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Halophily reloaded: new insights into the extremophilic life-style of *Wallemia* with the description of *Wallemia hederae* sp. nov

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Abstract *Wallemia* comprises air- and food-borne, mycotoxigenic contaminants including the halophilic *W. ichthyophaga*, xerotolerant *W. sebi* and xerophilic *W. muriae*. *Wallemia* isolates are easily overlooked and only a comparably small number of strains have been deposited in culture collections so far. In order to better understand the natural distribution of *Wallemia* spp. and to encounter their natural habitats, we tested more than 300 low-water-activity substrates and 30 air samples from a wide geographical coverage. We isolated more than 150 new *Wallemia* strains. *Wallemia sebi* and *W. muriae* were isolated mostly from hypersaline water, low-water-activity foods, plant materials and indoor. *Wallemia muriae* is the dominant *Wallemia* species in the air of natural and human influenced environments in Europe. New isolates of *W. ichthyophaga* were obtained from hypersaline environments such as brine, salt crystals, salty foods and $MgCl_2$-rich bitterns, and from the air of hay barns in Denmark. Five halotolerant strains were recognised as a hitherto un-described species *Wallemia hederae*, the phylogenetic sister of the halophilic *W. ichthyophaga*. *Wallemia* spp. show in-vitro growth on media that contain the chaotropic salt $MgCl_2$. *Wallemia ichthyophaga* can grow in liquid medium enriched with 2 M $MgCl_2$. Never before has a microorganism been grown on comparably high $MgCl_2$ concentrations. Tests of the activity of a wide range of extracellular enzymes in the presence of NaCl also suggested that *Wallemia* is well-adapted to substrates with a reduced water activity.

Keywords Chaophiles · Ecophysiology · Extracellular enzymes · Exudates · Machine learning · Taxonomy

Introduction

Wallemia sebi (Fr.) von Arx (1970), until 2005 the only known species of the ubiquitous and xerophilic fungal genus *Wallemia* Johan-Olsen (1887), is mainly known as a contaminant of food preserved with low-water-activity. It was originally described from salted products and later isolated from a wide range of foods preserved by either dehydration or enhanced salt or sugar levels including dry fruit, jam, cake, cereals, beans, nuts and confectionary (Pitt and Hocking 1997; Samson et al. 2002; Zalar et al. 2005). Although *W. sebi* is mycotoxigenic producing walleminol, walleminon (Wood et al. 1990; Frank et al. 1999), wallimidione (Desroches et al. 2014), and some other bioactive metabolites with antibacterial, antimycotic and anticancerogenic activities (Chamberlin et al. 1974; Takahashi et al. 1993; Peng et al. 2011), it was not considered as a threat to human health. At least walleminol (known also as walleminol A) was detected in jam and cake (Moss 1998). *Wallemia sebi* has been found in

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indoor air and dust (Takahashi 1997; Amend et al. 2010; Fröhlich-Nowoisky et al. 2009), and agriculture aerosols (Zeng et al. 2004; Kristiansen et al. 2012). Additionally, the species is suspected to cause respiratory diseases such as bronchial asthma and farmer's lung disease (Sakamoto et al. 1989; Reboux et al. 2001; Hanhela et al. 1995; Lappalainen et al. 1998). *Wallemia sebi* also became known as a rare human pathogen that occasionally causes cutaneous and subcutaneous infections (de Hoog et al. 2000; Guarro et al. 2008).

Species of *Wallemia* grow slowly on common microbiological media and are hence easily overlooked. The use of selective media for the isolation of xerophilic and halophilic fungi from different low-water-activity environments (Samson et al. 2002; Zalar et al. 2005) and ecological studies of fungi inhabiting hypersaline waters of solar salterns (Gunde-Cimermana et al. 2000), have resulted in discoveries of numerous *Wallemia* isolates, which in turn, have promoted ecological and natural biodiversity studies. In 2005, three species were recognized in *Wallemia*: *W. sebi*, *W. muriae* and *W. ichthyophaga*. Both *W. muriae* and *W. ichthyophaga* show their growth optimum in media with additional solutes, and they do not grow without these (Zalar et al. 2005; de Hoog et al. 2005; Kralj Kunčič et al. 2010, 2013). The segregation of these species was based on conidial size differences, their degree of xerotolerance, and supported by molecular ITS sequences. Although the three species have the same unique conidiogenesis and form conidia similarly disarticulating into four units (Moore 1986; Padamsee et al. 2012), *W. ichthyophaga* is separated from *W. sebi* and *W. muriae* by strongly differing ITS sequences (Zalar et al. 2005). As *W. ichthyophaga* requires at least 1.5 M NaCl in its culture medium for growth, but thrives also in saturated NaCl solutions (Zalar et al. 2005; Kralj Kunčič et al. 2010), it was considered as the most halophilic fungus known to date. In recent years, it has become an important model organism for the study of haloadaptation in eukaryotes (Kralj Kunčič et al. 2010; Lenassi et al. 2011; Konte and Plemenitaš 2013; Zajc et al. 2013, 2014).

The natural habitat of *Wallemia* spp. is solar salterns. *Wallemia* has been isolated from hypersaline brine with NaCl as the prevailing solute (0.5 to 5.2 M, 3–30 % [*v*/*w*]), as well as from bitterns that were rich in chaotropic ($MgCl_2$) and kosmotropic ($MgSO_4$) solutes (Javor 1984). The growth of the *Wallemia* spp. was determined on the kosmotropic NaCl. According to Kralj Kunčič et al. (2010), *W. ichthyophaga* grows on 9–30 % NaCl, whereas the *W. muriae* NaCl growth range is from 4 to 25 % NaCl.

Although *W. sebi*, *W. muriae* and the extremely rare *W. ichthyophaga* were sporadically isolated from natural and man-made low-water-activity environments, their individual ecophysiologies, and consequentially their pathogenic potentials, remained largely unknown. One of the foci of the present study was to broadly sample diverse low-water-activity environments, to better understand the distribution and ecology of the currently known *Wallemia* species and to characterize the obtained *Wallemia* isolates phylogenetically and physiologically. Additionally, the colony forming units per cubic metre (CFU/m^3) and percentage of the *Wallemia* spp. in 30 air samples of natural, indoor and outdoor environments were analyzed for connections with descriptive variables such as habitat, type of environment, and weather conditions, using a machine learning method. Selected strains were tested for growth on various carbon and nitrogen sources, and for their extracellular enzymatic activities on non-saline and saline media supplemented with 10, 17 and 25 % NaCl. Additionally, some strains were tested for their salt tolerance on the $MgCl_2$ chaotrope and the $MgSO_4$ kosmotrope. The study finally led to the recognition of a new *Wallemia* species that is described below as *Wallemia hederae*.

Materials and methods

Sampling of low-water-activity environments and substrates

In the period from April 2009 to June 2013, *Wallemia* strains were isolated from a wide variety of natural and man-made low-water-activity environments and substrates. This was achieved using general microbiological techniques and four isolation media for xerophilic and halophilic fungi: malt yeast with 50 % glucose agar (MY50G), malt yeast with 10 % NaCl and 12 % glucose agar (MY10-12; Pitt and Hocking 1997), dichloran glycerol (DG18) agar (Samson et al. 2002), and MEA (Gams et al. 1998) with 10 % NaCl (MEA10) and 17 % NaCl (MEA17) (Gunde-Cimermana et al. 2000). The antibiotic chloramphenicol (0.05 %) was added to all of the media used in the present study.

Additional unidentified *Wallemia* strains and taxonomic reference materials were obtained from the Ex Culture Collection of extremophilic fungi of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia), University of Alberta Microfungus Collection and Herbarium (UAMH, Alberta, Canada) and from the Belgian Co-ordinated Collections of Microorganisms (BCCM/MUCL, Louvain-la-Neuve, Belgium).

Air sampling of natural, indoor and outdoor environments

To define distribution and frequency of *Wallemia* propagules, air was sampled in natural and human influenced in- and outdoor environments. This was carried out from December 2012 to June 2013 at different locations in Slovenia and Denmark. Natural environments included forests, meadows and solar

salterns at the Adriatic coast. Indoor environments consisted of apartments or houses, stables for cattle or horses, and hay barns. The backyards of the indoor environments were considered as outdoor environments. During air sampling, relevant meteorological data were collected, including temperature, relative humidity, precipitation (clear, fog, rain, snow), direction and velocity of wind, air pressure, and presence of a cyclone or an anticyclone. These data were either recorded by direct measurements or were from measurements provided by the Slovenian Environment Agency, under the auspices of the Slovenian Ministry of Agriculture and Environment. In indoor environments, the indoor temperature and humidity was measured.

Air samples of 50, 100 or 500 L were collected approximately 1 m above the ground with a viable impaction sampler (Sas Super ISO, PBI International) and by using four different culture media in the sampler (MY50G, MY10-12, DG18, MEA17). Cultures were incubated at 24 °C for 20 day and inspected regularly. The concentrations of any fungi were calculated using actual plate counts, and expressed as colony forming units per cubic metre (CFU/m^3) air on the four media. For determination of the mean concentrations, only the CFU/m^3 air values from the MY50G, MY10-12 and DG18 media were used. Up to five *Wallemia* colonies were randomly selected from each medium plate and subcultured, kept as pure cultures, and identified as described below.

Machine learning analysis of air samples

The obtained air samples described with CFU/m^3 and percentage of occurrences of the *Wallemia* spp. on media MY50G, MY10-12, DG18 and MEA17 (considered as dependant or target variables) and the characteristics of the sample sites (considered as independent or descriptive variables) were subjected to a data mining analysis. The type of environment, habitat, time of sampling, humidity, temperature, pressure, cyclone, anticyclone, snow, rain, fog, clear and wind were used as descriptive variables and the obtained CFU/m^3 and percentages on the different media as a total of 8 numerical target variables. For the plates with confluent growth, the CFU/m^3 values were filled to 50,000 and percentage to 97 %. Two different scenarios for analysis were defined. The different scenarios used different descriptive variables, as follows: scenario A (all of the descriptive variables presented earlier) and scenario B (humidity, temperature, pressure, cyclone, anticyclone, snow, rain, fog, clear, wind).

To analyse the data, we used the machine learning tool CLUS (http://clus.sourceforge.net). We used predictive clustering trees (PCTs) for multi-target regression as models. PCTs are a generalization of regression trees – a machine learning approach commonly used for regression. PCTs, similarly as regression trees, are tree-like structures that have internal nodes and leaves. The internal nodes contain tests on the descriptive variables, while leaves represent the predictions of the target variables. PCTs can solve the more general task of structured output prediction, including the task of multi-target regression. The predictive clustering trees are able to exploit the mutual information and relation between the different target variables during the model construction. Furthermore, the PCTs are easily interpretable predictive models for multi-target regression. The method was settled as described earlier (Struyf and Džeroski 2006; Kocev et al. 2009, 2013; Kocev and Džeroski 2013).

Preservation of strains

Strains were maintained on MY50G and/or MEA10 or MEA17 agar slants and preserved under liquid nitrogen at −150 °C and below. The strains are deposited in the Ex Culture Collection of extremophilic fungi of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Infrastructural Centre Mycosmo, MRIC UL, Slovenia). Relevant reference material was also deposited at the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.

DNA extraction, PCR amplification and sequencing

DNA was extracted by mechanical lysis in cetyltrimethylammonium bromide buffer (Gerrits van den Ende and de Hoog 1999), from approximately 1 cm^2 of 7-day-old cultures grown on MEA (Gams et al. 1998) that was supplemented with 10 % NaCl. The sequencing of the ITS and SSU rDNA was performed after PCR amplification with the primer pairs ITS1/ITS4 and NS1/NS24 (White et al. 1990; Gargas and Taylor 1992). Genomic DNA was used as the template in 35 μL PCRs with 10× Dream Taq DNA polymerase (Fermentas), 1× Dream Taq buffer (Fermentas), 0.1 mM dNTPs, 0.8 μM forward primer, 0.8 μM reverse primer, under the following conditions: hot-start initial denaturation at 95 °C for 3 min, then 35 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, followed by final extension at 72 °C for 5 min using an Eppendorf thermal cycler (Westbury, New York). The PCR-amplified fragments were checked through agarose gel electrophoresis and sequenced automatically using the Sanger method, either at Macrogen (Seoul, Korea) or Microsynth (Vienna, Austria).

