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An atypical, pigment-producing *Metschnikowia* strain from a leukaemia patient

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A yeast strain was isolated from the sputum sample of a leukaemia patient in the *Spirito Santo* Hospital of Pescara, Italy. The fungus produced a pigment that formed a reddish halo around colonies, and was identified and deposited as a *Metschnikowia* spp. (accession number IHEM 25107-GenBank accession number JQ921016) in the BCCM/IHEM collection of biomedical fungi and yeasts (Bruxelles, Belgium). Although the physiology of the strain was close to that of *Metschnikowia sinensis*, the D1/D2 sequence did not correspond to any previously described *Metschnikowia* species. Phylogeny of the genus *Metschnikowia* is complex and requires far more analysis. We present the first non-*M. pulcherrima Metschnikowia* spp. isolate recovered from a human, and emphasize the role of man as a transient carrier of environmental yeasts, the pathogenicity of which still needs to be defined.

Keywords *Metschnikowia pulcherrima*, pulcherrimin, pigment

Introduction

The natural niches of the ascomycetous fungi belonging to the genus *Metschnikowia* include plants, flower-pollenating insects (bees, bumblebees) and lacewings [1,2]. Thirty-five species have been so far defined, with *Metschnikowia pulcherrima* being the only one that has been reportedly isolated from humans as a colonizer or a pathogen [1–11].

M. pulcherrima inhabits flowers, fruits, nectar, pests (bees, *Drosophila* spp.), excrements of fruit larvae, cider and wood [10,12]. It both causes fruit decay and is a bio-control agent as an alternative to pesticides by competing with post-harvest plant contaminants [13–15]. Finally, the yeast contributes to the final aroma and flavour of wine and sausages [16,17].

In humans, it has been isolated as a colonizer from sputum, nasopharynx, vagina, rectum, and blood [10,14,18]. Nevertheless, it is likely to play a role in pathologies like acne neonatorum, tinea pedis, diaper dermatitis and nail mycoses, as well as in infections of dog skin [10,19,20].

Materials and methods

Strain isolation

Patients admitted to the Haematology Department of the Pescara Civic Hospital are screened, on a weekly basis, for bacterial and fungal colonization, through the recovery of the latter in sputum, stool, and urine cultures. These studies are to detect, in a timely manner, opportunistic pathogens and monitor the spread of resistances among the isolates within the ward. In 2010, we collected a

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pigmented yeast (Fig. 1) from the sputum sample of a leukaemic host (few colony-forming units[CFUs]/plate) in the absence of any respiratory diseases. Identification as *M. pulcherrima* (anamorph, *Candida pulcherrima*) was made through the use of both the miniAPI system (ID 32 C) and the Vitek2 YST card (both by bioMérieux, Marcy l'Étoile, France). The strain was then sent to the 'BCCM/IHEM collection of biomedical fungi and yeasts' (Bruxelles, Belgium), for phenotypic and genome-based analyses.

DNA extraction, PCR and sequencing

DNA was prepared using the Invisorb Spin Plant Mini kit (Isogen Life Science, Temse, Belgium). The D1/D2 domain of the LSU rDNA was amplified by PCR with the primers LR0R and LR5 (Vilgalys Mycology Lab, http://www.biology.duke.edu/fungi/mycolab/). Purification of the PCR product was done with the Promega 'Wizard PCR Preps DNA Purification System' (Promega, Leiden, The Netherlands). Cycle sequencing was performed with the 'BIGDye terminator V3.1 Cycle sequencing Kit' (Life Technologies Europe BV, Gent, Belgium) and sequencing was accomplished with the ABI 3130xl Genetic Analyser (Life Technologies). Both forward and reverse strands were sequenced. The entire procedure of DNA extraction, PCR and sequencing was executed twice, from separate subcultures.

