Research Paper

Ultrastructural characteristics and variability of vegetative reproduction in *Fellomyces penicillatus*

Marie Kopecká[1], Wladyslav Golubev[2], Vladimíra Ramíková[1], Dobromila Klemová[3] and Ladislav Ilkovics[3]

[1] Department of Biology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[2] Russian Collection of Microorganisms, Institute for Biochemistry and Physiology of Microorganisms, Pushchino, Russia
[3] Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

The yeast strains VKM Y-2977 and VKM Y-2978, derived from the isolate Pa-202, were examined for their physiological properties and mycocin sensitivities and studied by light, phase-contrast, fluorescence, transmission and scanning electron microscopy. The cells of the first strain produced long stalk-like conidiophores, whereas the cells of the second one had the appearance of a typical budding yeast under the light microscope. Transmission and scanning electron microscopy showed the formation of stalk-like conidiophores and long necks in VKM Y-2977, similar in appearance to *Fellomyces fuzhouensis.* The actin cytoskeleton, microtubules and nuclei were similar as well, but due to presence of a capsule, they were not clearly visible. The second isolate, VKM Y-2978, had very short stalk-like conidiophores, and the neck, microtubules and actin cables were shorter as well. The actin patches, actin cables, and microtubules were similar in VKM Y-2977 and VKM Y-2978 and not clearly visible. The physiological characteristics and mycocin sensitivity patterns, together with the microscopic structures and ultrastructures, led us to conclude that both strains belong to *Fellomyces penicillatus*, even though they differ in the lengths of their stalk-like conidiophores and necks.

Abbreviation: RhPh, rhodamine-phalloidin

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Introduction

The mode of vegetative reproduction is an important characteristic in yeast taxonomy and identification. The mode of reproduction is diagnostic not only of the genus but sometimes also of the family (*e.g.* Schizosaccharomycetaceae). The majority of yeasts reproduce by multilateral budding (*e.g. Saccharomyces* Meyen ex Reess) whereas others do so by bipolar (*Nadsonia* Sydow) or monopolar budding on a broad base (*Malassezia* Baillon), by fission (*Schizosaccharomyces* Lindner) or by development of daughter cells on stalk-like conidiophores (*Fellomyces* Yamada et Banno). In some instances, however, the mode of reproduction is liable to variation. For example, budding may be both on a narrow and a broad base in species of the genus *Trichosporon* Behrend, and nonpolar buds may occur in polar budding yeasts (*Schizoblastosporion* Ciferri) [1].

During a survey of the yeast community colonizing dead needles of spruce litter, it has been found that several isolates produce new cells on stalk-like conidiophores in addition to budding ones. The presence of different modes of vegetative reproduction in these isolates can be attributed to contamination of these cultures or to variance in daughter cell formation. By replating on malt extract agar, two different colony forms were obtained from these isolates. Microscopically, one of them is largely characterized by budding,

Correspondence: Professor Marie Kopecká, MD, Ph.D., Department of Biology, Faculty of Medicine, Masaryk University, Kamenice 5, A6, 625 00 Brno, Czech Republic
E-mail: mkopecka@med.muni.cz
Phone: +420 54949 4778
Fax: +420 54949 1327
http://www.med.muni.cz/~mkopecka/publikations.php

but another form generates stalk-like conidiophore structures. The purpose of the current study is to examine cell reproduction in these two forms, and ultrastructural characteristics were compared with those found for *Fellomyces fuzhouensis* (Yue) Yamada et Banno [2] and *Cryptococcus laurentii* (Kuffrath) Skinner [3].

Materials and methods

Organisms and culture

The strains VKM Y-2977 and VKM Y-2978 were obtained from the Russian Collection of Microorganisms (www.vkm.ru). They were derived from the isolate Pa-202 [4]. For their identification, standard methods currently employed in yeast taxonomy were used [1]. The procedure for determining mycocin sensitivity patterns has been described previously [5]. Cell preparation for phase contrast, fluorescent and scanning electron microscopy has also been described previously [2, 6, 7]. For microscopy, the strains were grown on agar containing yeast extract (1%), peptone (2%) and dextrose (2%) at 23 °C for about 2 d. They were then inoculated into liquid YEPD medium and cultivated on a shaker (70 rpm) at 23 °C for 18 h.

