to produce endospores as well as ascospores in culture. We examined the two strains on their morphological, physiological, and biochemical characteristics and also on their molecular phylogeny. We found they were assignable to *Haseniaspora pseudoguilliermondii* Čadež, Raspor & M. T. Sm. However, the endospore formation had been reported before on *H. pseudosuilliermondii* or other species of the genus *Hanseniaspora*.

The genus *Hanseniaspora* is characterized by apiculate cell morphology and bipolar budding (Pijper 1928). The

genus now comprises 13 species (Jindamorakot et al. 2009). *Hanseniaspora* species have been characterized and identified by their physiological, morphological, and serological characteristics and also by DNA base composition and DNA–DNA hybridization (Cadez et al. 2003). However, it is difficult to identify the species due to lack of distinct phenotypic characteristics among the *Hanseniaspora* species. Although *H. meyeri* Čadež, Poot, Raspor & M. T. Sm., *H. guilliermondii* Pijper, *H. uvarum* (Niehaus) Shehata, Mrak & Phaff ex M. T. Sm., and *H. opuntiae* Čadež, Poot, Raspor & M. T. Sm. did not exhibit distinct physiological characteristics, they can be identified on the basis of differences in the internally transcribed spacer region (ITS) sequences or by polymerase chain reaction–restriction fragment-length polymorphism (PCR–RFLP) of ITS using two restriction enzymes (Cadez et al. 2003). *Hanseniaspora pseudoguilliermondii* originally described by Cadez et al. (2006) based on DNA relatedness resulted in DNA–DNA hybridization among *Hanseniapora* species, but physiological characteristics do not distinguish the two species (Cadez et al. 2003, 2006).

In this study, we observed details of vegetative growth in our two isolates of *H. pseudoguilliermondii* in order to characterize the endospores and understand their developmental process. Comparing them with other species of *Hanseniaspora* by examining their type strains, we tried to evaluate endospore formation as a taxonomic character in *Hanseniaspora*.

Methods

Strains and culture condition

The yeast strains used in this study are listed in Table 1. ST-398 and Y03-106-17 were isolated using the methods described by Jindamorakot et al. (2004) and Banno and Mikata (1981), respectively. We obtained five type strains of *Hanseniaspora* species from the Centraalbureau voor Schimmelcultures (CBS), The Netherlands.

For genomic DNA extraction, cells were cultured on yeast extract–malt extract (YM) agar (10 g/l glucose, 5 g/l peptone, 3 g/l yeast extract, 3 g/l malt extract, 15 g/l agar) and yeast–peptone–dextrose (YPD) broth (20 g/l glucose, 20 g/l peptone, 10 g/l yeast extract). Ascospores were produced by culturing on cornmeal agar (Difco, USA). For inducing endospore formation, cells were precultured in a malt broth (Banno and Mikata 1981) at 15°C for 1 week and transferred onto YM agar and incubated at 25°C for 1 week.

Morphological, physiological and biochemical examinations

Morphological, physiological, and biochemical characteristics of the strains were investigated according to the methods described by Yarrow (1998).

Sequencing the D1/D2 domain of 26S rDNA (D1/D2) and phylogenetic analysis

Genomic DNA was prepared by using previously described methods (Imanishi et al. 2007). The D1/D2 region was amplified by PCR according to previously described methods (Kurtzman and Robnett 1997). Purification of PCR products and determination of sequences were performed according to the methods described by Imanishi et al. (2007). The phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei 1987) using CLUSTAL W software package (Thompson et al. 1994). Bootstrap values (Felsenstein 1985) were calculated from 1000 trials. Nucleotide sequences of the D1/D2 region of strains Y03-106-17 and ST-398 have been deposited in the DNA Data Bank of Japan (DDBJ) under accession nos. AB525689 and AB538284, respectively.