Phylogenetic analysis

Reference sequences were retrieved from GenBank, with *Saccharomyces cerevisiae* chosen as outgroup. The alignment was executed with ClustalX [21] and manually edited with BioEdit v7.0.5.3 [22]. Ambiguously aligned regions were removed with Gblocks v0.91b [23] with settings that allow gaps in half the sequences and minimum block length set at 5. The resulting dataset was submitted to a ML analysis in RAxML v7.0.3 [24,25], by performing a rapid bootstrap algorithm for 1000 replicates applying the GTRMIX model of evolution, followed by a heuristic search for the best tree.

Morphology and physiology

Cultures were incubated for three weeks in YM (yeast malt) broth, and on several solid media at different temperature regimes to observe culture characteristics and cell morphology under different conditions. The latter consisted of YM agar and diluted V8 juice agar at 14°C, 25°C and 37°C, and 10× diluted Sabouraud's dextrose agar (S10) at 14°C and 25°C. Microscopic observations of the

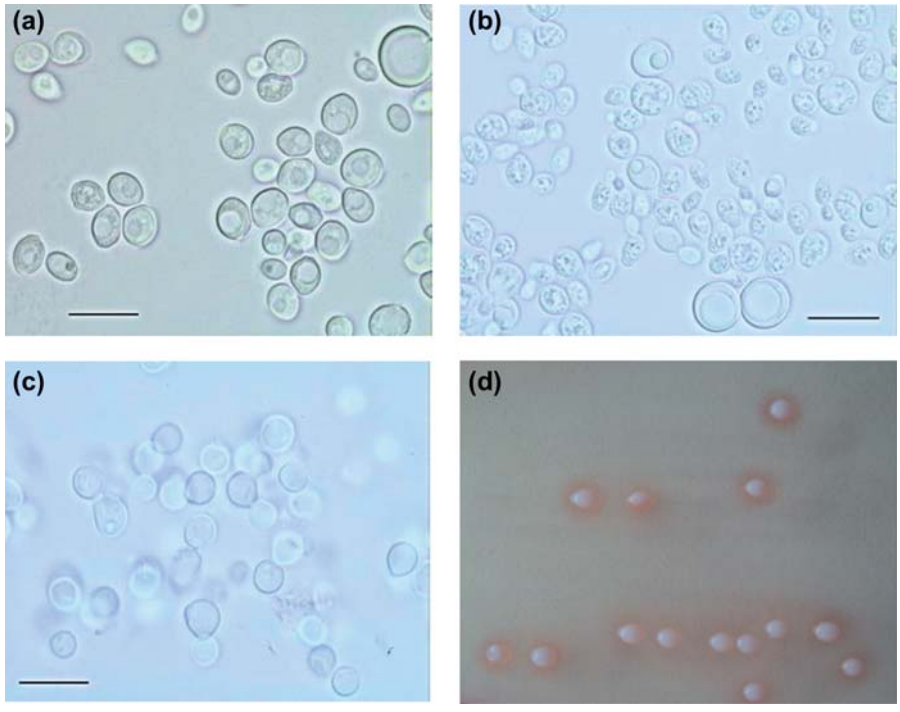

Fig. 1 Strain IHEM 25107 incubated for 3 weeks (a) in YM broth at 14°C, (b) in YM broth at 25°C and (c) on YM agar at 37°C (the scale bar represents 10 μm) – pigment visible around colonies after 24 h incubation at 36°C on Sabouraud dextrose agar (d). This Figure is reproduced in colour in the online version of *Medical Mycology*.

yeast cells were made in lactic acid. Cell dimensions are based on 30 measurements and given as averages (AVG) within an interval delimited by two times the standard deviation (SD): [AVG − 2 SD] – *AVG* – [AVG + 2 SD].

For assimilation testing, a cell suspension was added to a molten yeast carbon base (YCB) or yeast nitrogen base (YNB) agar medium, depending on the substrate to be tested and allowed to solidify. The different substrates employed in the studies are given in Table 1. Paper disks containing these substrates were placed on the surface of the solidified YCB or YNB agar containing the cell suspension. The presence of turbidity around the discs was evaluated after 48 h incubation at 25°C.