Fixation of cells

To visualize the cytoskeleton, the cells were fixed with 5.0% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), supplemented with ethylene glycol-bis(β-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and $MgCl_2$, at pH 6.9 for 10–90 min. Subsequently, three washes with PBS were performed [2, 8].

Visualisation of microtubules

To stain the microtubules, a modified method as described in [8] was used. Immediately after fixation, the cells were exposed to lysing enzymes of *Trichoderma harzianum* (Sigma) at a concentration of 1 mg/ml for 90 min at 37 °C. They were subsequently permeabilized with 1% (v/v) Triton X-100 in PBS for 5 min, and then 2.0% (w/v) bovine serum albumin was added for 30 min at 37 °C. For microtubule labeling, cells were incubated with the monoclonal anti-α-tubulin antibody TU 01 for 2–3 h at 37 °C [2]. After three washes with PBS at 5 min intervals, swine anti-mouse IgG-fluorescein-isothiocyanate (SwAM-FITC, Sigma) secondary antibody was added (2 h at 37 °C). Subsequently, the cells were washed three-times with PBS at 5 min intervals.

Visualisation of F-actin

The fixed cells were treated with 1.0% (v/v) Triton X-100 for 5 min. After two washes with PBS, actin was stained with rhodamine-phalloidin (RhPh; Molecular Probes, Inc.) for 1 h and subsequently the cells were washed with PBS.

Staining of nuclei

Nuclei were stained with 4′,6-diamidino-2-phenyl-indole dihydrochloride (DAPI) at a final concentration of 1 μg/ml of Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA 94010, USA).

Fluorescence microscopy

A Leica Laborlux S Leitz fluorescence microscope with standard filter blocks for violet (355–425 nm), blue (450–490 nm) and green (515–560 nm) emission light, including a Plan Phaco 3100/1.25 objective and equipment for phase contrast microscopy, was used. Photographic images were taken on Ilford 400 film.

Ultrathin sectioning

Cells were fixed in combined 3% glutaraldehyde and 1% paraformaldehyde in PBS for 3 h. After three 30 min washes with PBS, the cells were post-fixed in 1.5% $KMnO_4$ for 20 min. The fixed cells were washed with five 10 min washes with distilled water until a clear supernatant was achieved, and embedded in 2% (w/v) agar. The cells were dehydrated in a graded alcohol series (10–100%) and embedded in LR White resin. First, the samples were incubated for 2 d in a refrigerator, and then the LR White was allowed to polymerize for 3 d at 60 °C. Subsequently, ultrathin sections were produced. Some sections were contrasted with 2.5% uranyl acetate for 2 min and lead citrate for 1 min (see Fig. 5d); other sections were used without any further contrasting (see Figs. 5a–c, e, 11–e).

Transmission electron microscopy

The ultrathin sections were viewed and photographed with a MORGAGNI 268D (100 kV) microscope.

Scanning electron microscopy

The cells were fixed with 5% paraformaldehyde [2, 7], dehydrated with a graded alcohol series, dried using a CPD 030 critical point dryer (BAL-TEC) and coated with gold in a BALZERS SCD040 metal shadowing apparatus (5 min, 30 mA). The specimens were observed in Vega TS5136 XM (TESCAN) using digital microscopy imaging scanning equipment.

Results

Morphological and physiological characterization of the yeast strains

The appearance of a colony of the strain VKM Y-2977 is dull, white, rough, and dry. The cells in these colonies

develop thin tubular stalk-like conidiophores, at the end of which new ones are produced, separated by an end-break of the stalk. Capsules have not been observed in India ink preparations [9]. The strain VKM Y-2978 has glistening, greyish, smooth, and slimy colonies composed of budding capsulated cells. These forms are unstable; they can produce streak cultures with sectors of different cultural type upon long-term storage.

