UNIVERZA V LJUBLJANI
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FIZIOLOŠKE IN MOLEKULARNO-BIOLOŠKE PRILAGODITVE HALOFILNE GLIVE *Wallemia ichthyophaga* NA ŽIVLJENJE V OKOLJU Z VISOKIMI KONCENTRACIJAMI SOLI

DOKTORSKA DISERTACIJA

PHYSIOLOGY AND MOLECULAR BIOLOGY OF ADAPTATIONS TO LIFE IN HYPERSALINE ENVIRONMENTS OF THE HALOPHILIC FUNGUS *Wallemia ichthyophaga*

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Janja Zajc

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AI Bazidiomicetna gliva *Wallemia ichthyophaga* (rod Wallemia) je obligaten halofil, saj raste med 10 % in 32 % NaCl. V raziskavi smo določili, da je optimalno območje slanosti za rast 15-20 % NaCl, kar je najvišji optimum slanosti kadarkoli opisan med glivami. Ima izrazito preferenco po NaCl v primerjavi z glukozo za znižanje vodne aktivnosti gojišča. V svojih celicah kopiči organske topljence, kakršna sta poliola glicerol in arabitol. Glicerol je glavni osmotsko uravnavan topljenec, saj se njegova vsebnost poveča z naraščajočo koncentracijo NaCl ter se zmanjša po hipoosmotskem šoku. Skladno s strategijo kompatibilnih topljencev *W. ichthyophaga* ohranja relativno nizke vsebnosti kalija in natrija pri pogojih stalne koncentracije NaCl, pri hiperosmotskem šoku pa se vsebnosti obeh kationov znatno povečajo. Razmerje K^+/Na^+ v celicah nekoliko pada s slanostjo, tako zaradi večanja vsebnosti Na^+ kot tudi zmanjševanja vsebnosti K^+. Za razliko od ostalih gliv je rast *W. ichthyophaga* najboljša, ko je vsebnost Na^+ višja od K^+. Celična stena *W. ichtyhophaga* je v gojišču z glukozo tanjša kot v gojišču z NaCl, odebelitev pri višji koncentraciji glukoze pa manj izrazita kot v gojišču z NaCl. Glede na debelino celične stene, rastne parametre ter deleže celične stene v biomasi *W. ichthyophaga* preko celotnega slanostnega območja rasti trdimo, da je ojačenje celične stene povezano z uspešno rastjo *W. ichthyophaga* pri visokih slanostih. Majhen (9,6 Mb) in kompakten genom *W. ichthyophaga* ima predvidenih le 4884 protein-kodirajočih genov, ki pokrivajo skoraj tri četrtine celotnega zaporedja. Od 639 diferencialno izraženih genov, sta dve tretjini bolj izraženi pri nižji slanosti. Vrsta *W. ichthyophaga* je najverjetneje izgubila sposobnost spolnega razmnoževanja, saj nima določenega lokusa *MAT* in ima le majhen delež za mejozo specifičnih genov. Več proteinskih družin je značilno razširjenih ali skrčenih v genomu *W. ichthyophaga*. Med razširjenimi družinami so ATPazni kationski prenašalci tipa P in družina proteinov celične stene - hidrofobini. *W. ichthyophaga* je ekstremofilni specialist, ki kaže le nizko raven prilagodljivosti in genetske rekombinacije. To se odraža tako v značilnostih genoma, kot tudi v transkriptomskem odzivu na NaCl. Različne meritve in podatki iz genoma in transkriptoma kažejo na pomembno vlogo celične stene *W. ichthyophaga* pri njeni sposobnosti uspevanja pri tako visokih koncentracijah soli, ki so letalne za večino ostalih evkariontov.

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AB Basidiomycetous fungus *Wallemia ichthyophaga* (genus Wallemia) is obligate halophile as it grows from 10 % NaCl to 32 % (w/v) NaCl. We have determined its growth optimum to be between 15 and 20 % NaCl, which is the highest salinity optimum ever described among fungi. It prefers NaCl over glucose to reduce water activity of the growth medium. It accumulates compatible solutes, mainly polyols – glycerol and arabitol. Glycerol is the major osmotically regulated solute as its accumulation increases with salinity and is diminished after the hypoosmotic shock. In accordance with this strategy, *W. ichthyophaga* maintains relatively low intracellular amounts of potassium and sodium at constant concentrations of NaCl, but during the hyperosmotic shock, the amounts of both cations increased significantly. Intracellular K^+/Na^+ ratio slightly decreases with increasing concentration of NaCl due to increase in Na^+ content and decrease in K^+ content. In contrast to other fungi, the growth performance of *W. ichthyophaga* is the best, when Na^+ content exceeded K^+. The cell wall of *W. ichtyhophaga* is significantly thinner in media with glucose compared to media with NaCl, also its thickening at high glucose is less pronounced than at high NaCl. Considering the cell wall thickness, the content of cell wall in dry biomass and growth parameters across the salinity range, we claim that the reinforcement of the cell wall is linked to the successful growth of *W. ichthyophaga* at extremely high salinities. Its small (9,6 Mb) and compact genome has only 4884 protein-coding genes that cover almost three quartes of the sequence. Two thirds of 639 differentially expressed genes are more expressed at lower salinity. Several protein families are significantly expanded or contracted in the genome. Among the expanded families are the P-type ATPase cation transporters and cell wall proteins - hydrophobins. This species most likely lost its ability of sexual reproduction as no discernible *MAT* locus was found and only few meiosis specific genes were present. *W. ichthyophaga* is an extremophilic specialist for which only low levels of adaptability and genetic recombination are characteristic. This is apparent from its genome configuration and its transcriptomic response to salt. Different measurements and data from genome and transcriptome show the important role of the cell wall in its ability to thrive in conditions lethal to most other eukaryotes.

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KAZALO PRILOG

PRILOGA A: Slika S1: Klasifikacija predvidenih proteinov po kategoriji baze KEGG; Slika S2: Klasifikacija predvidenih proteinov v gruče ortolognih skupin (baza COG); Slika S3: Razporeditev pokritosti transkriptoma; Slika S4: Število različnih dogodkov alternativnega spajanja pri obeh slanostih.

PRILOGA B: Seznam proteinov *Wallemia ichthyophaga*, ki imajo 10 ali več predstavnikov določene domene Pfam.

PRILOGA C: Evolucija proteinskih družin. Rezultati analize po metodi CAFE. Za vsako družino je prikazana vrednost P njene obogatitve ali krčenja za celotno drevo, kot tudi za razvejitev, ki vodijo k *W. ichthyophaga*, *W. sebi*, in k *Wallemia* spp., ter Agaricomycotina. Predstavljene so zgolj družine z vrednostjo P manjšo od 0,01.

PRILOGA D: Dvanajst poti po KEGG bazi s signifikantno višjim deležem diferenčno izraženih genov kot pričakovano v primerjavi transkriptomov pri 10 % in 30 % NaCl (m/v).

PRILOGA E: Zbrana dovoljenja založnikov za objavo člankov v tiskani in elektronski verziji doktorske disertacije.

OKRAJŠAVE IN SIMBOLI

µl	mikroliter (10^{-6} litra)
µm, nm	mikrometer (10^{-6} metra), nanometer (10^{-9} metra)
AAS	**a**tomska **a**bsorpcijska **s**pektroskopija
AS	alternativno spajanje (angleško **a**lternative **s**plicing)
ATP	adenozin trifosfat
a_w	vodna aktivnost (angleško **w**ater **a**ctivity)
BGI	Pekinški genomski inštitut (**B**eijing **G**enomics **I**nstitute)
BLASTp	program za primerjavo (angleško **b**asic **l**ocal **a**lignment **s**earch **t**ool) proteinskega zaporedja s podatkovno bazo vseh zaporedij
CAFE	statistična bioinformatska analiza evolucije genskih družin (angleško **C**omputational **A**nalysis of gene **F**amily **E**volution)
cDNA	komplementarna DNA
DDBJ	Japonska podatkovna banka DNA (**D**NA **D**ata **B**ank of **J**apan)
EMBL	Evropski laboratorij za molekularno biologijo (**E**uropean **M**olecular **B**iology **L**aboratory)
DNA	deoksiribonukleinska kislina (angleško **d**eoxyribonucleic **a**cid)
GC	**g**vanin-**c**itozin
GPD1	gen za glicerol-3-fosfat dehidrogenazo (angleško **g**lycerol-3-**p**hosphate **d**ehydrogenase)
HD	**h**omeo**d**omena
HPLC	tekočinska kromatografija visoke ločljivosti (angleško **H**igh **P**erformance **L**iquid **C**hromatography)
JGI	oddelek za energijo Združenega genomskega inštituta (Department of Energy **J**oint **G**enome **I**nstitute)
KEGG	Kjotska enciklopedija genov in genomov (**K**yoto **E**ncyclopedia of **G**enes and **G**enomes)
M	molarna koncentracija (mol/l)
m/V	razmerje med maso in prostornino
$MgCl_2$	magnezijev diklorid
NaCl	natrijev klorid

nM	nanomolaren (10^{-9} mol/l)
NMR	**n**uklearna **m**agnetna **r**esonanca
PCR	verižna reakcija s polimerazo (angleško **P**olymerase **C**hain **R**eaction)
Pfam	podatkovna baza proteinskih družin (angleško **P**rotein **fam**ily) inštituta Sanger
RPKM	število odčitkov na kilobazni par na milijon (angleško **r**eads **p**er **k**ilo base per **m**illion)
SSH	**s**upresijska **s**ubtrakcijska **h**ibridizacija
YNB	gojišče s kvasno dušikovo osnovo (**y**east **n**itrogen **b**ase)

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

1.1 OKOLJA Z VISOKIMI KONCENTRACIJAMI SOLI

Okolja z visokimi koncentracijami soli (t.i. izjemno, ekstremno ali hiper-slana okolja) so tista, ki imajo koncentracijo soli (NaCl) bistveno višjo od morske vode, to je več kot 35 g/l (3,5 %) NaCl (Kerkar, 2005), običajno nad 10% NaCl. Pogosta so po vsem svetu, predvsem pa v vročih in suhih predelih. Koncentrirane raztopine soli najdemo v naravnih ekosistemih, kot so morski bazeni in slana močvirja, slana in alkalna jezera ter v umetnih ekosistemih, kot so soline (Trüper in Galinski, 1986). Izjemno slane vode glede na izvor delimo na (i) atalasohaline: to so tiste slane vode, katerih ionska sestava je odvisna od geološke podlage, zato zanje niso značilne visoke vsebnosti kloridnih in natrijevih ionov (na primer Mrtvo morje); in (ii) talasohaline: (z izhlapevanjem) skoncentrirana morska voda, v kateri prevladujejo natrijevi in kloridni ioni. Primer talasohalinih voda so (solarne) soline, kakršne so tudi Sečoveljske soline na Jadranski obali.

V solinah se proizvodnja soli začne z morsko vodo, ki izhlapeva preko vrste zaporednih evaporacijskih bazenov do končnega kristalizacijskega bazena, v katerem se oborijo NaCl in druge soli iz nasičene slanice. Glavna značilnost solinskega ekosistema je torej nekontinuirano naraščanje slanosti zaradi izhlapevanja morske vode od 2 % do 35 % NaCl oziroma do nasičenja. Grenčica, ki ostaja po kristalizaciji, je bogata z magnezijevim kloridom in predstavlja prav poseben habitat znotraj solin (Zajc in sod., 2012). Za življenje omejujoči dejavniki v solinah so poleg spreminjajoče se vodne aktivnosti, še nizka koncentracija kisika in visoka intenziteta svetlobe (Anton in sod., 2000). Slana okolja, kjer najdemo halofilne in/ali halotolerantne mikroorganizme, predstavlja tudi koža ljudi in živali, površine puščavskega grmičevja, ki izloča sol, in druge površine v aridnem podnebju, ki so izpostavljene periodičnemu izsuševanju (Galinski in Trüper, 1994).

Navsezadnje izjemno slana okolja predstavljajo tudi s soljo konzervirana živila. Sol se namreč že več tisočletij v mnogih kulturah uporablja za podaljševanje obstojnosti mesa, rib, oreščkov in celo živalskih kož v usnjarski industriji/obrti.

1.2 ŽIVLJENJE V IZJEMNO SLANIH OKOLJIH

Sprva so raziskovalci mislili, da so ta okolja poseljena izključno z arhejskimi in bakterijskimi halofilnimi predstavniki ter redkimi evkariontskimi predstavniki kot so alge vrste *Dunaliella salina* ter solinski rakci iz rodu *Artemia* (Rodriguez-Valera in sod., 1981; Javor, 1989; Abatzopoulos in sod., 2002; Oren, 2005). Po letu 1998 je skupina raziskovalcev pod vodstvom prof. dr. Nine Gunde-Cimerman prva na svetu osamila številne vrste gliv iz izjemno slane vode Sečoveljskih solin. Te glive, katerih glavni habitat je hiperslana voda, so uvrstili v umetno skupino halotolerantnih črnih kvasovk. Najbolj pogosti in preučevani predstavniki te skupine so sevi vrst *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum* in *Aureobasidium pullulans* (Gunde-Cimerman in sod., 2000).

Zatem so tako v Sečoveljskih solinah kot tudi v solinah po vsem svetu (soline La Trinidad v delti reke Ebro in Santa Pola na mediteranski obali Španije, Camargue v Franciji, soline na atlantski obali Portugalske ter tudi soline v Namibiji, Dominikanski republiki in v Portoriku) odkrili veliko pestrost gliv različnih filamentoznih rodov (*Cladosporium, Penicillium* in *Aspergillus* skupaj z njunima teleomorfnima oblikama *Eurotium* in *Emericella*) ter tudi skupine sorodnih črnih in nemelaniziranih kvasovk, pa tudi predstavnike bazidiomicetnega rodu *Wallemia* (Butinar in sod., 2005a; Butinar in sod., 2005b; Butinar in sod., 2005c; Zalar in sod., 2005a; Cantrell in sod., 2006; Butinar in sod., 2011). Tudi iz Mrtvega morja osamili številne vrste rodov *Cladosporium, Penicillium* in *Aspergillus*, katerih izolati so bodisi halotolerantni bodisi halofilni, in prenesejo do 20-25 % NaCl v gojišču (pregledano v Nazareth in sod., 2012; Oren in Gunde-Cimerman, 2012). Po letu 2006 so v izjemno slanih vodah spoznali tudi predstavnike različnih rodov protozojev, ki so izrazito halofilni. Ena izmed takšnih predstavnic je vrsta *Halocafeteria seosinensis*, ki je pogosto opažena v geografsko različnih hiperslanih okoljih tudi v solarnih solinah (Park in sod., 2006; Park in Simpson, 2010).

1.3 OPREDELITEV HALOFILNIH IN HALOTOLERANTNIH GLIV

Z naraščanjem topljencev bodisi anorganskih (soli) ali organskih (sladkorji) v življenjskem okolju, se zmanjšuje količina biološko dostopne vode oziroma vodna aktivnost (a_w). Takšna slana ali sladka okolja so torej sušna, organizmi, ki jih naseljujejo pa kserofilni ali kserotolerantni. Večina gliv ima splošen kserotolerantni fenotip (Northolt in sod., 1995), kar pomeni da rastejo pri a_w do 0,85 (ta vrednost ustreza 17 % NaCl ali 50 % glukoze v rastnem mediju) ne glede na vrsto topljenca. Nekatere redke vrste gliv imajo preferenco po soli (Scott in sod., 1957), kot na primer *Polypaecilum pisci* in *Scopulariopsis halophilica* (Wheeler in sod., 1988), druge pa rastejo izključno na sladkornih medijih, kot na primer *Xeromyces bisporus* (Pitt in Hocking, 1977). Za razliko od halofilnih arhej in nekaterih bakterij, v glivnem kraljestvu obligatnih zahtev po soli dolgo časa ni bilo odkritih. V začetkih raziskav so za halofilne glive smatrali tiste vrste, ki so jih redno in v visokem številu osamili iz okolja na selektivnih medijih s slanostjo nad 10 % in so bile sposobne rasti *in vitro* na gojišču z najmanj 17 % NaCl (Gunde-Cimerman in sod., 2000). Te glive rastejo večinoma bolje v prisotnosti soli, vendar je za rast ne potrebujejo nujno. Z odkritjem vrste *Wallemia ichthyophaga*, ki brez soli ne raste (Zalar in sod., 2005a), je bil termin halofilnosti pri glivah izzvan. Sedaj termin halotolerantnih ali ekstremno halotolerantnih vrst uporabljamo za glive, ki rastejo preko širokega razpona koncentracij soli (od 0 % pa celo do nasičenja s soljo), termin halofilnih gliv pa se uporablja izključno za tiste, ki brez soli ne rastejo (Zajc in sod., 2012).

Drugačen pogled na opredelitev sposobnosti gliv za življenje v ekstremnih okoljih je predstavil Gostinčar s sodelavci (2010). Glive so razdelili na tri ekološke tipe; mezofile, generaliste in specialiste. (1) **Mezofili** pretežno živijo v okoljih brez ekstremnih pogojev. (2) **Generalisti** se pretežno nahajajo v okoljih z zmerno stresnimi razmerami zaradi njihove omejene sposobnosti konkurence z mezofili in njihove nezmožnosti preživetja v najbolj ekstremnih pogojih,. V slanih okoljih je takšen generalist *A. pullulans*, ki tolerira do 18 % NaCl in optimalno raste brez NaCl. (3) Tretjo skupino predstavljajo **specialisti**, ki so izjemno tolerantni ali celo ekstremofilni, njihov rastni optimum je pomaknjen bližje ekstremnim pogojem. Večinoma naseljujejo ekstremne habitate, saj bodisi ne morejo tekmovati z generalisti bodisi preprosto niso sposobni preživeti v zmernih pogojih. Takšna

primera v izjemno slanih okoljih sta adaptivni specialist - izjemno halotolerantna *H. werneckii* s široko ekološko amplitudo (raste v celotnem razponu koncentracij NaCl), in obligatni specialist - halofilna *W. ichthyophaga* z ožjo ekološko amplitudo (za rast nujno potrebuje 10 % NaCl in raste tudi v nasičeni raztopini NaCl) (Gostinčar in sod., 2010).

1.4 ROD WALLEMIA: FILOGENIJA Z EKOFIZIOLOGIJO

Kozmopolitski rod gliv *Wallemia*, ki živijo v različnih okoljih z nizko a_w, je bil opredeljen v okviru razreda Wallemiomycetes z redom Wallemiales (Zalar in sod., 2005a). Molekularne analize (Zalar in sod., 2005a; Matheny in sod., 2006) skupaj s karakteristično zgradbo dolipor (Terracina, 1974; Padamsee in sod., 2012) so rod uvrstile v deblo Basidiomycota. Različne analize so filogenetski izvor rodu *Wallemia* postavljale na različna mesta v drevesu življenja; na bazo bazidiomicet (Zalar in sod., 2005a), kot *incertae sedis* (Hibbett, 2006), kot sestrsko skupino z Agaricomycotina in Ustilaginomycotina (Matheny in sod., 2006) ali kot sestrsko skupino z Agaricomycotina (Padamsee in sod., 2012).

Glede na morfološke, fiziološke in molekularne taksonomske analize izolatov iz sladke, slane in posušene hrane ter iz kristalizacijskih bazenov solin Mediteranskega, Karibskega in Mrtvega morja so v rodu *Wallemia* opredelili tri kserofilne oz. halofilne vrste: *W. sebi*, *W. muriae*, *W. ichthyophaga*. Sorodnost vrst rodu *Wallemia* je podkrepljena z morfološkimi značilnostmi. Pri vseh treh vrstah se na morfološko podobnih konidioforjih tvorijo artrosporam podobni konidiji združeni v enote po štiri, kar je edinstveno v kraljestvu gliv. Vrsto *W. ichthyophaga* lahko tako mikroskopsko (oblika in velikost celic), kot tudi makroskopsko (izgled in velikost kolonij) razlikujemo od ostalih dveh vrst, kar je skladno z njeno molekularno odmaknjenostjo (Zalar in sod., 2005a). Vrsta *W. ichthyophaga*, v nasprotju z micelijskima oblikama rasti *W. muriae* in *W. sebi*, raste v obliki večceličnih skupkov podobnim sarcinam, ki so sestavljene iz številnih nepravilno izodiametričnih celic (Zalar in sod., 2005a).

Do danes je iz izjemno slane vode solin, grenčice, slanega mesa ali morske soli osamljenih približno 20 sevov vrste *W. ichthyophaga* (Sonjak in sod.; Jančič in sod. neobjavljeni

podatki (Zalar in sod., 2005a). V raziskovalnem ospredju te doktorske naloge je tipski sev vrste *W. ichthyophaga* (EXF-994), ki je shranjen v mikrobiološki zbirki ekstremofilnih gliv EX na Oddelku za biologijo Biotehniške fakultete Univerze v Ljubljani. Sev je bil izoliran iz izjemno slane vode Sečoveljskih solin (Zalar in sod., 2005a).

Kljub temu, da je kserotoleranca pri bazidiomicetah redka (De Hoog in sod., 2005), je rod Wallemia eden najbolj kserofilnih taksonov gliv (Zalar in sod., 2005a). Dve od treh opisanih vrst rodu Wallemia, *W. muriae* in *W. ichthyophaga*, sta kserofilni, saj za rast potrebujeta medij z nizko a_w. Vrsta *W. sebi* lahko uspeva tudi brez dodatnih osmotsko aktivnih topljencev (Zalar in sod., 2005a) in lahko raste na širšem območju a_w (0,997-0,690) v gojiščih z glukozo in fruktozo ali glicerolom (Pitt in Hocking, 2009). V medijih z dodanim NaCl kot glavnim topljencem je najnižja vodna aktivnost, kjer *W. sebi* še raste, 0,81 (gojišče YNB z 25 % (m/v) NaCl) (Zalar in sod., 2005a). Razpona a_w (in koncentracije NaCl), ki omogočata rast vrst *W. muriae* in *W. ichthyophaga*, pa sta 0,98-0,81 (4 % - 25 % NaCl) in 0,96-0,77 (10 % - 32 % NaCl) (Zalar in sod., 2005a). Vse tri vrste rastejo boljše na gojišču z znižano a_w: vrsti *W. sebi* in *W. muriae* pri 0,96, *W. ichthyophaga* pa kar pri 0,88 (Zalar in sod., 2005a).

Med vrstami tega rodu je najbolj izjemna *W. ichthyophaga,* saj raste le v gojišču z visoko koncentracijo soli (nad 1,7 M ali 10 % (m/v) NaCl), uspeva pa tudi v gojišču z nasičeno raztopino NaCl. Vrsta *W. ichthyophaga* je najbolj halofilna vrsta, ne le v rodu *Wallemia*, temveč med vsemi do sedaj opisanimi glivami. Takšna ekstremna ekološka amplituda slanosti je značilna za specializirane arhejske halofile, v kraljestvu gliv pa je edinstvena.

1.5 ČRNE KVASOVKE: EKOFIZIOLOGIJA

Polifiletsko skupino črnih aksomicetnih kvasovk (Sterflinger et al., 1999), sprva osamljenih iz Sečoveljskih solin, predstavljata dve vrsti iz redu Capnodiales (*Hortaea werneckii*, *Phaeotheca triangularis)* ter vrsti iz redu Helotiales (*Trimmatostroma salinum*) in Dothideales (*Aureobasidium pullulans*). Zanje so značilne melanizirane celične stene, relativno počasna rast in razmnoževanje z endokonidiacijo ali sarcinsko konidiogenezo.

Pogosto so polimorfne (kvasna, filamentozna in meristematska rast) in imajo muriformne celice (Zalar in sod., 1999a; Zalar in sod., 1999b; Zalar in sod., 1999c; Gunde-Cimerman in sod., 2000).

Aureobasidium pullulans je kozmopolitska vrsta, ki jo najdemo v različnih okoljih s spreminjajočo se a_w – od filosfere, vode, hrane, površine kamnin (povzeto v Zalar in sod., 2008) do izjemno slane vode solin (Gunde-Cimerman in sod., 2000; Butinar in sod., 2005b). Analize genomov varietet A. *pullulans*, ki trenutno potekajo, so povzdignile vse štiri varietete na nivo vrst (Gostinčar in sod., članek v pripravi). Tako lahko rečemo, da je v slanih habitatih najpogostejša vrsta *A. pullulans*, ki tolerira do 18 % NaCl, a raste najbolje v odsotnosti NaCl (Butinar in sod., 2005b).

Trimmatostroma salinum je edina črna kvasovka, ki se je do sedaj pojavila le v izjemno slani vodi Sečoveljskih solin, predvsem na potopljenem lesu (Zalar in sod., 1999c; Zalar in sod., 2005b). Vrsta *Phaeotheca triangularis* je pogostejša, saj jo najdemo v solinah po vsem svetu (Butinar in sod., 2005b). Njuni slanostni območji rasti v pogojih *in vitro* sta od 0 do 24 % NaCl, nekoliko pa se razlikujeta v rastnem optimumu. Vrsta *T. salinum* ima optimum pri 2 - 8 % NaCl, vrsta *P. triangularis* pa kar pri 6 - 12 % NaCl (Butinar in sod., 2005b). Obe vrsti sta torej izjemno halotolerantni s širokim razponom rasti ter rastnim optimumom v gojišču z dodano soljo.

Hortaea werneckii je melanizirana kvasovka, ki je bila sprva poznana kot povzročiteljica površinske glivne okužbe človeške kože imenovane *tinea nigra* (Bonifaz in sod., 2008; Bonifaz in sod., 2010). Najdena je bila tudi na slani hrani in drugih substratih z znižano vodno aktivnostjo (povzeto v Lenassi in sod., 2013). Odkrita pa je bila kot najštevilčnejša prebivalka izjemno slane vode v pred-kristalizacijskih in kristalizacijskih bazenih solin po svetu (Gunde-Cimerman in sod., 2000; Cantrell in sod., 2006). Je edina črna kvasovka, ki je sposobna rasti preko celotnega razpona koncentracij NaCl, od 0 do skorajšnjega nasičenja (30 % NaCl), s širokim rastnim optimumom pri slanosti 6 - 14 % NaCl. Zato jo opredeljujemo kot izjemno halotolerantno. Njena visoka sposobnost prilagajanja na sol je zelo primerna lastnost za študije tolerance na sol pri evkariontih.

1.6 IZZIVI IN STRATEGIJE ADAPTACIJ V SLANEM OKOLJU

Okolja z visokimi koncentracijami soli predstavljajo organizmom težavo zaradi izgubljanja vode preko citoplazmatske membrane, kajti le-ta je prosto prepustna za vodo. Nizka a_w je glavni omejitveni dejavnik rasti mikroorganizmov, ker privede do izgube turgorja in posledične zaustavitve rasti. Dodaten stres pa predstavlja še toksičnost anorganskih ionov, ki vdirajo v celico (Murguia in sod., 1995). Za preživetje organizmov v takšnem okolju je nujno, da je v a_w citoplazme nižja od okolja. Tako voda vstopa v celico v smeri koncentracijskega gradienta, celični volumen ter razmerje med prosto in vezano vodo pa je ugodno za biokemijske procese. Sila, ki usmerja vodo v celico, je uravnana s turgorskim tlakom, ki nastane zaradi omejene sposobnosti celične membrane in predvsem celične stene za ekspanzijo (Blomberg in Adler, 1992; Hohmann, 2002). Zapleten proces osmoadaptacije vključuje tako zaznavanje zunajcelične koncentracije soli, kot tudi kontrolo pretoka ionov, spremembe v izražanju genov ter kopičenje osmotsko aktivnih snovi (osmolitov).

Halotolerantni in halofilni organizmi uporabljajo dve osnovni strategiji prilagoditve na visoko slanost v zunanjem okolju, da se izognejo izgubi znotrajcelične vode. Pri prvi strategiji celice vzdržujejo visoko znotrajcelično koncentracijo soli, ki je osmotsko najmanj ekvivalentna koncentraciji v zunanjosti (strategija vnosa soli), kar naj bi zahtevalo prilagoditev vseh znotrajceličnih sistemov. Pri drugi strategiji celice vzdržujejo nizko koncentracijo soli v citoplazmi, osmotski tlak okolja pa uravnotežijo z biosintezo in/ali s kopičenjem organskih kompatibilnih topljencev v citoplazmi (t.i. strategija kompatibilnih topljencev) (Oren, 1999; Oren, 2011). Poleg tega se celice prilagodijo tudi na nivoju sestave in lastnosti celične stene ter membrane, zlasti glive pa razvijejo še poseben tip rasti, imenovan ekstremofilni ekotip, ki ga označujejo posebna meristematska morfologija in pigmentacijia (Gunde-Cimerman in sod., 2009; Gostinčar in sod., 2011).

Večina študij molekularne biologije in fiziologije osmoadaptacije evkariontskih mikroorganizmov na povišane koncentracije soli, je bilo sprva opravljenih na na sol občutljivi kvasovki *Saccharomyces cerevisiae* (Rep in sod., 2000; Yale in Bohnert, 2001; Hohmann, 2002), na morski kvasovki *Debaryomyces hansenii* (Prista in sod., 1997) ter na

halofilni algi *Dunaliella salina* (Chen in sod., 2011; Narvaez-Zapata in sod., 2011). Odkritja velike biološke pestrosti gliv v hiperslanih okoljih (Gunde-Cimerman in sod., 2000) so sprožile potrebo po novih halotolerantnih in halofilnih modelnih organizmih. Dandanes je črna kvasovka, *H. werneckii,* najbolje opisana ekstremno halotolerantna gliva (Gunde-Cimerman in Plemenitaš, 2006; Plemenitaš in sod., 2008; Gostinčar in sod., 2011). S svojim razmeroma enostavnim načinom gojenja in z znanim zaporedjem genoma (Lenassi in sod., 2013), je primeren model za študije osmoadaptacije evkariontov.