Fermentation of glucose and galactose was also evaluated after incubation for 48 h at 25°C.

Antifungal susceptibility testing

Antifungal susceptibility of strain IHEM 25107 was evaluated by the Sensititre (TREK Diagnostic System, Ohio, USA) and an E-test (bioMérieux) on RPMI agar (Liofilchem®, Roseto degli Abruzzi, Italy). The Minimum Inhibitory Concentrations (MICs) were read after 24 h incubation and are reported in Table 2. Given the lack of specific criteria for interpretive categories for MIC results of *Metschnikowia* strains, we mentioned only MICs for the test strain and showed interpretive breakpoints for the antifungals and *Candida albicans*.

Table 1 Strain IHEM 25107 physiology.

Fermentation	
F1 D-Glucose)	+
F2 D-Galactose)	+ (weak and delayed)
Assimilation	
C1 D-Glucose	+
C2 D-Galactose	+
C3 L-Sorbose	+
C6 D-Xylose	+
C9 L-Rhamnose	−
C10 Sucrose	+
C11 Maltose	+
C12 α,α-Trehalose	+
C14 Cellobiose	+
C17 Melibiose	−
C18 Lactose	−
C19 Raffinose	+
C20 Melezitose	−
C24 Erythritol	+
C29 D-Mannitol	−
C31 myo-Inositol	−
N1 Nitrate	+
N3 Ethylamine	
Temperature	
25°C	+
35°C	+
37°C	+
45°C	−
No vitamins (Czapek agar)	

+, positive; −, negative.

Results

Phylogenetic position

The alignment for analysis (after exclusion of ambiguously aligned regions) consisted of 480 characters. The phylogenetic analysis firmly placed strain IHEM 25107 (GenBank accession number JQ921016) in the *M. pulcherrima* species group, distinct from the other described species (Fig. 2). There was 97% similarity between the LSU sequence of IHEM 25107 and the *M. sinensis* strain, 96% similarity with the *M. pulcherrima* strain, and 96% similarity between the *M. sinensis* and the *M. pulcherrima* strains. Nonetheless, physiological results were the same as *M. sinensis*, in spite of pulcherrimin-like pigment production that has not yet been observed in this species [11]. Therefore, the strain was cautiously labeled as a pigment-producing *Metschnikowia* species. Finally, although the isolate seemed to show susceptibility to fluconazole, voriconazole and amphotericin B (Table 2), definitive conclusions cannot be made as there are no specific breakpoints for *Metschnikowia* strains.

Morphology

YM broth (Fig, 1a and 1b). After 3 weeks' incubation, at 14°C and 25°C, there was sedimentation and a faint ring, whereas at 37°C, little sedimentation was observed. Cells were 4.4–*5.2*–6.0 × 3.4–*4.1*–4.9 μm at 14°C and 3.8–*4.5*–5.2 × 2.5–*3.3*–4.1 μm at 25°C. They were broadly ellipsoid, ovoid or subglobose, smooth, often contained a distinct vacuole (especially at 14°C), were surrounded by a thin (< 0.5 μm) and hyaline gelatinous sheath and multipolar budding, leaving broad scars. Chlamydospores were abundant, globose, 6–9 μm diameter, thick-walled, and strongly refractive (pulcherrima-like) with a very thin gelatinous-like outer layer. Filaments were absent.

YM agar (Fig. 1c). After 3 weeks' incubation, at 14°C, colonies were 3–7 mm in diameter, 1–1.5 mm high, broadly conical to flattened campanulate, white, smooth with a silky sheen, and diffusing a dark pink pigment into the medium. Similarly, at 25°C, colonies were pinkish cream with a diffusing dark pink pigment. At 37°C, colonies were 3–5 mm diameter, 0.5–1 mm high, faintly convex, dark pink in the colony centre, pale pink in the marginal zone, mat, and with diffusing dark pink pigment. Cells were 3.4–*3.95*–4.5 × 2.5–*3.0*–3.5 μm at

Table 2 Minimum Inhibitory Concentrations (mg/liter) expressed by strain IHEM 25107.