The cultures of both types were almost identical in physiological characteristics. They are urease positive, nitrate and nitrite negative, assimilate i-inositol and D-glucuronate, synthesize starch-like compounds and have no fermentative ability. They differed only in the rate of utilization of some carbon sources (Table 1). In addition, they have the same mycocin sensitivity patterns, *i.e.* they are sensitive to mycocins secreted by *Bullera alba* (Hanna) Derx, *Cryptococcus perniciosus* Golubev *et al.*, *C. pinus* Golubev et Pfeiffer, and *Filobasidium capsuligenum* Rodrigues de Miranda, but they are resistant to mycocins of *B. hannae* Hamamoto et Nakase, *B. sinensis* Li var. *lactis* Bai *et al.*, *B. unica* Hamamoto et Nakase, *C. laurentii*, *C. nemorosus* Golubev *et al.*, and *C. podzolicus* (Bab'eva et Reshetova) Golubev. Many distinctions in mycocin sensitivity patterns were found between *C. laurentii* and *F. penucillatus* (Table 2).

Phase contrast and fluorescence microscopy

Phase contrast (Figs. 1, 7) and fluorescent microscopy (Figs. 2–4, 8–10) of both strains showed long stalk-like conidiophores and necks in VKM Y-2977 (Figs. 1–4), similar to those previously described in *F. fuzhouensis* [2], while in VKM Y-2978 they were short (Figs. 7–10), similar to those previously described for *C. laurentii* [3] and *C. neoformans* [10].

Microtubule cytoskeleton

Microtubules were hardly visible in both strains (Figs. 2, 8), but were probably longer in VKM Y-2977 (Fig. 2a–d) than in VKM Y-2978 (Fig. 8–d). In interphase cells, the cytoplasmic microtubules were oriented towards the stalk (Fig. 2a, b) and extended into the bud (Fig. 8a, b) that emerged on the stalk-like conidiophores (Figs. 2c, 8c). During these two stages, the nucleus was in the mother cell (Figs. 3a–c, 9a–c). Before mitosis, the nucleus migrated into the daughter cell (Figs. 3d, e, 9d, e) and the cytoplasmic microtubules probably disintegrated in the mother cell, as diffuse fluorescence appeared in the daughter cell (Fig. 8c), similarly as in *F. fuzhouensis* [2]. During nuclear division, the mitotic spindle (Fig. 2c) delivered one nucleus back to the mother cell, while one daughter nucleus remained in the bud (Figs. 3f, g, 9f, g). After mitosis, the spindle dis-

Table 1. Phenotypic characteristics of *Fellomyces penicillatus* cultures.

	VKM Y-2977	VKM Y-2978
Fermentation	–	–
Assimilation of carbon compounds		
Glucose	+	+
Inulin	–	–
Sucrose	+	+
Raffinose	+	+
Melibiose	+	s
Galactose	+	+
Lactose	+	+
Trehalose	s	s
Maltose	+	+
Melezitose	+	+
α-Methyl-D-glucoside	–	–
Soluble starch	w	w
Cellobiose	+	+
Salicin	w	w
Arbutin	w	w
L-Sorbose	–	–
L-Rhamnose	+	+
D-Xylose	+	+
L-Arabinose	s	s
D-Arabinose	s	s
D-Ribose	s	s
Ethanol	–	–
Glycerol	–	–
Erythritol	–	–
Ribitol	w	w
L-Arabinitol	w	w
D-Xylitol	w	w
Galactitol	s	w
D-Mannitol	s	w
D-Glucitol	s	w
Inositol	s	s
D-Glucuronate	s	s
DL-Lactate	w	w
Succinate	s	s
Citrate	w	w
D-Gluconate	s	s
D-Glucosamine	s	s
N-Acetyl-D-glucosamine	s	s
Saccharate	w	w
Quinic acid	–	–
Allantoin	–	–
Orcine	–	–
Assimilation of nitrogen compounds		
Nitrate	–	–
Nitrite	–	–
Ethylamine	s	+
L-Lysine	+	+
Tryptophan	–	–
Cadaverine	–	–
Creatinine	–	–
Creatine	–	–
Additional tests and other growth characteristics		
Vitamin-free medium	–	–
50% (w/w) glucose	–	–
10% NaCl/5% glucose	–	–
Starch formation	positive	positive
Growth at 30 °C	–	–
Urease	positive	positive

+: growth, s: slow growth, w: weak growth, – no growth.