1.6.1 Strategija kopičenja kompatibilnih topljencev

Večina halofilnih in halotolerantnih mikroorganizmov doseže osmotsko ravnotežje s kopičenjem kompatibilnih topljencev (ali osmolitov), obenem pa v celici vzdržujejo nizko vsebnost ionov. Pri tej strategiji ni potrebe po specifično prilagojenih proteinih, saj ti topljenci tudi pri visokih (molarnih) koncentracijah omogočajo učinkovito delovanje celičnih funkcij, obenem pa lahko modulirajo aktivnost posameznih encimov (Oren, 1999; Roberts, 2005). Prednost strategije kopičenja kompatibilnih topljencev je visoka sposobnost prilagajanja celic na spreminjajoče se koncentracije soli v okolju, saj je znotrajcelična koncentracija kompatibilnih topljencev uravnavana glede na slanost okolja (Oren, 1999). Strategija je energetsko zelo draga, saj je poleg visokega energetskega vložka za sintezo visokih koncentracij organskih topljencev, potrebno tudi nenehno vlaganje energije v delovanje ionskih črpalk (Oren, 1999; Oren, 2002).

Nabor kompatibilnih topljencev, ki je zelo raznolik in stalno narašča, rutinsko odkrivajo s tekočinsko kromatografijo visoke ločljivosti (HPLC) in jedrsko magnetno resonanco (NMR) (Galinski, 1993). Običajno so to nizkomolekularne in dobro vodotopne molekule (Oren, 1999). Arheje prednostno kopičijo negativno nabite, bakterije in evkarionti pa predvsem nevtralne kompatibilne topljence (Roberts, 2005). Pogosto so to polioli (glicerol, arabitol in drugi) in njihovi derivati, sladkorji in derivati le-teh (saharoza, trehaloza, glukozilglicerol), aminokisline in derivati (prolin), kvartarni amini (glicin betain) (Galinski in Trüper, 1994; Oren, 1999; Roberts, 2005). Polioli so med glivami najbolj razširjeni kompatibilni topljenci. Preiskava 119 sevov kvasovk je pokazala, da jih večina proizvaja glicerol in arabitol, le nekatere med njimi tudi manjše količine eritritola. Kasneje pa je kemotaksonomska analiza tvorbe poliolov med 450 glivnimi vrstami pokazala, da vse

glive, razen oomicet, tvorijo poliole kot so glicerol, treitol, eritritol, ribitol, arabitol, ksilitol, sorbitol, manitol in galaktitol (Blomberg in Adler, 1992).

Glicerol, ki je kemijsko 1,2,3-propantriol, ima najenostavnejšo zgradbo od vseh poliolov, zato je njegovo kopičenje v visokih koncentracijah v izjemno slanih razmerah energetsko najcenejše (Oren, 1999). Poleg tega odlično ohranja aktivnost znotrajceličnih encimov tudi pri izjemno visokih koncentracijah. Razlog za to, da glicerol ni širše zastopan kot kompatibilni topljenec oziroma, zakaj je omejen le na domeno evkariontov, je verjetno v dejstvu, da je večina bioloških membran visoko prepustnih za nizko-molekularni glicerol (Oren, 1999). Celice morajo torej imeti posebne prilagoditve na nivoju membrane kot so to pokazali za algo *Dunalliela*, ki vsebuje več sterolov (Sheffer in sod., 1986) ali pa morajo imeti več energetsko zahtevnih transportnih sistemov za privzem glicerola kot so pokazali za *S. cerevisiae* (Hohmann, 2009).

1.6.1.1 Biološka vloga kompatibilnih topljencev

Poleg vzpostavljanja osmotskega ravnotežja, so številni poskusi pokazali (povzeto v Bolen in Baskakov, 2001), da kompatibilni topljenci povišujejo tudi stabilnost proteinov pred različnimi okoljskimi stresi, kot so visoke temperature, zmrzovanje in izsuševanje (Galinski in Trüper, 1994; Bolen in Baskakov, 2001; Roberts, 2005). Osmoliti imajo določene lastnosti, ki spodbujajo zvito strukturo proteinov, kljub nasprotnim - denaturacijskim učinkom okolja. Kompatibilni topljenci v visokih koncentracijah tekmujejo z vodnimi molekulami za interakcijo s površino proteina. Ker tvorijo močne strukture z vodo, so najverjetneje izločeni iz hidratacijskega ovoja proteina (Galinski, 1993), slednji pa so posledično prednostno hidratizirani, zato je koncentracija topljencev v okolici proteinov povišana. To posledično poviša površinsko napetost vode in s tem osmotski tlak, kar povzroči bolj kompaktno strukturo proteina. Hipoteza »prednostne izključitve« kompatibilnih topljencev tako razloži njihovo vlogo pri učinkoviti stabilizaciji hidratacijskega ovoja nativnega proteina (Roberts, 2005).

Poleg te razlage je Bolen s sodelavci ponudil še dopolnilni pogled, in sicer: stabilizacijski učinek kompatibilnih topljencev je tudi posledica njihove različne interakcije z zvitimi in denaturiranimi proteini, t. i. »osmofobni efekt«. Osmoliti imajo v primerjavi z vodo bolj

neugodne interakcije s peptidno hrbtenico proteina. Ta je bolj izpostavljena pri denaturiranih proteinih kot pri nativnih, zato osmofobni efekt prednostno poviša prosto energijo denaturiranega stanja, kar razmerje med stanjema proteina pomakne na stran nativno zvitih. Dobro poznane hidrofobne interakcije, vodikove vezi ter elektrostatične in disperzijske sile, ki vodijo zvijanje proteinov, dopolnjuje osmofobna sila/ oziroma osmofobni efekt, ki je še posebej pomemben v organizmih, katerih preživetje je odvisno od znotrajcelične prisotnosti osmolitov/kompatibilnih topljencev (Liu in Bolen, 1995; Bolen in Baskakov, 2001; Roberts, 2005).

1.6.2 Strategija kopičenja soli

Strategija privzema soli do visokih (molarnih) koncentracij za doseganje osmotskega ravnotežja med notranjostjo celice in njenim okoljem je značilna le za dve skupini mikroorganizmov: za halofilne arheje *Halobacteriaceae* in anaerobne halofilne bakterije redu *Halanaerobiales* (*Firmicutes*) (Oren, 1999). Pri tej strategiji so potrebne posebne strukturne prilagoditve celičnih komponent in encimov, saj je citoplazma izpostavljena visoki ionski jakosti (Galinski, 1995). Zaradi teh posebnih adaptacij pridobljenih skozi dolge in kompleksne evolucijske procese, so mikroorganizmi, ki imajo v svoji citoplazmi kisle halofilne encime, vezani na okolja s stalno prisotnostjo visokih koncentracij soli in kažejo le ozko sposobnost prilagajanja na spreminjajoče se razmere. Prednost te strategije je v nižjem energetskem vložku v primerjavi s strategijo sinteze molarnih koncentracij organskih topljencev (Oren, 1999).

1.6.2.1 Prilagoditve halofilnih proteinov

Halofilne proteine oz. encime je Madern s sodelavci opredelil kot tiste, ki so osamljeni iz organizmov, ki za optimalno rast potrebujejo vsaj 2,5 M NaCl (Madern in sod., 2000). Pri visokih koncentracijah soli, večina vodnih molekul obdaja ione in tako je za hidratacijo proteina na voljo manj vode. Posledično so lahko hidrofobni ostanki nehidratirani in proteini agregirajo. Visoke koncentracije soli torej ojačijo hidrofobne interakcije v proteinu, spreminjajo pa tudi elektrostatične interakcije med nabitimi aminokislinami, kar vodi v destabilizacijo proteinov zaradi njihovega razvijanja (Karan in sod., 2012). Halofilni proteini morajo zato imeti številne prilagoditve, ki jim omogočajo stabilno nativno strukturo.

Halofilni proteini so najbolje preučeni pri arhejah in bakterijah, o evkariontskih še ni znanega ničesar. Že zgodnje preučevanje njihove aminokislinske sestave je odkrilo, da imajo visok delež kislih aminokislin, manjšo vsebnost lizina in alifatskih aminokislinskih ostankov ter prisotnost manjših hidrofobnih aminokislin (Madern in sod., 2000). Novejša primerjalna študija proteomov halofilnih in nehalofilnih arhej in bakterij, je pokazala nekatere posebnosti, ki so jih avtorji poimenovali kar »makromolekularni podpisi haloadaptacije«: visoka vsebnost kislih aminokislinskih ostankov, posebej asparaginske kisline; nizka hidrofobnost, manjša vsebnost cisteinskih ostankov, nižja tendenca k tvorbi vijačnic in višja težnja k oblikovanju klobčičev (Paul in sod., 2008).

Glavna razlika med nehalofilnim in halofilnim proteinom je torej v večjem deležu kislih aminokislinskih ostankov, kot sta glutaminska in asparginska kislina, na površini halofilnega proteina (Zhang in sod., 2013). Ti negativni naboji omogočajo tekmovanje z ioni za vodne molekule, kar so pokazale tudi kristalne strukture halofilnih proteinov (pregledano v Reed in sod. 2013). Bioinformatske analize so pokazale tudi manjšo vsebnost serina, ki v tekmi za vodo z nabitimi ioni ni dovolj uspešen (Reed in sod., 2013). Alternativen pogled visoki vezavi vode kislih aminokislin na površini proteinov pa predvideva, da te aminokisline vežejo hidratirane katione, ki vzdržujejo hidratacijski ovoj okrog proteina (pregledano v Reed in sod. 2013). Manjši hidrofobni ostanki povišajo prožnost proteina pri visokih koncentracijah soli, saj preprečijo preveliko rigidnost hidrofobnega jedra proteina (Mevarech in sod., 2000).

Za razumevanje adaptacij proteinov na visoko slanost je nujno zavedanje, da halofilni proteini ne le preživijo visoke koncentracije soli, temveč da sol potrebujejo za svojo funkcijo. Odstranitev kationov namreč oslabi konformacijo proteina zaradi odbojev med izpostavljenimi negativnimi naboji na površini makromolekule (Mevarech in sod., 2000).

Novejše raziskave genomov povezavo med kislimi proteomi halofilnih organizmov in strategijo vnosa soli le delno potrjujejo, saj v določnih primerih obstaja odstopanje med analizo celokupnih proteinov, ki imajo izrazito kislo naravo, in analizo iz genoma predvidenih proteinov, ki teoretično niso posebej kisli (Oren, 2013). Glavni razlog za to je

lahko visoka vsebnost nevtralnih aminokislin, asparagina in glutamina, ki se med pripravo vzorcev s kislo hidrolizo deaminirata v kisli aminokislini aspartat in glutamat. Nasprotno, kisel proteom je lahko predviden tudi iz genomov zmerno halofilnih in morskih bakterij, ki sicer kopičijo organske kompatibilne topljence (Oren, 2013). Potrebna je torej večja previdnost pri povezovanju kislega proteoma z obligatno potrebo po soli in kopičenjem KCl.

1.6.2.2 Vzdrževanje nizke vsebnosti kationov pri glivah

V solinah, ki so habitat številnih halofilnih in halotolerantnih organizmov, nivo toksičnih Na^+ daleč presega nivo K^+ ionov. Celice morajo zato, da v teh pogojih vzdržujejo relativno stabilno in visoko razmerje K^+/Na^+, uporabljati veliko energije za pogon aktivnega transporta ionov preko membrane. To dosežejo s transporterji, ki imajo višjo afiniteto do privzema K^+ kot do Na^+, z učinkovitim iznosom toksičnih ionov in s selektivno kompartmentalizacijo kationov v celične organele (Ariño in sod., 2010). Transportni sistemi plazmatske membrane in membran organelov delujejo skupaj tudi zato, da vzdržujejo membranski potencial, regulirajo znotrajcelični pH in turgorski tlak (povzeto v Ariño in sod., 2010).

V plazmatski membrani *S. cerevisiae* so najpomembnejši sistemi za regulacijo vsebnosti K^+ Trk1 in Trk2, kanalčka za privzem (Ko in Gaber, 1991) in kanalček Tok1 za iztok K^+ (Ketchum in sod., 1995). Vzdrževanje nizke znotrajcelične koncentracije Na^+ pa je odvisno predvsem od delovanja dveh transportnih sistemov, in sicer ATPaze tipa P (Ena; iz lat. ***e**xitus **na**tru*), ki sklapljajo hidrolizo ATP za izmet Na^+ (in K^+) proti elektrokemijskem gradientu (povzeto v Ariño in sod., 2010), ter Nha antiporterja, ki uporablja transmembranski protonski gradient za izločanje Na^+. Ta dva sistema se dopolnjujeta, saj so ATPaze Ena pomembnejše pri višjih vrednostih pH, kjer Nha ne morejo delovati (Prior in sod., 1996). Pri *S. cerevisae* je ena glavnih determinant tolerance na sol ravno ATPaza Ena. Edini sistem za vnos Na^+ je simporter fosfata in Na^+ Pho89 (Martinez in Persson, 1998). Sistemi za dotok in iztok kationov v in iz celic so velikega pomena za razlago prilagoditev halofilnih/halotolerantnih evkariontov na izjemno visoko slanost. Sistemi ionskih transporterjev pri *W. ichthyophaga* še niso preučeni, so pa znani predvsem pri *S. cerevisiae* (Ariño in sod., 2010), halotolerantni *D. hansenii* in izjemno halotolerantni *H.*

werneckii (Prista in sod., 1997; Gorjan in Plemenitaš, 2006; Gunde-Cimerman in sod., 2009).

V primerjalni študiji meritev vsebnosti kationov v celicah treh ekofiziološko različnih vrst gliv, *H. werneckii, A. pullulans* in *D. hansenii* (Kogej in sod., 2005), je bilo ugotovljeno, da *H. werneckii* in *A. pullulans* vzdržujeta nizke koncentracije Na^+ in sta torej izključevalca Na^+ (angl. »Na^+-excluders«). Izmed vseh ekstremofilnih gliv je ionska homeostaza najbolje preučena pri halotolerantni kvasovki *D. hansenii*. Poleg sinteze kompatibilnih topljencev, veliko k njeni halotoleranci doprinaša zmožnost akumulacije Na^+ (Prista in sod., 1997). V celicah te kvasovke so bile izmerjene, v primerjavi z izjemno halotolerantno *H. werneckii*, nenavadno visoke koncentracije kationov (Larsson in sod., 1990; Kogej in sod., 2005). Tako *D. hansenii*, ki sicer uporablja kombinacijo strategije sinteze kompatibilnih topljencev in vnosa ionov v citosol, smatramo za »vključevalca Na^+« (angleško: »Na^+-includer«) (Gunde-Cimerman in sod., 2009). Prisotnost Na^+ v celicah, očitno ni toksična za *D. hansenii*, temveč lahko celo zaščiti celice pred dodatnimi stresnimi faktorji, na primer pred povišanimi temperaturami (Almagro in sod., 2000; Papouskova in Sychrova, 2007).

1.6.3 Prilagoditve halofilnih in halotolerantnih mikroorganizmov na nivoju celične stene

V izjemno slanem okolju so potrebne tudi dodatne prilagoditve na ravni sestave plazmatske membrane (Petrovič in sod., 1999; Turk in sod., 2004; Gostinčar in sod., 2008; Gostinčar in sod., 2009) in strukture celične stene (Kralj Kunčič in sod., 2010), ki predstavljajo prvo linijo obrambe pred okoljskim stresom (Mager in Siderius, 2002). Celična stena gliv je glavni organel, ki nudi celici zaščito pred mehanskimi poškodbami (Yin in sod., 2005), omogoča vzdrževanje notranjega turgorskega tlaka in je glavni medij interakcije glivne celice z njenim okoljem (Klis in sod., 2002). Glede na to, da je celična stena polifiletskega nastanka pri glivah in dejstvo, kako pestra je pojavnost gliv (kvasna, hifna oblika, spore in druge razmnoževalne strukture), je pričakovano, da je tudi celična stena gliv zelo raznolika.

Za izjemno halotolerantne askomicetne črne kvasovke *Hortaea werneckii*, *Phaeotheca triangularis* in *Trimmatostroma salinum* je značilna zelo debela in temno pigmentirana celična stena. Pigment, ki daje značilno temno-rjavo do črno obarvanost je 1,8-dihidroksinaftalen (DHN)-melanin, ki ga te glive tvorijo tako v pogojih visoke slanosti kot v okolju brez soli (Kogej in sod., 2004). V nadaljevanju je bilo ugotovljeno, da se pri višji slanosti pojavijo spremembe v prerazporeditvi melanina. Pri izjemno halotolerantni *T. salinum* so opazili, da so bile pri nižji slanosti melaninske granule manj organizirane, pri visokih slanostih pa so tvorile elektronsko gost povezan sloj okrog celic (Kogej in sod., 2006). Tudi pri pri *H. werneckii* so se granule melanina najtesneje organizirale pri optimalni slanosti. Melanizacijo celične stene so povezali z raziskavo kopičenja kompatibilnih topljencev pri *H. werneckiii* in ugotovili, da ima močno melanizirana celična stena pomembno vlogo v izjemno slanem okolju (Kogej in sod., 2007). Poleg znane vloge pri zaščiti pred UV sevanjem, temperaturnimi ekstremi in izsuševanjem, ima namreč melanin v celični steni *H. werneckii* tudi vlogo mehansko-osmotske zaščite. Pokazali so, da sodeluje pri zadrževanju glavnega kompatibilnega topljenca, glicerola (Kogej in sod., 2006; Kogej in sod., 2007), ki sicer dobro prehaja skozi lipidni dvosloj. Celice z melanizirano steno zadržijo več glicerola, saj lahko melaninske granule zmanjšajo permeabilnost celične stene z zmanjševanjem velikosti por, kot je bilo to pokazano pri *Cryptococcus neoformans* (Jacobson in Ikeda, 2005).

Debele celične stene predstavljajo pomemben oklep pred spremembami v slanem in izjemno slanem okolju (Clipson in sod., 1989; Kogej in sod., 2006). Debelo celično steno (0,5 do 1,0 um) imajo tako halotolerantne kvasovke rodu *Trimmatostroma* (Kogej in sod., 2006) kot tudi halofilna gliva *W. ichthyophaga* (Kralj Kunčič in sod., 2010). Podobno debelo celično steno so opisali tudi pri morski hifomiceti *Dendryphiella salina* (Clipson in sod., 1989). Rezultati meritev debeline celične stene gliv rodu *Wallemia* so pokazali zanimiv fenomen, in sicer debelina celične stene se pri višji slanosti statistično značilno odebeli. Odebelitev je bila še posebej povdarjena pri vrsti *W. ichthyophaga*, kjer je celična stena pri 25 % NaCl postala skoraj dvakrat debelejša v primerjavi s 15 % NaCl (Kralj Kunčič in sod., 2010).

1.6.4 Prilagoditve halofilnih in halotolerantnih gliv na nivoju morfologije celic in kolonij

Optimizacija razmerja med prostornino in površino (kvasna rast v vodnem okolju, hifna rast na trdnih medijih), skupaj z meristematsko rastjo, debelimi melaniziranimi celičnimi stenami, zunajceličnimi polimernimi snovmi, sposobnostjo adhezije in razmnoževanjem z endokonidiacijo, je pojmovana kot odgovor na stresne dejavnike, kot so nizka a_w, nizka dostopnost hranil in visoke oziroma nizke temperature (Sterflinger in sod., 1999). Takšen tip rasti imenujemo ekstremofilni ekotip in ima pomembno vlogo pri uspešni rasti črnih kvasovk v izjemno slanih pogojih (Kogej in sod., 2006; Kralj Kunčič in sod., 2010).

Študija morfologije Wallemia spp. pri zmerni in visoki slanosti je razkrila vpliv visokih koncentracij NaCl na celično morfologijo teh vrst (Kralj Kunčič in sod., 2010). Hifni predelki *W. sebi* in *W. muriae* so debelejši in krajši, njihovi micelijski prepleti (peleti) pa večji pri visokih slanosti. Filogenetsko oddaljena *W. ichthyophaga* se morfološko razlikuje od ostalih dveh vrst rodu Wallemia, saj ne raste v obliki hifnih pleteżev, temveč tvori mnogocelične skupke, ki so sestavljeni iz zelo kompaktno združenih celic. Velikost celic se ne spreminja glede na slanost, mnogocelični skupki pa postanejo statistično značilno večji pri visoki slanosti (Kralj Kunčič in sod., 2010). Podobne mnogocelične strukture pri visokih slanostih so značilne tudi za nekatere solinske črne kvasovke, kot so *H. werneckii* (Kogej in Gunde-Cimerman, neobjavljeni podatki), *P. triangularis* (De Hoog in sod., 1997; Zalar in sod., 1999b) in *T. salinum* (Kogej in sod., 2006). Rast v obliki tesnih skupkov celic najverjetneje omogoča zaščito celic v notranjosti. Z naraščanjem velikosti skupka, je razmerje med površino in prostornino manjše in tako je zmanjšano število celic, ki so v neposrednem stiku s stresnim okoljem. Sposobnost meristematske rasti v obliki skupkov naj bi tako močno povečala zmožnost preživetje mikroorganizmov v stresnih okoljih (Wollenzien in sod., 1995; Palkova, 2004; Palkova in Vachova, 2006).

Mnoge črne kvasovke, ki naseljujejo skale, tvorijo zunajcelične polimerne snovi (angl: »**e**xtracellular **p**olymeric **s**ubstances«, v nadaljevanju EPS), ki ima zaščitno vlogo pred stresnimi vplivi, predvsem pred cikli izsuševanja ter zmrzovanja in tajanja (Selbmann in sod., 2005). Tudi pri vseh treh vrstah rodu *Wallemia* so odkrili prisotnost EPS na površini

skupkov in celic, največ prav pri vrsti *W. ichthyophaga* v gojišču s 15% NaCl (Kralj Kunčič in sod., 2010). Podobno so pri črnih kvasovkah rodu *Trimmatostroma* opisali fibrozno plast EPS pri rasti v prisotnosti NaCl (Kogej in sod., 2006). Pri algi *Dunalliela salina* so z višanjem NaCl opisali povečano tvorbo EPS (Mishra in Jha, 2009). To kaže na to, da imajo EPS vlogo zaščite v izjemno slanem okolju (Kralj Kunčič in sod., 2010).

1.7 ZAZNAVANJE SPREMEMB V KONCENTRACIJI SOLI V OKOLJU IN KOPIČENJE GLICEROLA

Zaznavanje razlike v spremembi celične prostornine in/ali turgorskega tlaka ter razlike v koncentraciji soli, je ključno za sprožitev odziva s kopičenjem kompatibilnih topljencev. Med evkariontskimi organizmi so kaskade z mitogenom aktiviranih proteinskih kinaz (MAPK) vključene v številne celične procese, tudi v odziv na stres. Pri glivah, predvsem pri kvasovki *S. cerevisiae*, je najbolj preučena signalna pot HOG (angleško »**h**igh **o**smolarity **g**lycerol«), ki povzroči povišanje vsebnosti glicerola znotraj celice kot odgovora na povišanje osmolarnosti v okolju (Hohmann, 2009).

1.7.1 Signalna pot HOG pri halotolerantnih in halofilnih glivah

V signalni poti HOG *H. werneckii* (Plemenitaš in sod., 2003) je ključni protein za od soli odvisno izražanje *GPD1* homologov in posledično kopičenje glicerola MAPK HwHog1. Ta se poveže s promotojem in inducira izražanje *GPD1* gena, ko celice doživijo hiperosmotski šok (Petrovič in sod., 2002; Vaupotič in Plemenitaš, 2007). *H. werneckii* ima nekatere posebnosti v zaznavanju in odzivu na povišanje slanosti (Turk in Plemenitaš, 2002; Lenassi in Plemenitaš, 2005; Lenassi in sod., 2007; Fettich in sod., 2011). Medtem, ko se MAP kinaza Hog1 pri *S. cerevisiae* aktivira že pri nizkih slanostih, doseže homolog HwHog1 pri *H. werneckii* polno aktivnost le pri ekstremnih slanostih (Turk in Plemenitaš, 2002). Nekatere komponente poti HOG so odkrili tudi pri *W. sebi* (Padamsee in sod., 2012) in *W. ichthyophaga* (Konte in Plemenitaš, 2013). Pri *W. ichthyophaga* sta bila homologa WiHog1 nedavno podrobneje proučena. Zanimivo je, da je njun vzorec fosforilacije ravno obraten kot pri *S. cerevisiae*; pod optimalnimi slanostnimi pogoji sta konstitutivno fosforilirana, pod pogoji hipo- in hiperosmotskega šoka pa defosforilirana.

Poleg tega, je bilo njuno izražanje najvišje pri skrajnih pogojih slanosti, pri 10 in 30 % NaCl (Konte in Plemenitaš, 2013).

Pot HOG nadzoruje izražanje številnih osmotsko odzivnih genov (Hohmann, 2002). Raziskave sprememb ekspresije genov pri *H. werneckii* (Petrovič in sod., 2002; Vaupotič in Plemenitaš, 2007), so odkrile številne gene, ki se diferencialno izražajo pri različnih slanostih okolja in so odvisni od aktivacije s Hog1. Poleg tega pa so pokazale, da med celičnimi procesi osmoadaptacije pri halotolerantnih in na sol občutljivih glivah obstajajo pomembne razlike.

1.7.2 Kopičenje glicerola

Metabolizem glicerola ima pri *S. cerevisiae* pomembno vlogo pri uravnavanju celičnega metabolizma (Nevoigt in Stahl, 1997). Sintetizira se iz intermediata glikolize, dihidroksiaceton fosfata, v dveh reakcijskih korakih, ki jih katalizirata od nikotinamid adenin dinukleotid (NAD)- odvisna glicerol-3-fosfat dehidrogenaza (Gpd1) in glicerol-3-fosfataza (Gpp) (Albertyn in sod., 1994; Norbeck in sod., 1996). Njegovo razgradnjo pa vodita encima glicerol dehidrogenaza in dihidroksiaceton kinaza (Norbeck in Blomberg, 1997). Ta cikel je pomemben za pretvorbo NADH v reduciran NADPH, ki sodeluje pri boju z reaktivnimi kisikovimi substancami pod stresnimi pogoji (Hohmann, 2002). Anabolna pot glicerola, pri kateri je ključen Gpd2 encim, sodeluje tudi pri redoks uravnavanju celice, saj reoksidira višek NADH pod anaerobnimi pogoji (Ansell in sod., 1997). Tako je očitno, da morajo biti različne metabolne usode glicerola natančno regulirane, da celica preživi različne stresne pogoje.

Poleg biosinteze, pa celice *S. cerevisiae* ob hiperosmotskem šoku tudi aktivno privzemajo glicerol iz okolja s protonskimi simporterji (pri *S. cerevisiae* so to Stl1 glycerol/H^+ simporterji), akvagliceroporinski kanalčki (Fps1), ki delujejo v nasprotni smeri, pa se zaprejo. Ti med hipoosmotskim šokom omogočajo hiter izpust glicerola (Luyten in sod., 1995; Ferreira in sod., 2005).

1.8 GENOMIKA

V sedanjem času je zaradi napredka v tehnologiji, ki je skrajšal čas in zmanjšal stroške, sekvenciranje celotnih genomskih zaporedij organizmov dostopnejše kot kadarkoli prej (Pareek in sod., 2011; Zhang in sod., 2011). Leta 1996 je bil znan prvi evkariontski genom, in sicer genom kvasovke *S. cerevisiae* (Goffeau in sod., 1996). Poleg medicinsko in ekonomsko pomembnih gliv je znanih vse več genomov ekstremofilnih vrst. DOE Joint Genomic Institute ima v sklopu Genomske enciklopedije gliv celo projekt sekvenciranja preko 1000 genomov gliv, ki so bodisi pomembne v kmetijstvu (rastlinski patogeni, mikorizne vrste, vrste pomembne za biokontrolo), v biorafineriji (industrijske vrste gliv, glivni metabolizem sladkorjev in razgradnja lignoceluloze) ali pa so pomembne za razumevanje glivne biološke raznolikosti (Grigoriev in sod., 2011; Grigoriev in sod., 2012). Osem let za objavo genomskega zaporedja pekovske kvasovke, je postal znan tudi genom halotolerantne morske kvasovke *D. hansenii* (Dujon in sod., 2004; Kumar in sod., 2012). Šele leta 2012 pa je bil objavljen prvi genom izjemno halotolerantne glive *Wallemia sebi* (Padamsee in sod., 2012), nedavno pa tudi genom izjemno halotolerantne črne kvasovke *H. werneckii* (Lenassi in sod., 2013). Na spletni strani DOE Joint Genome Institute (Grigoriev in sod., 2011; Grigoriev in sod., 2012) so dostopni tudi genomi štirih varietet poliekstremotolerantne kvasovke *A. pullulans* (Gostinčar in sod., članek v pripravi).

Raziskovanje filogenetske in ekološke raznovrstnosti gliv s sekvenciranjem genomskega zaporedja predstavlja vir poznavanja potencialno dragocenih metabolnih poti in encimskih aktivnosti. Dostopnost genomskih zaporedij lahko privede tudi do boljšega razumevanja evkariontskega prilagajanja na slana okolja in odpira številne možnosti za njihov nadaljnji študij.

1.9 BIOTEHNOLOŠKI POTENCIAL HALOFILNIH GLIV

Eden glavnih razlogov za preučevanje esktremofilnih mikroorganimzmov je njihov biotehnološki potencial. Uporabnost halotolerantnih in halofilnih gliv izhaja iz študij osnovnih lastnosti in mehanizmov prilagajanja na ravni njihovih sekundarnih metabolitov, celične membrane, znotrajceličnih in zunajceličnih encimov in znotrajceličnih osmolitov-kompatibilnih topljencev. Slednji imajo širok spekter uporabe zaradi sposobnosti stabilizacije proteinov, nukleinskih kislin, membran in celic (Arakawa in Timasheff, 1985; Antranikian in sod., 1998; Kurz, 2008). So torej kemijski šaperoni, krioprotektanti, umetna sladila (polioli) in vlažilne komponente v kozmetičnih proizvodih (Roberts, 2005).