Table 1 Strains used in this study

Species	Strain no.	Other designations	Source	Origin
H. pseudoguilliermondii	Y03-106-17	NBRC 102099	Soil	Haha island, Japan
	ST-398		Flower of durian	Trad province, Thailand
	CBS 8772^T	NBRC 100529^T	Orange juice concentrate	Georgia, USA
H. lachancei	NBRC 100530^T	CBS 8818^T	Fermenting agave juice	Mexico
H. uvarum	NBRC 10833^T	CBS 314^T	Muscatel grape	USSR
H. meyeri	NBRC 100531^T	CBS 8734^T	Fruit of *Sapindus* sp.	Hawaii, USA
H. guilliermondii	NBRC 1411^T	CBS 465^T	Infected nail	South Africa
H. clermontia	NBRC 100528^T	CBS 8821^T	Rotting *Clermontia* sp.	Hawaii, USA
H. valbyensis	NBRC 10834^T	CBS 479^T	Soil	Denmark

DNA–DNA hybridization

Cell preparation, DNA isolation, and DNA–DNA hybridization were performed using a previously described method (Imanishi et al. 2007).

Chitin and nuclei staining and observation of cells

To observe development process of endospores, the following staining methods were employed. Cells were grown on YM agar at 25°C for 3 days. One loopful of cells was inoculated into 5 ml of YPD broth and grown at 25°C for 1 day with shaking at 120 rpm. Then, 1 loopful of culture was inoculated on YM agar and grown at 25°C for 2–3 days. One loopful of cultured cells was washed with 1 ml of distilled water and fixed with 50% (v/v) ethanol. Subsequently, the cells were stained with Fungiflora Y® (Biomate Inc., Japan), which stains the chitin components in the cell wall, according to the manufacturer's protocol. The nuclei were stained with VECTASHIELD® mounting medium containing 4′,6-diamidine-2-phenylidole dihydrochloride (DAPI) (Vector Laboratories Inc., USA), as described in the manufacturer's protocol. Cell morphology and stained bud scars and nuclei were observed using a microscope with an Axioplan 2 imaging system (Carl Zeiss, Germany).

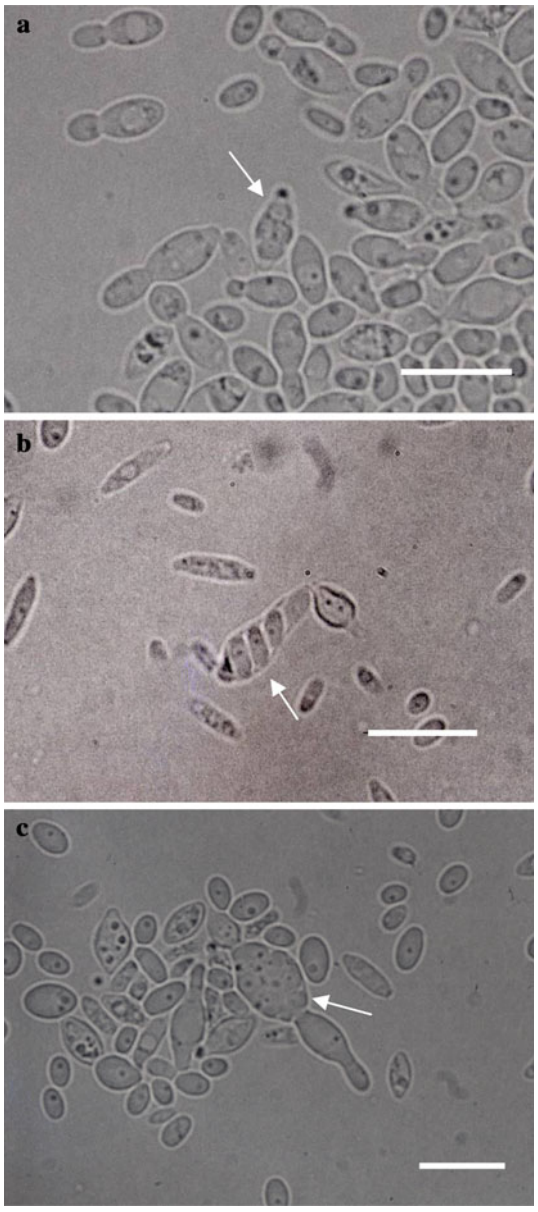

Fig. 1 Y03-106-17 cells. **a** Ascospores; *arrow* indicates an ascus containing four ascospores. **b, c** Endospores; *arrows* indicate endospores. *Bars* 10 μm