Table 2. Mycocin sensitivity patterns of *Fellomyces penicillatus* VKM Y-2977, VKM Y-2978 and *Cryptococcus laurentii* VKM Y-1665 cultures.

Mycocinogenic strains	VKM Y-2977	VKM Y-2278	VKM Y-1665^T
Bullera alba VKM Y-2829	+	w	+
B. hannae VKM Y-2832^T	–	–	w
B. sinensis var. *lactis* VKM Y-2826^T	–	–	+
B. unica VKM Y-2830^T	–	–	+
Cryptococcus laurentii VKM Y-1627, 1627, 1665^T	–	–	x
C. nemorosus VKM Y-2906^T	–	–	+
C. perniciosus VKM Y-2905^T, 2907	+	+	+
C. pinus VKM Y-2958^T	+	+	+
C. podzolicus VKM Y-2247, 2249, 2908	–	–	+
Cystofilobasidium bisporidii VKM Y-2700^T	–	–	–
Filobasidium capsuligenum VKM Y-1439	w	w	+

+: sensitive, w: weakly sensitive, –: non-sensitive, ^{T} – type strain.

integrated (Figs. 2d, 8d), and the cytoplasmic microtubules were gradually restored in both organisms. At the end of the cell cycle, the mother and daughter cells contained one nucleus each (Figs. 3f, g, 9f, g).

Actin cytoskeleton

The actin cytoskeleton was hardly visible in both strains (Figs. 4, 10). Actin patches were visible at the cell cortex; actin cables extended from the mother to the stalk-like conidiophores and bud. The actin cables were longer in VKM Y-2977 (Fig. 4) than in VKM Y-2978 (Fig. 10). During nuclear migration to the bud and in mitosis, the F-actin structures remained unchanged. At the beginning of cytokinesis, no actin cytokinetic ring appeared at the base of the bud.

Transmission and scanning electron microscopy

The ultrathin sections of the cells fixed by glutaraldehyde and contrasted by osmium tetroxide had a bad quality, probably due to interaction of the fixative and osmium tetroxide with the capsule. Therefore, we modified the fixation process and used 3% glutaraldehyde and 1% paraformaldehyde; then, $KMnO_4$ was used as post-fixation agent and for contrasting of the biomembranes. These ultrathin sections of cells observed by transmission electron microscopy (TEM) showed typical fungal organelles, including one nucleus, few vacuoles, often mitochondria and endoplasmic reticulum, and few Golgi apparatuses. Striking features of both organisms were cisternae of the endoplasmic reticulum, vesicles and mitochondria directing to sterigma and bud. The ultrastructure of the cell wall in both strains was lamellate (Figs. 5, 11), which is a characteristic feature of basidiomycetous yeasts. The first stage of daughter cell development in both organisms was the formation of the stalk-like conidiophores (Figs. 5a, b, 11a, b), which turned into a budding base when the bud emerged (Figs. 5c, 11c); organelles gradually migrated to the stalk-like conidiophores and the bud, and bud growth continued. Mitosis (not shown) was followed by eccentric cytokinesis occurring at the base of the bud. Then cytoplasm migrated out of the neck into the mother cell, and an empty neck appeared. Wall formation covered the cell surfaces of the mother and daughter cells (Figs. 5d, 11e). The main difference between the two strains investigated was the length of the stalk-like conidiophores and the neck; the one in VKM Y-2977 resembled the long stalk-like conidiophores and long neck seen in *F. fuzhouensis* [2], while the short stalk and short neck in VKM Y-2978 resembled those in *C. laurentii* [3] and *C. neoformans* [10]. After finishing cell division, empty necks without cytoplasm occurred in both organisms after break-off of the daughter cell (Fig. 5e).