	25°C Sensititre MICs	36°C Sensititre MICs	25°C E-test MICs	36°C E-test MICs	Breakpoints (*Candida albicans*) EUCAST version 3.0
AB	0.25	0.25	0.016	0.016	≤1 (S) – >1 (R)
FZ	0.5	0.25	0.5	0.5	≤2 (S) – >4 (R)
VZ	0.008	0.008	0.023	0.032	≤0.125 (S) – >0.125(R)

S, susceptible; R, resistant; AB, amphotericin B; FZ, fluconazole; VZ, voriconazole.

14°C, 3.2–*3.9*–4.6 × 2.5–*3.05*–3.6 µm at 25°C, and 4.3–*4.95*–5.6 × 3.8–*4.25*–4.7 µm at 37°C. The cell shape was ellipsoid or broadly ellipsoid to ovoid at 14 and 25°C, and more inflated and irregular at 37°C. Usually there were no distinct vacuoles present and pseudomycelium and hyphae were absent but short chains of 3–4 cells were often present at 37°C. The cell wall was smooth, refractive, mostly surrounded by thin (< 0.5 µm) and hyaline gelatinous sheath and multipolar budding was observed that left broad scars. Chlamydospores were abundant, as in YM broth. Filaments were absent.

S10. Colonies developed weakly as compared to YM agar. After 3 weeks' incubation at 14°C, colonies were

