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Competing interests

No competing interests have been declared.

Copyright notice© The Author(s) 2018. This is an Open Access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits redistribution, commercial and noncommercial, provided that the article is properly cited.**Citation**Chlebicki A, Spisak W, Saługa M. Halotolerance (ionic NaCl) and chaotolerance (ionic MgCl₂) of the human pathogen *Wallemia mellicola* isolated, for the first time, from indoor air in Poland. *Acta Mycol.* 2018;53(1):1104. <https://doi.org/10.5586/am.1104>**Digital signature**

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ORIGINAL RESEARCH PAPER

Halotolerance (ionic NaCl) and chaotolerance (ionic MgCl₂) of the human pathogen *Wallemia mellicola* isolated, for the first time, from indoor air in Poland

Andrzej Chlebicki^{1*}, Wojciech Spisak², Marta Saługa¹¹ W. Szafer Institute of Botany, Polish Academy of Sciences, Lubicz 46, 31-512 Cracow, Poland² Research and Development Center "Alcor" Ltd, Kępska 12, 45-130 Opole, Poland* Corresponding author. Email: a.chlebicki@botany.pl**Abstract**

We isolated the human pathogen *Wallemia mellicola* from the class Wallemiomycetes for the first time in Poland. The fungus was isolated from indoor dust in Cracow. The strain belonged to the genus *Wallemia*, as confirmed by molecular methods (i.e., ITS1-5.8S-ITS2 nrDNA sequencing). We compared the halotolerance and chaotolerance of *W. mellicola* to other those of halotolerant fungi (*Talaromyces diversus* and *Aureobasidium pullulans*) isolated from an anchialine cave. The *W. mellicola* strain tolerated up to 20% NaCl and up to 15% MgCl₂. As the concentration of NaCl in the culture medium increased, the colony diameter of *W. mellicola* decreased slightly. Dose–response curves for the two reference fungi *T. diversus* and *A. pullulans* revealed much lower tolerance of these fungi to increasing concentrations of NaCl. Our results indicated that *W. mellicola* is an advanced kosmotrope that is distinctly adapted to saline environments.

Keywords

airborne fungus; Wallemiomycetes; ITS1-5.8S-ITS2

Introduction

Fungi belonging to *Wallemia sebi* (Fr.) von Arx have been isolated from the air, soil, dried food, plant pollen, honey, salt, and hypersaline waters and are mostly xerophilic eukaryotes [1]. The genus *Wallemia* was first established by Johan Olsen in 1887 with *W. ichthyophaga* Johan Olsen as a type species (*Wallemia ichthyophaga* Johan-Olsen, Skr. Vidensk Selsk. Christiania, Kl. I, Math.-Natur. 12: 6. 1887). Previously, in 1832, Elias Fries in his famous work *Systema Mycologicum* described the fungus *Sporendonema sebi* Fr., which was transferred in 1970 to the genus *Wallemia* by von Arx as *Wallemia sebi* (Fr.) von Arx. This species is probably the most common taxon in the genus [2]. Nguyen et al. [2] and Jančíč et al. [3] sequenced various strains of *W. sebi* and divided the species complex into four new species: *W. canadensis* Jančíč, Nguyen, Seifert & Gunde-Cimerman, *W. tropicalis* Jančíč, Nguyen, Seifert & Gunde-Cimerman, *W. mellicola* Jančíč, Nguyen, Seifert & Gunde-Cimerman, and *W. sebi* sensu stricto. *Wallemia ichthyophaga* is halophilic, *W. sebi*, *W. tropicalis*, and *W. hederiae* are xerotolerant, *W. muriae* is xerophilic, and *W. mellicola* is halotolerant.

According to de Hoog et al. [4], species from the *W. sebi* complex may be human pathogens. Recently, the genomes of *W. ichthyophaga* and *W. mellicola* (strain CBS 633.66 under the name *W. sebi*) were sequenced [5,6], revealing that both species are characterized by a compact genome (9.6 and 9.8 Mb, respectively), among the smallest recorded for Basidiomycota [6].