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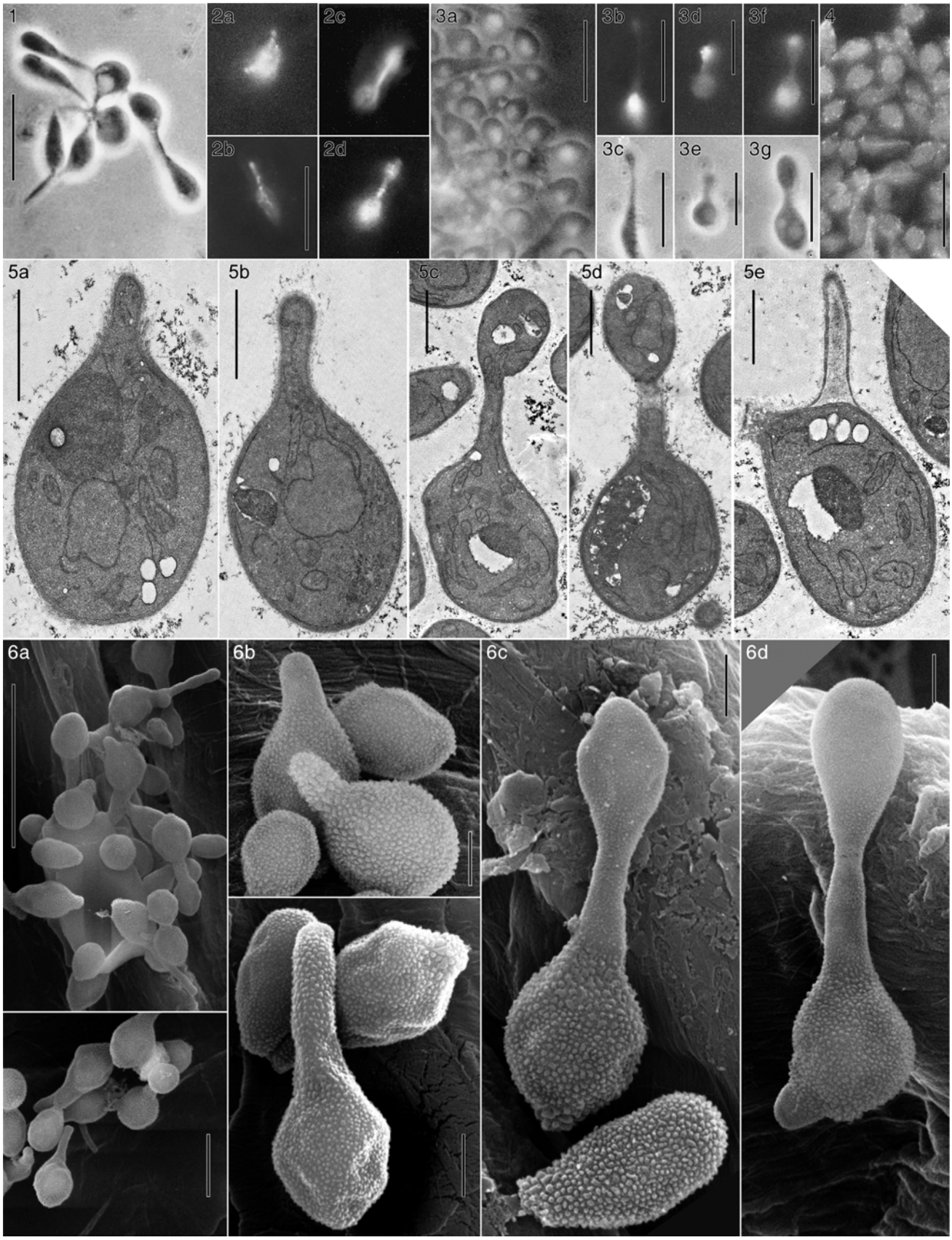
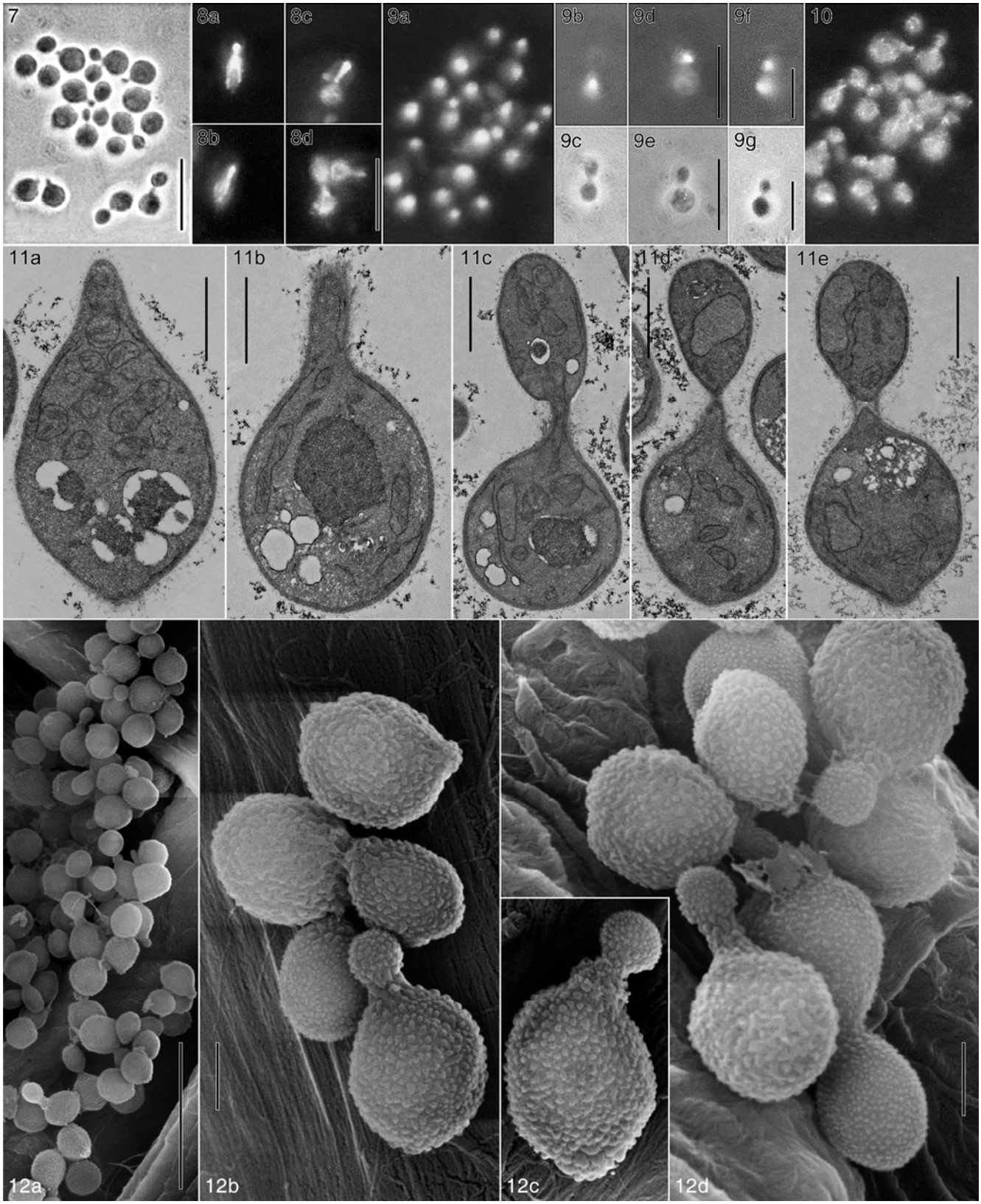
Plate I. Fig. 1. Phase contrast micrograph of living VKM Y-2977 cells at 18 h in YEPD medium. The bar corresponds to 10 µm. **Fig. 2.** Fluorescent micrographs of VKM Y-2977 cells at 18 h in YEPD medium. Cells were fixed and stained for microtubules using an anti-tubulin antibody. The bar corresponds to 10 µm. **Fig. 3** (a, b, d, f) Fluorescent micrographs of VKM Y-2977 cells at 18 h in YEPD medium. (c, e, g) Corresponding phase contrast micrographs of the same cells (b/c, d/e, f/g). The cells were fixed and the nuclei stained with DAPI. The bar corresponds to 10 µm. **Fig. 4.** Fluorescent micrograph of VKM Y-2977 cells at 18 h in YEPD medium. The cells were fixed and stained for actin with RhPh. The bar corresponds to 10 µm. **Fig. 5.** Transmission electron micrographs of VKM Y-2977 cells at 18 h in YEPD medium. The cells were fixed and processed for transmission electron microscopy by ultrathin sectioning technique. The bar corresponds to 1 µm. **Fig. 6.** Scanning electron micrographs of VKM Y-2977 cells at 18 h in YEPD medium. The cells were fixed and processed for scanning electron microscopy. The bar corresponds to 1 µm.