Sequence data, alignment and phylogenetic analysis

The newly generated ITS and SSU rDNA sequences were compared with sequences of other *Wallemia* taxa through BLAST searches (Altschul et al. 1990). The ITS of 67 *Wallemia* strains were automatically aligned to the published ITS sequences of *W. sebi* ex-neotype strain CBS 818.96 (AY328915) and strain CBS 202.33 (AY328912), *W. muriae*

ex-neotype strain MZKI B-952=CBS 116628 (AY302534), and *W. ichthyophaga* ex-neotype strain EXF-994=CBS 113033 (AY302523) (Zalar et al. 2005), using the MAFFT programme (Katoh et al. 2005). Similarly, newly generated SSU rDNA sequences of 13 *Wallemia* strains were aligned to AY741379 (*W. sebi* strain CBS 196.56), AY741380 (*W. sebi* strain EXF-757), AY741381 (*W. muriae* strain CBS 116628) and AY741382 (*W. ichthyophaga* strain CBS 113033; Zalar et al. 2005), and to AF548107 (*W. sebi* strain ALI 158; Wu et al. 2003), and to the sequence of *Geminibasidium donsium* H.D.T. Nguyen, N.L. Nickerson & Seifert strain CBS 113785 (JX242893; Nguyen et al. 2013). All of the sequences are deposited in GenBank. Phylogenetic relationships were determined via maximum-likelihood analysis using PhyML 3.0 (http://www.atgc-montpellier.fr/) (Guindon et al. 2010), by Bayesian analysis using MrBayes version 3.2.2 (Ronquist et al. 2012), and by maximum-parsimony analysis using PAUP* version 4.0b10 (Swofford 2003). The software jModelTest 2.1.4 (Darriba et al. 2012) was used for the selection of the appropriate models of sequence evolution under the Akaike information criteria. Maximum likelihood analyses were performed using bootstrap analysis with 1000 replicates. For Bayesian posterior probabilities, two independent Markov chain Monte Carlo runs were performed simultaneously. Each Markov chain Monte Carlo run covered 5 million generations, sampled every 100 generations. Bayesian posterior probabilities were calculated after the first 25 % of the trees were discarded as burn-in. The remaining trees were kept and combined into one 50 % majority rule consensus tree. The most parsimonious trees were determined through heuristic searches that adopted the characters of the alignments as unordered and equally weighted; gaps were treated as missing data. Starting trees were obtained via 100 stepwise, random addition sequences. Other parameters included a MaxTrees setting of 1000, the tree-bisection–reconnection branch-swapping algorithm, and the MulTrees option. Branch robustness was assessed by 1000 fast stepwise addition bootstrap replicates. Trees were visualised using the FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) or MEGA5 (Tamura et al. 2011) software.

Assimilation tests

Assimilation of 46 carbon (C) sources and four nitrogen (N) sources on agar media were performed with 22 strains, as described by Kurtzman et al. (2011). The tests were performed either with 17 % NaCl (*W. sebi*, *W. muriae*, *W. ichthyophaga*, *Wallemia* sp.) or without NaCl (*W. sebi*, *Wallemia* sp.). The pH of the media was adjusted to 5.5 before autoclaving. Before pouring the media into Petri dishes, filter sterilised C compound solution was added to a final concentration of 1 % (*w*/*v*). The final concentrations of the N sources were 1 % D-glucosamine, 0.056 % L-lysine, 0.078 % potassium nitrate, and 0.026 % sodium nitrite. Conidial suspensions of 7-day-old cultures grown at 24 °C on MY50G in saline were used for the three-point inoculations of the culture media. The cultures were incubated in the dark at 24 °C for 28 days.

Screening for extracellular enzymatic activity

Extracellular enzymatic activities were tested on solid media (2 % agar) without and with 10, 17 and 25 % NaCl (Paterson and Bridge 1994; Brizzio et al. 2007; Strauss et al. 2001). Strains were inoculated and incubated as described above. The basic medium for the testing of β-glucosidase, amylolytic and cellulotytic activities was 0.67 % yeast nitrogen base (YNB, Difco), with the addition of a specific C source: 1 % easculin (Sigma) for β-glucosidase activity, 0.2 % soluble starch for amylolytic activity, and 0.5 % carboxymethyl cellulose (Sigma) for cellulolytic activity. If not stated differently, the pH of all media was adjusted to 5.5 before pouring. Filter sterilised 1 % ammonium ferric citrate solution was added to the medium for the determination of the β-glucosidase activity, to a final concentration 0.02 % ammonium ferric citrate. Enzymatically active colonies were identified by the dark brown coloration of the media (Strauss et al. 2001). For detection of amylolytic activity, the plates were flooded after incubation with Lugol's iodine solution. A pale yellow zone around the colony in the otherwise blue medium indicated starch degradation (Brizzio et al. 2007). After incubation of plates for detection of cellulotytic activity, the colonies were stained with 0.03 % Congo red for 10 min, followed by destaining with 1 M NaCl. Activity was identified as a pale red halo around the colonies (Brizzio et al. 2007; Strauss et al. 2001). Proteolytic activity was detected on 0.5 % skimmed milk agar medium with 0.1 % yeast extract (Biolife). A clear zone in the otherwise opaque medium showed a positive reaction (Brizzio et al. 2007). Esterase activity was determined on Tween 80 agar medium, which consisted of 1 % Tween 80 (polyoxyethylene-sorbitan-monooleate), 1 % peptone (Merck), and 0.01 % $CaCl_2 \times 2H_2O$. The pH indicator bromocresol blue (12.5 mg/L) was added to the media before adjusting the pH to 5.4. After incubation, the ability to cleave the ester bond was detected as a precipitate and a purple halo around the colony in the yellowish-green medium (Brizzio et al. 2007).

Additionally the API ZYM® system (BioMèrieux) was used to detect the presence of 19 constitutive enzymes using chromogenic substrates. Five strains were selected and grown in liquid medium prepared without and with 10, 17 and 25 % NaCl. This liquid medium consisted of 2 % whey, 0.2 % yeast extract (Biolife), 1 % olive oil, 2 % glucose (Carlo Erba Reagents), 0.5 % peptone (Merck), 0.5 % glycerol, 0.5 % soluble starch (Sigma), 0.5 % chitin (Sigma), 0.2 % KH_2PO_4 (Sigma), 0.12 % $(NH_4)_2SO_4$ (Sigma), 0.03 % urea (Sigma), 0.003 % $CaCl_2$ (Sigma), 0.001 % $FeSO_4 \times 7H_2O$, 0.01 % $ZnSO_4 \times H_2O$,

0.01 % $MnSO_4 \times H_2O$, and 0.01 % $CoCl_2 \times 6H_2O$. The pH of the medium was adjusted to 5.5 before it was autoclaved. After 28 days of incubation at 24 °C on a rotary shaker (90 rpm; Innova® 42, Eppendorf), the cultures were centrifuged for 5 min (20,817× *g*), and the cell-free supernatant was used for further assays. The tests were performed according to the API ZYM® manufacturer instructions.

Based on the data from the API ZYM® screening, β-glucosidase (EC 3.2.1.21) activity was additionally measured in cell-free supernatants. β-Glucosidase activity was determined according to the manufacturer instructions (Sigma-Aldrich Enzyme Assays) by mixing 150 μL cell-free supernatant with 50 μL 500 mM acetate buffer (pH 5.0 at 37 °C) and 50 μL 5 mM 4-nitrophenyl-β-D-glucopyranoside (Sigma), in microtitre plates. The absorbance at 405 nm was read using a spectrophotometer (Multiskan spectrum, Thermo), over 30 min at intervals of 30 s, at 37 °C. One unit of β-glucosidase activity was defined as the amount of activity that releases 1 nmol 4-nitrophenyl-β-D-glucopyranoside to p-nitrophenol and D-glucose per h at pH 5.0 and 37 °C.

Growth in the presence of chaotropic and kosmotropic solutes

The growth of strains were checked at different concentrations of the chaotrope $MgCl_2$ and the kosmotrope $MgSO_4$. The basic medium consisted of 2 % malt extract (Biolife), 2 % glucose (Carlo Erba Reagents) and 0.1 % peptone (Merck). The media that contained up to 1.7 M $MgCl_2$ were solid (2 % agar), while the media with more than 1.7 M $MgCl_2$ and the media containing 1 M to 4 M $MgSO_4$ did not solidify and was liquid. Additionally, a solid medium was designed containing the three salts 1 M NaCl (58.44 g/L), 1 M $MgCl_2 \times 6\ H_2O$ (203.31 g/L) and 1 M $MgSO_4 \times 7H_2O$ (146.47 g/L). The water activity of the medium was 0.829 and its pH was adjusted to 5.5 before autoclaving for 15 min at 121 °C. Plates were inoculated with 10 μL of saline conidial suspension obtained from 7-day-old cultures grown at 24 °C on MY50G. The cultures were incubated in the dark at 24 °C for 28 days. The micromorphological characters of grown cultures were studied using differential interference contrast microscopy (Olympus BX51), with an attached digital camera (Olympus DP73).

Colony characteristics, microscopy and physiology

The macroscopic colony characters of *Wallemia* sp. were characterized including size and color of colonies, spreading characteristics of colonies, colony structure, production of exudates, and soluble pigment production. Eight media were used that had different water activities: MEA (a_w=0.998) (Gams et al. 1998); MEA10 (a_w=0.935); MEA17 (a_w=0.886) (Gunde-Cimermana et al. 2000); *Wallemia* morphology medium I (W-4) (a_w=0.950); *Wallemia* morphology medium II (W-10) (a_w=0.900) (Zalar et al. 2005); MY50G (a_w=0.890); MY10-12 (a_w=0.916) (Pitt and Hocking 1997); and DG18 (a_w=0.953) (Samson et al. 2002). The pH of the media was set to 6.5 prior autoclaving. The macroscopic characters were reported using a stereomicroscope (Leica EZ4), from point-inoculated media in plastic 9 cm-diameter Petri dishes incubated at 24 °C for 14 days in the dark. Micromorphological characters including hyphae, conidiophores, conidiogenous cells and conidia, were studied in slide cultures (Gams et al. 1998) using medium MY50G incubated at 24 °C for 14 days, with differential interference contrast microscopy (Olympus BX51) and a digital camera (Olympus DP73).

To determine the degree of halotolerance and xerotolerance of *Wallemia* sp., NaCl was used as the controlling solute. Ten μL of conidial suspensions were inoculated on top of a 0.22 μm filter placed on the top of MEA without and with additional NaCl at concentrations of 4 % (a_w=0.985), 8 % (a_w=0.948), 10 % (a_w=0.935), 12 % (a_w=0.917), 16 % (a_w=0.886), 20 % (a_w=0.859), 24 % (a_w=0.819), 28 % (a_w=0.779) and 30 % (a_w=0.756) NaCl. These cultures were incubated at 24 °C for 28 days. After incubation, the exudates were carefully collected. The volumes were measured using a microliter automatic pipette, and then they were analyzed by HPLC and API ZYM tests. The dry biomass of the remaining colonies was measured. For each medium, the data are means ±standard deviations of three replicates per strain.