Črna kvasovka *A. pullulans* proizvaja eksopolimer pululan, ki je zelo uporaben v živilski in farmacevtski industriji (Leathers, 2003; Cheng in sod., 2011). Črni kvasovki *T. salinum* in *H. werneckii* proizvajata zunajcelične hidrolitično encime, ki delujejo pri visokih koncentracijah soli in imajo potencialno vlogo v različnih industrijah (Zalar in sod., 2005b), predvsem v proizvodnji bioetanola. Mnoge halofilne in halotolerante glive, med njimi tudi vrste rodu *Wallemia*, sintetizirajo posebne bioaktivne metabolite le v stresnih razmerah povišane koncentracije soli (Sepčić in sod., 2011; Botić in sod., 2012).

Poleg tega predstavljajo halotolerantne in halofilne glive tudi bogat vir genov primernih za vnos v rastline in razvoj poljščin s povečano toleranco na slanostni stres. Velik problem sodobnega kmetijstva je namreč zasoljevanje namakalnih površin. Toleranca na stres je zaželena tudi pri mikroorganizmih, ki so udeleženi v številnih stresnih industrijskih postopkih (Gunde-Cimerman in sod., 2009). Z izražanjem gena *HAL2 H. werneckii*, ki kodira zapis za encim 3'-fosfoadenozin-5'-fosfatazo, ki je sicer močno občutljiv na natrij, so pokazali da lahko znatno povišajo toleranco *S. cerevisiae* na sol (Vaupotič in sod., 2007). Najnovejša študija pa je celo pokazala, da se z vnosom določene regije Hal2 proteina iz vrste *A. pullulans* v homolog tega proteina modelne rastline *Arabidopsis thaliana*, poviša toleranca te rastline na sol in sušo (Buh Gašparič in sod., 2013). Čedalje pomembnejša je tudi bioremediacija izjemno slanih okolij, kot so z olji onesnažena slana močvirja in slane odpadne vode, ki izvirajo iz industrijskih procesov (Margesin in

Schinner, 2001). Zaščitni mehanizmi tolerance halofilnih gliv na visoke koncentracije soli imajo torej široko komercialno uporabnost.

1.10 NAMEN RAZISKAV DOKTORSKE NALOGE

Znanje o fiziologiji prilagajanja gliv na rast pri izjemno slanih pogojih je za zdaj omejeno na na sol občutljivo kvasovko *S. cerevisiae*, na zmerno halotolerantno morsko kvasovko *D. hansenii* in izjemno halotolerantne askomicetne glive, kakršna je trenutno najbolje proučena črna kvasovka *H. werneckii* (obe vrsti povzeti v Gunde-Cimerman in sod., 2009; Gostinčar in sod., 2011). *W. ichthyophaga* je doslej edina gliva, ki kaže obligatno zahtevo po soli, zato bo preučevanje njene fiziologije pomembno dopolnilo dosedanje razumevanje prilagoditev in odzivov na slanost. Predstavljala bi lahko nov modelni organizem za raziskave pravih halofilnih, in ne zgolj halotolerantnih organizmov.

Preučili smo strategijo osmoadaptacije vrste *W. ichthyophaga* na rast pri slanih pogojih, in sicer tako, da smo določili vsebnost kompatibilnih topljencev in kationov glede na naraščajočo slanost in preverili, kako se vsebnosti spreminjajo ob hiperosmotskih in hipoosmotskih stresih. Podrobneje smo preučili izražanje gena za encim glicerol-3-fosfat dehidrogenazo (*GPD1*), ki sodeluje pri sintezi glicerola in je udeležen pri različnih stresnih odzivih organizmov. Genu smo določili celotno zaporedje, mu preverili funkcijo in ga biokemijsko okarakterizirali.

S poskusi gojenja gliv rodu *Wallemia* pri pogojih znižane vodne aktivnosti s soljo ali z glukozo smo odkrili pomembne rastne značilnosti ter spremembe v ultrastrukturi celične stene halofilne glive *W. ichthyophaga*. Z uporabo različnih metod smo poskušali identificirati proteine povezane s celično steno pri halofilni glivi *W. ichthyophaga* in pokazati, da so prilagojeni na izjemno slana okolja.

V zadnjem delu raziskav smo poskusili identificirati gene *W. ichthyophaga*, ki bi lahko izboljšali toleranco kvasovke *S. cerevisiae* na slanostni stres in analizirali njeno celotno genomsko zaporedje ter transkriptomski odziv pri dveh mejnih slanostih.

1.11 HIPOTEZE IN CILJI

- Halofilna gliva *W. ichthyophaga* najverjetneje uporablja strategijo kopičenja kompatibilnih topljencev za uravnavanje osmotskega stresa zaradi visokih koncentracij soli v okolju. Predvidevamo, da je glavni in inducibilni kompatibilni topljenec glicerol. Ob tem verjetno vzdržujejo relativno nizko koncentracijo ionov v citosolu.

- Podrobneje bomo preučili gen za encim glicerol-3-fosfat dehidrogenazo (Gpd1), ki sodeluje pri sintezi glicerola in je udeležen pri različnih stresnih odzivih organizmov, za katerega je bilo predhodno s pregledom subtrakcijske cDNA knjižnice ugotovljeno, da se povišano izražajo pri višji slanosti. Pri *S. cerevisiae* se nahaja v eni kopiji in se izraža v odvisnosti od slanosti okolja. Najverjetneje se nahaja v eni kopiji tudi pri glivi *W. ichthyophaga*, študija njegovega izražanje pa bo razkrila morebitne posebnosti v odzivu halofilne glive na slanostni stres. Izražanje gena *GPD1* v ustreznih mutantah za sintezo Gpd1 bo povrnilo njihovo toleranco na sol, njegovo okarakteriziranje pa bo razkrilo morebitne posebnosti citosolnega proteina halofilnega organizma.

- Presejanje genov *W. ichthyophaga*, odgovornih za toleranco na stres bo odkrilo kandidatne gene, primerne za izboljšavo tolerance na stres pri kvasovki *S. cerevisiae*.

- Predvidevamo, da se bodo karakteristike rasti in ultrastruktura celične stene glive *W. ichthyophaga* razlikovali v okolju, kjer je vodna aktivnost znižana z NaCl, od okolja, kjer je znižana z neionskimi topljenci. Poleg tega predvidevamo, da bomo, glede na pomembno vlogo celične stene in vanjo vključenih proteinov pri ohranjanju celične integritete, še zlasti v neugodnih pogojih, identificirali več proteinov povezanih z celično steno. Domnevamo tudi, da bo njihovo izražanje odvisno od koncentracije soli v okolju.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Strategija osmoadaptacije najbolj halofilne glive *Wallemia ichthyophaga*, ki raste optimalno pri slanostih nad 15 % NaCl

Naslov v originalnem jeziku: The osmoadaptation strategy of the most halophilic fungus *Wallemia ichthyophaga*, growing optimally at salinities above 15 % NaCl

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Izvleček:
Wallemia ichthyophaga je gliva iz starodavnega bazidiomicetnega rodu *Wallemia* (Wallemiales, Wallemiomycetes), ki raste v območju slanosti med 10 % NaCl (m/v) in nasičeno raztopino NaCl. Ta obligatna halofilnost je med glivami edinstvena. Glavni cilji študije so bili določiti optimalni razpon slanosti za rast halofilne *W. ichthyophaga* in odkriti njeno strategijo osmoadaptacije. Rezultati so pokazali, da je bila rast na trdnih gojiščih zelo počasna, kolonije pa majhne. Nasprotno, v tekočih šaržnih kulturah je bila specifična stopnja rasti *W. ichthyophaga* višja, proizvodnja biomase pa se je povečevala z višanjem slanosti. Optimalno območje slanosti za rast *W. ichthyophaga* je med 15 do 20 % (m/v) NaCl. Pri 10 % NaCl, sta proizvodnja biomase in hitrost rasti daleč najnižja med vsemi testiranimi slanostmi. Poleg tega je bila količina mase celične stene glede na suho biomaso izjemno visoka pri slanostih nad 10 % NaCl. Rezultati so pokazali, da je glicerol glavni osmotsko uravnavan topljenec, saj se je njegova vsebnost povečevala z naraščajočo slanostjo ter se je zmanjšala po hipoosmotskem šoku. Poleg glicerola, smo odkrili tudi manjše količine arabitola in sledove manitola. Skladno s strategijo kompatibilnih topljencev, je *W. ichthyophaga* ohranjala relativno nizke vsebnosti kalija in natrija pri pogojih stalne slanosti. Med hiperosmotskih šokom pa so se vsebnosti obeh kationov znatno povečale. Glede na naše rezultate in nedavno razpoložljivost zaporedja genoma, bi se lahko *W. ichthyophaga* začela uveljavljati kot novi modelni organizem za študij halofilije pri evkariontih.
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Osmoadaptation Strategy of the Most Halophilic Fungus, *Wallemia ichthyophaga*, Growing Optimally at Salinities above 15% NaCl

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Wallemia ichthyophaga **is a fungus from the ancient basidiomycetous genus *Wallemia* (Wallemiales, Wallemiomycetes) that grows only at salinities between 10% (wt/vol) NaCl and saturated NaCl solution. This obligate halophily is unique among fungi. The main goal of this study was to determine the optimal salinity range for growth of the halophilic *W. ichthyophaga* and to unravel its osmoadaptation strategy. Our results showed that growth on solid growth media was extremely slow and resulted in small colonies. On the other hand, in the liquid batch cultures, the specific growth rates of *W. ichthyophaga* were higher, and the biomass production increased with increasing salinities. The optimum salinity range for growth of *W. ichthyophaga* was between 15 and 20% (wt/vol) NaCl. At 10% NaCl, the biomass production and the growth rate were by far the lowest among all tested salinities. Furthermore, the cell wall content in the dry biomass was extremely high at salinities above 10%. Our results also showed that glycerol was the major osmotically regulated solute, since its accumulation increased with salinity and was diminished by hypo-osmotic shock. Besides glycerol, smaller amounts of arabitol and trace amounts of mannitol were also detected. In addition, *W. ichthyophaga* maintained relatively small intracellular amounts of potassium and sodium at constant salinities, but during hyperosmotic shock, the amounts of both cations increased significantly. Given our results and the recent availability of the genome sequence, *W. ichthyophaga* should become well established as a novel model organism for studies of halophily in eukaryotes.**

Wallemia is a genus of xerophilic fungi frequently involved in food spoilage and often isolated from indoor or outdoor air (1), soil (2), and sea salt (3). A phylogenomic approach has placed the Wallemiomycetes as a 250-million-year-old sister group of Agaricomycotina (4). This phylogenetically ancient basidiomycetous class comprises only the order Wallemiales with the single genus *Wallemia*. Until 2005, *Wallemia sebi* was the only known representative of the genus; later, three *Wallemia* species were recognized within the genus: *W. sebi*, *Wallemia muriae*, and *Wallemia ichthyophaga* (5). The last, *W. ichthyophaga*, a type strain isolated from the hypersaline water of the Sečovlje solar saltern, is the focus of this research. So far, only a limited number of strains of *W. ichthyophaga* have been isolated from hypersaline environments, such as water from solar salterns, bitterns (magnesium-rich residual solutions in salt production from seawater), salted meat (ham and prosciutto), and even salt crystals (5; S. Sonjak, S. Jančič, and N. Gunde-Cimerman, unpublished data).

The genus *Wallemia* represents one of the most xerophilic fungal taxa, although xerophily is rare both in the Basidiomycota and in the entire fungal kingdom. *W. ichthyophaga* requires growth media with reduced water activity (a_w) (0.959 to 0.771) (5). Recently, it was shown that no other basidiomycetous yeast was able to grow below an a_w of 0.9, adjusted either with NaCl or with sorbitol (6). Furthermore, *W. ichthyophaga* shows a preference for certain solutes that lower the a_w: it grows better on media with high concentrations of NaCl than on media with high concentrations of glucose (7, 8). This and the fact that it can thrive only in media with NaCl contents above 10% (wt/vol) and up to saturation (32% NaCl) make *W. ichthyophaga* the most halophilic fungus known to date (5, 7, 9).

Organisms living in environments with high concentrations of salts are challenged by osmotic stress and by the toxicity of specific ions (10). In the microbial world, the loss of internal water as a consequence of osmosis in hypersaline environments is obviated by two fundamentally different strategies of osmoadaptation. The energetically relatively favorable "salt-in" strategy, which results in molar concentrations of KCl in the intracellular environment, is used by the representatives of extremely halophilic *Archaea* and some bacteria, whereas most other halophilic and halotolerant microorganisms synthesize and/or accumulate small organic molecules called compatible solutes to achieve osmotic balance (11, 12). The mechanisms of salt tolerance in fungi have been studied mostly in salt-sensitive *Saccharomyces cerevisiae* (reviewed in reference 13), which cannot grow in hypersaline environments. Therefore, more suitable model organisms for halotolerance studies in eukaryotes were suggested, such as the extremely halotolerant black yeast *Hortaea werneckii*, the moderately halotolerant yeast *Debaryomyces hansenii*, and the halophilic *W. ichthyophaga* (all reviewed in reference 9) and the polyextremotolerant *Aureobasidium pullulans* (14, 15). *H. werneckii* can grow *in vitro* at a wide salinity range, from 0% to 32% (saturation) NaCl, and has a broad growth optimum, from 6 to 10% NaCl (16, 17), whereas the halotolerant *A. pullulans* grows best without NaCl but can tolerate up to 17% NaCl and the marine yeast *D. hansenii* grows better at low sodium and tolerates up to 24% NaCl (18–20). One of the

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most important adaptations of fungi to high salinity is the accumulation of a mixture of various compatible solutes, among which glycerol is the most common. Only the salt-tolerant *D. hansenii* maintains a relatively high internal concentration of sodium when coping with salt stress, and it is therefore considered an Na^+ includer organism (18, 19). Nevertheless, the production of compatible solutes, particularly glycerol, is crucial for its survival (19, 21). In spite of the obligately halophilic nature of *W. ichthyophaga*, its osmoadaptation (salt-in and/or compatible-solute) strategy was so far unknown.

Recently, a few morphological, as well as molecular-biological, studies of *W. ichthyophaga* were published (4, 7, 8, 22, 23). In liquid saline media, *W. ichthyophaga* grows meristematically. It forms sarcina-like structures or compact multicellular clumps (5). This morphology, which is believed to enhance survival in high-stress environments, is also typical of some other polyextremotolerant fungal species (24–26). A recent study of *W. ichthyophaga* has revealed specific morphological adaptations to high NaCl concentrations, such as a 3-fold thickening of the cell wall and an almost 4-fold increase in the size of multicellular clumps compared to growth at low salinity. These morphological phenomena are believed to have an important role in successful growth under extremely saline conditions (7). Furthermore, analysis of the *W. ichthyophaga* genome sequence revealed a significant enrichment of salt-responsive genes coding for hydrophobins (4), small cell wall proteins involved in diverse cellular functions, such as modification of the movement of solutes across the cell wall, and giving strength and rigidity to the cell wall (27, 28). This could be of great importance in hypersaline environments (4). In addition, *W. ichthyophaga* cells are abundantly covered with extracellular polysaccharides (7), which prevent rock-inhabiting fungi from desiccation (29) and might also have a protective function in *W. ichthyophaga* at high salinity (7). In addition to this, some molecular mechanisms of halotolerance in *W. ichthyophaga*, such as the adaptation of a gene crucial for glycerol production (*GPD1* [23]), an insight into the HOG pathway by the study of Hog1 mitogen-activated protein (MAP) kinase (22), and also genome and transcriptome sequence analysis (4), have been uncovered recently.

Neither the growth characteristics over the salinity range nor the osmoadaptation strategy of the halophilic *W. ichthyophaga* has been examined so far. Therefore, the objectives of this study were to determine the salinity growth optimum and the growth characteristics in liquid and solid saline media and to elucidate the strategy of osmoadaptation of the halophilic fungus *W. ichthyophaga* to the extremely high concentrations of NaCl by measuring the intracellular osmolyte compatible solutes and cations when cells are exposed to a series of constant salinities, as well as to hyperosmotic and hypo-osmotic shocks.

MATERIALS AND METHODS

Strain, media, and growth conditions. The strain used in this study was the type strain of the basidiomycetous fungus *W. ichthyophaga* EXF-994 (CBS 113033), originally isolated from hypersaline waters of the Sečovlje solar saltern on the eastern Adriatic coast of Slovenia (5). The strain is preserved in the Ex Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Slovenia) and in the Centraalbureau voor Schimmelcultures (CBS) (Utrecht, The Netherlands).

For the determination of growth curves and for the preparation of samples for osmolyte measurements, the fungal cultures were grown in the standard yeast nitrogen base (YNB) chemically defined medium composed of 0.17% (wt/vol) yeast nitrogen base, 0.08% (wt/vol) complete supplement mixture (both from Qbiogene, Heidelberg, Germany), 0.5% (wt/vol) ammonium sulfate, 2.0% (wt/vol) glucose in deionized water, with NaCl added to various concentrations (10%, 15%, 17%, 20%, 25% [wt/vol]), and the pH was adjusted to 7.0.

The cultures were grown at 24°C on a rotary shaker (180 rpm) in 500-ml Erlenmeyer flasks in liquid YNB. Media with identical NaCl concentrations were used for the inoculum and for sample cultures. One milliliter of a mid-exponential-phase inoculum culture was added per 100 ml of YNB to start the sample culture. Growth was monitored by measuring the dry biomass and the pH of the culture. The cells were harvested by filtration in the mid-exponential growth phase for determination of the intracellular cation content and in both mid-exponential and stationary growth phases for the compatible-solute determination, as described below.

Construction of growth curves and determination of growth parameters. The inocula of *W. ichthyophaga* for monitoring growth on solid YNB medium were prepared as cell suspensions in sterile spore suspension solutions (0.05% Tween 80, 0.05% agar, and distilled water) supplemented with the same concentration of NaCl as in the growth medium. Drops of the cell suspensions were inoculated onto solid media in triplicate. Growth on solid media was monitored every 2 to 5 days until day 40 by measuring the diameters of individual colonies. The differences among the mean colony diameters were compared by one-way analysis of variance (ANOVA).

The growth of *W. ichthyophaga* in liquid YNB medium was estimated from the dry biomass of the samples collected every 2 or 3 days. At the same time, the pH of the culture was measured. The cultures were filtered through a nitrocellulose filter (pore size, 1.2 μm), and the biomass was dried at 100°C to a constant weight. The growth curves were constructed from the results obtained in two independent experiments, each carried out in duplicate, and were used to determine the final fungal biomass yield. The growth rates were calculated from the doubling times obtained from the growth curves during the exponential growth phase.

In the same manner, the biomass yields were determined and the doubling times were calculated for the halotolerant *A. pullulans* at 10% and 17% NaCl and for extremely halotolerant *H. werneckii*, as well, at 10%, 17%, and 25% NaCl.

Preparation of samples of salt-adapted cells and of cells subjected to hyperosmotic and hypo-osmotic shock. The cultures of *W. ichthyophaga* for the determination of intracellular cation content after a hypo-osmotic shock were grown in triplicate in liquid YNB medium with 15% (wt/vol) NaCl. The cells subjected to extreme hyperosmotic shock were grown to the mid-exponential growth phase, and then NaCl was added to the culture to a final concentration of 25%. The samples (0.5 ml) for cation measurements were collected before the shock and periodically during the first 300 min after the shock ($t = 5$, 10, 30, 90, and 300 min).

The cultures of *W. ichthyophaga* for the analysis of intracellular compatible solutes after a hypo-osmotic shock were grown in triplicate in liquid YNB medium with 20% (wt/vol) NaCl (14, 17). Hypo-osmotic shocks were performed as follows; the cells were grown in YNB with 20% NaCl to the mid-exponential growth phase. The culture was then divided into three equal volumes and centrifuged at 2,700 × *g* (Centrifuge 5810 R; Eppendorf) and 24°C for 5 min. The cells were resuspended in three different dilutions of the growth medium (20, 50, and 80% [vol/vol]), which were prepared from the YNB medium with 20% NaCl by the addition of deionized water. The cells were then incubated at 24°C for 1 h, allowing the release of solutes, and then the samples for high-performance liquid chromatography (HPLC) analysis were prepared.

Determination of intracellular cation content in salt-adapted cells and in cells subjected to hyperosmotic shock. The cation contents of cells were determined in the mid-exponential growth phase by a modified procedure previously described (14). In short, Millipore membrane filters (0.8-μm pore size) were prewashed with 2% HCl and three times with MilliQ water. Samples of 0.5-ml cultures of *W. ichthyophaga* grown in liquid YNB media of various salinities were filtered through Millipore

membrane filters and washed with ice-cold 20 mM $MgCl_2$ and an isosmotic concentration of sorbitol. The washing procedure usually took less than 2 min. The cells collected on the filter were washed out onto a new filter in the same manner. Each sample was dried at 80°C to a constant dry weight. The cells on the filters were treated overnight with acid (4% HCl), and the cations were analyzed by atomic absorption spectrophotometry (AAS) (14, 18). Six samples were prepared per parallel experiment for each concentration of NaCl, and one-way ANOVA ($n = 18$) was employed to test the significant differences among various concentrations of NaCl and before and after the hyperosmotic shock.

Determination of intracellular compatible-solute content in salt-adapted cells and in cells subjected to hypo-osmotic shock. The preparation of samples for HPLC analysis was performed as previously described (14). In short, samples of cells grown to the appropriate growth phase or after the hypo-osmotic shock were filtered through Millipore membrane filters (0.8-μm pore size) and washed with an isosmotic (the same NaCl concentration as in the growth medium) ice-cold solution of the same composition as the respective growth medium lacking glucose. The cells collected on the filter were washed onto a new filter and washed again in the same manner. The biomass was scraped off the filter, frozen in liquid nitrogen, and freeze-dried (14).

The polyols were extracted from 30 mg of freeze-dried cells using the previously described method (17). The cells were suspended in 500 μl Bligh and Dyer solution (composed of methanol-chloroform-water [10:5:4]) and vigorously shaken for approximately 30 min (IKA-Vibrax VXR; Janke & Kunkel). Then, 130 μl chloroform and 130 μl demineralized water were added, and the suspension was again incubated for 30 min with shaking. The samples were centrifuged at 5,510 × *g* (Biofuge A; Heraeus Instruments) for 10 min for phase separation. The upper methanol-water phase containing the solutes was transferred to a 1.5-ml tube and frozen at −20°C until analysis. The samples were diluted with an appropriate volume of 80% (vol/vol) acetonitrile before analysis (17).

HPLC analysis was performed on an isocratic system (Thermo Separation Products) equipped with a P100 pump, UV 1000 UV detector, and Shodex RI-71 (Showa Denko) refractive index (RI) detector. HPLC separations were performed with a Grom-Sil Amino-1PR column (3 mm; 125 by 4 μm; LiChrocart system; Grom) with a LiChrospher 100 NH_2 guard column (Merck) at ambient temperature. Acetonitrile (Merck; 80% [vol/vol]) in water (HPLC quality) was used as a mobile phase at a flow rate of 1 ml min^{-1}. Then, a 20-μl sample was injected using a Rheodyne injector number 7125 (Rheodyne). Chromatogram analysis was performed using the chromatography software ChromQuest version 2.51 (Thermo Quest) (17).

Nuclear magnetic resonance (NMR) measurements were performed on a Bruker Avance 300DPX unit using D_2O as a lock signal and trimethylsilylpropionic acid sodium salt (TMSP) as the internal standard. Cell extracts from ~0.5 g dried cells were freeze-dried and resuspended in 1 ml D_2O (17).

The differences among the mean amounts of intracellular osmolytes after hypo-osmotic shock determined by HPLC analysis were compared by one-way ANOVA ($n = 3$).

Cell wall isolation. The harvested cells were ground into fine powder in liquid nitrogen with a precooled mortar and pestle and freeze-dried until further use for cell wall isolation and protein extraction. Cell walls were isolated according to a previously described protocol (30). In short, 100 mg of freeze-dried biomass powder was resuspended in 1 ml of ice-cold 10 mM Tris-HCl, pH 7.5, and disrupted using a Mixer Mill MM 400 (Retsch) with 5-mm steel beads in the presence of a protease inhibitor cocktail (Sigma-Aldrich) at 4°C. The cell walls were harvested by 10 min of centrifugation at 500 × *g* and 4°C. The pellet was extensively washed with 1 M NaCl to remove intracellular contaminants and extracted twice for 5 min at 100°C with 50 mM Tris-HCl, pH 7.8, containing 2% SDS, 100 mM Na-EDTA, and 40 mM β-mercaptoethanol. The SDS-treated cell walls were washed three times with water and freeze-dried. The cell wall fractions were stained with 1% (wt/vol) Congo red stain in aqueous solution and checked for the presence of intact cells by light microscopy. There were clumps present, but no obvious intact cells were observed. The samples were weighed, and the percentage of the cell wall versus the total dry biomass was calculated.

Protein extraction. Soluble proteins were extracted from 100 mg of freeze-dried biomass resuspended in 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.3 M sorbitol, 1 mM dithiothreitol) supplemented with an isosmotic amount of NaCl and in the presence of a protease inhibitor cocktail (Sigma-Aldrich). The biomass was disintegrated as described above. The homogenate was centrifuged (20,000 × *g*; 15 min; 4°C) to obtain the supernatant with soluble proteins. The protein concentration was estimated according to Bradford's method using bovine serum albumin as a standard (31).

RESULTS

Growth of *W. ichthyophaga* on agar surfaces is extremely slow and results in small colonies. The growth of the colonies of *W. ichthyophaga* was monitored by measuring the colony diameters. Log, deceleration, and stationary growth phases were clearly discernible, whereas the lag phase could not be determined exactly due to the slow growth, resulting in small colony size (Fig. 1 A). Three days after inoculation, the colonies grew more or less radially at an exponential growth rate. The durations of the growth phases were the same at 10%, 15%, and 17% NaCl. Here, the log phase lasted from 3 to 8 days after inoculation, while it was prolonged to 12 and 19 days at 20% and 25% NaCl, respectively. The stationary growth phase was reached after 19 days of growth at 10 to 20% NaCl, while at 25% NaCl, the stationary growth phase was reached only after 33 days (Fig. 2A).

The maximal radial-extension growth rate of *W. ichthyophaga* (Fig. 2B, primary *y* axis) increased with increasing salinity on solid YNB medium with up to 20% NaCl, where the highest exponential growth rate (radial-extension growth rate, 0.22 day^{-1}) was measured. At 25% NaCl, the radial-extension growth rate decreased to 0.14 day^{-1}. According to the calculated radial growth rates, the optimal salinity range for the growth of *W. ichthyophaga* on agar surfaces was between 15% and 20% NaCl, where the growth rates were comparable. Interestingly, the maximal colony diameter increased consistently over the whole salinity range. At 25% NaCl, the colonies were the largest, at 3.4-mm diameter, which is 1.8-, 1.6-, and 1.3-fold larger than at 10%, 15% and 17%, and 20% NaCl, respectively (Fig. 2B, secondary *y* axis). The differences among the final colony diameters at a specific salinity were statistically significant for all salinities ($P < 0.05$), except for the colony sizes at 15% and 17% NaCl, which did not differ.

The specific growth rates of *W. ichthyophaga* in submerged cultures are highest between 15% and 20% NaCl. In submerged shake flask liquid cultures, the growth of *W. ichthyophaga* exhibited all four distinguishable phases (lag, log, deceleration, and stationary growth phases) (Fig. 1B). The growth of *W. ichthyophaga* in liquid YNB medium with 10% NaCl was the slowest among all tested salinities (Fig. 1B and 2C and D). The lag phase (9 days) (Fig. 2C) was the longest and the biomass production (2.0 mg/ml of medium) was the lowest (Fig. 2D) at 10% NaCl. Similar growth characteristics (Fig. 2C and D) of *W. ichthyophaga* grown in media with 15% and 17% NaCl resulted in almost identical growth curves (Fig. 1B). At both salinities, the lag phase lasted 5 days (Fig. 2C) and the specific growth rates were the highest among all tested salinities (1.1 day^{-1}) (Fig. 2D, primary *y* axis). The final dry biomass at these salinities was among the highest, i.e., 5.0 and 5.1 mg/ml of growth medium for 15% and 17% NaCl, respectively

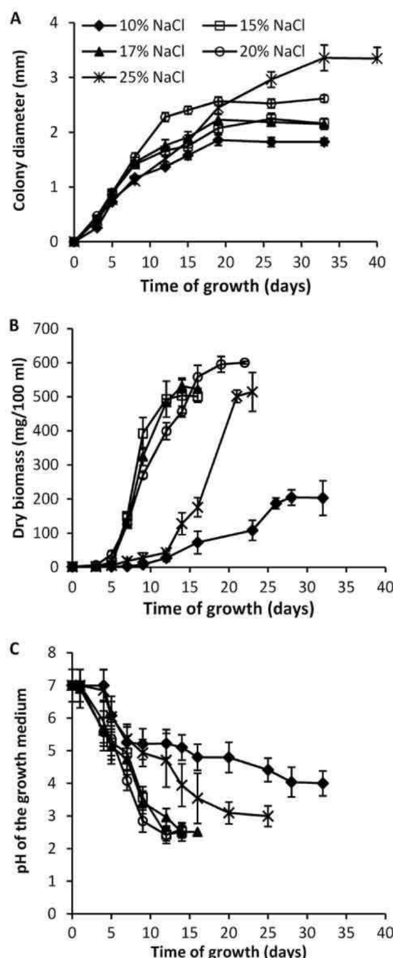

FIG 1 Growth curves of *W. ichthyophaga*. Shown are the growth curves of *W. ichthyophaga* grown on solid (A) and in liquid (B and C) YNB media supplemented with 10%, 15%, 17%, 20%, and 25% (wt/vol) NaCl. The growth curves were constructed by plotting the mean colony diameters of at least 10 colonies for each time point. The diameters of fungal cultures were measured on 3 replicate plates every 2 to 5 days for 40 days. The growth curves in panel B were determined from the dry fungal biomass. The fungal biomass was measured in two independent experiments, each carried out in duplicate, and the samples for biomass measurements were taken every 2 or 3 days. The curves in panel C were obtained by plotting the measured pH of the liquid fungal cultures, obtained when the samples for biomass were taken. The error bars indicate standard deviations of the means.