Species in the *W. sebi* complex produce toxic metabolites, i.e., walleminol A, walleminone [7,8], azasteroid [9], and wallimidione [10], which may cause subcutaneous infections referred to as phaeoerythromycosis [11]. Some reports have suggested that species in this complex could be the causal agents for lung disease in farmers in Scandinavia, France, and China and allergic activity [12]. These slow-growing fungi are easily overgrown by fast-growing hyphomycetes on agar plates and this could explain why they are rarely isolated by traditional cultivation techniques. Real-time PCR appears to be the most sensitive technique for detecting the *W. sebi* complex [13].

We isolated several microfungus strains from 90 flats in Cracow old town. The strains were temporarily identified as *Wallemia* sp. and were found in a single locality. Fungi from the genus *Wallemia* have not been reported in Poland. We examined the taxonomic and physiological status of the isolated strain.

Some culture features, including micromorphological and ecophysiological characters of the *Wallemia* strain enabled the correct identification of the species. The size of the cerebriform colony on YMA, relatively large conidia, growth at 34°C, degree of halotolerance, and growth on YMA with 13% MgCl₂ [14] indicated that the strain is *W. mellicola* Jančić, Nguyen, Seifert & Gunde-Cimerman.

To better describe the taxonomic position of the investigated strain, we performed ecophysiological tests [3], such as halotolerance (ionic NaCl) and chaotolerance (ionic MgCl₂) assays. We compared the results for *W. mellicola* to those for two other moderately halotolerant (Chlebicki and Jakus, unpublished data) species isolated from anchialine caves, *Aureobasidium pullulans* and *Talaromyces diversus*. Growth in highly saline environments requires specific adaptations [3]. We examined decreases in the size of the strain colony with increasing salinity to estimate halotolerance. Additionally, we used molecular methods to confirm the strain identification.

Material and methods

The investigated strain of *Wallemia* was isolated from the indoor dust of an old flat located in Cracow, Poland (Targowa Street). Air for mycological studies was collected using the Eco Mas 100 sampler (Microbial Air Sampler MBV Switzerland, distributed by Merck Eurolab, Darmstadt, Germany, V4, 100 liters) and Petri dishes contained PDA (potato dextrose agar). After sampling, the plates were stored at room temperature (ca. 22°C) in the dark. After 10 days, small colonies were transferred to the following media: PDA, MEA (malt extract agar), and MEA supplemented with 5% NaCl. Strains of *A. pullulans* and *T. diversus* used for comparison were isolated from anchialine caves located in Croatia (Chlebicki and Jakus, unpublished data). Halotolerance (ionic NaCl) and chaotolerance (ionic MgCl₂) were determined on YMA (yeast malt agar; Sigma-Aldrich, St. Louis, MO, USA). Conidia obtained from the cultivated strain were suspended in 0.9% NaCl and 0.05% agar and were point-inoculated in triplicate on each plate onto 9-cm Petri dishes with YMA medium supplemented with ionic MgCl₂ at 4%, 9%, 11%, 13%, 15%, and 17% and ionic NaCl at 10%, 12%, 14%, 16%, 18%, 20%, 24%, and 28%. Cultures in six replicates for each combination (in two series) were incubated in the dark at room temperature for 7, 14, and 28 days. Colony diameters were measured after 14 days. The fungal colonies were deposited in the fungal collection of the Department of Mycology, W. Szafer Institute of Botany in Cracow.

DNA was extracted using a modification of the CTAB protocol described by Särkinen et al. [15] and Healey et al. [16]. Amplification of the ITS region was performed by touchdown polymerase chain reaction (PCR). Amplification was performed in a 15-μL reaction volume containing 1× REDTaq PCR Reaction Buffer (Sigma-Aldrich), 0.2 mM dNTPs at an equimolar ratio, 0.01 mg/mL bovine serum albumin, 0.05 units/μL REDTaq DNA Polymerase (Sigma-Aldrich), and 0.2 μM each of the ITS1-F [17] and ITS4 primers [18]. Stock DNA (1 μL) was added to each reaction as a template. The PCR protocol was as follows: 3 min at 94°C, 10 cycles of 30 s at 94°C, 30 s at 60°C, reducing the annealing temperature by 1°C every successive cycle, and 1 min at 72°C, 25 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, followed by 7 min at 72°C. The reaction was held at 4°C until further processing. PCR products were purified using ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, CA, USA). Sequencing

reactions (3 min at 96°C and 30 cycles of 10 s at 96°C, 5 s at 50°C, and 2 min at 60°C) were performed using the primers mentioned above and the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA, USA) together with BDX64 Sequencing Enhancement Buffer (Nimagen, Lagelandseweg, Netherlands), according to the manufacturer's protocol (Nimagen) for a 32× dilution. Labelled fragments were separated on the POP-7 polymer, using an ABI Prism 3130 automated DNA sequencer.