Results

Sampling of low-water-activity environments and substrates

In total, 300 different low-water-activity environments and substrates were sampled (Table 1), the majority in Slovenia, but also in other European countries (Croatia, Germany, Iceland, Italy, the Netherland, Turkey), in Asia (Indonesia, Japan, Nepal), in Africa (Namibia, Tanzania, Tunisia) and in North (USA) and South (Argentina) America. Inventories of substrates and samples described in Table 1 yielded 42 *Wallemia* strains, of which 19 were identified as *W. muriae* (45.2 %), 12 as *W. sebi* (28.6 %) and 6 as *W. ichthyophaga* (14.3 %). Five strains (11.9 %) could not be identified and presented a hitherto un-described species. The most frequently occurring species, *W. muriae*, was mainly isolated from halophytes, saltpan silt, pollen, wheat seed, chocolate and indoor surfaces (walls, ceilings). *Wallemia sebi* prevailed in hypersaline waters of solar salterns, and in hay, maize seeds, jam, chocolate, and apiary and indoor surfaces (ceilings). The rarely occurring halophilic *W. ichthyophaga* currently known from hypersaline environments such as hypersaline water of salterns, salt crystals, and salty foods was also encountered from $MgCl_2$-rich

Table 1 Low-water-activity environments and substrates sampled for the isolation of *Wallemia* spp. Sources from which *Wallemia* was isolated are in bold. For the countries of origin 2-letter standard codes were used

Environment	Substrate	Details
Low-water-activity foods	Sugar-rich foods	Jam, SI; marmalade, SI; saturated sugar solution, SI; sugar, SI; dark chocolate, SI; milk chocolate, SI; white chocolate, SI; hazelnut chocolate, SI; chocolate with salt crystals, SI; chocolate cream, SI; cakes, SI; honey, SI, HR.
	Dry foods	Dry fruit (apricot, SI; fig, SI; pear, SI; apple, SI; mulberry, HR; plum, SI); cereals (barley, SI; winter wheat, SI; spring wheat, SI; triticale, SI; rice, JP, SI); seeds (grass, sunflower, sesame, maize, hemp), SI; flour (wheat, corn, buckwheat, rye), SI; spices (pepper, SI; salt, SI; cinnamon, SI; cloves, TA); teas (sage, dog rose, elder, linden), SI); coffee, SI and cacao, SI.
	Salted foods	Salted pork meat, SI; bacon, SI; salted ham, SI; sausages, SI; Hungarian salami, SI; porcine cracklings, SI; porcine rinds, SI; porcine fat.
Hypersaline environments	Solar salterns	Hypersaline water of solar salterns, SI[1], IT; bitterns, SI[1]; salt crystals, SI[1], IN; silt from the saltern ponds, SI[1]; flesh, feathers and bones from seagull cadaver, hypersaline water and silt around seagull cadaver, SI[1]; cuttlebones, SI[1]; mussel shells, SI[1]; insect cadavers, SI[1]; honeycomb, SI[1]; halophites (*Artemisia caerulescens*, *Arthrocnemum macrostachyum*, *Aster tripolium*, *Atriplex portulacoides*, *Inula crithmoides*, *Juncus maritimus*, *Limonium angustifolium*, *Salicornia europaea*) and other salterns plants, SI[1]; dry plant residues, SI[1].
	Salt lake	Hypersaline water, USA[1], TN; salt crystals, TN; pink salt crystals, USA[1]; salt with brine shrimps, USA[1]; salt with brine shrimp eggs, USA[1]; salt with fruit flies and other unknown insects, USA[1]; salt with sediments, USA[1]; oolites, USA[1]; dry plants and dry plant residues (blades of unidentified grass; unidentified wooden stems; unidentified sunflower-like seeds), USA[1].
	Evaporite	Dry plant residues (unidentified leaves, needles and berries, blades of unidentified grass, stems, wooden stems, unidentified flowers and inflorescences, cypress cones), USA[2]; soil, USA[2]; sand, USA[2], NA.
	Salt	Halite, SI; salt from salt mine, TR, AR; Himalayan salt, NP.
Agricultural environments	Livestock	Dry hay, SI; hay barn, SI; cereals, SI; compost, SI; maize silage, SI; animal (cow) hair, SI.
	Beekeeping	Plant flowers (*Hedera helix*), SI; pollen, SI, NL; pollen baskets (corbicula), SI; apiary, SI; beehive surfaces, SI; surrounding air, SI; surface of wooden block of honeycomb, SI; propolis on wooden block of honeycomb, SI; single honeycomb cells filled with honey or pollen, SI; live bees and bee cadavers, SI.
Indoor environments	Moldy surface	Basement, SI; Ceiling in the children's room, SI; ceiling in the bedroom, SI; wall in the kitchen, SI; wall in the living room, SI; bathroom, SI.
Other environments	Soil	Soil, SI, IS; silt and sediments around geyser, IS.
	Mushrooms	*Armillaria* sp., *Bulgaria* sp., *Clitocybe* sp., Coprinus sp., Entoloma sp., *Exidia* sp., Fomes sp., *Fomitopsis* sp., *Galerina* sp., *Hericium* sp., Hygrophorus sp., *Lactarius* sp., *Mycena* sp., *Oudemansiella* sp., *Peziza* sp., *Pholiota* sp., Pseudohydnum sp., *Pycnoporus* sp., *Stropharia* sp., *Trichordopsis* sp., *Xylaria* sp., SI.

SI[1], Slovenia, active solar salterns Sečovlje; USA[1], United States of America, Utah, Great Salt Lake; USA[2], Utah, Arches National Park

bitterns that remain after the precipitation of halite (NaCl) in crystallisation ponds of solar salterns. Representatives of *Wallemia* sp. were mainly plant-associated and isolated from ivy pollen, barley seeds, oak honey and air in a hay barn. No *Wallemia* strains were isolated from soil, salt lakes (e.g., Great Salt Lake, USA; Chott el Jérid, Tunisia), or from evaporite environments like Arches National Park in Utah, USA. Strains retrieved from culture collections were identified as *W. sebi* (17 strains), *W. muriae* (7) or *W. ichthyophaga* (1). The studied strains from the low-water-activity environments and substrates are listed in Table 2.

Analysis of air samples

All samples from Slovenia and Denmark (n=34) contained *Wallemia* propagules (Figs. 1 to 2). Their levels ranged from 10 to 10^6 CFU/m^3. The concentration of *Wallemia* propagules in the air of natural environments (n=12) such as forests, meadows and solar salterns was on average 250 CFU/m^3. These levels ranged from 10 to 10^3 CFU/m^3. The lowest levels were detected in the air of forests (10–70 CFU/m^3), while concentrations in the air above meadows ranged from 10 to 240 CFU/m^3. The levels in solar salterns were

Table 2 Strains studied, their original sources and GenBank accession numbers

Taxon	Culture		Source		GenBank accession number	
	EXF[c]	Other	Substrate	Origin	ITS	SSU
W. sebi	EXF-1278*		Hay storage	UK	KJ494635	–
	EXF-1441*		Unknown	Slovenia	KJ494640	–
	EXF-1442*		Unknown	Denmark	KJ494643	–
	EXF-1913*		Hypersaline water of salt lake	USA, Utah	KJ494637	–
	EXF-5746		Hypersaline water of solar salterns	Italy, Trapani	KJ494642	–
	EXF-5747		Hay	Slovenia, Begunje	KJ494634	–
	EXF-5752		Feathers of seagull cadaver	Slovenia, Sečovlje	KJ494639	–
	EXF-5860		Hypersaline water of solar salterns	Slovenia, Sečovlje	KJ494636	KJ494586
	EXF-5920		Maize seeds	Slovenia, Laško	KJ494641	–
	EXF-6148*	MUCL[d] 8703	Leaf (*Quercus* sp.)	Belgium, Egenhoven	KJ494633	KJ494578
	EXF-6155*	MUCL 46253	Flavoured mineral water	France	KJ494638	KJ494587
	EXF-277*		Hypersaline water of solar salterns	Spain	KJ494630	–
	EXF-1443*		Unknown	Denmark	KJ494622	–
	EXF-5675		Spoiled jam	Slovenia, Trebnje	KJ494618	–
	EXF-5677		Air in front of apiary	Slovenia, Ljubljana	KJ494616	KJ494588
	EXF-5828		White chocolate	Slovenia, Ljubljana	KJ494631	–
	EXF-5829		Milk chocolate	Slovenia, Ljubljana	KJ494624	–
	EXF-5830		Chocolate	Slovenia, Ljubljana	KJ494621	–
	EXF-5918		Ceiling in the children's bedroom	Slovenia, Kamnik	KJ494632	–
	EXF-5922		Chocolate	Slovenia, Ljubljana	KJ494629	–
	EXF-6149*	MUCL 15061, UAMH[e] 2817	Peat soil from cedar swamp	Canada, Ontario, Puslinch	KJ494615	KJ494582
	EXF-6150*	MUCL 40632	Dried coconut pulp (*Coco nucifera*)	Brazil, Para States, Belem	KJ494627	–
	EXF-6151*	MUCL 45615, CRGF[b] 494	Flower (*Verbena officinalis*), forest	Cuba, Guantanamo	KJ494620	–
	EXF-6152*	MUCL 45613, CRGF 503	Flower (*Clusia rosea*), forest	Cuba, Guantanamo	KJ494619	–
	EXF-6153*	MUCL 454611, CRGF 499	Flower (*Begonia relumbifolia*), bosque	Cuba, Sancti Spiritus	KJ494617	–
	EXF-6154*	MUCL 45614, CRGF 493	Flower (unidentified angiosperm)	Cuba, Pinar del Rio	KJ494626	–
	EXF-6156*	UAMH 2651	White bread	UK	KJ494628	–
	EXF-6157*	UAMH 2757	Soil	Canada, Edmonton	KJ494623	KJ494589
	EXF-6158*	UAMH 6689	Maple syrup	Canada, Fanny Bay	KJ494625	KJ494577
W. muriae	EXF-1271*		Indoor	UK	KJ494592	–
	EXF-2361*		Chocolate	Slovenia, Ljubljana	KJ494606	–
	EXF-2362*		Chocolate	Slovenia, Ljubljana	KJ494612	–
	EXF-2363*		Chocolate	Slovenia, Ljubljana	KJ494591	–
	EXF-3554*		Saturated sugar solution	Slovenia, Ljubljana	KJ494605	–
	EXF-4957*		Hypersaline water of Dead sea	Israel, Eilat	–	–
	EXF-4967*		Hypersaline water of Dead sea	Israel, Eilat	KJ494610	KJ494584
	EXF-5673		Pollen basket (*corbicula*) of honey bee	Slovenia, Krško	KJ494604	–
	EXF-5674		Pollen basket (*corbicula*) of honey bee	Slovenia	KJ494608	–

Table 2 (continued)

Taxon	Culture		Source		GenBank accession number	
	EXF[c]	Other	Substrate	Origin	ITS	SSU
	EXF-5678		Halophyte	Slovenia, Sečovlje	KJ494603	–
	EXF-5679		Halophyte	Slovenia, Sečovlje	KJ494599	–
	EXF-5680		Halophyte	Slovenia, Sečovlje	KJ494595	–
	EXF-5681		Halophyte	Slovenia, Sečovlje	KJ494600	–
	EXF-5682		Halophyte	Slovenia, Sečovlje	KJ494598	–
	EXF-5683		Halophyte	Slovenia, Sečovlje	KJ494597	–
	EXF-5684		Halophyte	Slovenia, Sečovlje	KJ494602	–
	EXF-5685		Halophyte	Slovenia, Sečovlje	KJ494601	–
	EXF-5686		Halophyte	Slovenia, Sečovlje	KJ494596	–
	EXF-5750		Spring wheat	Slovenia, Begunje	KJ494607	–
	EXF-5751		Saltpans silt	Slovenia, Sečovlje	KJ494590	–
	EXF-5914		Wall in the kitchen	Slovenia, Radomlje	KJ494611	–
	EXF-5915		Wall in the living room	Slovenia, Vrhnika	KJ494593	KJ494583
	EXF-5916		Wall in the kitchen	Slovenia, Ljubljana	KJ494594	–
	EXF-5917		Ceiling in the bedroom	Slovenia, Kamnik	KJ494613	–
	EXF-5919		Ceiling in the children's bedroom	Slovenia, Kamnik	KJ494614	–
	EXF-5921		Milk chocolate	Slovenia, Trebnje	KJ494609	–
W. ichthyophaga	EXF-3555*		Salted ham	Slovenia, Ljubljana	KJ494655	KJ494579
	EXF-5676		Sea salt crystal	Slovenia, Sečovlje	KJ494654	KJ494581
	EXF-6065		Hypersaline water of solar salterns	Slovenia, Sečovlje	KJ494653	–
	EXF-6067		Hypersaline water of solar salterns	Slovenia, Sečovlje	KJ494651	–
	EXF-6068		Bittern	Slovenia, Sečovlje	KJ494650	–
	EXF-6070		Bittern	Slovenia, Sečovlje	KJ494652	–
	EXF-6200		Hypersaline water of solar salterns	Slovenia, Sečovlje	KJ494649	–
W. hederae	EXF-5748		Barley seeds	Slovenia, Šentjur	KJ494645	–
	EXF-5753[a]	CBS[f] 136982	Ivy (*Hedera helix*) flowers (pollen)	Slovenia, Krško	KJ494646	KJ494585
	EXF-7704		Oak honey (*Quercus* sp.)	Croatia, Kutjevo	KJ494647	KJ494580
	EXF-8357		Air in hay barn	Slovenia, Laško	KJ494644	–
	EXF-8358		Air in hay barn	Slovenia, Laško	KJ494648	–

Strains obtained during sampling

* strains obtained from EXF, MUCL and UAMH

[a] Ex-type strain

[b] CRGF, Collection of Fungal Genetic Resources, Cuba

[c] EXF, Culture Collection of Extremophilic Fungi, Ljubljana, Slovenia

[d] MUCL, Mycotheque de l'Universite Catholique de Louvain, Louvain la Neuve, Belgium

[e] UAMH, The University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada

[f] CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

considerably higher, and ranged from 200 to 1000 CFU/m^3. Indoor concentrations of *Wallemia* propagules ranged from 20 up to 10^6 CFU/m^3. While levels in the apartments were moderate (20–500 CFU/m^3), *Wallemia* propagules reached the highest levels (500–10^6 CFU/m^3) in the air of stables and hay barns, both in Slovenia and Denmark. The occurrence of *Wallemia* propagules in the air samples of stables and hay barns was also confirmed by light microscopy. Figure 1 shows straight or bent chains of spherical conidia (1.5–4.0 μm) that are characteristic of *Wallemia*. Outdoor levels of *Wallemia* propagules ranged from 10 air to 300 CFU/m^3 air.