(Fig. 2D, secondary *y* axis). When *W. ichthyophaga* was grown in the medium with 20% NaCl, it entered the log phase only 3 days after inoculation of the media. The log phase was the longest among all salinities (Fig. 1B and 2C). Also, the specific growth rate had the second highest value (0.95 day^{-1}), and the final biomass was the highest (6.0 mg/ml), almost 3 times the biomass obtained at 10% NaCl. The growth of *W. ichthyophaga* at 25% NaCl was characterized by a longer lag phase (7 days) than at 15 to 20% NaCl but shorter than at 10% (Fig. 2C). Similarly, the specific growth rate (0.6 day^{-1}) was lower than at 15 to 20% NaCl but higher than at 10%. The final biomass (5.1 mg/ml) was comparable to those obtained when *W. ichthyophaga* grew at 15% or 17% NaCl (Fig. 2D).

The doubling time of *W. ichthyophaga* is shortest (15 h) at the optimal 17% NaCl, and it is 2.7- and 1.9-fold longer at 10% and 25% NaCl, respectively. The doubling times of *A. pullulans* (9.7 and 29.5 h at 10% and 17% NaCl, respectively) and *H. werneckii* (8.6, 22.4, and 56.9 h, respectively, for 10%, 17%, and 25% NaCl) increased with increasing salinity (see Fig. 5A). The biomass production of *A. pullulans* (8.2, 4.6, and 1.7 mg/ml, respectively, for 0%, 10%, and 17% NaCl) strictly decreases with increasing salinity, whereas in *H. werneckii*, comparable biomass values were measured in the range of 0% to 17% NaCl (from 8.6 to 9.3 mg/ml), when the biomass production steeply decreased (see Fig. 5B).

***W. ichthyophaga* growing at optimal NaCl concentrations acidified the growth medium the most.** To monitor the growth of *W. ichthyophaga* over the range of tested salinities, the pH of the growth medium in the submerged cultures was measured. During the growth of *W. ichthyophaga*, the initial pH 7.0 of the growth medium gradually decreased (Fig. 1C). The curves of pH decrease versus time were similar at 15%, 17%, and 20% NaCl and were easily distinguishable from the curves at 25% and 10% NaCl, where the slopes of pH decrease were slower. At 15, 17, and 20% NaCl, the pH of the medium reached pH 2.5 at the end of growth, whereas at 25% NaCl, the pH was 3.0, and at 10% NaCl, the pH was 4.0. The decrease in pH was inversely related to the increase in the biomass: the higher the biomass, the lower the pH of the medium (compare Fig. 1B and C).

The cell wall content of the dry biomass of *W. ichthyophaga* is extremely high at salinities above 10%. The mass of the cell wall of *W. ichthyophaga* grown in the medium with 10% NaCl represented 26.1% of the dry biomass weight of the fungus. In the growth media with higher concentrations of NaCl, the share of the dry cell wall per total dry biomass increased significantly. In the media with 15% and 17% NaCl, the cell wall represented 53.0% of the dry weight, whereas in the media with 20% and 25% NaCl, it was as high as 57.0% and 58.1%, respectively.

The ratio of intracellular potassium and sodium ion contents in *W. ichthyophaga* decreased with increasing salinity. The preadapted cells of *W. ichthyophaga* were grown at various constant salinities to the mid-exponential growth phase, when the cultures were analyzed for intracellular amounts of potassium and sodium ions by AAS. Measurements of cation contents in the cells have shown that the amounts of K^+ and Na^+ in *W. ichthyophaga* changed according to the NaCl concentration of the medium (Fig. 3A). When *W. ichthyophaga* was grown in YNB medium with 10% NaCl, the cells contained the largest amount of K^+ and the smallest amount of Na^+. With increasing NaCl concentrations in the medium, the amount of K^+ gradually decreased with a statistically significant difference ($P < 0.05$), and it reached its minimal value at 20% NaCl. Unlike K^+, a clear tendency of increasing Na^+ content with increasing salinity was observed, although it was not statistically significant.

The intracellular potassium and sodium ion contents in *W. ichthyophaga* increased after hyperosmotic shock. The salt-adapted cells of *W. ichthyophaga* were subjected to a hyperosmotic shock by a sudden increase in the salt concentration of the medium from 15% NaCl to 25% NaCl. The intracellular cation contents were measured at 5 different times within the first 300 min after the shock (Fig. 3B). The contents of K^+ and Na^+ significantly

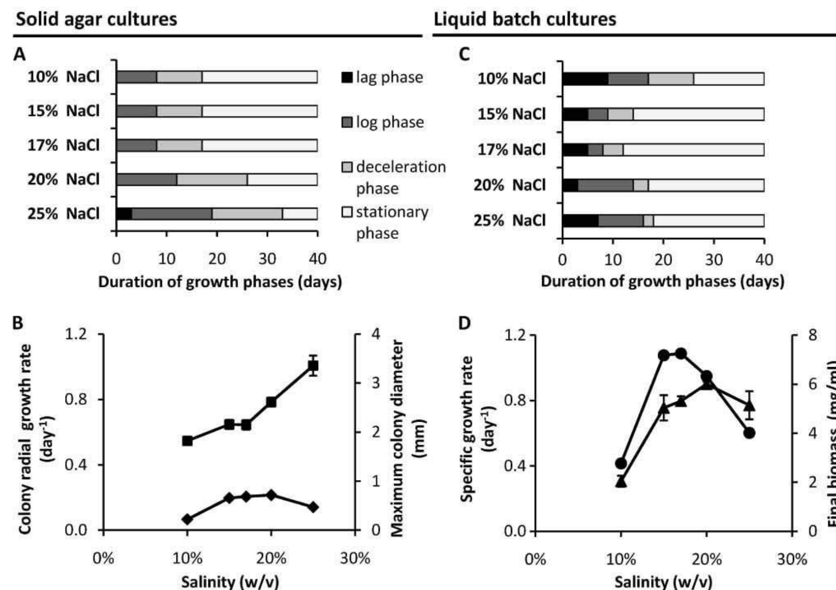

FIG 2 Growth parameters of *W. ichthyophaga*. Shown are the growth parameters of *W. ichthyophaga* cultures grown on solid (A and B) and in liquid (C and D) YNB media at various concentrations of NaCl. (A and C) Durations of the lag, log, deceleration, and stationary growth phases on solid (A) and in liquid (C) growth media. (B and D) Colony radial growth rates (diamonds) and maximal colony diameters (squares) calculated from data obtained on solid growth media (B) and specific growth rates (circles) and final biomasses (triangles) in liquid growth media (D). The error bars indicate standard deviations of the means.

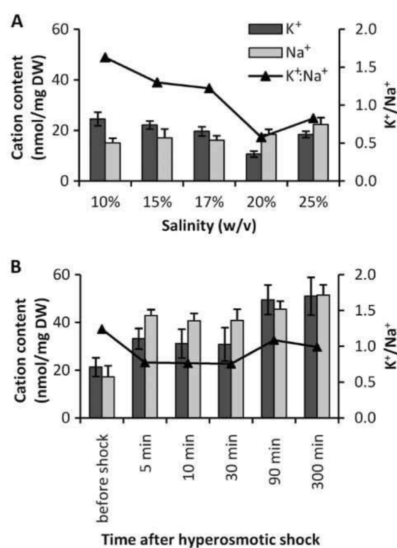

FIG 3 Intracellular amounts of K^+ and Na^+ in *W. ichthyophaga* grown at constant salt concentrations (A) and after hyperosmotic shock (B). The fungus was grown in a defined NaCl-amended YNB liquid medium at 24°C and 180 rpm on a rotary shaker to the mid-exponential growth phase. The hyperosmotic shock was performed by the addition of 10% (wt/vol) NaCl to the mid-exponential-phase cultures grown at 15% NaCl. The samples were collected before the shock and periodically within the first 300 min after the shock. Six samples from each of the three parallel flasks were taken for cation measurements by AAS. The values shown are means and standard errors of the mean ($n = 15$). The K^+/Na^+ ratio is represented by the triangles. DW, dry weight.

increased within the first 5 min after the shock and then remained approximately the same for up to 30 min after the shock. Ninety minutes after the shock, another significant increase in the contents of both cations occurred. At the end of the experiment, the amounts of K^+ and Na^+ were significantly higher than the amounts measured in the *W. ichthyophaga* cells grown in the medium with a steady 25% NaCl (compare Fig. 3A and B). The K^+/Na^+ ratio dropped from an initial 1.2 to 0.8 30 min after the shock, and it started to rise 90 min after the shock. At the end of the experiment (300 min after the shock), the K^+/Na^+ ratio (1.0) almost reached the initial value before the shock, but the absolute amounts were significantly higher than before the shock (Fig. 3).

Glycerol is the main compatible solute in *W. ichthyophaga*. The preadapted cells of *W. ichthyophaga* were grown to the mid-exponential growth phase, when the cells were extracted and analyzed for organic compounds with a possible osmotic role.

Two different HPLC methods were applied for screening the cell extracts. The first method, which was optimized to detect uncharged compatible solutes, including polyols, detected only glycerol, arabitol, and mannitol in the cells of *W. ichthyophaga* (Fig. 4). The main polyol was glycerol, whereas arabitol was present in smaller amounts and mannitol only in trace amounts. All three were also confirmed by NMR (data not shown). The second method, 9-fluorenylmethoxy carbonyl (FMOC) derivatization of amino acids, revealed very small amounts of glutamate, alanine, and proline signals (data not shown).

The amounts of polyols in the cells varied and depended upon the salinity of the growth medium and upon the growth phase of the fungal culture (Fig. 4A and B). In the mid-exponential growth phase, as well as in the stationary growth phase, there was significant correlation ($P < 0.05$) between the intracellular

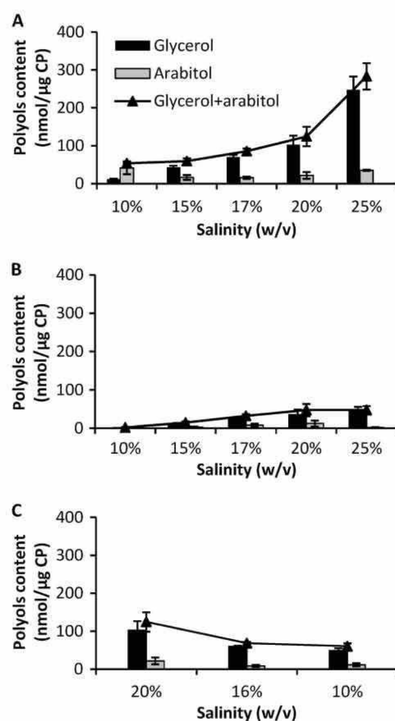

FIG 4 Intracellular amounts of glycerol and arabitol in *W. ichthyophaga* grown to the mid-exponential growth phase (A) and stationary phase (B) at constant salt concentrations and after hypo-osmotic shock (C). The triangles represent the total polyol content at each of the salinities. (A and B) *W. ichthyophaga* was grown in liquid YNB with various salinities at 24°C and 180 rpm on a rotary shaker in three parallel flasks to the appropriate growth phase, sampled, extracted, and analyzed by HPLC as described in Materials and Methods. (C) *W. ichthyophaga* was grown in liquid YNB with 20% (wt/vol) NaCl and suddenly exposed to diluted media with lower salinities (16% and 10%) for 1 h. The cell samples were extracted and analyzed by HPLC as for panels A and B. The values shown are means and standard errors of the mean ($n = 9$). CP, cell proteins.

amounts of glycerol and the salinity of the growth medium. Arabitol did not correlate with salinity either in the mid-exponential or in the stationary phase. The largest amounts of arabitol were detected in the cells of *W. ichthyophaga* grown to the mid-exponential growth phase in YNB with 10% NaCl and 25% NaCl, whereas it remained at similarly low values in the cells grown in the media with 15%, 17%, and 20% NaCl (Fig. 4A). The total amount of polyols correlated with the increasing salinity, entirely due to the increases in the amount of glycerol at all tested salinities. Glycerol was the main polyol detected at all tested salinities except 10% NaCl, where arabitol mainly contributed to the total amount of polyols. The correlation between the combined amounts of glycerol and arabitol and salinity was less apparent in the stationary phase, where the total amount of polyols in the cells was also much lower than at exponential phase (Fig. 4B).

Both glycerol and arabitol are lost from the cells of *W. ichthyophaga* during hypo-osmotic shock. In order to test the osmotic roles of glycerol and arabitol, salt-adapted mid-exponential-phase cells of *W. ichthyophaga* grown at optimal salinity (20% NaCl) were exposed to two drastic hypo-osmotic shocks, performed by dilution of the growth medium (from 20% to 16% NaCl for a moderate shock and 20% to 10% NaCl for a drastic shock) (Fig. 4C). The intracellular amounts of polyols were measured 1 h after the shocks. The hypo-osmotic shock caused a decrease in the intracellular amounts of both glycerol and arabitol. However, the decrease of glycerol correlated with the dilution of the initial growth medium, i.e., the decrease in salinity, whereas the decrease of the arabitol content did not. In conclusion, drastic hypo-osmotic shock of mid-exponential-phase cells of *W. ichthyophaga* grown at 20% NaCl caused the loss of glycerol and also, to a lesser extent, of arabitol.

DISCUSSION

W. ichthyophaga is the most halophilic, not only of the three species of the basidiomycetous genus *Wallemia*, but also of all fungi. It is one of the rare fungal species that strongly prefers high concentrations of ionic (e.g., salt) over nonionic (e.g., sugar) solutes. It requires at least 10% NaCl (or some other osmolyte for an equivalent a_w) for *in vitro* growth and grows over a wide range of salinities up to saturated NaCl solution (5, 7). As the physiology of this unique species has so far received only little attention, we studied the basics of its halophilic behavior, its growth characteristics in growth media of various salinities, and its strategy of osmoadaptation to the level of intracellular contents of organic compatible solutes and inorganic potassium and sodium.

As fungi are most often grown on solid media for a variety of purposes, such as isolation from environmental samples, identification, and different screenings (32), we have characterized the growth of *W. ichthyophaga* on solid growth medium supplemented with NaCl. The growth rate was low, and the colonies were relatively small at all tested salinities (Fig. 1A and 2A and B). Similar shapes of growth curves and comparable growth kinetics (the highest colony radial growth rates) were observed at 15, 17, and 20% NaCl. This broad salinity range represents the optimum for the growth of *W. ichthyophaga* on YNB agar plates. At 10% NaCl, which is the lowest salinity limit for growth of *W. ichthyophaga*, the growth rate was the lowest and the colonies were the smallest, whereas the largest colonies were observed at 25% NaCl. The increased colony size of *W. ichthyophaga* on the extremely saline medium suggests that the salinity is favorable for its growth. The colonies spread on the surface of the growth medium and do not reduce their contact area with the medium by forming smaller, convex colonies, as observed in less halophilic/halotolerant species at the highest tolerated salinities (33). This is also in accordance with the previous study, in which only the final colony diameters of various *W. ichthyophaga* strains were measured at various NaCl concentrations (5). The extremely slow growth and the resulting small colonies of *W. ichthyophaga* on agar plates, which can be quickly overgrown by contaminants at moderate salinity, are probably the main reasons for the low numbers of isolates and the late identification of the species. The above-mentioned growth characteristics need to be taken into account when studying the ecology of *W. ichthyophaga*. Therefore, growth media with extremely high salinities (such as 15 to 20% NaCl) and prolonged incubation (at least 14 days) are required for the successful isolation of *W. ichthyophaga* strains from natural environments.

In a previous study of the morphological response to hypersaline environments, *W. ichthyophaga* was cultivated in submerged shake flask cultures in liquid YNB media at 15% and 25% NaCl

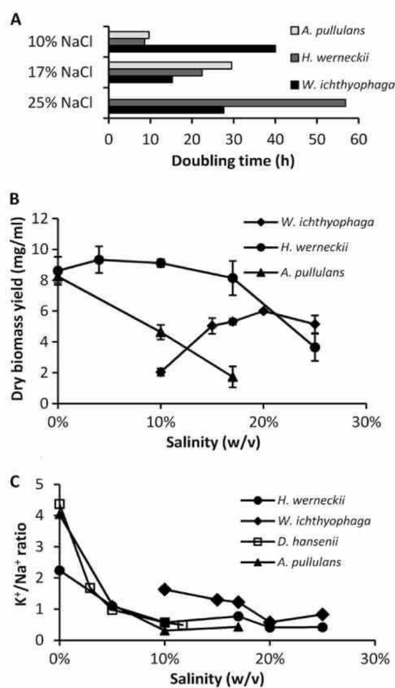

FIG 5 Comparison of doubling times (A), dry biomass production (B), and K^+/Na^+ ratios (C) of *W. ichthyophaga*, *H. werneckii*, *A. pullulans*, and *D. hansenii* at various salinities. The data on K^+/Na^+ ratios for *H. werneckii*, *A. pullulans*, and *D. hansenii* in panel C are from Kogej et al. (14). The error bars indicate standard deviations of the means.

(7), but the optimal NaCl concentration had not been determined. Four growth phases (lag, log, deceleration, and stationary growth phases) were discernible during the growth of *W. ichthyophaga* in the liquid YNB, and their duration changed according to the salinity (Fig. 1B and 2C and D). The growth parameters (the lag phases, the biomass production, and the specific growth rate) of *W. ichthyophaga* at 15 and 17% NaCl were practically indistinguishable and were comparable to those at 20% NaCl. These three growth parameters indicate that the growth optimum in liquid YNB medium of *W. ichthyophaga* is extremely saline and relatively broad, from 15% to 20% NaCl, whereas at 25% and 10% NaCl, growth is suboptimal.

A comparison of the doubling times of *W. ichthyophaga* with the doubling times of the halotolerant *A. pullulans* and the extremely halotolerant *H. werneckii* shows that at lower salinity (10% NaCl), the doubling time of *W. ichthyophaga* is more than 4 times longer, whereas at higher salinity (17% and 25% NaCl), it is the shortest (Fig. 5A) (14). Also, the biomass of *W. ichthyophaga* is by far the lowest at 10% NaCl, but on the other hand, at 25% NaCl, it is the highest among the three fungi (Fig. 5B). According to our results, it is obvious that *W. ichthyophaga* is well adapted to life at salinities higher than 10% NaCl and that it has, to our knowledge, the highest growth optimum ever described in the fungal kingdom (9, 20, 32, 34, 35).

The external medium acidified during the growth of *W. ichthyophaga*. The fastest pH decrease occurred in the media with cultures with the fastest growth kinetics, and the higher the biomass, the lower the final pH of the growth medium. Therefore, measuring the pH of the growth medium can indicate the growth phase of the *W. ichthyophaga* culture at a specific salinity. This is especially useful because *W. ichthyophaga* grows in clumps, and thus, optical density measurements cannot be used to monitor its growth. The nature of this pH change is not supported by any additional measurements. As previously shown, the glucose-induced acidification of the growth medium may occur due to an extrusion of organic acids during the progress of growth and/or due to the activity of the plasma membrane H^+-ATPase (36). However, the possible connection between the acidification and the salinity of the medium might be found in the fact that the acidification is a consequence of the activity of the plasma membrane H^+-ATPase, which generates the electrochemical gradient of H^+ across the plasma membrane. This powers all the secondarily active symporters and antiporters necessary for the maintenance of homeostasis of intracellular K^+ and Na^+. Hence, the lower pH at higher salinity might occur due to higher demands for the proton motive force (37, 38).

The fungal cell wall, which is a highly dynamic structure and is regulated depending on the internal and external stimuli (39), provides physical, as well as osmotic, protection (40). A previous study showed that the relatively thick cell wall of *W. ichthyophaga* was almost 3-fold thicker at high salinity (7). Determination of the cell wall content in the dry biomass of *W. ichthyophaga* at various salinities revealed that the dry biomass contained 26% cell wall at the lowest salinity, which is in accordance with the measurements from literature (e.g., in *S. cerevisiae*, the cell wall makes up from 15 to 30% of the dry weight of the cell, at most [41]). At higher salinities, the cell wall content of the biomass steeply increased to over 50%. Similarly, this trend was also observed for the halotolerant *D. hansenii* (J. Ramos, unpublished data). At extremely high salinities, the cell maintains a higher positive turgor pressure; hence, the extreme strengthening, observed as an increase in the thickness of the cell wall, is expected (40, 42, 43). The growth of *W. ichthyophaga* at 10% NaCl is obviously impaired, and the cell wall share in the biomass of *W. ichthyophaga* is lower than at higher salinities. Therefore, we speculate that the cell wall strengthening is linked to the successful growth of *W. ichthyophaga* at high salinities.

The "salt-in" strategy of osmoadaptation, employed by the extremely halophilic archaea and certain halophilic bacteria, involves the accumulation of a molar concentration of potassium and chloride, and it also requires a specific adaptation of the intracellular enzymatic machinery. Microorganisms using this strategy generally cannot survive in low-salt media (44). Since *W. ichthyophaga* requires a substantial amount of NaCl for growth, a trait frequently found in prokaryotes but exceptional in eukaryotes, and furthermore, it is also a phylogenetic maverick in the phylum Basidiomycota, we assumed that *W. ichthyophaga* might accumulate large amounts of cations (the "salt-in" strategy of osmoadaptation) to maintain positive turgor pressure at high salinity. The present study was performed in the same manner as the previous study, with measurements of cations in the cells of ecophysiologically different fungal species: the extremely halotolerant *H. werneckii* and the halotolerant *A. pullulans* and *D. hansenii* (14). Therefore, direct comparison of the results is possible. In all three previously studied fungal representatives, *H. werneckii*, *A. pullulans*, and *D. hansenii*, the K^+/Na^+ ratio decreased with in-

creasing salinity (Fig. 5C) (14). As expected, in *W. ichthyophaga* at higher NaCl concentrations, the K^+/Na^+ ratio also decreased, but the growth of the organism improved. It must also be noted that *W. ichthyophaga* kept the K^+/Na^+ ratio higher than the other three fungi over the whole salinity range of growth (Fig. 5C). In *W. ichthyophaga*, the lowest K^+/Na^+ ratio was calculated in the cells grown at 20% NaCl. Under those conditions, the specific growth rate was near the optimum and the biomass was highest (Fig. 2D). On the other hand, the highest K^+/Na^+ ratio was calculated for the cells grown at 10% NaCl, where the growth was slowest and the biomass production lowest. This is in contrast to what happens in *S. cerevisiae* or in halotolerant fungi, and it implies that higher Na^+ than K^+ levels are somehow favorable for growth of *W. ichthyophaga*. It could thus be an Na^+ includer organism, similar to *D. hansenii* (18) or a number of basidiomycetous yeasts, when osmotically stressed (6). However, *W. ichthyophaga* is unique in its better growth performance when the intracellular Na^+ content exceeds that of K^+. When *W. ichthyophaga* was exposed to a major hyperosmotic shock from 15% NaCl to 25% NaCl, the contents of K^+ and Na^+ increased and the amounts of cations far exceeded the amounts measured in the *W. ichthyophaga* cells grown in the media with a steady 25% NaCl. However, at the end of the experiment, the established K^+/Na^+ ratio was the same as the one measured at steady 25% NaCl.

The recent genome analysis of *W. ichthyophaga* revealed the relatively low numbers of alkali metal transporters (4) compared to those found in the genome of the extremely adaptable *H. werneckii* (45). The increase of both sodium and potassium contents in the cells of *W. ichthyophaga* after hyperosmotic shock is in accordance with the transcription data: the small number of transporter-encoding genes is transcriptionally unresponsive to high salinity and shows low levels of expression (4). At steady NaCl concentrations, the cation content in *W. ichthyophaga* is small and more or less constant compared to the major decreases of the intracellular K^+/Na^+ ratio across the salinity range in other fungi (Fig. 5C).

In environments with high concentrations of salt, fungi mainly employ the "compatible-solute strategy," i.e., they exclude salt from their cytoplasm and biosynthesize and/or accumulate a variety of organic molecules compatible with cellular functions to maintain the positive turgor pressure required for growth (44). In the cell extracts of *W. ichthyophaga*, a mixture of polyols was also detected. Glycerol, unsurprisingly, was found in the largest amounts and arabitol in smaller amounts, and only trace amounts of mannitol were measured. In accordance with this, the key enzyme for glycerol biosynthesis, glycerol-3-phosphate dehydrogenase (Gpd1), was previously shown to be salt inducible and therefore contributed to the osmoadaptation of *W. ichthyophaga* (23). The composition of the polyol pool did not change according to the growth phase (log versus stationary phase), as was previously shown for halophilic eubacteria (46) and the black yeast *H. werneckii* (17), but the size of the polyol pool did. In the stationary growth phase, smaller amounts of polyols than in the exponential growth phase were measured, probably due to cell death and/or substrate depletion. We have preliminarily measured (data not shown) the cation content in the stationary growth phase to see whether the low polyol content was due to the higher cation content. However, the sodium and potassium contents were even lower in the stationary growth phase than in the log phase. Glycerol is the main compatible solute of *W. ichthyophaga*, since its amounts are the largest of all the solutes, and its content increased with increasing salinity. This is in accordance with previous studies on halotolerant ascomycetous (17) and basidiomycetous (6) yeasts. Besides glycerol, which is energetically the cheapest (11), other polyols (erythritol, inositol, arabitol, xylitol, and mannitol) and various other compatible solutes are found among fungal representatives (17, 47–49). The cells of *W. ichthyophaga* exposed to a sudden hypo-osmotic shock responded by reducing the amounts of glycerol and arabitol. The balancing of the sudden change in osmotic pressure occurred primarily due to the expulsion of glycerol, confirming the role of glycerol as the main compatible solute. From the molecular perspective, reduced levels of mRNA of *GPD1* in *W. ichthyophaga* after the hypo-osmotic shock were observed (23). Also, in the recently assembled genome of *W. ichthyophaga* (4), homologues of key enzymes for the biosynthesis of the three polyols, as well as for the glycerol proton symporters for active accumulation of glycerol (50) and aquaglyceroporin-related channels for quick glycerol expulsion (51, 52), were found.

To sum up, the optimal salinity range for the growth of *W. ichthyophaga* is the highest (from 15% to 20% NaCl) ever described for a eukaryotic organism. In this salinity range, the growth rates and the biomasses are the highest. Instead of determining the dry biomass production over time, the growth progression of this meristematically growing fungus is easily monitored by measurement of the pH of the growth medium. This improves the reproducibility of the experiments involving cultivation to a certain growth point, since the optical density measurements usually employed are useless because of the specific growth form of *W. ichthyophaga*. According to our results, *W. ichthyophaga* is well adapted to life at salinities above 10% NaCl. This study also revealed that the osmoadaptation strategy of *W. ichthyophaga* involves the accumulation of glycerol as the main compatible solute and that the levels of sodium and potassium also change with changing salinity. However, the cell wall thickening at high salinities is a unique adaptation of *W. ichthyophaga*, as the thickened cell wall represents the highest proportion of the fungal biomass so far reported. Although *W. ichthyophaga* is an obligate halophile, which is exceptional in the whole fungal kingdom, its basic strategy of osmoadaptation with glycerol and fine tuning with cations is similar to those of other halophilic and halotolerant fungi studied so far. Our experimental results, coupled with the standardization of cultivation and with the available genomic resource for *W. ichthyophaga*, are expected to accelerate research into the osmoadaptation of this special fungus.