Results and discussion

Identification of the two isolates ST-398 and Y03-106-17 as *H. pseudoguilliermondii*

The two strains grew by bipolar budding. Asci were formed directly from vegetative cells and each produced four

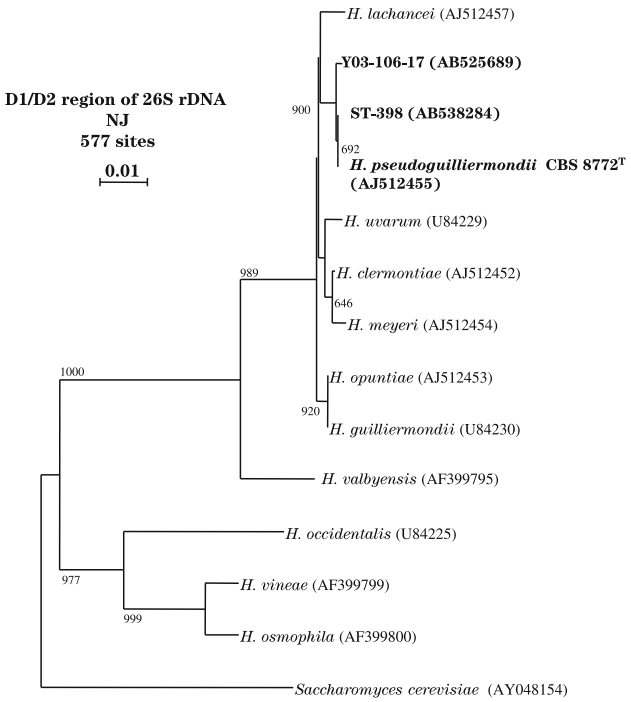

Fig. 2 Phylogenetic relationships of *Hanseniaspora* species inferred from the D1/D2 nucleotide sequences by using the neighbor-joining method. Bootstrap values were calculated from 1000 replicates, and values <50% were omitted. All sequences of type strains were edited to the longest common region (577 bp). The GenBank sequence accession numbers are shown in *parentheses*. The *bar* indicates a sequence dissimilarity value of 0.01 substitutions/site. *Saccharomyces cerevisiae* was used as an outgroup

hat-shaped ascospores (Fig. 1a). On the basis of these characteristics, the two strains appeared to belong to the genus *Hanseniaspora*. According to the results of blast search by the nucleotide sequence of D1/D2, both of Y03-106-17 and ST-398 were closely related to CBS 8772^T strain of *H. pseudoguilliermondii*. Phylogenetic analysis showed these three strains were grouped into 1 clade and separated from other *Hanseniaspora* species (Fig. 2). The nucleotide sequences of CBS 8772 and ST-398 are 100% (572 bp) identical, and that of Y03-106-17 was 1 bp different from them. In the DNA–DNA hybridization experiment, three strains were supported by high values (79–99%), whereas lower values (>47%) were detected when they were paired with other species (Table 2). By the physiological examination, these three strains had the same abilities to ferment 7 carbon sources and assimilate 44 carbon and 5 nitrogen substrates, although only a few differences were observed among *Hanseniaspora* species (Table 3). These results supported identification of the two strains of Y03-106-17 and ST-398 as *H. pseudoguilliermondii*.

Table 2 DNA relatedness among taxa of *Hanseniaspora* and isolates resulted in DNA–DNA hybridization

	Source of DNA	DNA relatedness (%)						
		1	2	3	4	5	6	7
1	Y03-106-17	100	99	79	26	47	38	12
2	ST-398	–	100	83	20	38	26	11
3	*H. pseudoguilliermondii* (CBS 8772^T)	–	–	100	29	25	27	14
4	*H. guilliermondii* (CBS 465^T)	–	–	–	100	28	29	21
5	*H. lachancei* (CBS 8818^T)	–	–	–		100	26	9
6	*H. uvarum* (CBS 314^T)	–	–	–			100	15
7	*H. meyeri* (CBS 8734^T)	–	–	–				100