From the air samples, 130 strains were isolated and pure cultured (Supplementary information, Table S1). The most commonly recovered species from the air in all of the natural, indoor and outdoor environments was *W. muriae* (114 strains; 87.7 %). Furthermore, *W. muriae* was the only *Wallemia* species that occurred in indoor air, and in air of meadows and solar salterns. Surprisingly, four strains of *W. sebi* (3.1 %) were isolated from air samples, with three originating in a forest, one outdoors and none indoors, none in apartments, stables or hay barns. Two strains (1.5 %) of the un-described *Wallemia* sp. were isolated from the air of hay barns in Slovenia, on MEA17. Both of these strains (EXF-8357, EXF-8358) were included in the phylogenetic analysis based on ITS. Ten strains (7.7 %) of *W. ichthyophaga*, which was previously isolated exclusively from hypersaline environments, were isolated in air of horse stables and hay barns in Denmark. Cerebriform colonies (Fig. 2) of this extremely halophilic species were detected on MEA17 after 14 days of incubation at 24 °C.

Machine learning

Values of CFU/m^3 and the percentage of *Wallemia* propagules in the air of natural, indoor and outdoor environments for each of the media used (MY50G, MY10-12, DG18 and MEA17) were analyzed for connections with descriptive variables such as habitat, type of environment, and weather conditions, using a machine learning. The machine learning analysis (Supplementary information, Figure S1) indicate that habitat is the most important variable that influences the CFU/m^3 scores and the *Wallemia* species composition in the air. More specifically, the analysis confirmed that *Wallemia* propagules are most frequently present in stables and barns. This is even more pronounced when the sampling was not performed in winter – the largest CFU/m^3 and percentage scores overall are obtained in spring. Large values for the CFU/m^3 scores and percentage are obtained also in the solar salterns (Supplementary information, Figure S1a).

The PCT given in (Supplementary information, Figure S1b) was constructed by removing the habitat and environment variables from the set of descriptive variables. The most important variable in this scenario is the presence of anticyclone: if there is an anticyclone (stable/good weather conditions) the values for the CFU/m^3 and percentage scores for all media are relatively low as compared to the samples taken in unstable weather conditions (cyclone). The highest values for the CFU/m^3 and percentage scores are encountered at unstable weather conditions, within the temperature range of 10 °C and 17 °C. In these specific conditions, the increase in humidity above 61 yields lower scores for three media (DG18, MY10-12 and MY50G) and higher scores for MEA17. At temperatures lower than 10 °C, and unstable weather conditions, the decisive factor seems to be the pressure (the two right most leaves from the PCT). Pressure value lower than 1015 increases the CFU/m^3 scores for the colonies grown in MY50G and MEA17 (for MY50G this comes at a price of decrease of the percentage score), while simultaneously it decreases the CFU/m^3 and percentage scores for samples grown in DG18 media. Contrary to this, at temperatures higher than 17 °C and humidity higher than 45, pressure values lower than 1015 increase the CFU/m^3 and percentage scores for the colonies grown in all four media. Finally, at temperatures higher than 17 °C and humidity lower than 45 the CFU/m^3 and percentage scores are relatively low for all media. Precipitation (clear, fog, rain, snow), direction and velocity of the wind are not important parameters for the presence of the *Wallemia* propagules in the air (they do not appear in the tree).

Phylogeny

The dataset of ITS and SSU rDNA was used for determination of the phylogenetic relationships, using maximum-likelihood, maximum-parsimony, and Bayesian phylogenetic analyses. The general time reversible (GTR) nucleotide substitution model was selected for both the ITS and the SSU rDNA partitions. *Geminibasidium donsium* was chosen as a root of the tree and Basidioascus as a possible sister taxon of *Wallemia* (Nguyen et al. 2013). The SSU rDNA sequences of *W. sebi* ranged from 1634 to 1639 bp. Those of *W. muriae*, *W. ichthyophaga* and *Wallemia* sp. were 1636, 1640, and 1642, respectively. The SSU rDNA alignment consisted of 1649 alignment positions that comprised 96 parsimony-informative characters, 110 parsimony-uninformative variable characters, and 1443 constant characters. Parsimony analyses identified a single tree, which had a consistency index of 0.965, a retention index of 0.970, a rescaled consistency index of 0.935, and a homoplasy index of 0.035. A General Time Reversible plus Gamma model and gamma distribution of rate variation (GTR+G) was selected for the SSU rDNA dataset. The gamma distribution shape parameter was α=0.021 across sites. In modeltest analyses, base frequencies were calculated as 0.28728, 0.17885, 0.24826, 0.28561 for A, C, G, T, respectively; substitution rates were AC=1.31271, AG=2.65245, AT=1.78348, CG=

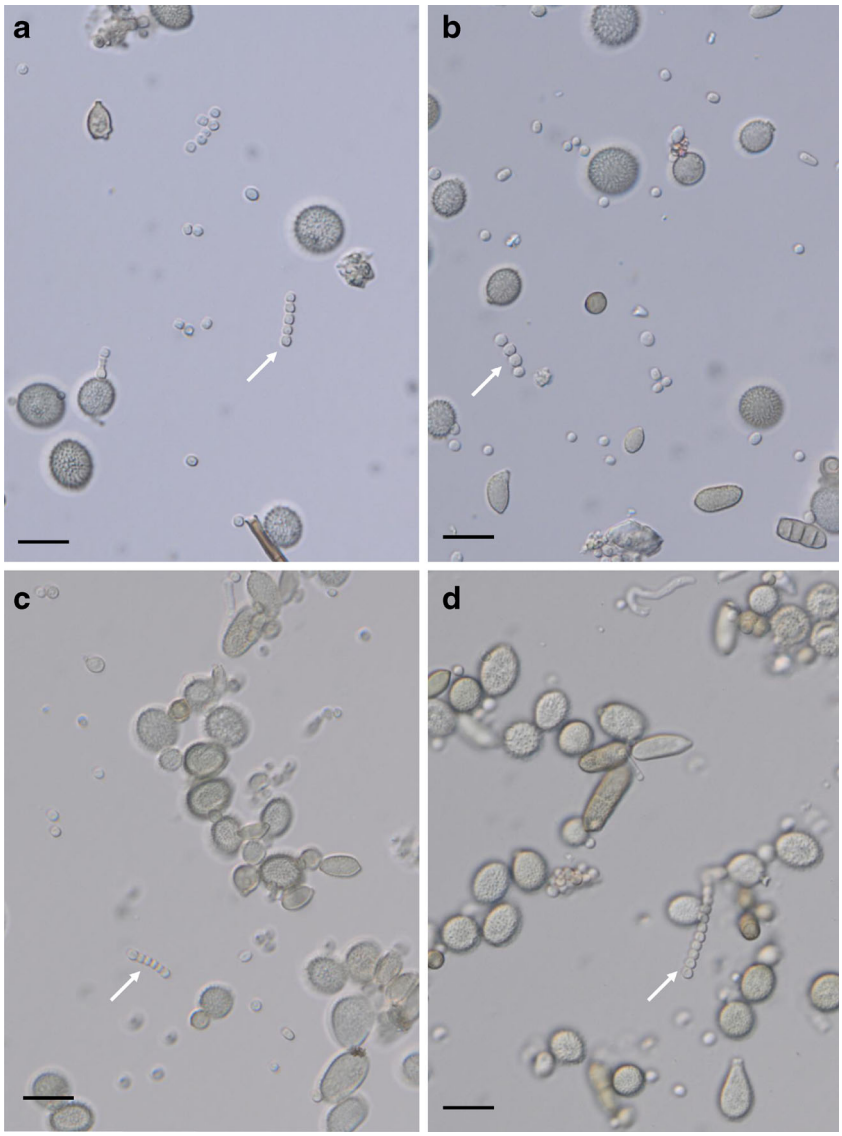

Fig. 1 Light microscopy of *Wallemia* spp. conidia and other fungal and non-fungal particles in air samples from hay barns in Denmark (**a** and **b**) and Slovenia (**c** and **d**). Spherical conidia arranged in unique chains (*white arrows*) indicate *Wallemia* spp. Scale bars: 10 μm (**a** to **d**)

1.00098, CT=4.94978, GT=1.00000. The most negative likelihood (−lnL) score was −3508.36003. Overall identical topologies were encountered in the maximum likelihood (Fig. 3), MrBayes consensus (not shown), and maximum-parsimony (not shown) phylograms. Nodes were considered as at least moderately supported when the maximum parsimony (MP-BPs) and maximum-likelihood bootstrap values (ML-BPs) were≥65 % and the Bayesian posterior probabilities (B-PP) were≥95 %. The Wallemiales received strong support (MP-BPs, 100; ML-BPs, 100; B-PP, 100). The *W. sebi*/*W. muriae* aggregate formed a strongly supported group (100/100/100). *Wallemia ichthyophaga* and *Wallemia* sp. formed a strongly supported monophyletic group in ML and MP analyses; however, this group received no support in Bayesian analyses (96/65/74). The species nodes for *W. ichthyophaga* and *Wallemia* sp. were equally strongly supported (100/100/100).

ITS sequences were analyzed phylogenetically for segregating infraspecific groups within the so far delineated species clades (Fig. 4). ITS sequences of *Wallemia* sp. differed strongly from those of the other described species. Those of *W. ichthyophaga* also differed strongly from the ITS of *W. sebi* and *W. muriae*. The sequences of *W. sebi* and *W. muriae* were well alignable. ITS sequences of *W. sebi* ranged from 492 to 498 bp. Those of *W. muriae*, *W. ichthyophaga* and *Wallemia* sp. ranged from 491 to 498, 563–571, and 502–503, respectively. The alignment consisted of 574 characters and comprised 172 parsimony-informative characters, three parsimony-uninformative variable characters, and 399 constant characters. Parsimony analyses identified a single tree, which had a consistency index of 0.916, a retention index of 0.989, a rescaled consistency index of 0.905, and a homoplasy index of 0.084. A General Time Reversible plus Gamma model (GTR) was selected for the ITS dataset. The gamma