ACKNOWLEDGMENTS

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2.1.2 Prilagoditev glicerol-3-fosfat dehidrogenaze Gpd1 na visoko slanost pri izjemno halotolerantni glivi *Hortaea werneckii* in halofilni glivi *Wallemia ichthyophaga*

Naslov v originalnem jeziku: Adaptation of the glycerol-3-phosphate dehydrogenase Gpd1 to high salinities in the extremely halotolerant *Hortaea werneckii* and halophilic *Wallemia ichthyophaga*

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Izvleček
Identificirali in okarakterizirali smo glicerol-3-fosfat dehidrogenazne (*GPD1*) gene iz izjemno halotolerantne (*Hortaea werneckii*) in halofilne (*Wallemia ichthyophaga*) glive. Črna kvasovka *H. werneckii* in nemelanizirana bazidiomicetna gliva *W. ichthyophaga* naseljujeta podobna slana okolja, vendar imata dve različni strategiji haloadaptacije na nivoju sinteze glicerola preko Gpd1. Izjemno halotolerantna *H. werneckii* ima dva na sol odzivna gena *GPD1*, ki kažeta podobno regulacijo genskega prepisovanja in imata 98 % identičnost aminokislinskega zaporedja. Kjub temu imata različne učinke, ko sta heterologno izražena v *gpd* mutantah kvasovke *Saccharomyces cerevisiae*. Le HwGpd1B izooblika dopolnjuje funkcijo glicerol-3-fosfat dehidrogenaze v *gpd1* mutanti, nobena od HwGpd1 izooblik pa ne reši osmotske občutljivosti *gpd1gpd2* dvojnih mutant. Obligatno halofilna *W. ichthyophaga* ima le en *GPD1* paralog, katerega prepisovanje je v primerjavi s homologi *H. werneckii* v manjši meri odvisno od soli. Heterologno izražanje *WiGPD1* v *S. cerevisiae* povrne osmotoleranco tako *gpd1* kot tudi *gpd1gpd2* mutiranih sevov, kar je verjetno posledica visoke splošne aminokislinske podobnosti Gpd1 proteinov pri *W. ichthyophaga* in *S. cerevisiae*. Filogenetska analiza aminokislinskih zaporedij razkriva, da evolucijski izvor vseh treh novih encimov ustreza filogeniji glivnih vrst, iz katerih so bili določeni geni.
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Adaptation of the glycerol-3-phosphate dehydrogenase Gpd1 to high salinities in the extremely halotolerant *Hortaea werneckii* and halophilic *Wallemia ichthyophaga*

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ABSTRACT

We report the first identification and characterisation of the glycerol-3-phosphate dehydrogenase (GPD) genes from extremely halophilic fungi. The black ascomycetous yeast *Hortaea werneckii* and the non-melanised basidiomycetous fungus *Wallemia ichthyophaga* inhabit similar hypersaline environments, yet they have two different strategies of haloadaptation through Gpd1-regulated glycerol synthesis. The extremely halotolerant *H. werneckii* codes for two salt-inducible *GPD1* genes that show similar gene transcription regulation and have 98 % amino-acid sequence identity between paralogues; however, they have distinct effects when expressed heterologously in *Saccharomyces cerevisiae gpd* mutants. Only the HwGpd1B isoform complements the function of Gpd in the *gpd1* mutant, whereas none of the Gpd1 isoforms can rescue the salt sensitivity of the *gpd1gpd2* double mutant. The obligate halophile *W. ichthyophaga* codes for only one *GPD1* orthologue, the transcription of which is less affected by salt when compared to the *H. werneckii* homologues. Heterologous expression of *WiGPD1* in *S. cerevisiae* recovers halotolerance of the *gpd1* and *gpd1gpd2* mutant strains, which is probably due to the overall high amino-acid similarity of the Gpd1 protein in *W. ichthyophaga* and *S. cerevisiae*. Phylogenetic analysis of amino-acid sequences reveals that the evolutionary origins of all of these three novel enzymes correspond to the phylogeny of the fungal species from which the genes were identified.

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Introduction

The extremely halotolerant *Hortaea werneckii* is a black yeast from the ascomycetous order Capnodiales, whereas the halophilic *Wallemia ichthyophaga* is a non-melanised basidiomycetous fungus from the xerophilic order Wallemiales. They were both first isolated from the Sečovlje solar salterns (Gunde-Cimerman *et al.* 2000), an environment that is

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Abbreviations: aLRT, approximate likelihood ratio test; BLAST, basic local alignment search tool; GPD, glycerol-3-phosphate dehydrogenase; HOG, high osmolarity glycerol; MAPK, mitogen-activated protein kinase; NAD(P)(H), nicotinamide adenine dinucleotide (phosphate) (reduced); OD_{600}, optical density at 600 nm; ORF, open-reading frame; PCR, polymerase chain reaction; PTS2, peroxisomal targeting sequence; SSC, saline-sodium citrate buffer; UV, ultraviolet; YNB, yeast nitrogen base; YPD, yeast extract, peptone, dextrose

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characterised by high concentrations of NaCl, occasional rapid changes in water activity, low oxygen concentrations, and high UV radiation (Anton *et al.* 2000). *Hortaea werneckii* is the most salt-adaptable fungus known, as it can grow without NaCl and in almost saturated NaCl solutions. On the other hand, *W. ichthyophaga* has an obligate halophilic character, as it grows only in media with NaCl above 1.7 M (Kralj Kunčič *et al.* 2010; Zalar *et al.* 2005), which is a rare exception in the fungal kingdom. Thus, both of these species represent unique model organisms for studies of eukaryotic halophiles.

One of the most important adaptations of fungi to low water activity is the accumulation of different compatible solutes, among which glycerol is the most significant. The metabolism of glycerol has an important role in controlling and balancing the cellular metabolism in *Saccharomyces cerevisiae* (Hohmann 2002; Nevoigt & Stahl 1997). Glycerol is produced from the glycolytic intermediate dihydroxyacetone phosphate through two reaction steps catalysed by nicotinamide adenine dinucleotide (NAD)-dependent glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp) (Albertyn *et al.* 1994; Norbeck *et al.* 1996). The reverse pathway is controlled by two enzymes, glycerol dehydrogenase and dihydroxyacetone kinase, which appear to be involved in glycerol degradation (Norbeck & Blomberg 1997). This cycle might be important for conversion of NADH to nicotinamide adenine dinucleotide (phosphate) (reduced) (NADPH), which helps yeast to combat reactive oxygen species when under stress (Hohmann 2002). Glycerol anabolic pathways also have a role in redox balancing, as they reoxidise excess NADH under anaerobic conditions (Ansell *et al.* 1997). The key enzyme in this process is Gpd2, the expression of which is stimulated under anaerobic or microaerobic conditions (Hohmann 2002). Glycerol is also an important precursor for phospholipid biosynthesis (Daum *et al.* 1998). Thus, it appears that these different metabolic fates of glycerol must be tightly regulated for a cell to survive diverse stress conditions.

To date, the most studied function of glycerol in *S. cerevisiae* is its intracellular accumulation as a response to osmotic shock. This is the result of either the *de novo* expression of the Gpd1 enzyme and the corresponding increased enzyme activity (Andre *et al.* 1991; Blomberg & Adler 1989) or the reversed transport of glycerol into the cell by the membrane-protein channels (Hohmann 2002). As Gpd1 has a rate-limiting role in the flux from glucose to glycerol (Nevoigt & Stahl 1996), an increased activity of Gpd1 is sufficient to increase glycerol production. The induced transcription of the *GPD1* gene is triggered by the extracellular osmolarity, through the high osmolarity glycerol (HOG) pathway (Albertyn *et al.* 1994; Yale & Bohnert 2001).

Glycerol accumulation as the main compatible solute is also one of the main responses of both the extremely halotolerant *H. werneckii* and the halophilic *W. ichthyophaga* to increased extracellular salinity (Kogej *et al.* 2007; Petrovič *et al.* 2002; our unpublished data). In *H. werneckii*, the glycerol concentrations correlate with increases in salinity, with concentrations of up to 1.5 M NaCl inducing robust glycerol accumulation, whereas at higher salinities, intracellular glycerol increases only slightly. This is probably due to rearrangements of the melanin granules on the outer parts of the cell wall of *H. werneckii*, which at lower salinities form a distinct layer, thereby reducing the permeability of cell wall to glycerol. At higher salinities, melanisation is reduced, resulting in the escape of glycerol from the cell (Kogej *et al.* 2007).

As indicated above, the pathway connecting changes in extracellular salinity to intracellular glycerol accumulation in *H. werneckii* is the HOG pathway (Vaupotič & Plemenitaš 2007), for which many components have already been identified (Lenassi & Plemenitaš 2007; Lenassi *et al.* 2007; Turk & Plemenitaš 2002; our unpublished data). The key protein in the salt dependency of the *H. werneckii GPD1* homologue is the mitogen-activated protein kinase (MAPK) HwHog1, which associates with its promoter and induces gene expression when cells are shifted from 3.0 M to 4.5 M NaCl (Vaupotič & Plemenitaš 2007). HwHog1 is itself also regulated by environmental salinity, as it shows maximal transcript numbers at the optimum salinity for *H. werneckii* (1 M) and at the extreme salinity of 4.5 M (Lenassi *et al.* 2007), and full kinase activity only at extremely high salt concentrations (Turk & Plemenitaš 2002). In contrast to *H. werneckii*, where responses to osmotic stress have been extensively studied, studies of the HOG pathway in *W. icthyophaga* are only at an early stage.

In the present study, we have identified the *H. werneckii* and *W. ichthyophaga* homologues of the *GPD1* gene, which encodes Gpd, an enzyme that is crucial for the synthesis of glycerol. We have examined their salt-dependent expression and their functional involvement in glycerol production in *GPD1* and *GPD1/GPD2* deficient *S. cerevisiae* strains. Our data show significant differences in the Gpd responses not only between *H. werneckii* and *W. ichthyophaga*, but also compared to the salt-sensitive *S. cerevisiae*. These differences appear to contribute to the diverse salt responses of these three fungal species.

Materials and methods

Strains and growth conditions

Hortaea werneckii (EXF 225; MZKI B-736) and *W. ichthyophaga* (EXF 994) used in this study were isolated from extremely saline water of Sečovlje solar saltern and preserved in the culture collections of the Department of Biology, Biotechnical Faculty, University of Ljubljana (EXF) and the Slovenian National Institute of Chemistry (MZKI). The wild-type *Saccharomyces cerevisiae* BY4741 (*MATa his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and the *S. cerevisiae GPD1* deletion mutant (BY4741; *YDL022w::kanMX4*) strains were obtained from the Euroscarf Yeast Deletion Strain Collection, Frankfurt, Germany. The *S. cerevisiae gpd1gpd2* double-mutant (W303-1A; *gpd1Δ::TRP1; gpd2Δ::URA3*) strain was derived from W303-1A (*MATa leu2-3/112; ura3-1; trp1-1; his3-11/15; ade2-1; can1-100; GAL; SUC2*), and was kindly provided by Dr. Johan Thevelein, VIB Institute, Belgium.

The cells were grown at 28 °C in a rotary shaker (180 rpm) in defined YNB medium of 0.17 % (w/v) yeast nitrogen base (YNB), 0.08 % (w/v) complete supplement mixture (both Qbiogene), 0.5 % (w/v) ammonium sulphate, 2.0 % (w/v) glucose in deionised water, with NaCl added to different concentrations, and pH adjusted to 7.0. The cells were harvested in the mid-exponential growth phase by 10 min centrifugation at $4000 \times g$. For hypersaline stress, *H. werneckii* cells were grown

in YNB medium with 1 M NaCl to an optical density at 600 nm (OD_{600}) of 1.0, harvested, and then resuspended in YNB medium with 3 M NaCl. For hyposaline stress, cells grown in YNB medium with 3 M NaCl were resuspended in YNB medium without NaCl. In the case of *W. ichthyophaga*, the cells were initially grown in YNB medium with 1.8 M or 4.5 M NaCl. They were then harvested in the mid-exponential growth phase and subjected to saline stress by resuspension in YNB medium with 4.5 M NaCl or 1.8 M NaCl, respectively. Aliquots of cell suspension were removed at indicated times before and after the stress. The cells were separated from the growth medium by fast filtration through a 0.45 μm-pore filter and frozen in liquid nitrogen.

DNA and RNA isolation

Highly purified fungal genomic DNA was isolated from mid-exponential-phase cells grown in YNB media with 15 % (w/v) NaCl salt by the phenol/chloroform/isoamyl alcohol method, modified for DNA isolation from filamentous fungi, as described previously (Rozman & Komel 1994). The total RNA content of the *Hortaea werneckii* and *Wallemia ichthyophaga* cells was isolated using the TRI Reagent (Sigma–Aldrich), according to the manufacturer instructions. The RNA was extracted from mid-exponential-phase cells grown in YNB medium with the indicated NaCl concentrations, or from cells exposed to hypersaline and hyposaline shock. Possible DNA contamination was degraded with DNAse I (Fermentas), and the integrity and purity of the RNA was evaluated spectrophotometrically and by capillary electrophoresis (Agilent 2100 Bioanalyser). The cDNA from total *W. ichthyophaga* RNA was synthesised using RevertAid™ H Minus First-Strand cDNA Synthesis kits (Fermentas).

Cloning and sequencing of GPD1 genes from the genomes of H. werneckii and W. ichthyophaga

A partial sequence of the *GPD1* gene from *Hortaea werneckii* had already been described in a previous study (Petrovič *et al.* 2002). For *Wallemia ichthyophaga*, a partial sequence of the *GPD1* gene was discovered in a suppression subtractive hybridisation study (our unpublished data). The complete sequences of the genes were obtained using GenomeWalker™ Universal kit and SMARTer™ RACE cDNA Amplification kit (both Clontech Laboratories), according to the manufacturer instructions. The nucleotide sequences have been deposited in the EMBL-Bank and GenBank under the following accession numbers: *WiGPD1* [FR686467], *HwGPD1A* [HQ188302] and *HwGPD1B* [HQ188303].

Real-time polymerase chain reaction (PCR)

Approximately 100 ng cDNA was used as template for real-time PCR with the following gene-specific oligonucleotides: 5′-GCACGGCGTATGAGGTTAAT-3′ and 5′-ACTTGCCCTC CAAAATATTA-3′ for *HwGPD1A*; 5′-GGTACGGCCTACGAGGT CAA-3′ and 5′-GTACTTGCCCTCGAGGATCAA-3′ for *HwGPD1B*; and 5′-CCATCGCTTGTGGTTTCAACGA-3′ and 5′-TGCAGGTGT TGCCGATCTCATT-3′ for *WiGPD1*. The thermal profile for the reaction was the following: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 59 °C and 30 s at 72 °C, followed by a dissociation curve. The reaction mix was prepared using the Power SYBR® Green PCR Master Mix (Applied Biosystems), according to the manufacturer instructions. The reactions were performed in an ABI 7900 real-time PCR instrument, and analysed with Sequence Detection Systems 2.2.2 software (Applied Biosystems). The expression values of the *GPD1* genes were standardised to the amount of the 28S rRNA gene fragment, the expression of which remains unchanged under these different environmental conditions (Lanišnik Rižner *et al.* 1999). The optimal final concentration of primers used in the real-time PCR was found to be 300 nM, at which none of them formed observable dimers.

Southern blotting

To design the probe, a 360 bp fragment of the *Hortaea werneckii GPD1* gene (Petrovič *et al.* 2002) was labelled with [^{32}P]dCTP using Prime-it random primer labelling kits (Stratagene), according to the manufacturer instructions. For Southern blotting, 15 μg high purity genomic DNA was digested with the restriction enzymes EcoRI and HindIII (New England Biolabs), followed by electrophoresis on a 1 % agarose gel. The Southern blot transfer and the hybridisation were processed as previously described (Turk & Plemenitaš 2002).

A 402 bp *WiGPD1* product was amplified from genomic DNA of *Wallemia ichthyophaga* by PCR using the primers 5′-TTGAAGACGCAACACTCCTG-3′ and 5′-TACCGTCGTT GAAACCACAA-3′. The probe was labelled using Biotin DecaLabel™ DNA Labelling kits (Fermentas), based on the random-oligonucleotide DNA labelling method. Highly purified genomic DNA (30 μg) was digested with the restriction enzymes EcoRI, BamHI and HindIII, and a combination of EcoRI and HindIII (Fermentas), and followed by electrophoresis on 1 % agarose gels. Nucleic acids were transferred to a positive nylon membrane (Tropilon-Plus™ Membrane; Applied Biosystems) by capillary blotting with 10× saline-sodium citrate buffer (SSC), and UV-cross-linked to the membranes. Hybridisation and chemiluminescent detection of the probe DNA were carried out according to the manufacturer protocols (Southern-Star™ Systems, Applied Biosystems), under low stringency conditions.

Construction of the recombinant vector

For expression of the *Hortaea werneckii GPD1* homologues in *Saccharomyces cerevisiae*, the corresponding open-reading frames (ORFs) were amplified from *H. werneckii* cDNA using the primers 5′-ATAGGATCCATGGCCTCGCTCTCCGCCCACT-3′ and 5′-CCGAAGCTTTTAATCCTTCTGCTCAATCAA-3′ for *HwGPD1A* and 5′-ATAGGATCCATGGCCCCGCTATCCACC CACT-3′ and 5′-CCGAAGCTTTTAATCTTTCTGCTCAATCAG-3′ for *HwGPD1B*, containing BamHI and HindIII restriction sites (underlined), respectively. The resulting products were cloned into BamHI/HindIII sites of the low-copy-number plasmid pRD53 (*CEN*, *ARS*, *URA3*, GAL1/10 promoter, *Amp*^{R}), providing in the plasmids pRD53-HwGPD1A and pRD53-HwGPD1B. The cloned HwGPD1A/B sequences were verified by sequencing.

The pYX142 shuttle vector with Leu selection marker and triosephosphate isomerase (TPI) promoter was used as the expression vector for *WiGPD1* (kindly provided by Dr. Stefan

Hohmann, Göteborg University, Sweden). The ORF of Wi*GPD1* was prepared by touchdown PCR using *Wallemia ichthyophaga* cDNA as template, Phusion™ Hot Start High-Fidelity DNA Polymerase (Finnzymes), and the following primers: 5′-CGAATTCATGGTTAAAGAATCTGTTGCAGT-3′ and 5′-CGGATCCTTAAAGTCTAGAAGTAAGCTCCTTAGCA-3′, containing EcoRI and BamHI restriction sites (underlined), respectively. The resulting 1029 bp PCR fragment from the pJET1.2/blunt cloning vector (Fermentas) was subcloned into EcoRI/BamHI sites of the pYX142 plasmid, resulting in the pYX142-Wi*GPD1* construct. The correct orientation was verified by sequencing and by restriction analysis with EcoRI and BamHI.

Functional expression of GPD1 homologous in S. cerevisiae and salt tolerance assays

Yeast cells were grown in yeast extract, peptone, dextrose (YPD) medium (1 % yeast extract, 2 % peptone, 2 % glucose; pH 7.0) at 28 °C and 180 rpm, to mid-exponential growth phase, and then transformed with 1 μg pRD53-HwGPD1A, pRD-HwGPD1B or pYX142-WiGPD1 plasmids, using Alkali-Cation Yeast Transformation kits (Qbiogene). Transformants were selected on YNB-Ura or YNB-Leu plates (Formedium). Positive colonies were grown in YNB-Ura or YNB-Leu medium to mid-exponential phase, and adjusted to an OD_{600} of 0.5 for stress-tolerance assays. Tenfold serial dilutions (10^0–10^4) of the transformants with the corresponding media were prepared and spotted in 3 μl aliquots onto YNB-Ura or YNB-Leu plates supplemented with the indicated NaCl concentrations. The plates were incubated at 30 °C for 3–5 d, and then scanned.

Measurement of glycerol content

Wild-type (W303-1A and BY4741) and mutant (*gpd1* and *gpd1gpd2*) cells transformed with either empty plasmid (pRD53 and pYX142) or plasmid containing *HwGPD1A*, *HwGPD1B* and *WiGPD1* were grown in YNB-Ura or YNB-Leu medium to mid-exponential phase, harvested and resuspended in the respective medium supplemented with 0.5 M NaCl. After a 3 h incubation at 30 °C and 180 rpm, the total glycerol concentration of the samples was determined as described previously (Larsson *et al.* 1990), using Free Glycerol Reagent (Sigma–Aldrich). All of the measurements were performed in duplicate.

Sequence and structure analysis

Sequence similarity searches were conducted using the BLAST algorithm on the non-redundant protein database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The ExPASy ProtParam programme (http://www.expasy.ch/tools/protparam.html) was used to analyse the physicochemical parameters of the amino-acid sequences. Sequence alignment was performed with ClustalW (www.ebi.ac.uk/clustalw/) (Chenna *et al.* 2003) using the default settings. Domain definitions were retrieved using InterProScan (http://www.ebi.ac.uk/InterProScan/) (Zdobnov & Apweiler 2001).

Gene phylogeny reconstruction

Homologues of the Gpds were identified by protein–protein BLAST searches (Altschul *et al.* 1997) against a GenBank non-redundant protein database, with E values <0.01. Representative protein sequences were aligned using the L-INS-i method in the MAFFT software (Katoh *et al.* 2005; Katoh & Toh 2008). The gene trees were generated with MrBayes software, applying Bayesian inference (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Three parallel runs were performed for one million generations with mixed amino-acid models, the default temperature, and six chains. The trees were sampled every 100 generations. The trees were sampled before the analysis reached stationarity of likelihood values, and those sampled before the average standard deviation (SD) of the split frequencies reduced below 1 % were excluded from the final analysis. The stationarity of the likelihood values was checked using the Tracer software (Rambaut & Drummond 2007). A second set of trees was generated with the PhyML software (Guindon & Gascuel 2003) with aLRT implementation, for the calculation of branch supports as a minimum of SH-like and Chi^2 based support (Anisimova & Gascuel 2006) (data not shown). The analyses were run using a WAG model of amino-acid substitution, the optimised proportion of invariable sites, and the six substitution rate categories with an optimised gamma distribution parameter.

Results

The GPD1 gene homologue has two copies in the genome of H. werneckii and one in the genome of W. ichthyophaga

Given that many genes associated with adaptation to life in hypersaline environments exist in two copies in the genome of *Hortaea werneckii* (Gunde-Cimerman & Plemenitaš 2006), and given the lack of any such data in the case of *Wallemia*

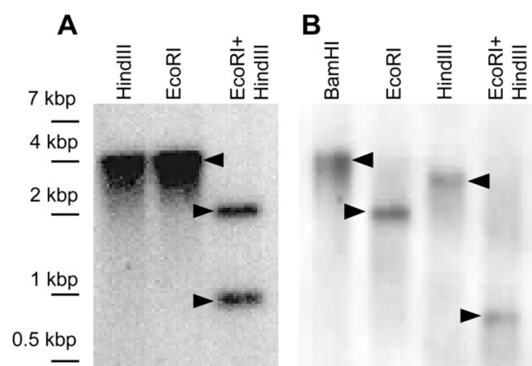

Fig 1 – Determination of the *GPD1* gene copies in *Hortaea werneckii* (A) and *Wallemia ichthyophaga* (B). Southern blotting of different restriction enzyme digests (EcoRI, HindIII, BamHI and EcoRI + HindIII; as indicated) of the genomic DNA with radio-labelled (A) and biotin-labelled (B) probes specific for the *GPD1* gene. None of the endonucleases used have the cutting site within the probe sequence.

ichthyophaga, we assessed the *GPD1* gene-copy number by the Southern blotting of the digested genomic DNA. The partial sequence of a *H. werneckii GPD1* gene homologue was obtained previously by PCR (Petrovič et al. 2002), while the *WiGPD1* gene fragment was detected by suppression subtractive hybridisation with mRNA populations from *W. ichthyophaga* grown at 1.8 M and 4.5 M NaCl. Hybridisation under low stringency conditions was performed using a 360 bp, [^{32}P]dCTP-labelled fragment of the *HwGPD1* gene (Petrovič et al. 2002), and a 402 bp, biotin-labelled *WiGPD1* fragment as probes. All of the selected restriction endonucleases had their cleavage sites outside the probe sequence.

Although only one band appeared after digestion of genomic DNA of *H. werneckii* with EcoRI and HindIII, the digestion with the combination of both restriction endonucleases gave two visible bands of different sizes, suggesting that the *GPD1* gene has at least two copies in the genome of *H. werneckii* (Fig 1A). When the genomic DNA of *W. ichthyophaga* was digested with BamHI, EcoRI, HindIII and EcoRI + HindIII, Southern blotting revealed only single fragments with homology to the *GPD1*-derived probe (Fig 1B), which indicates that *W. ichthyophaga* has only one copy of the *GPD1* gene.

Cloning and sequencing of the GPD1 gene homologue from the genome of H. werneckii and W. ichthyophaga

To identify *GPD1* homologues in *Hortaea werneckii*, the partial sequence obtained in a previous study was used for the *H. werneckii* genome and cDNA library DNA walking. Analysis of the amplified products revealed two highly similar intron-less gene sequences, which were named *HwGPD1A* (GenBank [HQ188302]) and *HwGPD1B* (GenBank [HQ188303]). The ORFs of *HwGPD1A* and *HwGPD1B* contain 1281 bp, which encode proteins of 427 amino acids that differ in only nine-amino-acid residues (Fig 2, black arrows). HwGpd1A and HwGpd1B have predicted molecular weights of 46.35 kDa and 46.37 kDa, and theoretical isoelectric points 5.94 and 6.04. The BLAST search of HwGpd1A and HwGpd1B showed 65 % identity with Gpd1 from *Pyrenophora tritici-repentis* (XP_001933128.1), 63 % identity with the putative Gpd1 orthologue from *Neosartorya fischeri*

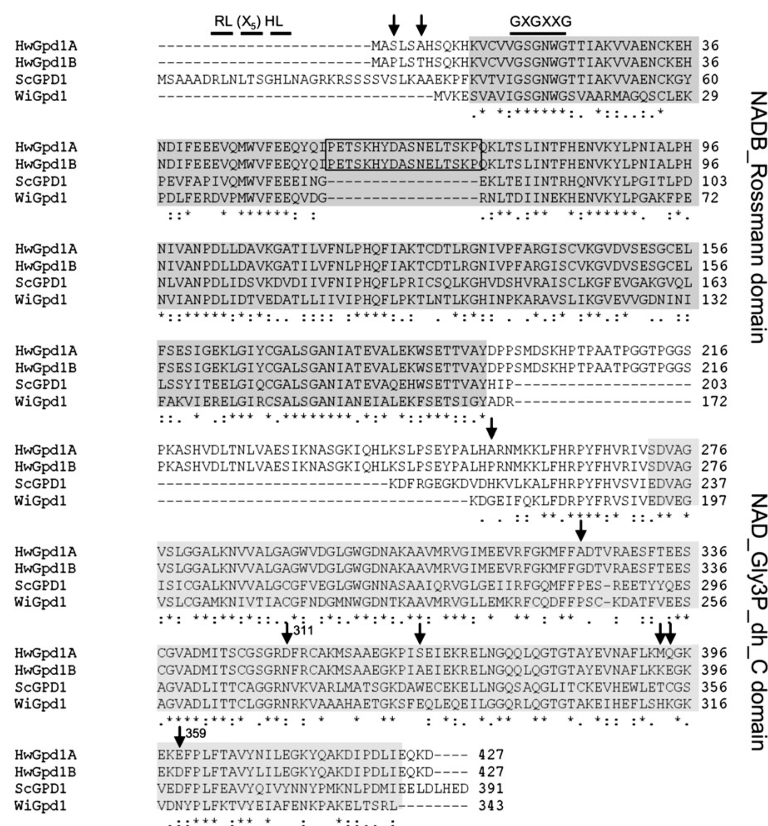

Fig 2 – Sequence alignment of *H. werneckii* and *W. ichthyophaga* Gpds. Amino-acid sequence alignment of HwGpd1A [HQ188302], HwGpd1B [HQ188303], WiGpd1 [FR686467] and ScGpd1 [X76859.1] in ClustalW. The consensus sequence RL (X_5) HL of the peroxisomal targeting signal type 2 (PTS2) is indicated by a dashed bar. The NADB_Rossmann domain [cl09931] is shaded in dark grey, and includes the consensus NAD(P)/NAD(P)H– binding pattern GXGXXG (indicated by a bar) and a framed amino-acid segment in HwGpd1A and HwGpd1B with no homology to WiGpd1 or ScGpd1. The catalytic NAD_Gly3P_dh_C domain [cl08454] is shaded in light grey. Black arrows indicate amino acids that differ between HwGpd1A and HwGpd1B, and the numbers indicate the residue positions.

NRRL 181 (XP_001265436.1), and 42 % identity with *Saccharomyces cerevisiae* Gpd1 (X76859.1).

Similarly, the sequence of the *Wallemia ichthyophaga GPD1* homologue was obtained. The *WiGPD1* gene sequence contains a 45-bp-long and a 49-bp-long intron at positions +35 and +87, respectively. The *WiGPD1* ORF (1029 bp) encodes a protein of 343 amino acids with a deduced molecular weight of 37.81 kDa and an isoelectric point of 5.45. The BLAST search of WiGpd1 showed 61 % identity with the *Ustilago maydis* hypothetical protein (UM00555.1) and 52 % identity with the *S. cerevisiae* Gpd1 orthologue (X76859.1).

Sequence alignment of HwGpd1A, HwGpd1B and WiGpd1 with ScGpd1 (X76859.1) (Fig 2) revealed two putative conserved domains: the N-terminal NAD(P)H/NAD(P)(+) binding Rossmann fold domain (Fig 2, NADB_Rossmann) with its universally conserved NAD(P)-binding motif GXGXXG, and the C-terminal substrate-binding domain (NAD_Gly3P_dh_C domain) that catalyses the interconversion of dihydroxyacetone phosphate and L-glycerol-3-phosphate. The consensus sequence GSGNWG was highly conserved in all four aligned amino-acid sequences, whereas a 17-amino-acids-long region inserted in the conserved NADB_Rossmann domain was only found in the *H. werneckii* orthologues, HwGpd1A and HwGpd1B. Interestingly, no N-terminal type 2 peroxisomal targeting sequence (PTS2) was found in the Gpd1 homologues of the halotolerant/halophilic fungi studied.

Expression of the HwGPD1A, HwGPD1B and WiGPD1 genes is salt dependent

The expression of *GPD1* is known to be regulated by the HOG pathway, which is activated by high environmental osmolarity, and results in glycerol accumulation in the cell (Albertyn et al. 1994; Yale & Bohnert 2001). Many previous studies on *Hortaea werneckii* have supported the dependence of *GPD1* expression on environmental salinity (Vaupotič & Plemenitaš 2007), therefore *H. werneckii* and *Wallemia ichthyophaga* cells were subjected to different salinities, hypersaline and hyposaline shock; and the *GPD1* gene transcript levels were analysed by real-time PCR.

The *HwGPD1* isogenes both showed salt-dependent expression (Fig 3A), but with slight differences. The transcript levels of *HwGPD1A* were comparable at salinities up to 3 M NaCl, with a 5-fold increase at 1.8 M NaCl. Similarly, the expression of *HwGPD1B* was not up-regulated in the 0 M to 3 M salinity range, with the exception of transient up-regulation at 1.0 M NaCl. Interestingly, a steep increase in mRNA levels of both of these paralogues was observed at 4.5 M NaCl, which reached almost 15-fold the basal level. A sudden shift of the cells from 0 M to 3 M NaCl (Fig 3A) led to 40 min and 60 min lag phases before induction of *HwGPD1B* and *HwGPD1A* transcription occurred, respectively. The mRNA level of both of these genes increased with time up to 120 min after the stress.