Endospore formation as phenotypic characteristics of *H. pseudoguilliermondii*

The two strains were found to produce endospores (Fig. 1b, c) that were previously unknown in *H. pseudoguilliermondii*. We obtained a type strain of *H. pseudoguilliermondii* CBS 8772^T and observed endospore formation in these three strains. Morphological examination revealed that they all produced endospores in addition to ascospores (Fig. 1). We examined culture conditions suitable for endospore formation. Culturing cells on YM agar at 25°C after preculture with malt broth or YPD broth induced endospore formation, but the cells cultured on cornmeal agar or 5% malt agar did not produce endospores. Endospores were not observed when cells were subcultured several times on the YM plates. Therefore, endospores might be formed in response to changes in the environmental conditions around cells. As only the following yeast genera *Trichosporon, Candida, Cryptococcus, Oosporidium, Cystofilobasidium, Leucosporidium* have been reported to produce endospores (Yarrow 1998), endospores are peculiar structure in yeasts. We examined endospore formation of the type strains of six other *Hanseniaspora* species, which were cultured under the same condition in which endospore formation of *H. pseudoguilliermondii* was induced. However, we could not observe any endospores in any type strain of the six *Hanseniaspora* species. Endospore formation seems to be a species-delimiting phenotypic characteristic of *H. pseudoguilliermondii*.

Table 3 Physiological characteristics of our two isolates and *Hanseniaspora* species

Species/strains	Fermentation			Assimilation						Growth temperature (°C)		
	G	Su	Cell	G	Cell	Sa	D-Glt	Su	Ma	30	34	37
Y03-106-17	+	−	+	+	+	+	+	−	−	+	+	+
ST398	+	−	+	+	+	+	+	−	−	+	+	+
H. pseudoguilliermondii CBS 8772^T	+	−	+	+	+	+	+	−	−	+	+	+
H. guilliermondii	+	−	+	+	+	+	v	−	−	+	+	+
H. lachancei	+	−	+	+	+	+	+	−	−	+	+	+
H. uvarum	+	−	+	+	+	+	v	−	−	+	N.D.	−
H. meyeri	+	−	+	+	+	+	+	−	−	+	+	−
H. clermontiae	+	−	+	+	+	+	+	−	−	+	−	−
H. opuntiae	+	−	+	+	+	+	+	−	−	+	+	+
H. valbyensis	+	−	+	+	+	+	−	−	−	+	−	−
H. vineae	+	−	+	+	+	+	−	v	+	+	+	−

Data of *Hanseniaspora* species from Smith (1998)

+ positive, *v* variable, − negative, *N.D.* not determined, *G* glucose, *Su* sucrose, *Ma* maltose, *Cell* cellobiose *Sa* salicin, *D-Glt* D-gluconate

Description

Proposal of new characteristics of Hanseniaspora pseudoguilliermondii Cadez, Raspor & M. T. Sm.

Cells were grown in malt broth for 1 week at 15°C; subsequently, a loopful of culture was inoculated on YM agar. Endospore formation was noted after incubation at 25°C for 1 week. The Latin diagnosis of *H. pseudoguilliermondii* Cadez, Raspor & M. T. Sm: In agaro YM medio post 7 ad 25°C, endosporae formantur.

Characterization of endospore

Endospores are defined as a group of cells enveloped in a membrane. *In sensu stricto*, endospore dose not include ascospores, although they are enveloped in an ascus wall. We observed spore formation in detail to find differences between ascospore and endospore in this species. Ascospores formed well on cornmeal agar in all three strains, including the type strain. A vegetative cell became directly an ascus, which produced four hat-shaped ascospores (Fig. 1a). On the other hand, the shape of the endospores of these strains was elliptic and oval (Fig. 1b, c). Endospores were produced when a vegetatively growing mother cell failed to separate properly from its daughter cell after budding, and the mother cell budded the second daughter cell into the unseparated daughter cell, forming an endospore wrapped in its sister cell. Sometimes, multiple endospores per vegetative cell were observed (Fig. 1b, c). Figure 1b, c shows endospores formed by the strain Y03-106-17, and the other two strains of *H. pseudoguilliermondii* were also observed to form endospores in the same manner.