The morphological characters of the living fungi were examined in water and cotton blue in lactophenol by light microscopy (Nikon SMZ 1500, Nikon Eclipse 800; Nikon, Tokyo, Japan) equipped with a digital camera for microphotography documentation. For scanning electron microscope (SEM) studies, the mycelium was fixed in 3% buffered glutaraldehyde (pH 7), washed two times in buffer for 10 min and subsequently dehydrated in ethanol and acetone. The prepared mycelium was coated with gold and images were obtained using a LEO 1430 VP microscope (SEM; Zeiss, Oberkochen, Germany) with a working distance of ca. 10 mm. The nomenclature for fungal species followed Index Fungorum.

Results and discussion

Wallemia mellicola Jančíč, Nguyen, Seifert & Gunde-Cimerman

In Jančíč, Nguyen, Frisvad, Zalar, Schroers, Seifert & Gunde-Cimerman, PLoS One 10(5): e0125933, 14. 2015.

Description. Colonies were initially punctiform after spreading on the agar, grew on MEA without additional solutes, purplish brown and brown in reverse, cerebriform (Fig. 1), surface was velvety, irregular in shape, powdery, with margins darker than the

colony (Fig. 1B). Conidia were initially cubic, then spherical, verruculose, $(2.5)2.9\text{--}3.5 \times 1.8\text{--}2.2 \mu\text{m}$ (Fig. 2). Conidia were the largest in the genus *Wallemia* suggesting *W. mellicola*. Similar species, e.g., *W. sebi* and *W. canadensis*, have smaller conidia and cerebriform colonies on YMA. The most similar species *W. sebi* has distinctly smaller conidia than those of *W. mellicola*. Moreover, only *W. mellicola* can grow on media without NaCl among all known *Wallemia* species.

Material examined. Poland, Cracow, Targowa Street, December 12, 2014, coll. et det. A. Chlebicki, collection PKA-35, GenBank accession number No. KX977321.

We used a DNA sequence analysis to confirm the correct strain identification. The ITS1-5.8S-ITS2 sequences of our strain PKA-35 were 583 bp. In Blast searches, two strains (GenBank accession numbers KJ409882.1 and AY302532.1) had 100% query coverage with our strain. *Wallemia mellicola* strain DAOM 242695 = KJ409882.1 [2] was chosen as the leading sequence. There were no differences between DAOM 242695 and PKA-35 = KX977321 sequences. Therefore, based on culture features, micromorphological data, as well as the ITS sequence, we may conclude that the investigated strain belongs to the species *W. mellicola*.