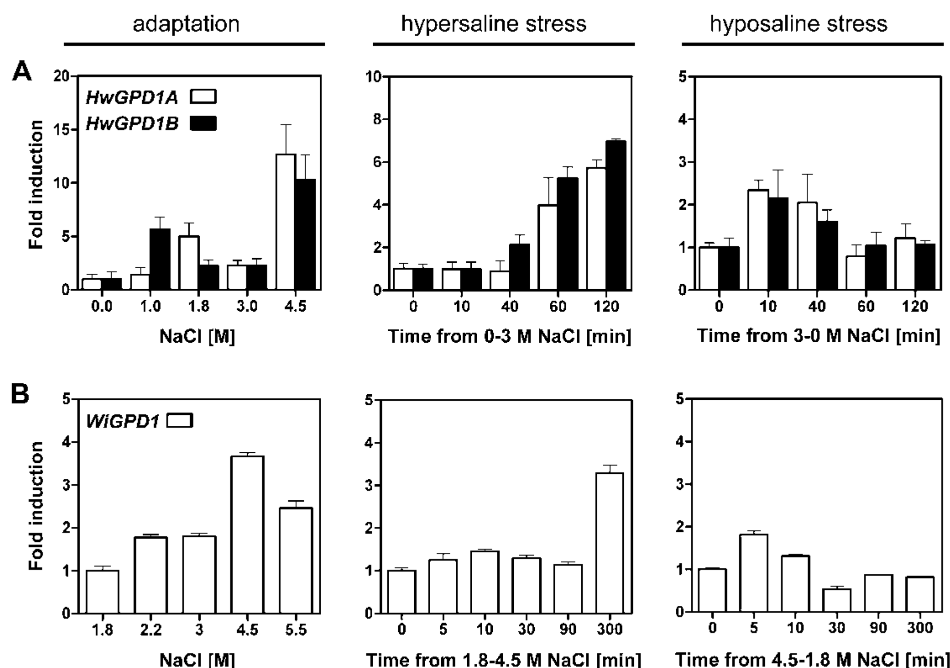

Fig 3 – *GPD1* homologue gene expression in adaptation and under stress conditions. (A) Expression analysis of *HwGPD1A* (white blocks) and *HwGPD1B* (black blocks) in salt-adapted (adaptation) and NaCl-stressed (hypersaline or hyposaline stress) *H. werneckii* cells at the indicated NaCl concentrations and times. The relative fold-inductions of *HwGPD1* isogenes on cDNA levels are presented as means (±SD) of two real-time experiments, each carried out in duplicate, relative to 0 M NaCl in adapted cells, or relative to the zero time in stressed cells. (B) Expression analysis of *WiGPD1* in *W. ichthyophaga* cells as for (A), except for the *WiGPD1* gene levels in the adapted cells presented relative to 1.8 M NaCl.

Hyposaline stress caused a sudden transient increase in expression of both *HwGPD1A* and *HwGPD1B*, which declined to the starting levels within 60 min.

As shown in Fig 3B, the level of *WiGPD1* mRNAs was lowest at 1.8 M NaCl, only slightly higher at 2.2 M and 3 M NaCl (1.8-fold induction in both cases), and highest at 4.5 M NaCl (3.7-fold induction), which indicated salt-dependent expression of *WiGPD1*. Interestingly, expression of *WiGPD1* at saturated (5.5 M) NaCl was only weakly (2.5-fold) induced. Hypersaline shock caused by a sudden shift from 1.8 M to 4.5 M NaCl led to late induction of *WiGPD1* expression, as 3.3-fold increase in *WiGPD1* mRNA levels was seen only 5 h after the shock. In the case of the hyposaline shock caused by a shift from 4.5 M to 1.8 M NaCl, *WiGPD1* mRNA levels transiently increased by 1.8-fold within 5 min, and then reduced by 0.5-fold 30 min after exposure.

HwGPD1 and WiGPD1 genes complement the osmoprotective role of Gpd1 in S. cerevisiae

To determine whether *HwGPD1A*, *HwGPD1B* and *WiGPD1* are functional homologues of *Saccharomyces cerevisiae GPD1*, we performed functional complementation assays. Wild-type BY4741 and W303-1A *S. cerevisiae* strains and the derived *GPD*-mutated strains were transformed with an empty plasmid or a plasmid carrying either *HwGPD1A*, *HwGPD1B* or *WiGPD1*. The growth patterns of the transformants were monitored on amino-acid-drop-out YNB media supplemented with the indicated NaCl concentrations and compared to growth on plates without NaCl (Fig 4).

The wild-type *S. cerevisiae* strain heterologously expressing *HwGPD1A* or *HwGPD1B* showed similar growth on plates with added NaCl compared to an empty-plasmid control (Fig 4A). The growth of the *gpd1* mutant was improved only by the expression of *HwGPD1B*, and not by *HwGPD1A*, which was best seen on plates with 0.8 M NaCl. Interestingly, expression of *HwGPD1A* or *HwGPD1B* in the *gpd1gpd2* double mutant did not rescue the salt sensitivity of the mutant strain. As can be seen in Fig 4B, there was no difference in growth of wild-type cells and wild-type cells expressing *WiGPD1*, whereas the *gpd1* and *gpd1gpd2* mutants transformed with *WiGPD1* both showed improved salt tolerance, compared to the strains transformed with the empty vector. The *gpd1* strain grew poorly on plates with 0.8 M NaCl and did not grow at 1 M NaCl unless it was transformed with *WiGPD1*. The double-mutant strain *gpd1gpd2* grew only on media without NaCl unless it was transformed with *WiGPD1*, following which it grew, even at NaCl concentrations as high as 1.5 M.

Under certain conditions, glycerol overproduction can result in a shortage of NADH for acetaldehyde reduction, which can result in cell-growth defects (Remize *et al.* 1999). To exclude acetaldehyde-induced growth inhibition of the *HwGPD1A*- or *HwGPD1B*-expressing *gpd1* and *gpd1gpd2* mutants, and to confirm the results from the functional complementation assays, we measured total glycerol contents in the wild-type and corresponding mutant strains transformed with the *GPD1* homologue genes (Fig 4C–F). In the wild-type strains transformed either with empty plasmid or plasmids containing *HwGPD1A*, *HwGPD1B* or *WiGPD1*, the glycerol concentrations were comparable (Fig 4C–F). Low glycerol production of the *gpd1* mutant was increased to the level of the wild type only when cells were expressing *HwGPD1B* or *WiGPD1* (Fig 4C, D), whereas the expression of *HwGPD1A* increased the glycerol levels only slightly (Fig 4C). The *S. cerevisiae gpd1gpd2* strain, which itself does not produce detectable glycerol (Albertyn *et al.* 1994; Ansell *et al.* 1997), reached wild-type glycerol levels when transformed with *WiGPD1*. This was not seen for the *HwGPD1A*- and *HwGPD1B*-expressing mutants, where the glycerol levels remained low. Taken together, *HwGpd1B* and *WiGpd1* rescued the salt sensitivity of the *gpd1* mutant, whereas only *WiGPD1* expression conferred salt tolerance to the *gpd1gpd2* double-mutant strain. In contrast, the growth of mutant strains expressing *HwGPD1A* was similar to the strains transformed with the empty plasmids.

Gene phylogeny reconstruction

The amino-acid sequences of the newly identified Gpds were compared with representative sequences of homologous genes in the databases, to position the *Gpd1* of *Hortaea werneckii* and *Wallemia ichthyophaga* in the fungal kingdom. Good convergence of the runs was reached when constructing the gene tree with MrBayes. The likelihood values reached a plateau after approximately 2000 generations, while the average SDs of the split frequencies declined below 1 % after approximately 10 000 generations. The first 100 trees were discarded as burn-in. The posterior probabilities for the amino-acid models were 1 for the WAG model (Whelan & Goldman 2001). The topologies of the trees constructed with MrBayes and PhyML were the same. The two deduced proteins from *H. werneckii* (HwGpd1A, HwGpd1B) shared the greatest similarities with homologous enzymes from fungi belonging to Pezizomycotina, while the protein from *W. ichthyophaga* (WiGpd1) was positioned between ascomycetes and basidiomycetes (Fig 5).

Discussion

In the present study, we have identified and characterised the homologues of *Saccharomyces cerevisiae* GPD from the extremely halotolerant *Hortaea werneckii* and the halophilic *Wallemia ichthyophaga*. Although both of these fungi inhabit similar hypersaline environments, they use two different strategies for adaptation of their Gpd1-regulated glycerol synthesis to extreme saline conditions. *Hortaea werneckii* codes for two salt-induced *GPD1* genes, with similar gene transcription regulation and with 98 % amino-acid sequence identity between these paralogues, while *W. ichthyophaga* has only one *GPD1* gene, the transcription of which is less affected by salt when compared to the *H. werneckii* homologues. The functionality of the newly isolated Gpds in the context of the *S. cerevisiae GPD* mutant background differed between the orthologues, as shown by the functional complementation and glycerol measurement studies. The HwGpd1B and WiGpd1 isoforms both complemented the Gpd1 function of the *gpd1* mutant, whereas only WiGpd1 rescued the salt tolerance of the *gpd1gpd2* double mutant. Interestingly, HwGpd1A did not complement the glycerol synthesis function in any of the *S. cerevisiae GPD* mutants. Phylogenetic analysis of amino-acid sequences revealed that HwGpd1A and HwGpd1B

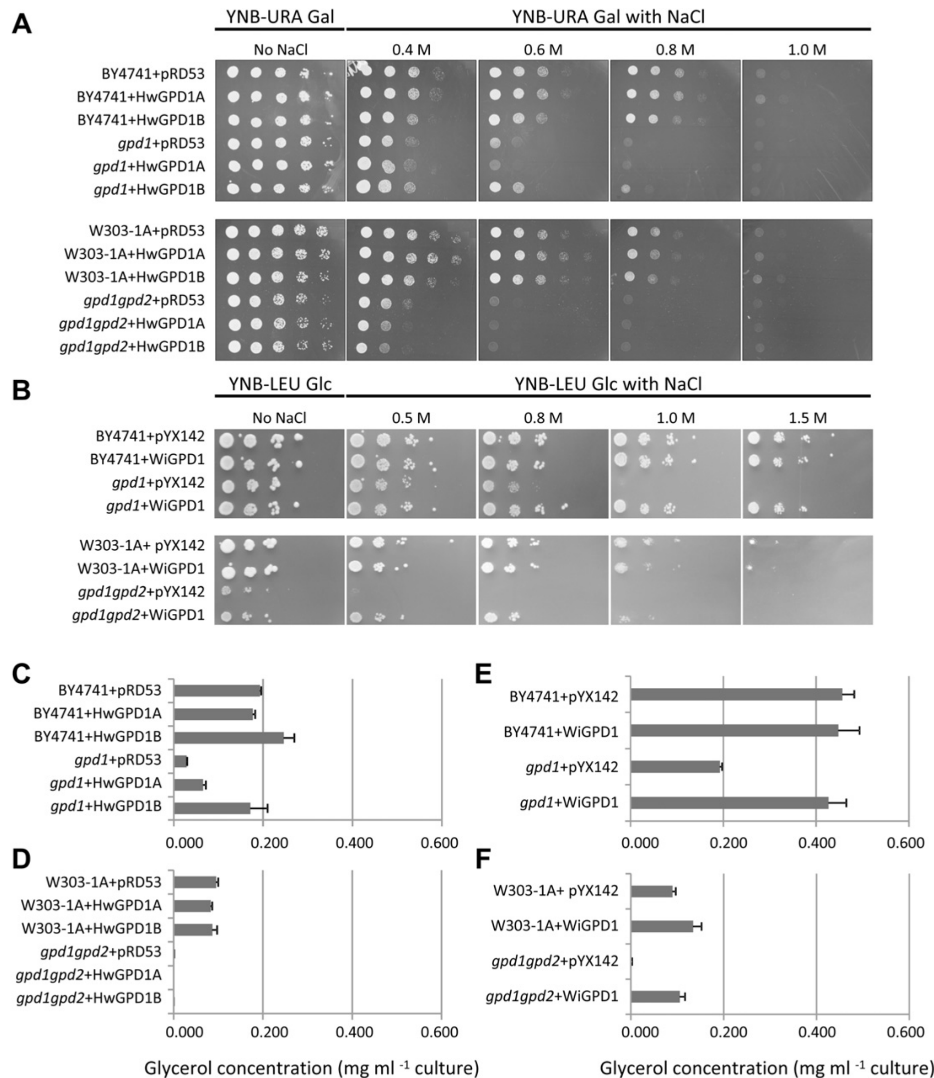

Fig 4 – Functional complementation of the *gpd1* and *gpd1gpd2* mutants with the Gpd1 homologues from *H. werneckii* (A, C, D) and *W. ichthyophaga* (B, E, F). (A, B) Tenfold serial dilutions of cultures of cells transformed with empty plasmid as control (pRD53 and pYX142) and plasmid carrying the indicated *GPD1* isogenes were spotted onto YNB-Ura Gal (A) and YNB-Leu Glc (B) plates without NaCl and supplemented with indicated concentrations of NaCl. (C–F) Quantification of the total glycerol concentrations for the wild-type BY4741 and *gpd1* mutant cells (C, E) and W303-1A wild-type cells and *gpd1gpd2* cells (D, E) transformed with either the empty plasmids (control) or the plasmids carrying the *GPD1* homologues from *H. werneckii* (C, D) and *W. ichthyophaga* (E, F). Glycerol was analysed in cultures incubated in YNB with 0.5 M NaCl. Data are means (±SD) of two experiments, each carried out in duplicate.

share the greatest similarity with homologous enzymes from fungi belonging to Pezizomycotina, while WiGpd1 was positioned between ascomycetes and basidiomycetes, which corresponds to the phylogeny of the fungal species.

The Gpd1 protein is essential to confer halotolerance by glycerol production and redox balancing in fungi. Its function is therefore conserved in many organisms that have been analysed for glycerol production to date (Chen *et al.* 2008; Fillinger *et al.* 2001; Furukawa *et al.* 2007; Lee *et al.* 2008; Peng *et al.* 2010; Thome 2004; Watanabe *et al.* 2008; Yan *et al.* 2008). So far, the most extensively studied are the *S. cerevisiae* Gpd1 (Albertyn *et al.* 1994) and Gpd2 enzymes (Eriksson *et al.* 1995), which have similar kinetic characteristics, although with differing specificities regarding cellular distribution and regulation of gene expression. Both are located in the cytosol, but Gpd1 can also be sequestered to peroxisomes, due to its PTS2

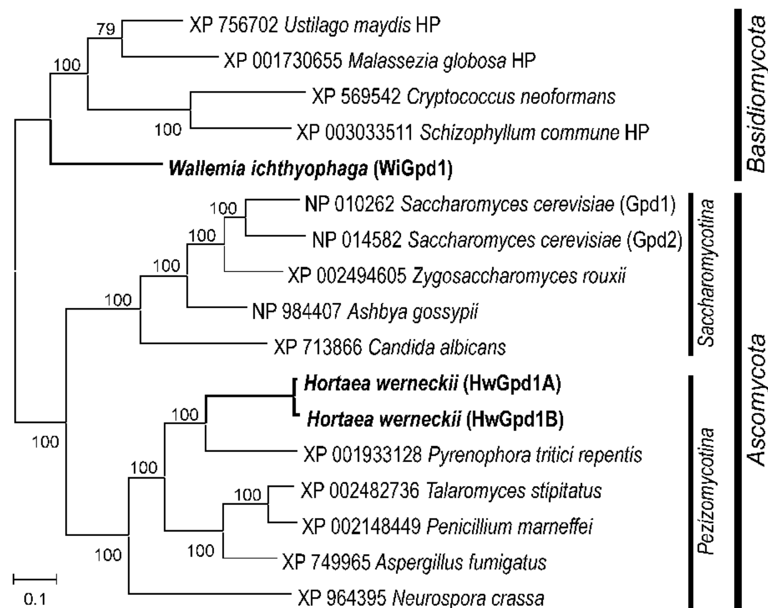

Fig 5 – Unrooted gene tree of the Gpd enzymes, constructed from alignment of the representative protein sequences retrieved from the GenBank database with the BLAST programme. Alignments were analysed with the MrBayes software, in three runs, with one million generations and six chains each. A mixed amino-acid model was used, and the first 1 % of trees was excluded from the final consensus tree. GenBank accession numbers of proteins are shown before the species names. Hypothetical proteins are marked with "HP".

peroxisome-targeting sequence, while Gpd2 is found partly in mitochondria in non-respiring cells (Jung *et al.* 2010; Valadi *et al.* 2004). Although Gpd2 can complement the Gpd1 function when overexpressed, expression of only Gpd1 is induced under hyperosmotic stress (Rep *et al.* 1999). Therefore, the Gpd1 isoform has the major role in osmoadaptation of wild-type *S. cerevisiae* cells.

While all of the studies on the Gpd1 enzymes to date have been performed with salt-sensitive or moderately halotolerant fungi, the present study is the first report of the Gpd1 homologues from fungi isolated from extremely saline environments. When comparing HwGpd1A, HwGpd1B and WiGpd1 to Gpd1 from *S. cerevisiae*, the N-terminal PTS2 sequence that is important for peroxisome localisation is lacking in all of the homologues isolated from both *H. werneckii* and *W. ichthyophaga*. The physiological role for Gpd1 in peroxisomes is probably linked to regeneration of NAD+ from the NADH produced by β-oxidation of fatty acids (Jung *et al.* 2010; Valadi *et al.* 2004), whereas its function in osmostress is dependent on the cytosol and nuclear fractions of Gpd1 (Jung *et al.* 2010). Thus, it appears that the constant cytosolic localisation of the Gpd1 homologues is beneficial for organisms living in extremely saline environments.

Apart from PTS2, there is good overall conservation of the amino-acid sequences of the conserved NAD(P)H+/NAD(P) binding and catalytic domains between the studied Gpd1 homologues. It was therefore surprising that only HwGpd1B and WiGpd1 rescued the salt sensitivity of the *gpd1* mutant strain, and WiGpd1 alone of the *gpd1gpd2* double mutant. The reason for these differences in the functioning of the *H. werneckii* and *W. ichthyophaga* Gpd1 homologues might be connected to the insertion of a 17-amino-acid segment into the conserved NAD(P)H+/NAD(P) binding domain of the *H. werneckii* paralogues. Similar amino-acid insertions in conserved protein domains have also been seen for other *H. werneckii* proteins that are important for salt tolerance, like HwHal2 (Vaupotič *et al.* 2007), HwPbs2 and HwSho1 isoforms (our unpublished data), indicating some form of general adaptation of the *H. werneckii* proteins to saline environments. However, the specific role of this amino-acid segment in the HwGpd1 isoforms is not known. We can infer from our data that a functional Gpd2 isoform is critical for the HwGpd1B rescue of the salt sensitivity of the yeast *gpd1* mutant. For Gpd1 with a deletion in the putative PTS2 sequence, it was previously shown that the presence of wild-type Gpd1 was necessary for localisation of the mutant protein to peroxisomes, which suggested that dimerisation of the proteins restored function to the mutant protein (Jung *et al.* 2010). Other studies have additionally supported the dimerisation of the Gpd1 proteins under physiological conditions (Berrada *et al.* 2002; Ou *et al.* 2006). Altogether, we can assume that Gpd2 dimerises with HwGpd1B, which compensates for some of the missing characteristics of HwGpd1B regarding its correct functioning in *S. cerevisiae*. Even more surprisingly, there is only a nine-amino-acid difference between the *H. werneckii* Gpd1 isoforms, which are mostly localised to the conserved catalytic domain, and which prevent HwGpd1A from complementing Gpd1 function. Among these, the most likely residues critical

for the function of Gpd1 are Asn at position 311 and Asp at position 359, which are identical between Gpd1 and HwGpd1B, but different in HwGpd1A.

Although important differences between HwGpd1A and HwGpd1B were observed, they clustered closely together in the unrooted gene tree, which indicates recent gene duplication. Gene duplication has already been accepted as one of the mechanisms of adaptation to various stresses. In yeast, for example, most of the duplicated genes code for membrane transporters and proteins involved in stress responses (Kondrashov *et al.* 2002). These duplications appear to have adaptive roles in compensation for functions that are compromised by genetic, environmental or stochastic perturbations. This was recently shown for *GPD2*, which was up-regulated in a *S. cerevisiae gpd1* mutant grown in a hyperosmotic environment (DeLuna *et al.* 2010). Several other genes that are involved in salt-stress responses are also duplicated in the genome of *H. werneckii*, like *HwHAL2* (Vaupotič *et al.* 2007), *HwHHK7* (Lenassi & Plemenitaš 2007), *HwSHO1* and *HwPBS2* (our unpublished data) and others (Gorjan & Plemenitaš 2006; Gostinčar *et al.* 2009), which probably provide evolutionary benefit for *H. werneckii* living in environments that have fluctuating salt concentrations. Interestingly, the halophilic *W. ichthyophaga* has developed a different mechanism of adaptation, as duplication of genes involved in halotolerance has not been observed to date (this study, and our unpublished data). It is also worth noting that this is the first report of fungal *GPD1* gene duplication, as only one copy of the salt-regulated gene has been reported for the genomes of sequenced fungi.

The difference between *HwGPD1* isogenes and *WiGPD1* is also in the transcriptional response to different environmental salinity conditions. The *HwGPD1* isogene mRNA started accumulating 40 min after exposure of cells to hypersaline stress, and increased with time to a certain point, as with *S. cerevisiae GPD1* (Rep *et al.* 1999). In contrast, *WiGPD1* showed no gradual response for the mRNA transcript, and was only induced after 300 min exposure. All three genes responded quickly to hyposaline stress; however, the expression profiles of the *HwGPD1* genes were of higher amplitude. When looking at the levels of mRNA in cells adapted to different salinities, *WiGPD1* showed a gradual increase in the transcript at higher salinities, with the maximum at 4.5 M NaCl. This is similar to *S. cerevisiae*, where *GPD1* mRNA levels slowly increase with increase in solute concentrations (Ansell *et al.* 1997). The *HwGPD1* isogenes, on the other hand, showed almost no increase in transcript levels up to around 3 M NaCl, while a sudden increase in transcript numbers occurred at 4.5 M NaCl, probably reflecting the additional need for glycerol synthesis to counteract its loss through the cell walls (Kogej *et al.* 2007). Interestingly, we observed a transient up-regulation of *HwGPD1A* transcripts at 1.8 M NaCl and of *HwGPD1B* transcripts at 1.0 M NaCl. This corresponds to the expression profile of the *HwHOG1* gene, which is up-regulated at 1 M and 4.5 M NaCl (Lenassi *et al.* 2007), additionally supporting the role of the *H. werneckii* HOG pathway in the regulation of the *HwGPD1* isogenes. The 3-fold difference between the transcript levels of the *HwHOG1* (5-fold induction) and *HwGPD1* isogenes (15-fold induction) could be explained by posttranslational regulation of activity of HwHog1, which is fully functional only at salinities of more than 3.5 M NaCl (Turk & Plemenitaš 2002). Altogether, it appears that HwHog1-regulated expression of *HwGPD1* genes is much more dynamic when compared to *WiGPD1*.

In conclusion, the data in the present study on the HwGpd1A, HwGpd1B and WiGpd1 homologues confirm previous observations that the extremely halotolerant black yeast *H. werneckii* and the halophilic *W. ichthyophaga* have evolved two fundamentally different strategies to combat stress induced by the high salinity of their natural environment. The differences described between adaptive and obligate halophiles and salt-sensitive fungi at the molecular level also contribute to our understanding of the adaptation of eukaryotes to environments with elevated salt concentrations.

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2.1.3 Sekvenciranje genoma in transkriptoma halofilne glive *Wallemia ichthyophaga*: prisotne in odsotne haloadaptacije

Naslov v originalnem jeziku: Genome and transcriptome sequencing of the halophilic fungus *Wallemia ichthyophaga* : haloadaptations present and absent.

Avtorji:
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Izvleček:
Bazidiomicetna gliva *Wallemia ichthyophaga,* ki je umeščena v filogenetsko ločen razred Wallemiomycetes, je najbolj halofilna gliva znana do sedaj. Za rast potrebuje najmanj 10 % NaCl in uspeva celo v nasičeni raztopini soli. Z namenom raziskovanja genomske osnove tega izjemnega fenotipa, smo *de-novo* pridobili genomsko zaporedje tipskega seva vrste in analizirali transkriptomski odziv na pogoja, ki sta blizu spodnje in zgornje meje njenega slanostnega območja rasti. Nenavadno kompakten genom je velik 9,6 Mb in vsebuje le 1,67 % ponavljajočih se zaporedij. Zgolj 4884 predvidenih protein-kodirajočih genov pokriva skoraj tri četrtine celotnega zaporedja. Od 639 diferencialno izraženih genov, sta dve tretjini bolj izraženi pri nižji slanosti. Filogenomska analiza, ki temelji na največjem možnem naboru podatkov (celoten proteom), uvršča razred Wallemiomycetes kot 250 milijonov let staro sestrsko skupino Agaricomycotina. V nasprotju s tesno sorodno vrsto *Wallemia sebi*, je *W. ichthyophaga* najverjetneje izgubila sposobnost spolnega razmnoževanja. Več proteinskih družin je značilno razširjenih ali skrčenih v genomu. Med razširjenimi družinami so ATPazni kationski transporterji tipa P, ne pa tudi družina izmenjevalcev natrija in vodika. Prepisovanje vseh, razen treh prenašalcev kationov, ni odvisno od soli. Analiza kaže tudi znatno obogatitev v družini hidrofobinov. To so proteini celične stene z več celičnimi funkcijami. Polovica le-teh je diferenčno izraženih in večina vsebuje nenavadno visoko število kislih aminokislin. To odkritje je še posebej zanimivo zaradi široke uporabnosti hidrofobinov gliv v industriji, farmaciji in medicini.

W. ichthyophaga je ekstremofilni specialist, ki kaže le nizko raven prilagodljivosti in genetske rekombinacije. To se odraža tako v značilnostih genoma, kot tudi v transkriptomskem odzivu na sol. Nič nenavadnih lastnosti v običajnih mehanizmih tolerance na sol, na primer v transportu anorganskih ionov ali sintezi kompatibilnih topljencev, nismo našli. Namesto tega različni podatki kažejo vlogo celične stene *W. ichthyophaga* v odgovoru na sol. Pričakujemo, da bo razpoložljivost genomskega zaporedja olajšala nadaljnje raziskave te edinstvene vrste in omogočila boljši pogled na prilagoditve, ki *W. ichthyophaga* omogočajo uspevanje v pogojih, ki so smrtni za večino drugih evkariontov.

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BMC Genomics

RESEARCH ARTICLE Open Access

Genome and transcriptome sequencing of the halophilic fungus *Wallemia ichthyophaga*: haloadaptations present and absent

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Abstract

Background: The basidomycete *Wallemia ichthyophaga* from the phylogenetically distinct class Wallemiomycetes is the most halophilic fungus known to date. It requires at least 10% NaCl and thrives in saturated salt solution. To investigate the genomic basis of this exceptional phenotype, we obtained a *de-novo* genome sequence of the species type-strain and analysed its transcriptomic response to conditions close to the limits of its lower and upper salinity range.

Results: The unusually compact genome is 9.6 Mb large and contains 1.67% repetitive sequences. Only 4884 predicted protein coding genes cover almost three quarters of the sequence. Of 639 differentially expressed genes, two thirds are more expressed at lower salinity. Phylogenomic analysis based on the largest dataset used to date (whole proteomes) positions Wallemiomycetes as a 250-million-year-old sister group of Agaricomycotina. Contrary to the closely related species *Wallemia sebi*, *W. ichthyophaga* appears to have lost the ability for sexual reproduction. Several protein families are significantly expanded or contracted in the genome. Among these, there are the P-type ATPase cation transporters, but not the sodium/ hydrogen exchanger family. Transcription of all but three cation transporters is not salt dependent. The analysis also reveals a significant enrichment in hydrophobins, which are cell-wall proteins with multiple cellular functions. Half of these are differentially expressed, and most contain an unusually large number of acidic amino acids. This discovery is of particular interest due to the numerous applications of hydrophobines from other fungi in industry, pharmaceutics and medicine.

Conclusions: *W. ichthyophaga* is an extremophilic specialist that shows only low levels of adaptability and genetic recombination. This is reflected in the characteristics of its genome and its transcriptomic response to salt. No unusual traits were observed in common salt-tolerance mechanisms, such as transport of inorganic ions or synthesis of compatible solutes. Instead, various data indicate a role of the cell wall of *W. ichthyophaga* in its response to salt. Availability of the genomic sequence is expected to facilitate further research into this unique species, and shed more light on adaptations that allow it to thrive in conditions lethal to most other eukaryotes.

Keywords: *Wallemia ichthyophaga*, Wallemiomycetes, Genome, Transcriptome, Phylogeny, Haloadaptation, Halotolerance, Hypersaline, Extremophile, Hydrophobin

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Background

Wallemia Johan-Olsen (Wallemiales, Wallemiomycetes) is a genus of cosmopolitan xerophilic fungi that are found in a wide variety of environments characterised by low water activity (a_w) [1,2]. According to the characterisation of dolipore septa [3,4] and to molecular analysis [2,5], *Wallemia* was placed in the phylum Basidiomycota. Through various studies, its inferred phylogenetic origin varied from the root of basidiomycetes [2], to *incertae sedis* [6], to being a sister group of the Agaricomycotina and Ustilaginomycotina [5] or only of the Agaricomycotina [4]. Previously, the genus contained only one species, but it was later segregated into three species: *Wallemia ichthyophaga*, *Wallemia sebi* and *Wallemia muriae* [2].