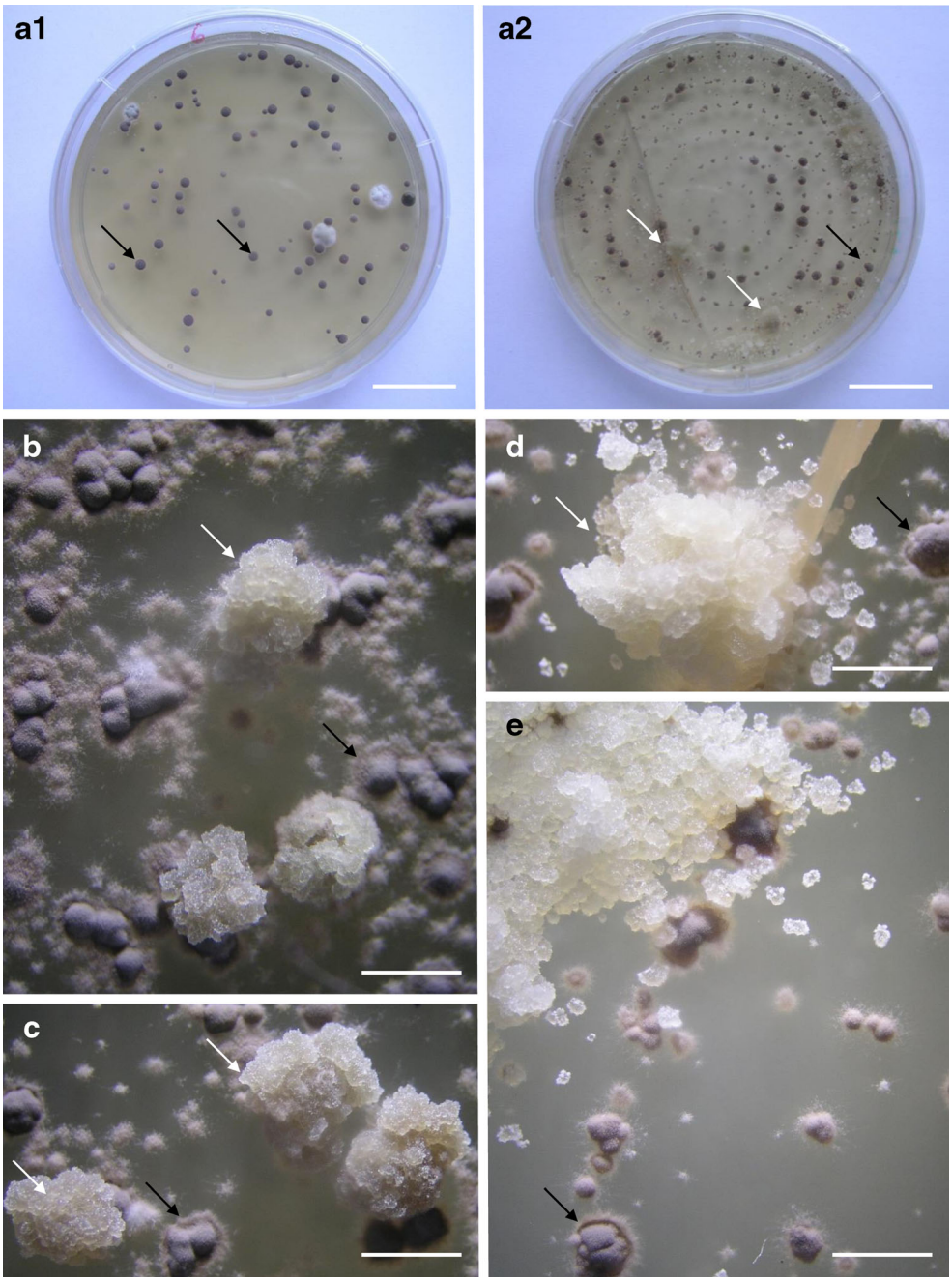

Fig. 2 Colonies of *Wallemia* spp. encountered on MEA17 NaCl plates used for air samples in Denmark. **a** Colonies of *Wallemia* spp. (*black arrows*) and of *W. ichthyophaga* (*white arrows*) from air in horse stable and hay barn. **b** to **e** *W. ichthyophaga* (*white arrows*) and other *Wallemia* spp. (*black arrows*) in air samples. Scale bars: 2 cm (**a**); 2 mm (**b** to **e**)

distribution shape parameter was $\alpha=0.352$ across sites. In modeltest analyses, base frequencies were calculated as 0.33041, 0.15777, 0.17562, 0.33620 for A, C, G, T, respectively; substitution rates were AC=1.59975, AG=4.38586, AT=4.31520, CG=0.50098, CT=7.78541, GT=1.00000. The most negative likelihood (−lnL) score was −1804.48311. Two independent Markov chain Monte Carlo analyses (Fig. 4a) and 1000 heuristic bootstrap replicates of the ITS yielded trees with highly concordant topologies, while the 1000 maximum-likelihood tree was different and is shown as an unrooted tree without support values in Fig. 4b. Nodes were considered as at least moderately supported when MP-BPs and ML-BPs were≥75 %, and Bayesian posterior probabilities≥95 % (≥75/≥75/≥95). The relatedness and monophyly of *Wallemia* sp. and *W. ichthyophaga* is strongly supported in MP and Bayesian analyses (ML-BP=100, B-PP=100), but was not evident in maximum-likelihood analysis. The ITS sequences of *Wallemia* sp. and *W. ichthyophaga* differed from those of the other *Wallemia* taxa, which formed a well-supported monophyletic clade. The phylogenetic structure of the *W. sebi*/*W. muriae* aggregate form a paraphyletic group, consisting of four subgroups, could not be resolved on the basis of ITS sequences. One of these was strongly supported (100/99/100) and represented *W. muriae* including the ex-type

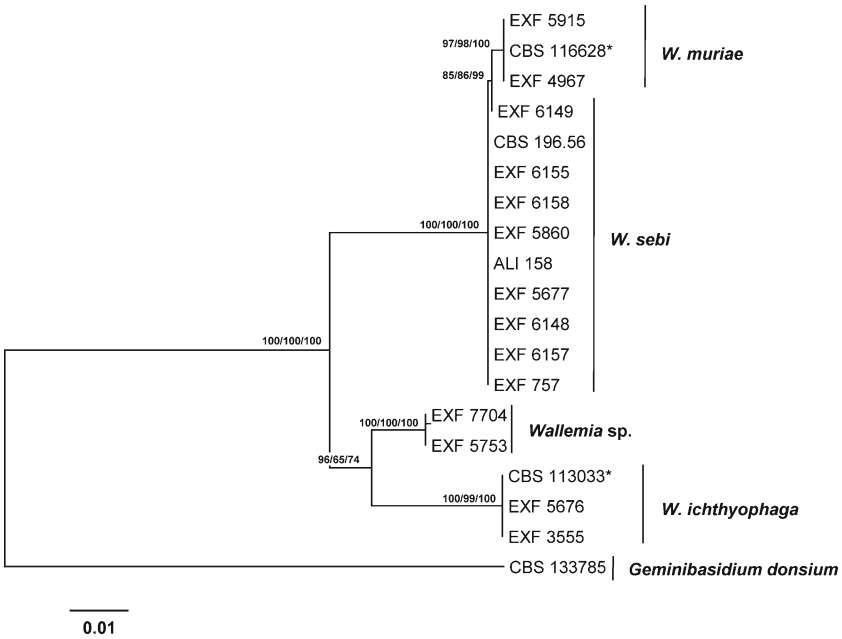

Fig. 3 Maximum-likelihood phylogram inferred from SSU rDNA sequences rooted with the sequence of *G. donsium*. The values given at the nodes of the tree represent bootstrap support values from maximum-parsimony analysis and maximum-likelihood analysis, and Bayesian posterior probabilities. The relatedness and monophyly of *Wallemia* sp. and *W. ichthyophaga* is strongly supported in maximum-parsimony analysis (bootstrap=96 %), moderately supported in the maximum-likelihood (bootstrap=65 %) but not supported in Bayesian analyses. The relatedness of *W. sebi* and *W. muriae* is highly supported. Stars (*) indicate ex-type strains

strains of *W. muriae* (CBS 116628). The other three subgroups accommodated strains morphologically and physiologicaly identified as *W. sebi. Wallemia sebi* subgroup 1 was strongly supported (92/93/100) containing the ex-neotype strain of *W. sebi* (CBS 818.96). *Wallemia sebi* subgroups 2 and 3 did not receive any support in this analysis. They may represent currently unresolved phylogenetic species of the *W. sebi*/*W. muriae* aggregate.

Physiology

Assimilation profiling for *W. sebi*, *W. muriae*, *W. ichthyophaga* and *Wallemia* sp. was performed under hypersaline conditions on 17 % NaCl and for *W. sebi* and *Wallemia* sp. as these can also grow on non-saline media. All four *Wallemia* species assimilated glucose, sucrose, fructose, raffinose, melibiose, galactose, maltose, trehalose, melezitose, cellobiose, L-sorbose, D-xylose, L-arabinose, *N*-acetyl-D-glucosamine, glycerol, erytritol, D-mannitol, D-sorbitol, myo-inositol, galactitol, D-gluconate and succinate as their sole source of carbon under hypersaline conditions. *Wallemia* sp. and *W. sebi* were also able to assimilate the same set of C sources under non-saline conditions. Weak growth occurred on ribitol and no growth on D-glucosamine, D-arabinose, L-rhamnose, D-ribose, lactose, lactate, soluble starch, citrate, methanol, ethanol, D-glucuronate and urea.

The tested taxa developed strongly on the N providing D-glucosamine and L-lysine for all species under hypersaline (all species) and non-saline conditions (*Wallemia* sp. and *W. sebi*). Assimilation of nitrate was weakly positive for *W. sebi* and *Wallemia* sp. under non-saline conditions, and for *W. sebi*, *Wallemia* sp. and *W. muriae* under hypersaline conditions. No growth of *Wallemia* spp. was observed when nitrite was provided as the sole nitrogen source, neither under hypersaline nor non-saline conditions.

Activities of extracellular hydrolytic enzymes of *W. sebi*, *W. muriae*, *W. ichthyophaga* and *Wallemia* sp. that we obtained with different methods are provided in Table 3. These data show that *W. sebi*, *W. muriae*, *W. ichthyophaga* and *Wallemia* sp. express β-glucosidase, esterase (C4), α-glucosidase, α-galactosidase, N-acetyl-β-glucosaminidase, acid phosphatase and alkaline phosphatase activities. No cellulolytic, amylolytic and proteolytic extracellular enzymatic activities were observed and the enzymes lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, β-manosidase and α-fucosidase were not detected. Esterase lipase (C8) activity was observed in *W. muriae*, *W. ichthyophaga* and *Wallemia* sp., but not in *W. sebi*. Finally, activities of α-chymotrypsin and β-galactosidase were detected only in *Wallemia* sp., which appears to be the most enzymatically active *Wallemia* species. The intensity of extracellular enzyme activities in all of these species was highest when 10 and 17 % NaCl. Only *W. sebi* and *Wallemia* sp. showed extracellular enzyme activities under non-saline conditions, while the most

Fig. 4 ITS phylogeny delimiting clearly the *W. sebi*/*W. muriae* aggregate from *Wallemia* sp. and *W. ichthyophaga*. **a** The phylogram inferred from the aligned sequences of the ITS rDNA using Bayesian analysis. The values given at the nodes of the tree represent bootstrap support values of maximum-parsimony and maximum-likelihood analyses followed by Bayesian posterior probabilities. The relatedness and monophyly of *Wallemia* sp. and *W. ichthyophaga* is highly supported in the maximum-parsimony and Bayesian (ML-BP=100) analyses, but not evident from maximum-likelihood analysis. Stars (*) indicate ex-type strains. **b** Unrooted radial tree based on the maximum-likelihood analysis