As light microscopy did not show any capsules in the strain VKM Y-2977, the cells were further studied at higher magnification by scanning electron microscopy (Fig. 6), in comparison to VKM Y-2978 (Fig. 12). Capsules of almost identical ultrastructure were identified in both yeast strains, covering the mother cells, necks and buds. Transmission electron microscopy also showed the capsules as a thin layer on the external surface of the cell wall, densely decorated by $KMnO_4$ electron-dense particles (Figs. 5, 11).

Transmission (Figs. 5, 11) and scanning (Figs. 6, 12) electron microscopy proved the existence of stalk-like conidiophores in both strains, but the lengths of the stalks differed. Generally, the stalk-like conidiophores (Figs. 5b, 6a, b) and necks (Figs. 5c, d, 6c, d) were longer than 1 μm in the strain VKM Y-2977, whereas the stalks (Figs. 11b, 12a) and necks (Figs. 11c–e, 12a–d) were usually shorter than 1 μm in VKM Y-2978.

Discussion

The morphological and physiological properties (Table 1) assigned the studied cultures to the genera *Fellomyces* or *Cryptococcus* Vuillemin. As deduced from phylogenetic analyses, the genus *Fellomyces* and many *Cryptococcus* species are placed within the order Tremellales (Basidiomycota, Agaricomycotina, Tremellomycetes) (Lopandic *et al*., 2011 [11]). According to taxonomic keys and standard criteria used for differentiation [1, 12], the strain VKM Y-2977 was identified as *F. penicillatus* (Rodrigues de Miranda) Yamada et Banno whereas the strain VKM Y-2978 can be assigned to the *C. laurentii* complex [13] because its stalk-like conidiophores were too small under the light microscope and mainly budding cells were observed (Fig. 7). However, short denticles or stalks were demonstrated in this strain by electron microscopy (Figs. 11a, b, 12a), which allows us to identify the strain VKM Y-2978 as *F. penicillatus* as well. Almost identical physiological and mycocin sensitivity profiles (Tables 1, 2) confirm this identification.

It is pertinent to note that, according to light microscopy observations, other *Fellomyces* species are also characterized both by budding and by forming conidia on stalks [12]. The predominant mode of reproduction depends greatly on the strain and the growth conditions, and in some cases isolates with reduced sterigmata cannot be distinguished from budding yeasts, especially from cryptococci. Since the production of stalked conidia is not always stable, this criterion is not very reliable for the definition of genera, and other characteristics should supplement it.