To date, only a limited number of strains of *W. ichthyophaga* have been isolated from hypersaline water of solar salterns, bitterns (magnesium-rich residual solutions in salt production from sea water) and salted meat (ham: *prosciutto*) [2] (Sonjak et al., unpublished data). In addition to differences in phylogenetic DNA markers, *W. ichthyophaga* is also distinguished from the other two representatives of the genus by its characteristic morphology and halophilic physiology [2,7]. Although xerotolerance is rare in the Basidiomycota, all three *Wallemia* spp. are among the most xerophilic fungal taxa [2]. However, while *W. sebi* and *W. muriae* strongly prefer high concentrations of non-ionic solutes (for example sugars) over those of NaCl (although they can also tolerate up to 4.6 M and 4.3 M NaCl, respectively [8]), the opposite is true for *W. ichthyophaga* [2]. *W. ichthyophaga* requires at least 1.5 M NaCl for *in-vitro* growth (or some other osmolyte for an equivalent a_w), and it even thrives in saturated NaCl solution. It also tolerates high concentrations of other salts, such as $MgCl_2$ (Sonjak et al., unpublished data). Such a narrow ecological amplitude is common for specialised archaeal halophiles, but in the fungal kingdom it is an exception. Even the most salt-tolerant fungal species do not normally require salt for growth, and they frequently have their growth optimum in the absence of salt. Because of this, *W. ichthyophaga* is a rare fungal example of an obligate extremophilic specialist [9], and it is considered to be the most halophilic fungus known to date.

Studies of haloadaptation mechanisms of *W. ichthyophaga* began relatively recently and are thus still at early stages. The fungus counterbalances the osmotic pressure caused by high concentrations of salt in the surrounding medium by intracellular accumulation of a mixture of polyols, among which glycerol is the major osmotically regulated solute (Zajc et al., unpublished data). It was previously published that *W. ichthyophaga* has a glycerol-3-phosphate dehydrogenase gene (*WiGPD1*) that encodes the key enzyme in the biosynthesis of glycerol. Its expression elevated at high concentrations of salt. Comparisons of Gpd1 from the salt-sensitive *Saccharomyces cerevisiae* to WiGpd1 have shown high overall amino-acid similarity; however, *WiGPD1* lacks the N-terminal peroxisomal targeting (PTS2) sequence, which is important for its peroxisome localisation [10,11]. The consequent constant cytosolic localisation of Gpd1 might thus be beneficial for organisms that live in extremely saline environments [11].

High-osmolarity glycerol (HOG) signalling pathway in fungi is responsible for the sensing of osmolarity changes and for the facilitation of adaptation of cells to hypersaline environment in *S. cerevisiae* [12]. This is also the case in the extremely halotolerant black yeast *Hortaea werneckii* [13]. Several, but not all, of the genes of this pathway have been found in the genome of *W. sebi* [4]. In *W. ichthyophaga* the homologues of MAP kinases Hog1 have recently been studied in detail [14]. Two homologues were found (WiHog1A and WiHog1B), but only one of them was able to complement the *hog1Δ* strain of *S. cerevisiae* and activate the HOG-responsive glycerol-3-phosphate dehydrogenase (GPD) promoter. The transcription of both genes was lowest at optimal salinity (20% NaCl), while at limiting salinities (10% and 30% NaCl) the transcription increased by at least 2- and up to 6-fold. The proteins were dephosphorylated after exposing the cells to both hypo- and hyper-osmotic shocks, a pattern opposite to that of *S. cerevisiae* [14].

High concentrations of salt trigger substantial morphological changes to *W. ichthyophaga* cells. These are believed to have important adaptive roles under hypersaline conditions. This fungus grows in the form of sarcina-like structures, or compact multicellular clumps [2]. This morphology can be observed in several phylogenetically distant polyextremotolerant species, and it is believed to enhance survival in high-stress environments [15-17]. The cells have an abundant cover of extracellular polysaccharides [8], which have been reported to protect against desiccation in rock-inhabiting fungi [18], and which might also have a protective role at high salinity. Apart from an almost four-fold increase in the size of cell clumps, the most striking morphological response of *W. ichthyophaga* to high salinity is a three-fold thickening of the cell wall, which results in a substantially decreased functional cell volume [8].

In environments such as salterns, which are believed to be the primary habitat of *W. ichthyophaga*, the levels of toxic sodium ions (Na^+) far exceed those of potassium ions (K^+). Under these conditions, the cells must use a lot of energy on active transmembrane transport of ions, to maintain a stable and high intracellular K^+/Na^+ ratio. This is achieved by transporters that have higher affinity for K^+ than for Na^+ at the level of influx, by efficient efflux of toxic or surplus cations from the cells, and also by selective compartmentalisation of cations in organelles (reviewed in [19]). The transport systems that

mediate these alkali-metal cation fluxes at both the plasma and organelle membranes function together not only to maintain K^+ homeostasis and to eliminate toxic Na^+ (or Li^+), but also to preserve membrane potential, regulate intracellular pH, and maintain positive turgor inside the cell, which is necessary for plasma-membrane/ cell-wall expansion and cell division and to cope with osmotic stress (reviewed in [19]). Hence, the alkali-metal cation influx and efflux systems of these halotolerant eukaryotes are of great interest for the explanation of osmoadaptation to extremely saline environments. Nevertheless, no studies that focus on *W. ichthyophaga* ion transporters have been published to date.

The mating behaviour of the *Wallemia* spp. is also unclear. To date, no teleomorphs or fruiting bodies have been observed in any of the *Wallemia* spp. The existence of a single mating type locus and an almost complete set of meiosis genes encoded in the genome of *W. sebi* suggest the capability for sexual reproduction [4]. However, genetic evidence for sexual reproduction in *W. ichthyophaga* has not yet been assessed.

Our knowledge of the mechanisms underlying the exceptional ability of *W. ichthyophaga* to thrive at salt concentrations that are lethal to the vast majority of eukaryotes and all but the most adapted prokaryotes is only starting to expand. This is partly due to the relatively recent taxonomic description of this species, although it is also a consequence of the significant experimental input needed for even the most basic discoveries. To alleviate this problem and to facilitate further work with *W. ichthyophaga*, results of *de-novo* sequencing of its whole genome and transcriptomes of the cells grown at two limiting salinities are presented here. The characteristics of the genome and the predicted proteome and transcriptomes are described and discussed in light of the halophilic nature of *W. ichthyophaga*, together with the apparent inability for sexual reproduction of this species. The phylogenetic position of *Wallemiomycetes* is determined with excellent support values on the basis of the whole proteome, which is the largest dataset used for this purpose, so far.

Results and discussion

Genome sequencing, assembly and gene prediction

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession [GenBank: APLC00000000]. The version described in this paper is the first version, [GenBank:APLC01000000]. For all genes and proteins discussed here, GenBank accession numbers are provided in the text. The total assembly size of the *W. ichthyophaga* genome is 9.6 Mb, and it is assembled into 101 contigs and 82 scaffolds (Table 1). This genome size is even smaller than for the closely related species *W. sebi* (9.8 Mb; Figure 1, Table 1) [4]. While even smaller genomes exist (*Malassezia globosa* at 9.0 Mb), most basidiomycetous haploid genomes are at least twice as large, and range even up to more then 40-times larger [20]. In line with its small size, the genome of *W. ichthyophaga* is very compact. It contains only 1.67% repetitive sequences, and almost three quarters of the genome is covered by the coding DNA sequences. The overall GC content is 45.35%, and it is characteristically lower in the mitochondrial DNA (Figure 2). While relatively low, this value is still higher than *W. sebi*, which contains only 40.01% GC. The gene density is 514 genes/ Mb scaffold, which is higher than in *M. globosa* (476 genes/ Mb) and only slightly lower than *W. sebi* (538 genes/ Mb). The absolute number of predicted proteins (4884) is also unusually small and in the range observed for *Escherichia coli* [21]. For *W. sebi* 5284 proteins were predicted, and 4285 for *M. globosa*, while >10000 proteins are not uncommon in other basidomycetes). Despite the reduction in genome size and gene number the number of introns is not unusually small as is seen in some other fungi with small genomes [22]: on average, the predicted genes contain 2.41 introns that are 61 bp long (Table 1). In other fungi the average intron densities range from just over 1.0 intron/ kb coding sequence (cds) in *Schizosaccharomyces pombe* to approximately 5.0 introns/ kb cds in *Cryptococcus neoformans* [23]. For all of the predicted proteins, 3715 had hits in the SwissProt database (e-value cut-off, 10^{-6}), 3278 genes were mapped in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database, and 2603 were classified in the Clusters of Orthologous Groups (COG) database (Additional file 1: Figures S1 and S2).

Alignment of the whole genomic sequence with the genome of *W. sebi* reveals long syntenic regions (Figure 3A). According to BLASTp analysis (e-value cut-off, 10^{-6}), 93.9% of the proteins from *W. ichthyophaga* have homologues in the proteome of *W. sebi* (Figure 3B). Among unique proteins, only a quarter can be classified into at least one protein family, according to the Pfam database (Figure 3C). The large overlap between these species is not surprising given their close phylogenetic proximity. Both of these fungi were originally classified as *W. sebi*, until this species was segregated into *W. ichthyophaga*, *W. sebi* and *W. muriae*, based on differences in conidial size, xerotolerance, and sequence data [2]. Proteins present in *W. ichthyophaga* but absent in *W. sebi* include several proteins related to DNA processing and DNA damage: two 5'-3' exoribonucleases [GenBank:EOR04230, GenBank:EOQ98776]; a telomerase reverse transcriptase [GenBank:EOR00183]; a DNA repair protein RAD50 [GenBank:EOR01216] and a DNA polymerase similar to mu polymerase [GenBank:EOR02079], both of which are involved in non-homologous end joining repair; an ATP-dependent DNA helicase [GenBank:EOR02849]; and a DNA damage-inducible protein 1 [GenBank:EOR00413].

Table 1 *Wallemia ichthyophaga* (EXF-994) genome assembly statistics and comparison with *Wallemia sebi* (adapted from [4] or calculated from the genomic data published online [76])

Statistic	*W. ichthyophaga*	*W. sebi*
Coverage	>270×	71×
Genome assembly size (Mb)	9.625	9.82
Number of scaffolds	82	56
Scaffold N50 (Mb)	0.44	0.34
Number of contigs	101	114
Contig N50 (Mb)	0.35	
CDS total length (Mb)	7.083 (73.59% of genome)	6.701 (68.39% of genome)
CDS average size (bp)	1450	1268
Predicted protein-coding genes (n)	4884	5284
Predicted proteins, average length (aa)	483	423
Exon total length (Mb)	7.083	
Exon total number	16670	
Exon average length (bp)	424	410
Introne total length (Mb)	0.719	
Introne total number	11786	
Introne average length (bp)	61	55
GC content (%)	45.35	40.01
GC content of CDS (%)	47.51	42.06
Repeat content (kb)	160.929 (1.67% of genome)	
- Tandem repeats (kb)	85.47	
- DNA transposons (kb)	84.77	
tRNA (kb)	11.55	

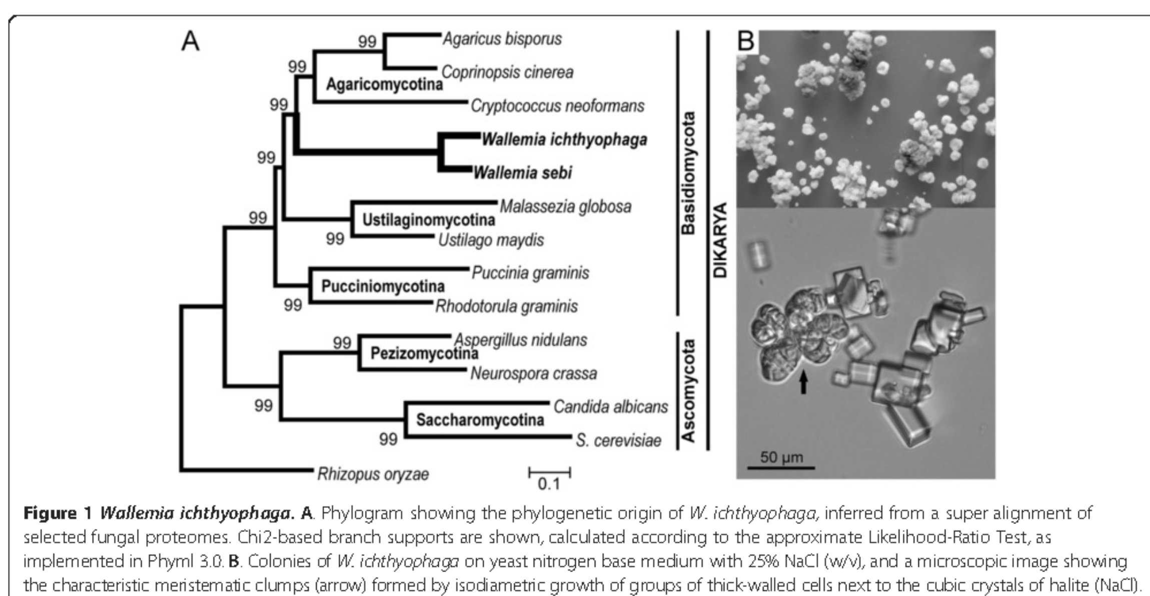

Figure 1 *Wallemia ichthyophaga*. A. Phylogram showing the phylogenetic origin of *W. ichthyophaga*, inferred from a super alignment of selected fungal proteomes. Chi2-based branch supports are shown, calculated according to the approximate Likelihood-Ratio Test, as implemented in Phyml 3.0. **B**. Colonies of *W. ichthyophaga* on yeast nitrogen base medium with 25% NaCl (w/v), and a microscopic image showing the characteristic meristematic clumps (arrow) formed by isodiametric growth of groups of thick-walled cells next to the cubic crystals of halite (NaCl).

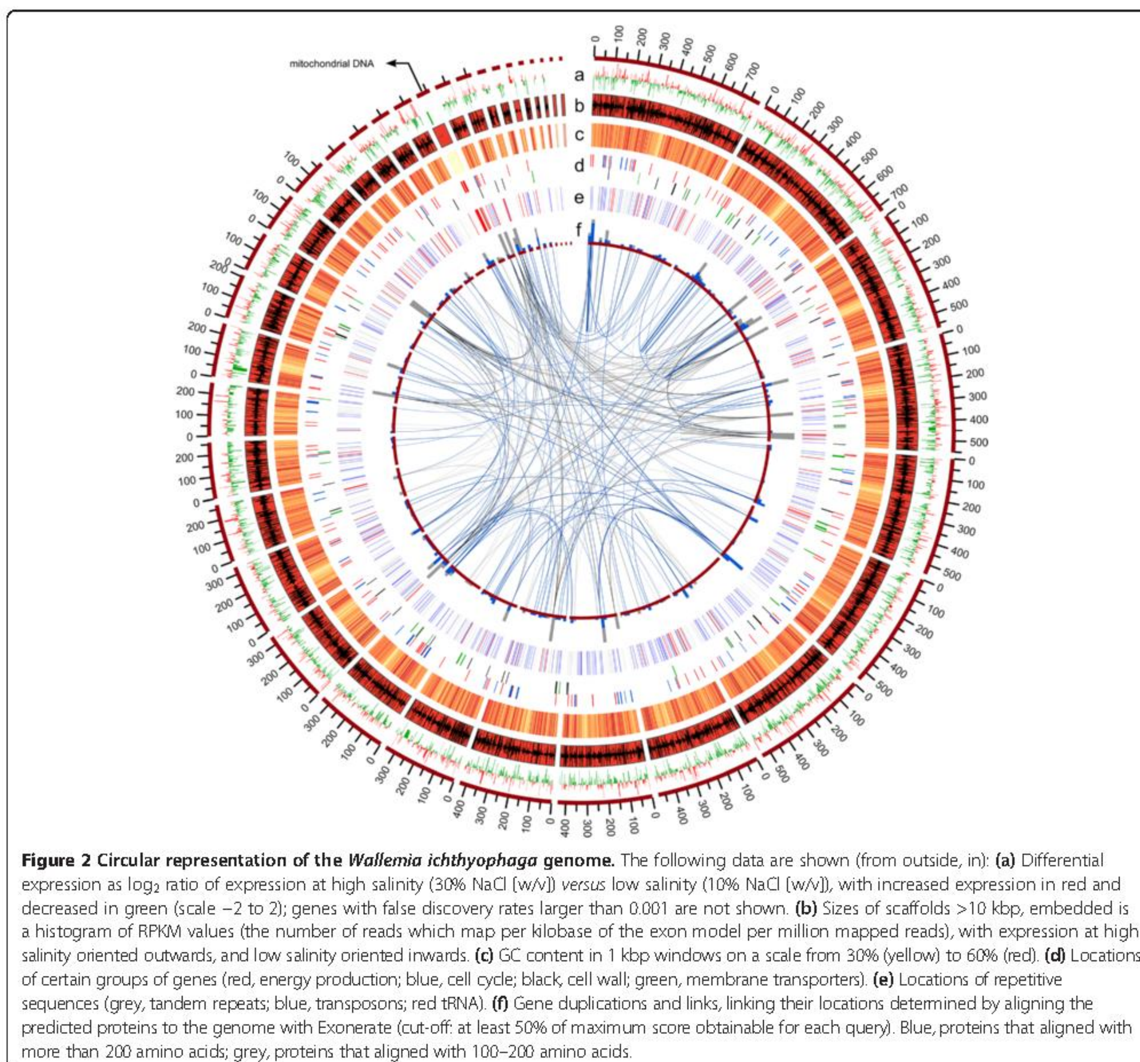

Figure 2 Circular representation of the *Wallemia ichthyophaga* genome. The following data are shown (from outside, in): **(a)** Differential expression as $\log_2$ ratio of expression at high salinity (30% NaCl [w/v]) *versus* low salinity (10% NaCl [w/v]), with increased expression in red and decreased in green (scale −2 to 2); genes with false discovery rates larger than 0.001 are not shown. **(b)** Sizes of scaffolds >10 kbp, embedded is a histogram of RPKM values (the number of reads which map per kilobase of the exon model per million mapped reads), with expression at high salinity oriented outwards, and low salinity oriented inwards. **(c)** GC content in 1 kbp windows on a scale from 30% (yellow) to 60% (red). **(d)** Locations of certain groups of genes (red, energy production; blue, cell cycle; black, cell wall; green, membrane transporters). **(e)** Locations of repetitive sequences (grey, tandem repeats; blue, transposons; red tRNA). **(f)** Gene duplications and links, linking their locations determined by aligning the predicted proteins to the genome with Exonerate (cut-off: at least 50% of maximum score obtainable for each query). Blue, proteins that aligned with more than 200 amino acids; grey, proteins that aligned with 100–200 amino acids.

Non-homologous recombination may offer *W. ichthyophaga* an additional mode of mitotic recombination, which would be important in light of the fact that this species (contrary to *W. sebi*) appears to be incapable of meiosis and sexual reproduction, as discussed below.

Phylogenetic position of Wallemiomycetes

The phylogenetic position of Wallemiomycetes in published studies has been determined as at the base of Basidiomycota, as a sister group to either Agaricomycotina or Ustilaginomycotina or both, or simply as *incertae sedis* within Basidiomycota [2,4-6]. However, these analyses used only limited sets of genes or proteins. In the present study, the whole proteomes of 14 fungi were aligned, and the resulting phylogeny resolved the position of the *Wallemia* spp. as a sister group to (or the earliest diverging lineage of) Agaricomycotina (Figure 1). This also supports Pucciniomycotina as the earliest diverging lineage within the Basidiomycota analysed. These results are in agreement with phylogenetic positioning of *W. sebi* published by Padamsee et al. [4].

Previously published calibration points were used to construct the chronogram [24]: *Rhizopus oryzae*–Dykaria split 495 million years ago (mya), Ascomycota–Basidiomycota split 452 mya, Pezizomycotina crown 215 mya, Basidomycota crown 340 mya. Under these assumptions, the split between *W. ichthyophaga* and *W. sebi* is estimated as 11.9 mya, and that between Wallemiomycetes and Agaricomycotina as 250 mya. As the calibration of the fungal tree of life remains uncertain due to scarcity and poor preservation of

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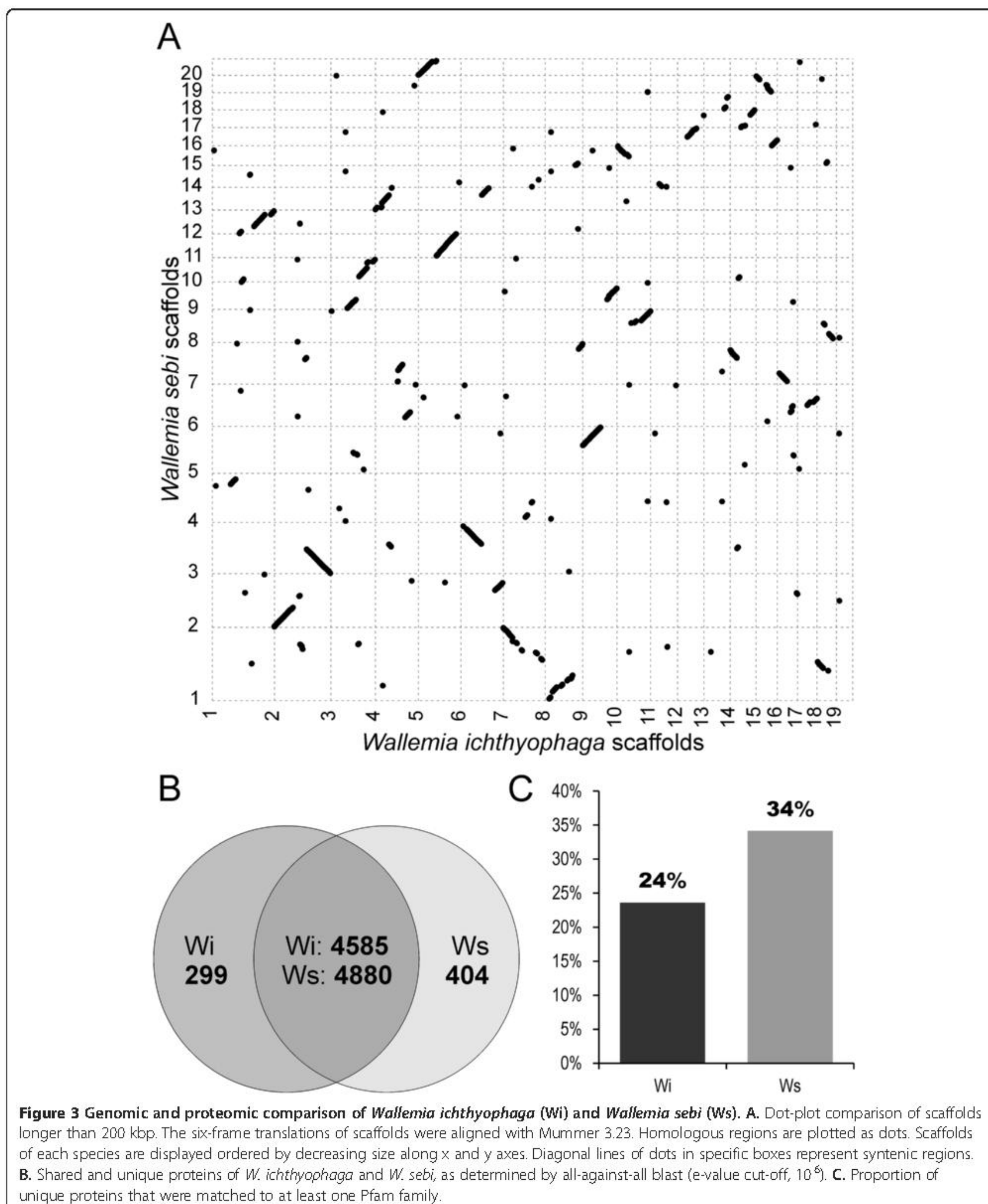

Figure 3 Genomic and proteomic comparison of *Wallemia ichthyophaga* (Wi) and *Wallemia sebi* (Ws). A. Dot-plot comparison of scaffolds longer than 200 kbp. The six-frame translations of scaffolds were aligned with Mummer 3.23. Homologous regions are plotted as dots. Scaffolds of each species are displayed ordered by decreasing size along x and y axes. Diagonal lines of dots in specific boxes represent syntenic regions. **B.** Shared and unique proteins of *W. ichthyophaga* and *W. sebi*, as determined by all-against-all blast (e-value cut-off, 10^{-6}). **C.** Proportion of unique proteins that were matched to at least one Pfam family.

fossil material, these values can only be considered as rough estimates.

Expansions and contractions of the protein families

Of the predicted proteins, 3924 (80.3%) contain at least one of the 2678 Pfam domains in *W. ichthyophaga* (Additional file 2: Table S1). Among the families represented by the most proteins, there are several connected to transport functions in the cell: e.g., major facilitator family (PF07690), mitochondrial carriers (PF00153), ABC transporters (PF00005, PF00664) and others (PF00083). Seven protein families are significantly expanded and 19 are contracted in *W. ichthyophaga*, while 17 are significantly changed in the predicted last common ancestor of *W. ichthyophaga* and *W. sebi* (Additional file 3: Table S2). Among the proteins that are enriched, there are hydrophobins (PF01185; Figure 4A) and P-type ATPases (PF00690; Figure 4B).

Hydrophobins

The hydrophobins are cell-wall proteins that are secreted by filamentous fungi and have roles in a broad range of processes in growth and development [25]. Hydrophobins are characterised by their small size (≤20 kDa) and amphipathic nature, with their hydrophobic and hydrophilic domains [26]. There are multiple hydrophobin genes in the genome of individual fungi, due to possibly different functional roles or differential expression, or to different environmental conditions or developmental stages. In the predicted last common ancestor of *W. ichthyophaga* and *W. sebi*, 15 hydrophobins are estimated. A significant enrichment of the hydrophobin protein family occurred in *W. ichthyophaga* (to 26 representatives), and on the contrary, a significant contraction occurred in *W. sebi* (to 12 representatives; Figure 4A). Among all of the protein family expansions in *W. ichthopyhaga*, this is the most significant.

As previously shown [26], and as can be seen from our multiple sequence alignment of all of the hydrophobins from the list of basidiomycetous species analysed (Figure 5B, bottom), the DNA sequence similarities for the different hydrophobins are usually low between the different species. The most important feature of the primary sequence, which is common to all hydrophobins, is the characteristic pattern of conserved spacing of eight cysteine residues that form four disulphide bridges [26,27]. These are also conserved in the hydrophobins of *W. ichthyophaga*. Interestingly, the hydrophobins of *W. ichthyophaga* and its closest relative *W. sebi* are similar, and they share a large fraction of conserved positions of their amino acids (Figure 5B, bottom). They also both contain a high proportion of acidic amino acids, as compared to other fungi (Figure 5B, top). Higher proportion of acidic amino-acids is characteristic of proteins exposed to high concentrations of salt as has been noted in halophilic proteins of Archaea [28]. As hydrophobins are likely to be directly exposed to the external high concentrations of NaCl in *W. ichthyophaga*, the unusually high proportion of acidic amino acids is not surprising. Acidic amino acids on a protein surface enable the binding of large amount of salts and water under solvent conditions, and in this way they maintain soluble and active conformations in an environment that is generally detrimental to other proteins [29]. Moreover, it has been shown that halophilic proteins are characterised by low hydrophobicity and under-representation of cysteines [30]. Therefore, it is interesting that halophilic hydrophobins have at least moderate levels of hydrophobicity and are cysteine rich, although, at the same time, they show salinity-biased amino-acid compositions.

The property of the hydrophobins to spontaneously assemble at hydrophobic–hydrophilic interfaces to form amphipathic monolayers governs their diverse functions in the growth and development of filamentous fungi. For instance, the hydrophobins allow these cells to breach the air–water interface, prevent water-logging while maintaining permeability to gaseous exchange, enable attachment to hydrophobic surfaces, modify the movement of solutes across the cell wall, and give strength and rigidity to the cell wall [25,31]. Some of these functions would also be beneficial in hypersaline environments. Modulation of cell wall permeability could be of great importance in an environment where toxic salt ions are constantly leaking into the cell, while strengthening and rigidifying the cell wall would be useful during changes of environmental osmolarity. Given that certain hydrophobins are responsible for microconidial chain formation in *Fusarium verticillioides* [32], hydrophobins in *W. ichthyophaga* might additionally be involved in the aggregation of these cells into compact clumps, as is characteristic of this fungus. Formation of meristematic clumps is observed as a stress-response in many unrelated halotolerant fungi [9]. Both the changes in the cell wall and the formation of multicellular structures are reported to be among the main adaptations of *W. ichthyophaga* to hypersaline environments [8].