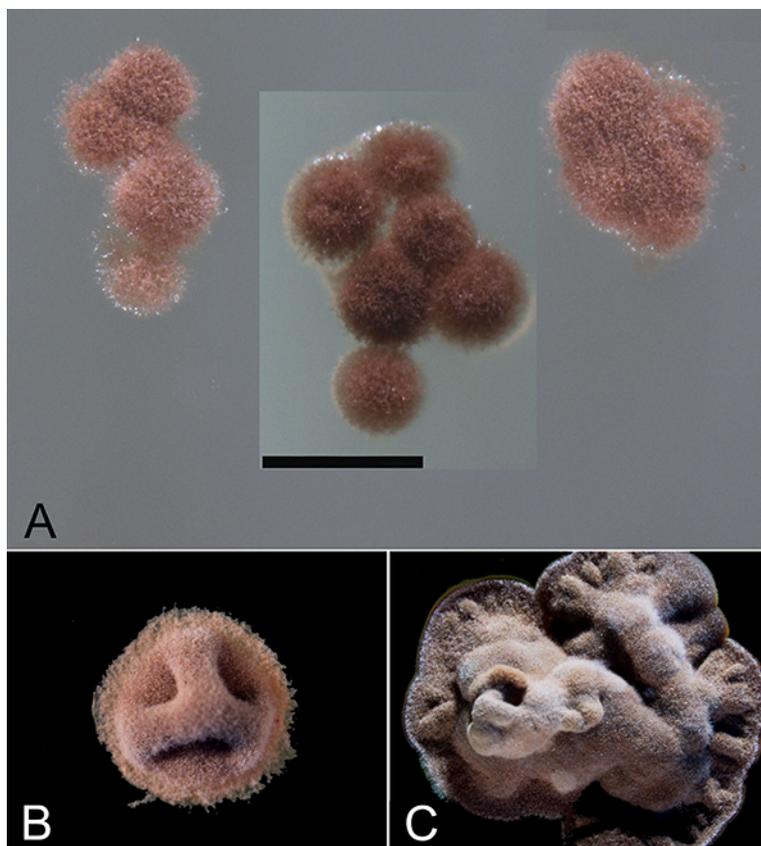


Fig. 1 *Wallemia mellicola* strain PKA-35: colonies grown on PDA for 10 days (A) and on YMA for 1 month (B) and 2 months (C). Scale bar: 1 mm.

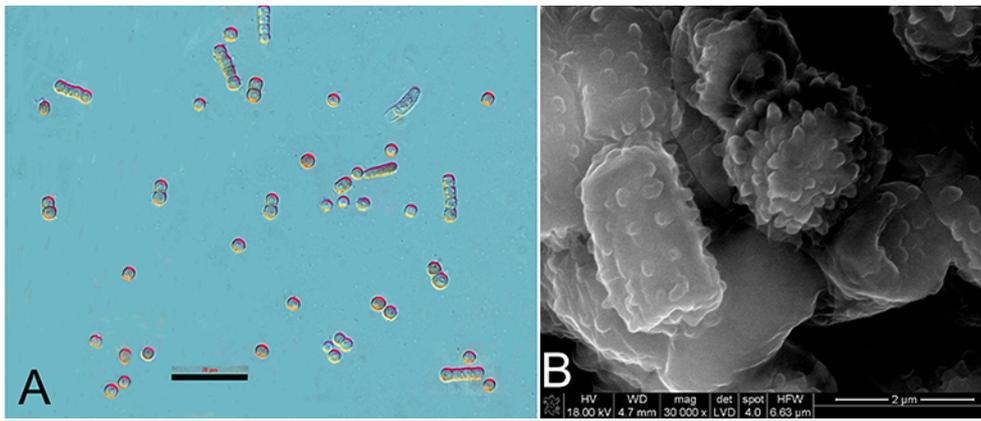


Fig. 2 *Walleimia mellicola* strain PKA-35: (A) spores observed by light microscopy (scale bar: 20 μm); (B) spores observed by SEM (scale bar: 2 μm).