The ultrastructural study provided important data: (i) Both strains produce stalk-like conidiophores before bud development. (ii) Both strains have identical capsules on mother cells, necks and buds. (iii) Both strains have multilayered cell walls. In addition, fluorescence microscopy of nuclear staining by DAPI proved nuclear migration to the bud before mitosis in both strains. These cytological characteristics testify that both strains belong to basidiomycetous yeasts, even though they differ in the lengths of their stalks, and the physiologic findings specify *F. penicillatus*. The related *F. fuzhouensis* differs from *F. penicillatus* by the presence of a capsule only on mother cells, but not on the long necks or conidia, and this capsule has a different appearance (Fig. 2a2 in [7]), and probably therefore the actin cytoskeleton and the microtubules were more clearly visible [2]. It is interesting to note that the application of a microtubule inhibitor (methyl benzimidazol-yl carbamate (BCM)) induces the generation of *F. fuzhouensis* cultures without stalk-like conidiophores and necks; only neckless conidia form on the mother cells, and the capsules on these conidia [7] are similar by morphology to the capsules in the strain VKM Y-2978. In contrast, the actin inhibitor latrunculin A blocked the development of both stalk-like conidiophores and buds. These data show that both the microtubules and the actin cytoskeleton are necessary for stalk-like conidiophore development, while for bud development only the actin cytoskeleton is necessary but not the microtubules [6, 7]. Actin is known to be required also for yeast cytokinesis [8] and for correct microtubule, spindle and nuclear positioning [14–16], while the microtubules are required for yeast mitosis [17]. Nevertheless, the detection of actin and microtubule cytoskeletons in both strains investigated was very difficult. We presume that this is caused by (i) non-specific linkage of rhodamine-

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Plate II. Fig. 7. Phase contrast micrograph of living VKM Y-2978 cells at 18 h in YEPD medium. The bar corresponds to 10 μm. **Fig. 8.** Fluorescent micrographs of VKM Y-2978 cells at 18 h in YEPD medium. The cells were fixed and stained for microtubules using an anti-tubulin antibody. The bar corresponds to 10 μm. **Fig. 9.** (a, b, d, f) Fluorescent micrographs of VKM Y-2978 cells at 18 h in YEPD medium. (c, e, g) Corresponding phase contrast micrographs of the same cells (b/c, d/e, f/g). The cells were fixed and the nuclei stained with DAPI. The bar corresponds to 10 μm. **Fig. 10.** Fluorescent micrographs of VKM Y-2978 cells at 18 h in YEPD medium. The cells were fixed and stained for actin with RhPh. The bar corresponds to 10 μm. **Fig. 11.** Transmission electron micrographs of VKM Y-2978 cells at 18 h in YEPD medium. The cells were fixed and processed for transmission electron microscopy by ultrathin sectioning technique. The bar corresponds to 1 μm. **Fig. 12.** Scanning electron micrographs of VKM Y-2978 cells at 18 h in YEPD medium. The cells were fixed and processed for scanning electron microscopy. The bar corresponds to 1 μm.

phalloidin for actin staining to the capsules in both strains and (ii) non-specific linkage of antibodies for the detection of microtubules to the capsules, similar to the binding of $KMnO_4$ to the capsules in TEM (Figs. 5, 11). Also large clumps of many cells can prevent effective procedures for cytoskeleton visualization and cell biology studies. Nevertheless, except for longer microtubules and actin cables, we did not find any differences in the actin cortical patches between the two strains.

Conclusions

The cytological characteristics of the *Fellomyces* cultures studied are typical of basidiomycetous yeast fungi. Their specific features occur at the first stage of daughter cell formation when the mother cell develops stalk-like conidiophores, at the end of which the bud appears, and then the stalk becomes the neck between the bud and the parent cell. Since the length of the stalk-like conidiophores can vary significantly among the isolates studied, their formation is not very reliable for defining the genus, and other characteristics should supplement it. The nucleus migrates to the bud before mitosis, which is initiated eccentrically in the bud. Mitosis is followed by the unique eccentric cytokinesis that occurs at the base of the bud, and it strongly differs from the cytokinesis in budding and fission yeasts by the absence of the actin cytokinetic ring and a septum, and by the generation of an empty neck joining the bud and the mother cell; they form a new wall layer separating the bud/parent from the empty neck. These features are identical to those in the "long-neck yeast", *Fellomyces fuzhouensis*, and demonstrate a unique manner of reproduction among yeasts. The morphological and physiological characteristics and the mycocin sensitivity patterns of the studied strains validate their identification as *Fellomyces penicillatus.*

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Conflict of Interest Statement

The authors declare that they have no competing interests.

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