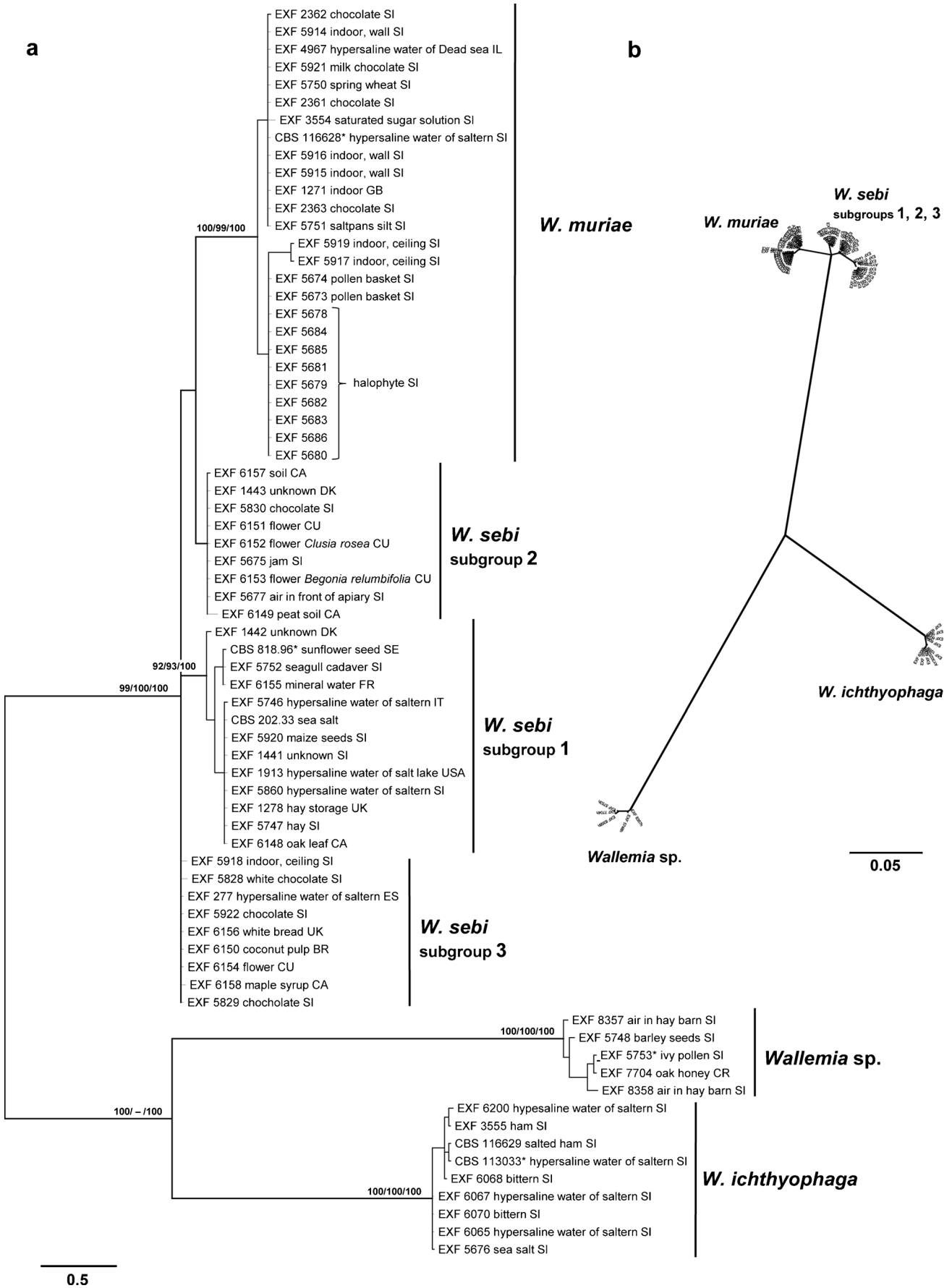
a
EXF 2362 chocolate SI
EXF 5914 indoor, wall SI
EXF 4967 hypersaline water of Dead sea IL
EXF 5921 milk chocolate SI
EXF 5750 spring wheat SI
EXF 2361 chocolate SI
EXF 3554 saturated sugar solution SI
CBS 116628* hypersaline water of saltern SI
EXF 5916 indoor, wall SI
EXF 5915 indoor, wall SI
EXF 1271 indoor GB
EXF 2363 chocolate SI
EXF 5751 saltpans silt SI
EXF 5919 indoor, ceiling SI
EXF 5917 indoor, ceiling SI
EXF 5674 pollen basket SI
EXF 5673 pollen basket SI
EXF 5678
EXF 5684
EXF 5685
EXF 5681
EXF 5679
EXF 5682
EXF 5683
EXF 5686
EXF 5680
halophyte SI
W. muriae
100/99/100
EXF 6157 soil CA
EXF 1443 unknown DK
EXF 5830 chocolate SI
EXF 6151 flower CU
EXF 6152 flower Clusia rosea CU
EXF 5675 jam SI
EXF 6153 flower Begonia relumbifolia CU
EXF 5677 air in front of apiary SI
EXF 6149 peat soil CA
W. sebi subgroup 2
EXF 1442 unknown DK
CBS 818.96* sunflower seed SE
EXF 5752 seagull cadaver SI
EXF 6155 mineral water FR
EXF 5746 hypersaline water of saltern IT
CBS 202.33 sea salt
EXF 5920 maize seeds SI
EXF 1441 unknown SI
EXF 1913 hypersaline water of salt lake USA
EXF 5860 hypersaline water of saltern SI
EXF 1278 hay storage UK
EXF 5747 hay SI
EXF 6148 oak leaf CA
W. sebi subgroup 1
92/93/100
99/100/100
EXF 5918 indoor, ceiling SI
EXF 5828 white chocolate SI
EXF 277 hypersaline water of saltern ES
EXF 5922 chocolate SI
EXF 6156 white bread UK
EXF 6150 coconut pulp BR
EXF 6154 flower CU
EXF 6158 maple syrup CA
EXF 5829 chocholate SI
W. sebi subgroup 3
EXF 8357 air in hay barn SI
EXF 5748 barley seeds SI
EXF 5753* ivy pollen SI
EXF 7704 oak honey CR
EXF 8358 air in hay barn SI
100/100/100
Wallemia sp.
EXF 6200 hypesaline water of saltern SI
EXF 3555 ham SI
CBS 116629 salted ham SI
CBS 113033* hypersaline water of saltern SI
EXF 6068 bittern SI
EXF 6067 hypersaline water of saltern SI
EXF 6070 bittern SI
EXF 6065 hypersaline water of saltern SI
EXF 5676 sea salt SI
100/100/100
W. ichthyophaga
100/ – /100
0.5
b
W. sebi subgroups 1, 2, 3
W. muriae
W. ichthyophaga
Wallemia sp.
0.05

Table 3 Activities of selected extracellular enzymes expressed by *Wallemia* spp. on media with and without NaCl

Enzyme activity	Presence (+) or absence (−) of enzymatic activity, according to species and NaCl concentration (%)															
	W. sebi				*W. muriae*				*W. ichthyophaga*				*Wallemia* sp.			
	0	10	17	25	0	10	17	25	0	10	17	25	0	10	17	25
β-Glucosidase[a]	−	+	+	−	−	+	+	−	−	+	+	+	−	+	+	−
Esterase[a]	+	+	+	−	−	+	+	−	−	+	+	+	+	+	+	−
Cellulolytic[a]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Amylolytic[a]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Proteolytic[a]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Alkaline phosphatase[b]	+	+	+	−	−	+	−	−	−	+	+	+	+	+	+	−
Esterase (C4)[b]	−	+	+	−	−	+	−	−	−	+	+	−	+	+	+	−
Esterase Lipase (C8)[b]	−	−	−	−	−	+	−	−	−	+	+	−	+	+	+	−
Lipase (C14)[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Leucine arylamidase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Valine arylamidase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Cystine arylamidase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Trypsin[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Chymotrypsin[b]	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−
Acid phosphatase[b]	+	+	+	−	−	+	+	−	−	+	+	+	−	+	+	−
Naphthol-AS-BI-phosphohydrolase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Galactosidase[b]	+	+	+	−	−	+	+	−	−	+	+	+	−	+	+	−
β-Galactosidase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
β-Glucuronidase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Glucosidase[b]	+	+	+	−	−	+	+	−	−	+	+	+	+	+	+	−
β-Glucosidase[b]	−	+	+	−	−	+	+	−	−	+	+	+	+	+	+	−
N-Acetyl-β-glucosamidase[b]	−	+	+	−	−	+	+	−	−	+	+	+	+	+	+	−
β-Manosidase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Fucosidase[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Screening of extracellular enzyme activities on agar media using specific substrates

[b] detection of extracellular enzymes using API ZYM® system (bioMèrieux)

halophilic *W. ichthyophaga* showed weak β-glucosidase, α-glucosidase, α-galactosidase, N-acetyl-β-glucosaminidase, esterase (C4), acid phosphatase and alkaline phosphatase activities even under extremely hypersaline conditions (25 % NaCl). Spectrophotometrical assessments in cell-free supernatants under different salinities (Supplementary information, Figure S2) confirmed that *W. sebi*, *W. muriae* and *W. ichthyophaga* had strongest β-glycosidase activity at 10 % NaCl. Surprisingly, *W. sebi* showed hardly any activity under non-saline conditions, while *Wallemia* sp. had strongest β-glucosidase activity of all tested species. *Wallemia* sp. and *W. ichthyophaga* showed high activity also under extremely hypersaline conditions (25 % NaCl).

The growth response of *W. sebi*, *W. muriae*, *W. ichthyophaga* and *Wallemia* sp. to various concentrations of $MgCl_2$ and $MgSO_4$ on solid or liquid malt extract media is summarized in Table 4. *Wallemia sebi*, *W. muriae* and *Wallemia* sp. grew on media with 0 to 3.5 M kosmotropic $MgSO_4$. *Wallemia ichthyophaga* showed growth on media with 1.5 M to 2.5 M $MgSO_4$. Abundant sporulation was observed at 2 M $MgSO_4$ for *W. ichthyophaga* (Fig. 5c). *W. sebi* and *Wallemia* sp. (*W. muriae*) tolerated 1.7 (1.5) M $MgCl_2$, while growth of *W. ichthyophaga* was observed also at 2.0 M $MgCl_2$, without the addition of a kosmotropic salt to compensate the chaotropic effect of the medium. At maximal $MgCl_2$ concentrations *W. ichthyophaga* formed thick-walled multicellular clumps (Fig. 5a to b). All *Wallemia* species grew well on MEA supplemented with a mixture of NaCl, $MgCl_2$ and $MgSO_4$, each at 1 M (Fig. 5d to e).

Discussion

The uniqueness of the genus *Wallemia* is reflected by its phylogenetic position (Zalar et al. 2005; Hibbett 2006; Matheny et al. 2007; Padamsee et al. 2012; Zajc et al. 2013), however,

Table 4 Tolerance of *Wallemia* species to different concentrations of $MgCl_2$ and $MgSO_4$ expressed as the number of strains compared to the total number of tested strains that show colony growth at specified conditions

Salt	Concentration (M)	Species			
		W.sebi	*W.muriae*	*W.ichthyophaga*	*Wallemia* sp.
$MgCl_2$	1.0	10/10	5/5	10/10	2/2
	1.2	9/10	5/5	10/10	2/2
	1.3	8/10	5/5	10/10	2/2
	1.5	7/10	2/5	10/10	2/2
	1.7	4/10	0/5	10/10	2/2
	1.9	0/10	0/5	10/10	0/2
	2.0	0/10	0/5	6/10	0/2
	2.1	0/10	0/5	0/10	0/2
	2.3	0/10	0/5	0/10	0/2
$MgSO_4$	1.0	10/10	5/5	0/10	2/2
	1.5	10/10	5/5	2/10	2/2
	2.0	10/10	5/5	9/10	2/2
	2.5	10/10	5/5	6/10	2/2
	3.0	10/10	5/5	0/10	2/2
	3.5	9/10	5/5	0/10	2/2
	4.0	0/10	0/5	0/10	0/2
$MgCl_2/MgSO_4/NaCl$	1.0 of each	10/10	5/5	10/10	2/2

also because of its extremophily. Representatives of the genus *Wallemia* can be defined as salt-tolerant halophiles, sugar-tolerant osmophiles, and xerophiles that tolerate low water activity in general (Wheeler et al. 1988; Gock et al. 2003; Zalar et al. 2005; Kralj Kunčič et al. 2010, 2013; Zajc et al. 2014). Xerotolerance, and even more so, xerophily, is relatively rarely observed in the fungal kingdom, specifically in Basidiomycota (de Hoog et al. 2005).

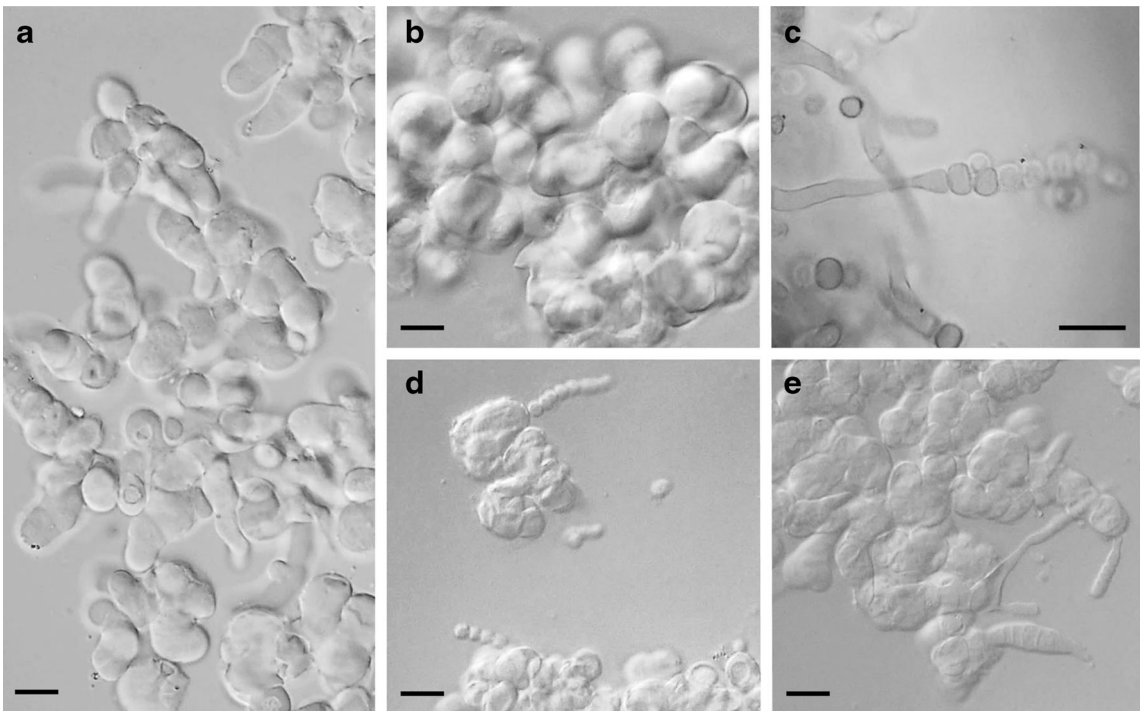

Fig. 5 Micromorphology of *W. ichthyophaga* in media with $MgCl_2$, $MgSO_4$ and NaCl. **a** and **b** Thick-walled cells forming meristematic clumps on MEA with 2 M $MgCl_2$ (**a** EXF-994; **b** EXF-5676). **c** Conidiogenous cell producing conidia on agar medium with 2 M $MgSO_4$ (EXF-994). **d** and **e** Meristimatic growth and conidiogenesis on agar medium with 1 M NaCl, 1 M $MgCl_2$ and 1 M $MgSO_4$. Scale bars: 10 μm (**a** to **e**)

Wallemia spp. is present in low-water-activity environments and substrates

The specific ecology of these three *Wallemia* species is not yet fully understood. The present study initially focused on the identification of primary ecological niches amongst different sampled low-water-activity environments and substrates. We mainly sampled different low-water-activity foods following Grant (2004), fodder such as hay and silage, natural hypersaline and desiccated environments such as solar salters, salt lakes and desserts, and partly complex samples such as soil, pollen and mushrooms. The majority of *Wallemia* strains were isolated from sugar-rich and dry foods, solar salterns, agricultural environments, and indoor surfaces, while none were retrieved from soil, salty foods, sampled hypersaline lakes and evaporite environments. Our phylogenetic and ecophysiological studies were based on 42 strains from low-water-activity environments, 26 strains obtained from different culture collections and 2 strains obtained from air sampling. Another 128 strains from the air in different natural (forests, meadows and solar salterns), indoor (residential interiors), agricultural farm (stables, hay barns), and outdoor (backyards) environments were not included in these analyses. According to ITS barcode analyses 87.7 % of air-borne strains were identified as *W. muriae* and only 12.3 % as *W. sebi*, *W. ichthyophaga* and *Wallemia* sp.