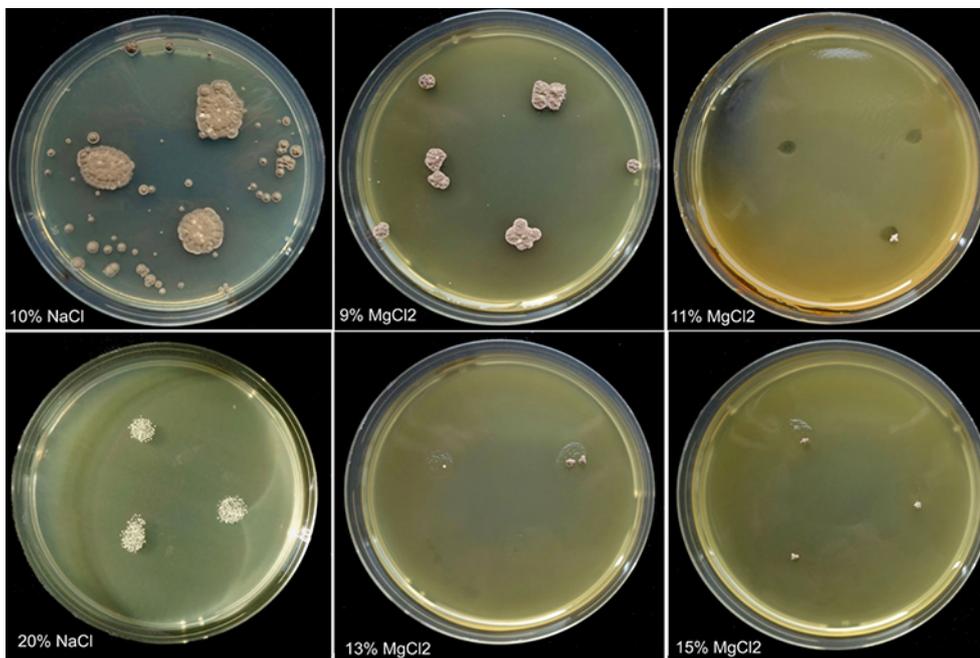


Fig. 3 Halotolerance and chaotolerance of *Walleimia mellicola* strain PKA-35 on YMA after 30 days.

It is worth noting that *W. mellicola*, isolated and cultured from an old flat in Cracow, has not been previously recorded in Poland. It is also the first species of Wallemiomycetes noted in Poland.

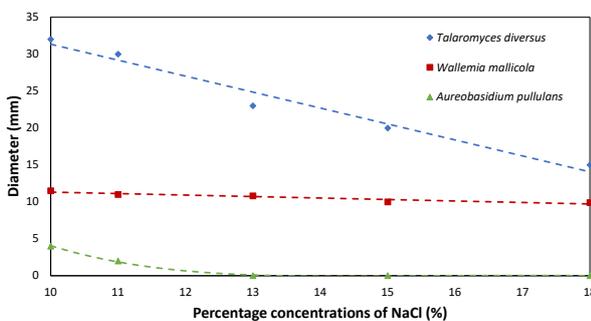


Fig. 4 Dose–response curves for the fungi *Talaromyces diversus*, *Walleimia mellicola*, and *Aureobasidium pullulans* on YMA medium with various concentrations of NaCl after 3 weeks.

The tested strain of *W. mellicola* grew at up to 20% NaCl and up to 15% MgCl₂ (Fig. 3, Fig. 4). According to Zajc et al. [19], the type of salt added to media affects the growth of strains. Extensive studies have identified fungi that thrive on media with NaCl. In contrast, few strains are able to growth in the presence of MgCl₂ [19].

Jančíč et al. [14] noted a halotolerance of 4–24% NaCl and chaotolerance of 4–13% MgCl₂ for *W. mellicola*. The most closely related *W. canadensis* exhibits no growth at 34°C on MEA and no growth on YMA with 13% MgCl₂ [14]. With increasing concentrations of NaCl in the culture medium, the colony diameter of our *W. mellicola* strain decreased only slightly (Fig. 4). The growth curves obtained for *T. diversus* and *A. pullulans* indicated much greater declines than that for *W. mellicola* (Tab. 1). These results suggest that *W. mellicola* is more adapted to saline

Tab. 1 Halotolerance of different fungi.

Species	NaCl concentration						
	10%	12%	14%	16%	18%	20%	24%
<i>Talaromyces diversus</i>	+	+	+	+	+	+	±
<i>Aureobasidium pullulans</i>	+	+	+/-	-	-	-	-
<i>Wallemia mellicola</i>	+	+	+	+	+	+	-

“+” – growth of strain; “±” – very weak growth; “-” – lack of growth.

environments than the other two fungal species. The examined strains of *A. pullulans* and *T. diversus* were isolated from anchialine caves (Chlebicki and Jakus, unpublished data). Its distinct adaptation to a saline environment suggests that the strain is a long-standing and evolutionary advanced kosmotrope.

Wallemia has a unique type of conidiogenesis and arthrospore-like conidia [20,21] (meristematic arthroconidia; Fig. 2). However, Moore [22] suggested that the “conidia” of *W. sebi* are in fact meiospores. Conversely, Padamsee et al. [6] evaluated conidiogenesis in *W. sebi*, and observed a developmental pattern suggesting that meiosis is extremely unlikely during conidia formation. This pattern does not suggest cryptic sexual reproduction. *Wallemia* strains have a variant of the *Tremella*-type of septal pore apparatus [6]. Moreover, *Wallemia* fungi are basidiomycete anamorphs that belong to the new order Wallemiales and the new class Wallemiomycetes [1]. According to Padamsee et al. [6], Wallemiomycetes are the earliest diverging lineage of Agaricomycotina.

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