Nuclei behavior was observed by staining with DAPI. In the ascospore formation, generally, four nuclei produced by miosis are observed in an ascus (Fig. 3b). In the endospore formation, Fig. 3d shows two nuclei per vegetative cell, and one of them appears to belong to the first daughter cell. Figure 3f shows that the nuclei synthesized in the mother cell were transferred into an endospore cell through the neck of the budding cells. The nucleus of the endospore appeared to be doubled in the mother cell and was transferred to the second daughter cell, which eventually became the endospore.

By Fungiflora Y® staining, the locality of the chitin component was observed. Ascospores were strongly dyed (Fig. 3h); therefore, their cell walls were considered to be composed of chitin. On the other hand, endospores and vegetative cells were not dyed (Fig. 3j), suggesting that a main component of these cell walls was not chitin. In other *Hanseniaspora* species, ascospores were stained, but vegetative cells were not strained with Fungiflora Y®. These

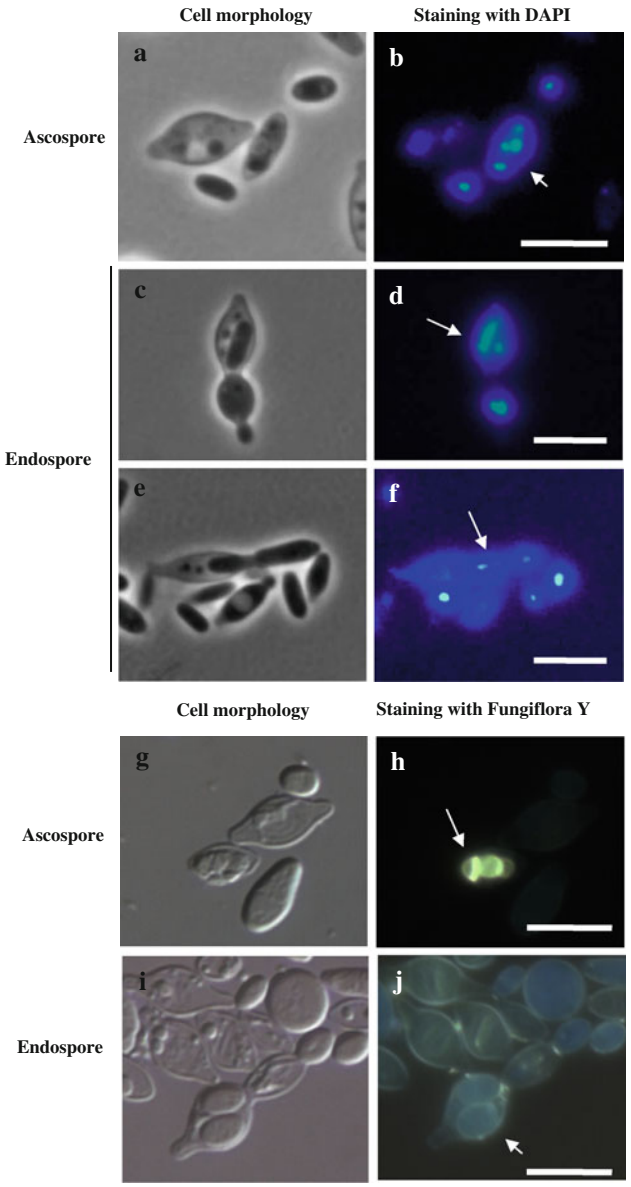

Fig. 3 Y03-106-17 cells. **a, b, g, h** asci. **c, d, e, f, i, j** endospores. Cells were stained by 4′,6-diamidine-2-phenylidole dihydrochloride (DAPI) (**b**, **d**, **f**) or Fungiflora Y® (**h**, **j**) and observed under ultraviolet light. The same cells were observed under a bright field for comparison. *Arrows* indicate an ascus (**b**), the first daughter cells containing 1 endospore (**d**), transfer of nucleus through the neck of budding cells (**f**), ascospores (**h**), and the first daughter cell containing two endospores (**j**). *Bars* 10 μm

differences between ascospores and endospores allow us to easily identify endospores. The function of the endospore of *H. pseudoguilliermondii* remains unclear. The optimum culture conditions for inducing endospore formation were not found, and we failed to obtain sufficient endospores for the relevant examinations. But the detection of endospores in *H. pseudoguilliermondii* can encourage future studies to clarify the function of endospore formation in yeast.

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