Habitat and weather conditions are the most important variables that influences the CFU/m^3 scores and the percentages of *Wallemia* propagules in the air

The machine-learning analysis used in our study indicates that the CFU measures collected from air volumes and the percentage of *Wallemia* spp. grown on the different media MY50G, MY10-12, DG18 and MEA17 are primarily influenced by the explored habitats. Stables and hay barns emerged as most important habitats of *Wallemia* spp. The high (up to 10^6 CFU/m^3) concentrations of *Wallemia* propagules observed in these environments were comparable to those reported by Zeng et al. (2004). Also, high values for the CFU/m^3 (up to 10^3) were obtained from the air of solar salterns, which may present another natural ecological niche of *Wallemia* spp. The lowest CFU/m^3 and percentage scores were obtained from samples taken in natural environments such as forests and meadows. Our findings from air samples analysis suggest that *Wallemia* spp. may have become adapted to man-made environments. To our surprise, stable/good weather conditions (anticyclone conditions) or unstable weather conditions (cyclone conditions) emerged as a key weather parameter that can influence the number of CFU/m^3 and percentage of *Wallemia* propagules in the air. High frequency of *Wallemia* propagules in the air matches with their global presence in the soil. Wallemiomycetes seem to be present with>1 % (Tedersoo et al. 2014), which seems incredibly much for just 4 species.

Strains morphologically identified as *W. sebi* have highly varible ITS sequences

High proportion of strains that originated from low-water-activity environments and substrates were identified as the xerotolerant *W. sebi*. As *W. sebi* can grow only slowly on media without water-activity-lowering solutes, *W. sebi* generally grows better on media with non-ionic solutes such as glucose and glycerol (a_w below 0.850). *Wallemia sebi* can tolerate up to 4.3 M NaCl (Wheeler et al. 1988; Zalar et al. 2005; Kralj Kunčič et al. 2010, 2013). The ITS sequences of *W. sebi* strains included in the present study show high molecular variability suggesting that *W. sebi* presents a species complex (see also Zalar et al. 2005). Only the ex-neotype subgroup of *W. sebi* ex-neotype including 11 environmental strains and the clinical isolate CBS 196.56 from a human chronic ulcerative skin lesion (Zalar et al. 2005) received considerable support in ITS based phylogenetic analyses. It is clear that sequences of other gene loci need to be generated and analyzed to resolve the *W. sebi* species complex that also included *W. muriae* in our analyses. The ITS sequence of the clinical isolate CBS 196.56 was identical to the ITS sequences of two new strains isolated Zalar et al. (2005), the first from a seagull cadaver and the second from flavoured mineral water. Other strains in this clade mostly originated from environments that had reduced water activities, such as hypersaline water, dried plant materials (corn seed, hay and oak leaf) and from indoor environments. Strains from the two other statistically unsupported subclades were isolated from similar habits.

Wallemia muriae is the most commonly recovered *Wallemia* spp. in the air of sampled sites (central Europe)

Wallemia muriae is an obligate xerophile that, like *W. sebi*, grows better on media with lowered water activity due to non-ionic solutes (Zalar et al. 2005; Kralj Kunčič et al. 2010, 2013). Previously, the strains of *W. muriae* were obtained mainly from sugar-rich, dry and salty food, hypersaline water of salterns and from human influenced interiors (Zalar et al. 2005). In addition, we isolated *W. muriae* also from chocolate, pollen baskets, and surfaces of plants including halophytes and others growing in salterns. Plant associated strains from pollen and halophytes formed an unsupported subclade, while the other *W. muriae* strains that were mainly isolated from low-water-activity foods, solar salterns and indoors, grouped into the other clade. Air sampling suggested that *W. muriae* could be the most abundant *Wallemia* species in residential buildings (100 %), stables and hay barns (59 %), and outdoors (96 %) in central Europe. Previously, only *W. sebi* was reported from air, and it was considered as the causative agent of

respiratory allergies (Reboux et al. 2001; Hanhela et al. 1995; Lappalainen et al. 1998). Only *W. sebi* has so far been identified as an agent of respiratory diseases including the so called farmer's lung disease and other allergological problems. Its high prevalence in human influenced environments suggests that *W. muriae* should be considered as a causal agent of such respiratory problems as well.

Wallemia ichthyophaga is well-adapted to substrates with a reduced water activity

Wallemia ichthyophaga has been characterized as an obligate halophile because it requires at least 10 % NaCl for in-vitro growth although it can grow at least slowly on media with high concentrations of sugars (Zalar et al. 2005; Kralj Kunčič et al. 2010, 2013; Zajc et al. 2014). As *W. ichthyophaga* is an extremophilic specialist (Gostinčar et al. 2010; Zajc et al. 2013), it has been selected as a model organism for the study of adaptations to hypersaline conditions in eukaryotes. By using hypersaline medium (MEA17) and a prolonged incubation time of 14 days we could isolate 10 additional strains of this species that earlier was represented by three strains only (Zalar et al. 2005). Most were obtained from the air in horse stables and hay barns in Denmark. The sampled hay had been salted to prevent moulding and heating (Roussel et al. 2005) and it is possible that this special agricultural practice presented the selective factor for *W. ichthyophaga*. Six other strains of this species were obtained from salted meat, hypersaline water, sea salt crystals, and, surprisingly, from bitterns. The bitterns accumulate high concentrations of $MgCl_2$ and had been considered extremely hostile for microbial growth (Javor 1984). However, our subsequent physiological tests indeed proved that *W. ichthyophaga* can well grow on $MgCl_2$-rich substrates. Habitats with high salt concentrations including NaCl and $MgCl_2$, as encountered in solar salterns, may present the natural niche for *W. ichthyophaga*.

Chaotolerance in *Wallemia*: *W. ichthyophaga* grows even at 2 M $MgCl_2$

In their natural habitats such as the hypersaline water of solar salterns, *Wallemia* might be subjected to low-water-activity conditions as well as high concentrations of chaotropic ($MgCl_2$) and kosmotropic (NaCl, $MgSO_4$) stressors. Chaotropes, like high temperatures, promote disorder of cellular structures, while kosmotropes and low temperatures have strong stabilising effects (Williams and Hallsworth 2009). Concentrations above 1.26 M $MgCl_2$ were previously thought to totally inhibit microbial growth (Hallsworth et al. 2007). To our surprise, all of the *Wallemia* species can grow very well at concentrations of around 1 M $MgCl_2$. *Wallemia sebi* can grow in up to 1.7, *W. muriae* in up to 1.5, *Wallemia* sp. in up to 1.7, and *W. ichthyophaga* in up to 2 M $MgCl_2$, even in the absence of other compensating solutes. These are the highest concentrations tolerated by any microorganism. In the absence of compensating solutes such as NaCl or $MgSO_4$, the highest levels of $MgCl_2$ that can still support life, might be about 2.3 M (Hallsworth et al. 2007). To thrive under such hostile conditions, *W. ichthyophaga* forms unique meristematic clumps with thick-walled cells (Kralj Kunčič et al. 2010). By means of contrast, *W. ichthyophaga* grows in a comparably narrow range of concentrations of the kosmotropic $MgSO_4$ (1.5–2.5 M), while the other *Wallemia* species grow in a wider range (0–3.5 M $MgSO_4$). Accordingly, the genus *Wallemia* and its species present a suitable model taxon for the study adaptations to extremely dry and hypersaline conditions. In addition, it may serve also for the study of adaptations to chaotropic conditions. This recently proposed ecophysiological grouping (Williams and Hallsworth 2009) is described as chaotolerant and chaophilic fungi.

Wallemia spp. show some extracellular enzyme activities under hypersaline conditions

Little is known about the extracellular enzymes produced by fungi under hypersaline conditions. Our results have shown that the activities of the extracellular enzymes from *Wallemia* spp. are dramatically affected by the salinity. All species showed highest extracellular enzymatic activities when 10–17 % NaCl was added to the medium. *Wallemia sebi* and *Wallemia* sp. are the only two species that do not have an obligate halophilic nature. They showed at least weak extracellular enzymatic activities when grown on media without added NaCl. On the other hand, the halophilic *W. ichthyophaga* showed at least weak extracellular enzymatic activities even at 25 % NaCl. While we have studied the activity of selected enzymes, it is clear that *Wallemia* species may express unique enzymes at osmotic stresses with unknown functions, not described yet. Accordingly, the genus *Wallemia* presents an interesting target for the exploitation of unique enzymes useful for biotechnological processes including the biological treatment of highly saline wastewater in fish processing or of the petroleum and leather industries (Lefebvre and Moletta 2006).

SSU rDNA phylogeny reveals that halophilic *W. ichthyophaga* is the sister taxon of a hitherto un-described *Wallemia* species

The SSU rDNA phylogenetic reconstruction reflects the accepted taxonomic position of the genus *Wallemia* within the class Wallemiomycetes (Zalar et al. 2005). Recently, the xerotolerant and heat-resistant genera *Basidioascus* and *Geminibasidium* were included, both of which belong to the order Geminibasidiales (Nguyen et al. 2013). Description of the class was further emended by genome-scale phylogenetic