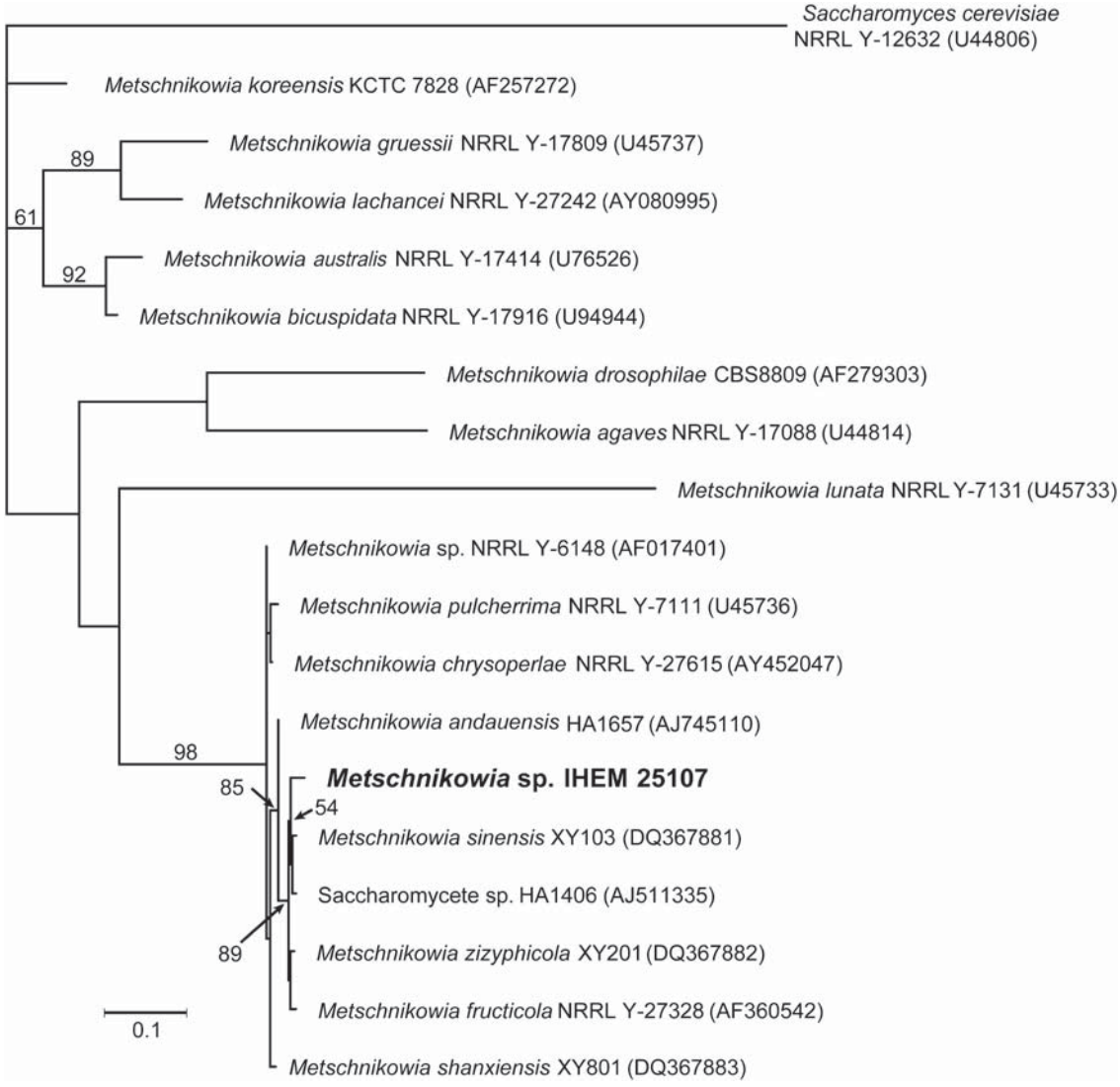

Fig. 2 Strain IHEM 25107 position in the phylogenetic tree, produced with a ML analysis. Bootstrap support values of higher than 50% are given.

1.5–2 mm diameter, less than 0.5 mm high, smooth and shiny, cream-coloured although some were very pale pink. The colony reverse was white with a faint pink tinge. At 25°C, colonies were 1–2 mm diameter, cream to pale greyish-pink especially at the colony margin, with the reverse being pale pink. Cells and chlamydospores were similar to those seen on YM agar. Chlamydospores were extremely abundant at 25°C.

Diluted V8 agar. No ascospores were produced during 5 weeks' incubation at 14°C, 25°C and 37°C, although this was expected due to the heterothallic nature of *Metschnikowia*.

Physiology

The physiology of strain IHEM 25107, summarized in Table 1, corresponds to that of *M. sinensis* [11], except for pigment production [2–28].

Discussion

The genus *Metschnikowia* includes environmental, plant-related yeasts. Until now, *M. pulcherrima* was the only *Metschnikowia* species known to have been isolated from a human patient. The LSU-based phylogenetic analysis of strain IHEM 25107 confirms its placement in the genus and although physiological tests identify it as *M. sinensis*, LSU data sets it apart from all known species.

Metschnikowia strains have been observed to produce the pigment 'pulcherrimin' which is the ferric salt of pulcherrimic acid, and forms a chelate complex with iron ions in the growth medium, inhibiting bacterial and fungal survival, as well as spore germination [26,28]. The molecule forms reddish halos around colonies (Fig. 1) that contain a water-insoluble complex of ferric ions and pulcherrimic acid [26,27]. To date, the role of pulcherrimin is unclear, but it might be a defense reaction against iron excess [26,28,29]. It is shown that pulcherrimin is not essential for growth and its expression is strain-dependent [29]. Furthermore, it is also produced by *Kluyveromyces* species (*K. marxianus*), but it is not related to the carotenoid pigment of the red yeasts *Rhodotorula* spp. and *Sporobolomyces* spp. [26,30,31].

To conclude, it is clear that the taxonomy of this group of fungi needs further study and the phylogenetic analysis should be elaborated with additional genes. In addition, these organisms' adaptation to the growth environment (*in vivo* and *in vitro*) along with their evolution still represents an almost unexplored field. In this situation, we could believe that besides exerting antimicrobial activity, pulcherrimin could offer an advantage for the survival in a hostile environment, like the human host is to a yeast.

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Note

'Stat rosa pristina nomine, nomina nuda tenemus.' (Umberto Eco, Il Nome della Rosa).

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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