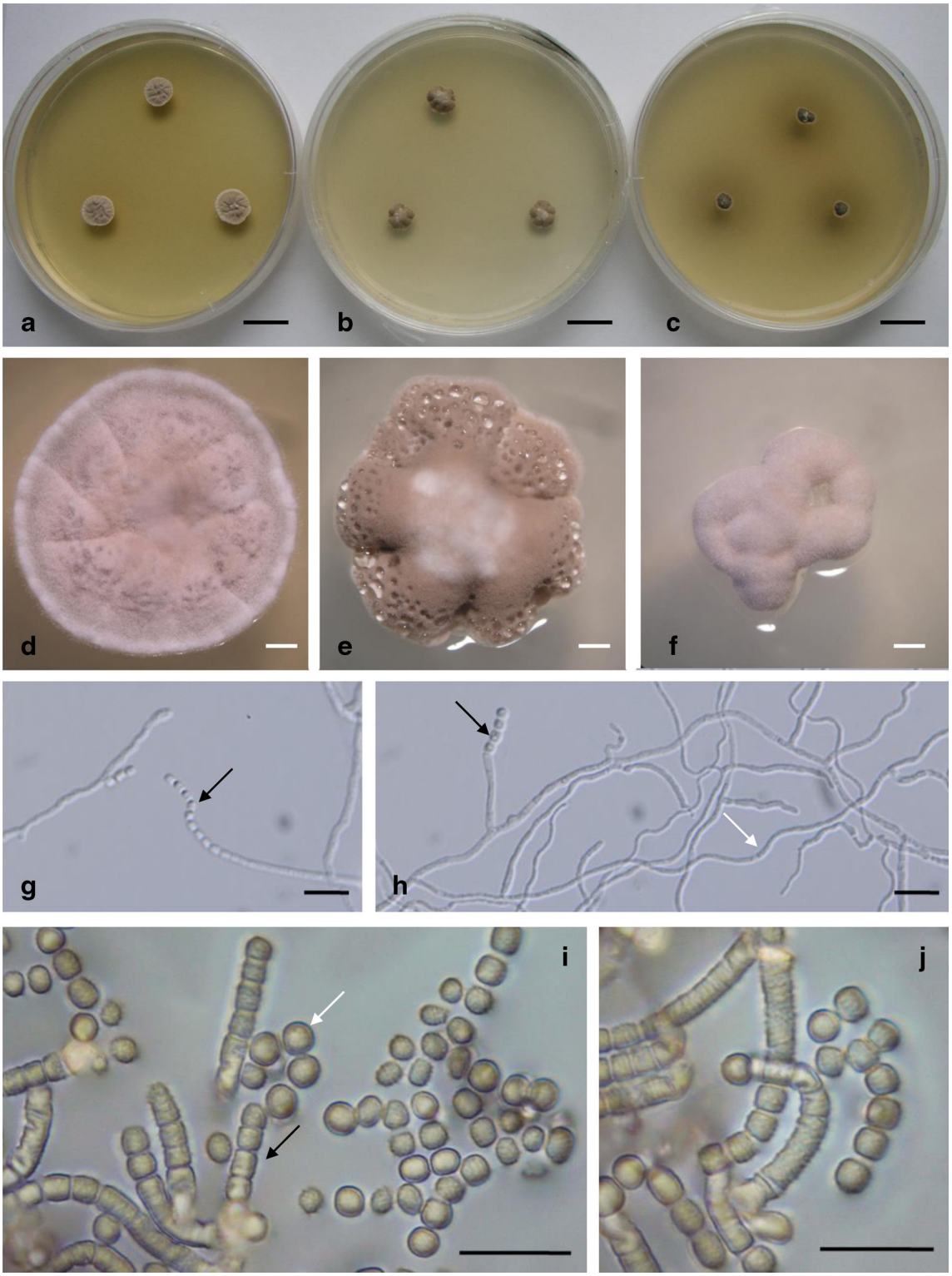

Fig. 6 *Wallemia hederae* colonies, conidiogenous structures and conidia. **a** to **c** Colonies on MY50G (**a**), MY10-12 (**b**) and MEA17 (**c**). **d** to **f** Powdery colony on MY50G (**d**), on MY10-12 (**e**) and on MEA without exudate droplets (**f**). **g** Conidiophore and conidiogenous cell producing conidia in a chain (*black arrow*). **h** Hyphae (*white arrow*), a conidiophore with an assemblage of 4 conidia (*black arrow*). **i** and **j** Short cylindrical (*black arrow*) and spherical one-celled conidia (white arrow) and conidia chains. Scale bars: 1 cm (**a** to **c**); 1 mm (**d** to **f**); 10 μm (**g** to **j**)

analyses of *W. sebi* and *W. ichthyophaga*. These analyses suggest that the Wallemiomycetes represent the earliest diverging lineage of the Agaricomycotina (Padamsee et al. 2012; Zajc et al. 2013).

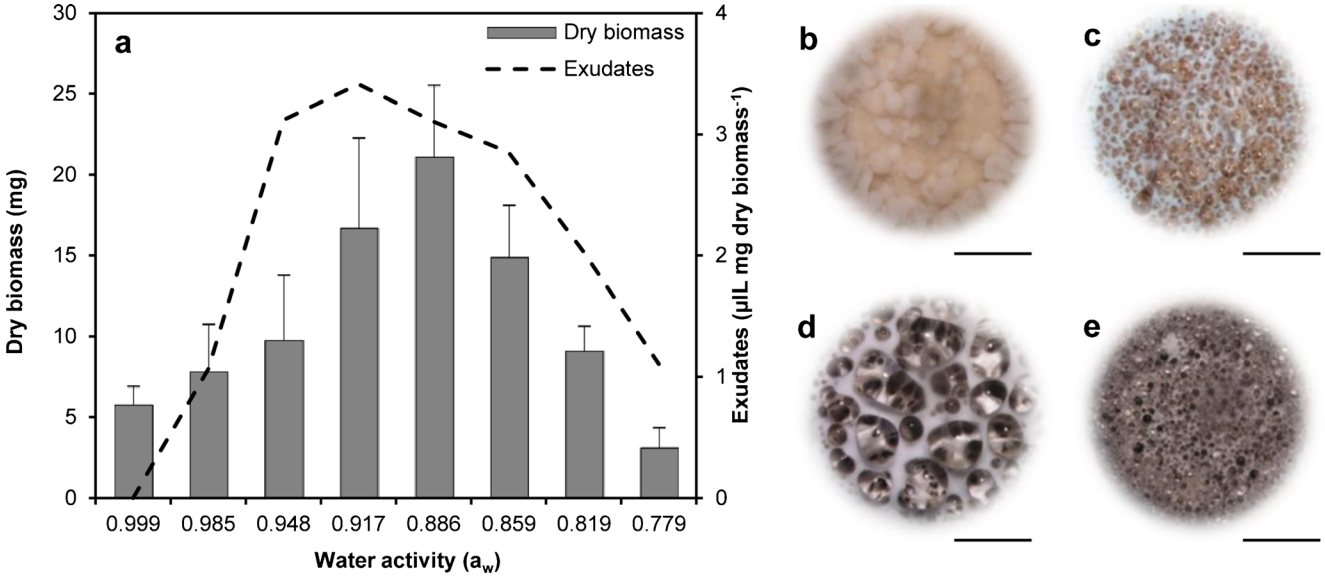

Fig. 7 *Wallemia hederae*. **a** Production of dry biomass in mg on media with different water activities due to various amounts of NaCl in means+ standard deviation and amount of exudate production in µl per mg dry biomass, dashed curve. **b** Colony on MEA without exudate production and colonies on MEA with 8 % (**c**), 16 % (**d**) and 24 % NaCl (**e**) showing exudate production. Scale bar: 0.5 cm (**b** to **e**)

Large molecular distances in the SSU rDNA (and also the ITS) distinguish *W. ichthyophaga* from the members of the *W. sebi*/*W. muriae* aggregate. Zalar et al. 2005 speculated that the clear gap between these two taxa is due to currently unknown or extinct *Wallemia* species. The here described *Wallemia* sp. confirmed this hypothesis. However, its rDNA sequences differ strongly from both *W. ichthyophaga* and the *W. sebi*/*W. muriae* aggregate although it forms a strongly supported monophyletic group with *W. ichthyophaga* when conserved SSU rDNA sequences are analyzed. The new species carries morphological features that clearly suggest *Wallemia*. Arthrospore-like conidia are formed in units of four and are produced from conidiophores only known from *Wallemia* species through basauxic conidiogenesis (Madelin and Dorabjee 1974; Zalar et al. 2005; Padamsee et al. 2012). Conidia of the new species range from 2.5 to 3.5 µm and differ from conidia of *W. sebi* (1.5–2.5 µm) and *W. ichthyophaga* (3.5–5.0 µm) (Zalar et al. 2005).

The production of guttation droplets emerged in this study as an interesting feature for the species from the genus *Wallemia*. Information on the function of such droplets in fungi and their ecological role is scarce. They might function as a water reservoir to enable constant growth of the hyphae (Jennings 1991), as reserves of metabolic by-products, enzymes and secondary metabolites including mycotoxins (Hutwimmer et al. 2009; Gareis and Gareis 2007). In *Wallemia*, guttation droplets may also present yet-undescribed morphophysiological reactions to osmotic stress and could be artefacts of the applied in-vitro conditions. Preliminary analyses of *Wallemia* sp. exudates when grown under hypersaline conditions have revealed the presence of some still unknown secondary metabolites, as well as hydrolytic enzymes with various activities.

On the basis of phylogenetic inferences and comparative analyses of morphological and physiological characters a new species of *Wallemia* described below as *W. hederae* was encountered in the context of our ecological studies.

Taxonomy

Wallemia hederae Jancic, Zalar & Gunde-Cimerman, sp. nov.
MycoBank MB806046
Fig. 6

Etymology: After the host/substrate of the ex-type: common ivy (*Hedera helix*).

Cultural characteristics: Colonies on MEA cerebriform, compact, 3.5–4.5 mm diam. after 14 days at 24 °C, spreading deeply into the agar, pale brown, without sporulation and exudates, margin irregular, reverse grey. On MEA17 punctiform, compact, 3.5–5.0 mm diam., spreading deeply into the agar, pale brown to white, without sporulation, with numerous exudates visible as dark droplets that cover most parts of the colony surface, margin irregular, without exudate droplets, reverse dark grey. On MY10-12 strongly punctiform or powdery, compact, 7.0–8.5 mm diam., spreading deeply into the agar, walnut brown, weak sporulation exudates present as clear and transparent droplets only on the margin, margin irregular, reverse dark grey. On MY50G, colonies weakly punctiform to powdery due to strong sporulation, compact, 9.5–12.0 mm, walnut to pale brown, exudates randomly

distributed as yellowish droplets, margin regular, white or of same colour as the colony, reverse dark brown.

Microscopy: Hyphae hyaline, smooth, thin-walled, 1.5–2.5 μm wide, forming a compact mycelium. Conidiophores erect, densely packed in parallel orientation, subhyaline, unbranched or sometimes sympodially elongating, smooth-walled, slightly constricted near the tip and below a darker integument when maturing. Conidiogenous cells cylindrical, verruculose, basauxically extending, basipetally disarticulated into packages of four arthrospore-like conidia. Conidia one-celled, pale brown, initially short cylindrical, becoming spherical, verrucose, thick-walled, 2.5–3.5 μm diam.

Physiology: Growth independent on type of solute, also on media without additional solutes at a_w=0.999, growth optimum at a_w=0.917–0.859 or at (12–)16(−20) % NaCl, maximum at a_w=0.779 or at 28 % NaCl, extremely halotolerant (Fig. 7), growing also on media with 0–1.7 M $MgCl_2$, 0–3.5 M $MgSO_4$ or with a mixture of 1 M NaCl, 1 M $MgCl_2$ and 1 M $MgSO_4$.

Habitats and distribution: Common ivy flowers (pollen), oak honey, barley seeds, hay (Europe: Slovenia, Croatia).

Typification: Holotype of *Wallemia hederae* designated here: **Slovenia**, Krško, from pollen of *Hedera helix* (common ivy), collected by J. Božič, isolated by S. Jančič, Nov. 2009 (CBS H-21963, freeze dried living deposit consisting of mycelium from MEA plus 16 % NaCl); culture **ex-type** preserved as CBS 136982 and EXF-5753.

Additional strains examined: EXF-5748, EXF-7704, EXF-8357, EXF-8358.

Diagnostic characters: halotolerance (growth on MEA with addition of 0–28 % NaCl); conidial size range (2.5–3.5 μm in diam.), production of exudates on MEA17.

Notes: *Wallemia hederae* differs from the other described *Wallemia* species in its prolific production of a large amount of exudate droplets (guttation) (Fig. 7), especially on media supplemented with high concentrations of NaCl. Guttation is known to frequently depend on the composition of the cultivation media (Hutwimmer et al. 2009), however, NaCl has not yet been reported to trigger exudate formation. Colonies of *W. sebi* and *W. muriae* produce exudates randomly and can occasionally produce yellow guttation droplets (Zalar et al. 2005), but typically these species do not produce exudates on hypersaline media. Guttation on MEA17 NaCl is therefore a diagnostic character for *W. hederae*. Osmophilic traits place *W. hederae* in an intermediate position between *W. sebi* and *W. ichthyophaga*. Although it is clearly halophilic as *W. ichthyophaga*, it can grow, albeit slowly, as *W. sebi* on media without added solutes (Zalar et al. 2005; Kralj Kunčič et al. 2010, 2013). Its optimal range of water activity range from 0.917 to 0.859 (12–20 % NaCl), which has so far been observed only in the obligate halophilic species *W. ichthyophaga*.

Dichotomous key to currently recognised Wallemia species (see also Zalar et al. 2005):

Macromorphological characters used in the key are from colonies grown on MEA, MEA17 and MY50G incubated at 24 °C for 14 days. Micro-morphological characters including conidia are from slide cultures using medium MY50G incubated at 24 °C for 14 days.

1A Colony growth observed only on media with additional solutes (NaCl, glucose) at 24 °C after 14 days; conidia 2.5–5.0 μm in diam. … 2

1B Colony growth on media such as MEA without additional solutes at 24 °C; conidia 1.5–3.5 μm in diam. … 3

2A Colonies on MY50G dark brown at 24 °C after 14 days, with a cerebriform surface; conidia 3.5–5.0 μm in diam. … *W. ichthyophaga*

2B Colonies on MY50G walnut brown at 24 °C after 14 days, with a powdery surface; conidia 2.5–3.0 μm in diam. … *W. muriae*

3A Conidia 1.5–2.5 μm in diam.; exudate droplets absent on MEA17 at 24 °C after 14 days … *W. sebi*

3B Conidia 2.5–3.4 μm in diam.; large amount of exudates on MEA17 at 24 °C after 14 days … *W. hederae*

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