Over the last few years, the hydrophobins have received a lot of attention from biotechnologists. Their ability to reverse the hydrophilic–hydrophobic character of a surface and/or their surfactant capacity has many potential applications. They can be used as surfactants and emulsifiers in food processing, in anti-fouling coatings, surface coating of biomaterials, such as surgical instruments and medical implants, and immobilisation of various substances. They might even have a role as functional coatings of drug nanoparticles ([26,27,33]. The differences in the amino-acid compositions of the hydrophobins from the halophilic *W. ichthyophaga* (and

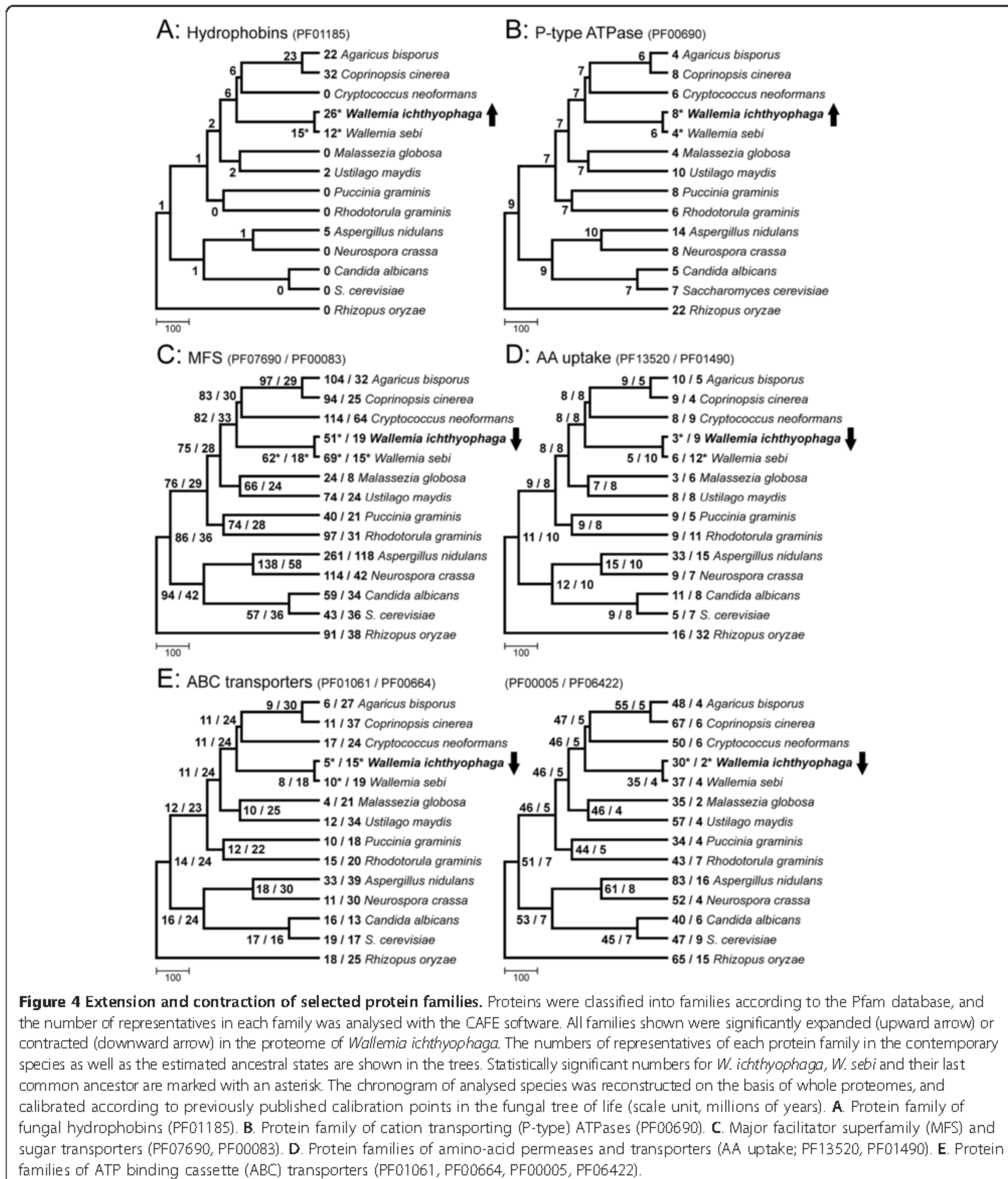

Figure 4 Extension and contraction of selected protein families. Proteins were classified into families according to the Pfam database, and the number of representatives in each family was analysed with the CAFE software. All families shown were significantly expanded (upward arrow) or contracted (downward arrow) in the proteome of *Wallemia ichthyophaga*. The numbers of representatives of each protein family in the contemporary species as well as the estimated ancestral states are shown in the trees. Statistically significant numbers for *W. ichthyophaga*, *W. sebi* and their last common ancestor are marked with an asterisk. The chronogram of analysed species was reconstructed on the basis of whole proteomes, and calibrated according to previously published calibration points in the fungal tree of life (scale unit, millions of years). **A**. Protein family of fungal hydrophobins (PF01185). **B**. Protein family of cation transporting (P-type) ATPases (PF00690). **C**. Major facilitator superfamily (MFS) and sugar transporters (PF07690, PF00083). **D**. Protein families of amino-acid permeases and transporters (AA uptake; PF13520, PF01490). **E**. Protein families of ATP binding cassette (ABC) transporters (PF01061, PF00664, PF00005, PF06422).

especially the high number of acidic amino acids) might give these proteins unique properties. These are well worth investigating too, as they might expand the scenarios of hydrophobin use in novel applications or in known applications under specific conditions, such as those with high salt concentrations.

Transporters of alkali metal cations

The significant enrichment in *W. ichthyophaga* of the protein family of cation-transporting ATPases (PF00690) might contribute to its halophilic ecotype. This protein family of cation proteins is represented by three H^+ and two Na^+ P-type ATPases in the plasma membrane, one

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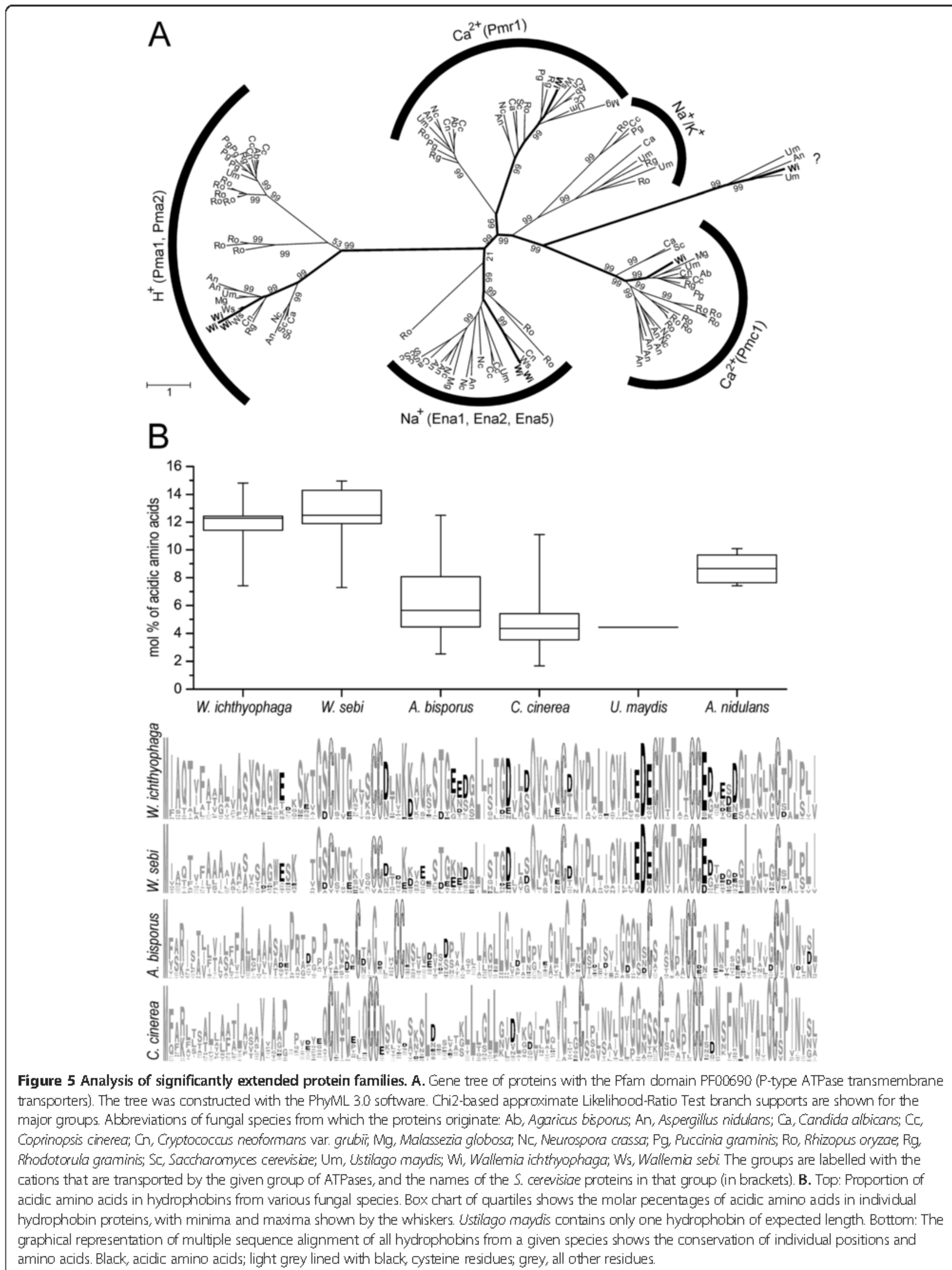

Figure 5 Analysis of significantly extended protein families. A. Gene tree of proteins with the Pfam domain PF00690 (P-type ATPase transmembrane transporters). The tree was constructed with the PhyML 3.0 software. Chi2-based approximate Likelihood-Ratio Test branch supports are shown for the major groups. Abbreviations of fungal species from which the proteins originate: Ab, *Agaricus bisporus*; An, *Aspergillus nidulans*; Ca, *Candida albicans*; Cc, *Coprinopsis cinerea*; Cn, *Cryptococcus neoformans* var. *grubii*; Mg, *Malassezia globosa*; Nc, *Neurospora crassa*; Pg, *Puccinia graminis*; Ro, *Rhizopus oryzae*; Rg, *Rhodotorula graminis*; Sc, *Saccharomyces cerevisiae*; Um, *Ustilago maydis*; Wi, *Wallemia ichthyophaga*; Ws, *Wallemia sebi*. The groups are labelled with the cations that are transported by the given group of ATPases, and the names of the *S. cerevisiae* proteins in that group (in brackets). **B.** Top: Proportion of acidic amino acids in hydrophobins from various fungal species. Box chart of quartiles shows the molar pecentages of acidic amino acids in individual hydrophobin proteins, with minima and maxima shown by the whiskers. *Ustilago maydis* contains only one hydrophobin of expected length. Bottom: The graphical representation of multiple sequence alignment of all hydrophobins from a given species shows the conservation of individual positions and amino acids. Black, acidic amino acids; light grey lined with black, cysteine residues; grey, all other residues.

of each of the two groups of Ca^{2+} P-type ATPases in vacuoles (Pmc1) and the Golgi apparatus (Pmr1), and a protein with unknown specificity (Figure 5A, Table 2).

In the extensively studied model of yeast *S. cerevisiae*, the two H^+-exporting P-type (Pma1, 2) and several Na^+-exporting Ena ATPases (i.e., *exitus natru*: exit of Na^+), are crucial for the maintenance of homeostasis of intracellular K^+ and Na^+ [34]. In *S. cerevisiae*, Pma pumps are the most abundant plasma-membrane protein. These consume at least 20% of the cellular ATP [35], to generate the electrochemical gradient of H^+ across the plasma membrane, which is indispensable for all secondary active symporters and antiporters [19]. The *W. ichthyophaga* genome encodes three putative Pma proton pumps [GenBank:EOR02126, GenBank:EOR02128, GenBank:EOR00565], which are highly similar to the only two Pma ATPases from *W. sebi* (over 91% identity).

The maintenance of low concentrations of toxic Na^+ occurs via two types of Na^+ efflux systems in the plasma membrane. Ena P-type ATPases couple the hydrolysis of ATP to the export of Na^+ (or K^+) against the electrochemical gradient, while Nha antiporters export Na^+ by using the transmembrane H^+ gradient. These systems have complementary functions: Ena ATPases are more important at high pH, which does not allow for the correct functioning of Nha antiporters. In *S. cerevisiae* the ENA cluster, particularly *ENA1*, is a major determinant of salt tolerance in this yeast (reviewed in [19]). The two Ena ATPases of *W. ichthyophaga* [GenBank:EOR00553, GenBank:EOR04078] are 92% identical and are particularly different from *ScENA1* (36% and 37% identity). Interestingly, in *W. sebi*, there is only one ENA ATPase.

P-type ATPases are not the only ones responsible for the maintenance of the cellular ion homeostasis. A variety of other secondary active transporters contribute to keeping the intracellular concentrations of highly toxic Na^+ low, while at the same time maintaining a constant level of K^+. These tasks are particularly problematic in the environments predominated by the high concentrations of Na^+. By searching the genome of *W. ichthyophaga* for homologues of known transporters from *S. cerevisiae* [19] and unconventional yeast [36], we identified several plasma-membrane (Nha1, Trk1, Pho89, Ena and Pma) and intracellular (Kha1, Pmr1, Nhx1, Vnx1, Vma1, Pmc1, Mrs7/Mdm37) homologues, as summarised in Table 2. Apart from the above mentioned enrichment of Ena and Pma transporters the number of other genes does not differ from the numbers of genes found in the less halotolerant *W. sebi*. *Wallemia ichthyophaga* therefore appears to be using a different salt-combating strategy then the extremely halotolerant ascomycete *H. werneckii*. A recent genome analysis in that case revealed significantly increased numbers of most of the alkali cation transporters [37]. For example, *H. werneckii* contains eight inward K^+

Table 2 Major plasma membrane and intracellular transporters of *Wallemia ichthyophaga* (Wi) and *Wallemia sebi* (Ws)

Cellular location[a]	Transporter type	Substrate specificity/ main function	Name of the Sc[b] homologue	Number of homologues in Wi[c]	Number of homologues in Ws[d]
PM	Channel	K^+ efflux	Tok1	0	0
	Uniporter	K^+ uptake	Trk1,2	1	1
	P-type ATPase	Na^+ (and Li^+) efflux	Ena1,2,5	**2** ↓[c]	**1**
	Antiporter	Na^+, (K^+)/ H^+ exchange	Nha1	2	2
	Antiporter	Na^+/H^+ exchange	/	1	1
	Symporter	Na^+/P_i cotransporter	Pho89	1 ↑[d]	1
	P-type ATPase	H^+ export	Pma1,2	**3**	**2**
unknown	Permease	Ca^{2+} permease	/	1	1
	Antiporter	Ca^{2+}/H^+ exchange	/	1	1
	P-type ATPase	cation transporting, unknown specificity	/	2 ↓[e]	2
GA	Antiporter	K^+/H^+ exchange	Kha1	2	2
	P-type ATPase	Ca^{2+} and Mn^{2+} transport into GA	Pmr1	1	1
LE	Antiporter	Na^+, (K^+)/ H^+ exchange	Nhx1	1	1
	Antiporter	Na^+, K^+ /H^+ exchange	Vnx1	1	1
VAC	V-type ATPase	H^+ in vacuole	Vma1	1	1
	P-type ATPase	depleting cytosol of Ca^{2+} ions	Pmc1	1	1
MTH	Antiporter	K^+/H^+ exchange	Mrs7/Mdm38	1	1

[a]*PM* plasma membrane, *GA* Golgi apparatus, *LE* late endosomes, *VAC* vacuole, *MTH* mitochondria.
[b]Sc, *S. cerevisiae*; bold numbers mark differences in numbers of transporters between *W. ichthyophaga* and W. sebi.
[c,d,e] Differential expression of the transporter homologues in *W. ichthyophaga* (log_2 ratios: c: ↓ -1.00; d: ↑ +1.52; e: ↓ -1.27).

(Trk) transporters and four outward (Tok) K^+ channels (only one Trk and no Tok homologues are found in *W. ichthyophaga*), eight Nha $Na^+(K^+)$ proton antiporters (two in *W. ichthyophaga*), and six Pho89 Na^+/P_i symporters (only one in *W. ichthyophaga*) [37].

In addition to passive K^+ channels active processes for K^+ import may be beneficial in hypersaline environments. These can be carried out by $K^+(Na^+)$-ATPase (alkali cation uptake, Acu, transporters) or K^+-H^+symporter (Hak symporters) [36,38]. While no Hak homologues are identified in *W. ichthyophaga*, there are two possible homologues of the otherwise rare Acu ATPases [GenBank: EOQ99826, GenBank:EOR03958], one of which has a clearly recognisable P-type ATPase PF00690 domain.

Intracellular transporters comprise mainly membrane alkali metal cation/ H^+ antiporters of the vacuole (Vnx1), endosomes (Nhx1), and Golgi apparatus (Kha1), and also a mitochondrial membrane crucial K^+/H^+ antiport exchange mechanism (Mdm38 or Mrs7) [19]. All of these intracellular transporters have also been identified in the *W. ichthyophaga* proteome, with all at one copy number except for Kha1, which is present as two copies.

Low numbers of transporters in the proteome of *W. ichthyophaga* and the absence of specific transporters may be connected to life at constant (albeit high) salinity. The absence of, for example, the outward K^+ channel Tok, may be harmless if the organism is not exposed to severe hypoosmotic shocks and the subsequent need to quickly release the surplus K^+. Furthermore, continuous removal and/or compartmentalization of sodium in these conditions might not even be feasible due to the high ATP demand. The opposite is true for *H. werneckii*, which can efficiently adapt to a whole range of salinities and contains an abundance of both inward and outward K^+ channels.

Other transporters

In contrast to the alkali-metal ion transporters, there are statistically significant contractions for several proteins that are involved in transport of other molecules (Figure 4C-E). Major facilitator superfamily transporters (Figure 4C) are the largest family of secondary transporters, with their wide substrate specificity ranging from ions to carbohydrates, lipids, amino acids and peptides, nucleosides and other molecules [39]. Amino-acid permeases and transporter proteins (Figure 4D) facilitate the cellular uptake of amino acids. Finally, ATP binding cassette (ABC) transporters (Figure 4E) are one of the largest protein superfamilies [40]. Both the ABC and the major facilitator superfamily transporters have been extensively studied due to their roles in the development of multidrug resistance in fungal pathogens and tumour cells. Together, they account for approximately half of all of the genes that encode transporters in fungal genomes [41]. The contraction of both families might indicate that in its environment, *W. ichthyophaga* experiences reduced need for this type of defence against internally produced or external toxins. This latter might be caused by the limited competition with other species, due to the extreme salinity conditions that are preferred by *W. ichthyophaga*. On the other hand, the structure and composition of its unusually thick cell wall that is formed as a response to the high salt concentrations might limit the diffusion of problematic compounds before they even reach the plasma membrane. However, proteins of both superfamilies also have numerous functions other than the export of toxins [41]. Their loss may thus be the consequence of other factors as well, such as little need to export secondary metabolites or to sequester heavy metals, nutritional specialisation for a limited number of substrates or even avoidance of accidental export of osmoprotectants by transporters with broad specificity.

Genes involved in management of compatible solutes

W. ichthyophaga contains genes for several proteins involved in the synthesis and accumulation of the compatible solutes. All of them except Gpp (glycerol-3-phosphatase) are present in more then one copy. Key enzymes for the biosynthesis of three polyols, which are present in *W. ichthyophaga* cells (Zajc et al., unpublished data) were identified: glycerol, arabitol and mannitol. Glycerol is synthesized from dihydroxyacetone phosphate, glycolitic intermediate, via two reaction steps catalyzed by (NAD)-dependent glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp) [42]. As previously shown *W. ichthyophaga* contains a GPD1 homologue, WiGPD1 [EMBL:FR686467, GenBank: EOR01876], the expression of which is salt-induced [11]. A second homologue was found by searching the genome [GenBank: EOR02702]. Expectedly, Gpp [GenBank: EOR02702] and both copies of Gpd are well conserved.

The synthesis of D-mannitol could be perfomed from fructose via a reduction step catalyzed by two NADP-dependent mannitol dehydrogenases [GenBank:EOR01463, GenBank:EOQ99146] as was previously described also for the basidiomycete *Agaricus bisporus* [43]. In fungi arabitol is produced from D-ribulose-5-phosphate, an intermediate of pentose phosphate pathway, by two D-arabinitol-2-dehydrogenases (homologues in *W. ichthyophaga* are [GenBank:EOR02008, GenBank:EOQ98686]). Homologues of these enzymes were identified also in *W. sebi* (in even more copies): four mannitol dehydrogenases and three D-arabinitol-2-dehydrogenases.

When the cells of *S. cerevisiae* are subjected to hyperosmotic shock, leaking of glycerol is counteracted by active import. This is performed by glycerol/ proton symporters of the plasma membrane, Stl1 [44]. In these conditions the aquaglyceroporin channel Fps1 is closed. It opens during a hypoosmotic shock and thus allows quick glycerol

expulsion [45]. In *W. ichthyophaga* four homologues of Stl1 were found [GenBank:EOR04223, GenBank:EOQ99617, GenBank:EOR01226, GenBank:EOR01982], as well as three aquaglyceroporin related proteins [GenBank:EOR00599, GenBank:EOR04990, GenBank:EOQ99141]. *W. sebi* contains the same number of homologues, all of them highly similar to those of *W. ichthyophaga*.

Mating and meiosis genes

In organisms that show no observable behavioural and morphological traits that are characteristic of sexual reproduction, searching for the mating and meiosis homologues in the genome can shed some light on their reproductive cycles [46]. In basidomycetous fungi sexual reproduction is genetically governed by a tetrapolar *MAT* locus with pheromone/ pheromone receptors and homeodomain (HD)-containing transcription factors encoded by two unlinked loci. The *MAT* loci have expanded in some cases, and in others they have fused, which results in a tetrapolar-to-bipolar transition [47].

BLASTp searches of homeodomain protein sequences from different basidiomycetous fungi in the proteome of *W. ichthyophaga* identified five genes that encode putative HD-motif transcription factors located on different scaffolds [GenBank:EOR04835, GenBank:EOR03936, GenBank:EOR03899, GenBank:EOR01329, GenBank:EOQ99399]. Only one of these, EOR03899 (hypothetical WiSxi1) was similar (e-value, 10^{-5}) to the HD genes involved in mating in basidiomycetes, as shown by the comparison of the *W. ichthyophaga* HD proteins against GenBank. It [GenBank:EOR03899] is most similar to Sxi1D alpha from *Cryptococcus neoformans var. neoformans* [GenBank:ABR67867]. Together with Sxi2a, Sxi1D is required for the initiation of dikaryon formation in *C. neoformans* [48]. In addition, BLASTp searches for pheromone response factors reveal three putative mating-related DNA-binding proteins with high-mobility group (HMG-box) domains [GenBank:EOR04027, GenBank:EOQ98813, GenBank:EOQ98831]. All three of these are somewhat similar to basidiomyceteous HMG-box transcription factors (most similar to the HMG-box transcription factor from *Ustilago hordei* [GenBank:CCF49140]; e-value approx. 10^{-13}). No discernible *MAT* locus was identified. Furthermore, no pheromone receptors or pheromone precursors were found.

The results of the investigation of the proteome of *W. ichthyophaga* for the meiosis-specific genes [49] also suggest that *W. ichthyophaga* cannot outcross. Only three (putative Dmc1, Mer3 and Msh4; e-values, $<10^{-40}$) out of eight meiosis-specific homologues were identified in *W. ichthyophaga*, meaning that it does not have a complete set of meiotic machinery. Since the presence of a representative set of meiotic genes provides strong inferences about meiosis and sex [46], it appears that *W. ichthyophaga* is incapable of sexual reproduction. This is in agreement with the fact that there are no reports on sexual reproduction for this species in the existing literature.

Contrary to this, in the closely related *W. sebi* the two mating-type genes are located near to each other (Sxi1 and Ste3 pheromone receptor homologue; ~20 kb apart) [4]. The inspection of the region between these two genes identified two other putative mating-type genes; a pheromone and a transcription factor that encodes a HMG DNA-binding motif. In addition, the genome of *W. sebi* contains a near-complete set of meiosis genes, which lacks only the homologue of Hop1. Therefore, it appears that *W. sebi* is capable of sexual reproduction, although this part of its life cycle remains cryptic. It appears to have a bipolar mating system with two mating types, analogous to some other Basidiomycota [4].

The lack of sexual reproduction in *W. ichthyophaga* is not entirely surprising. In stable extreme environments, asexual reproduction might be advantageous, as it avoids the energy expenditure needed for producing gametes and attractants. Strict asexuality in specialised and adapted local populations would also help in the preservation of well-adapted genomic configurations [50]. Without sexuality, genetic drift can rapidly fix alleles in fragmented small populations that are adapted to the extreme habitats. These might all contribute to the mainly mitotic life style that many extremophilic fungal species have [9].

Transcriptome of *Wallemia ichthyophaga* at limiting salinities

Mapping more than 50 million EST sequences to the *W. ichthyophaga* genome aligned 83.96% and 83.70% of reads (transcriptome grown at 10% and 30% NaCl (w/v), respectively; Additional file 4: Table S3). Of these, over 99% matched to unique locations in the genome. At each salinity, approximately 95% of the predicted genes were more than 90% covered by the ESTs (Additional file 1: Figure S3), which indicates the high quality of the sequencing and mapping.

Changes in salinity have been reported to trigger drastic remodelling of the transcriptome of *S. cerevisiae*. However, many of these changes are only transient, and they soon return to near basal levels [51], which indicates that they are not involved in long-term survival at high salt concentrations. On the other hand cDNA subtraction analysis of *H. werneckii* cells adapted to 17% and 25% NaCl revealed a long-term differential expression of 95 genes. More then one third of them were shown to interact with the high osmolarity glycerol pathway [52]. Our purpose was therefore not to investigate the temporary transcriptional perturbations of *W. ichthyophaga* under osmotic stress, but the transcriptome differences in cells growing at the lower and upper salinity limits that are tolerated by *W. ichthyophaga*. Of the total 4884

genes in *W. ichthyophaga*, 639 (13.1%) were differentially expressed when compared across the transcriptomes of cells grown at these salinities. The number of genes with increased expression was twice as high (425) at lower salinity compared to higher salinity (214; Figure 6A and Additional file 5: Table S4). Of these, 72% and 79% (low and high salinity, respectively) were matched to at least one Pfam family (Figure 6C). Around 200 novel transcripts were

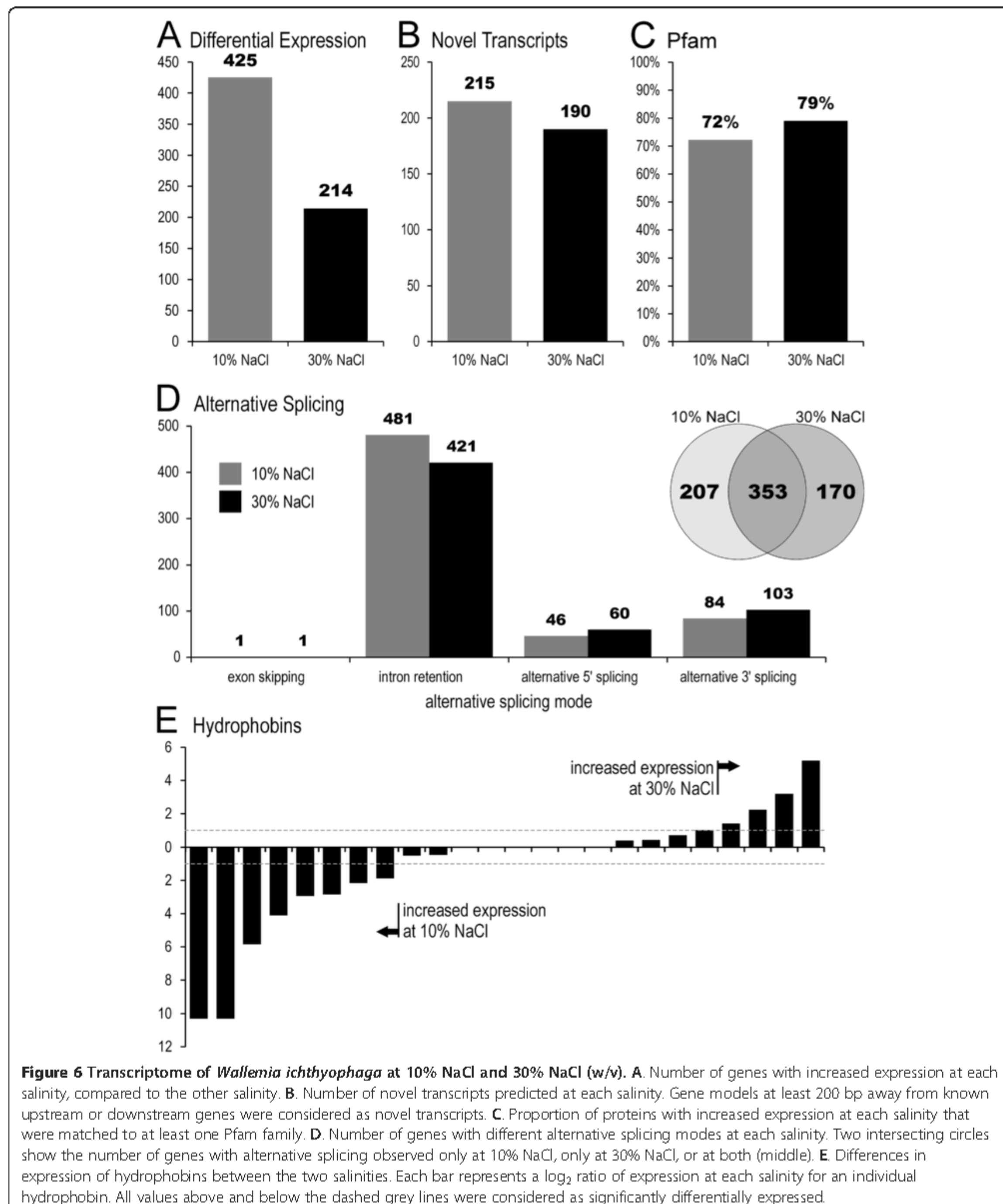

Figure 6 Transcriptome of *Wallemia ichthyophaga* at 10% NaCl and 30% NaCl (w/v). A. Number of genes with increased expression at each salinity, compared to the other salinity. **B**. Number of novel transcripts predicted at each salinity. Gene models at least 200 bp away from known upstream or downstream genes were considered as novel transcripts. **C**. Proportion of proteins with increased expression at each salinity that were matched to at least one Pfam family. **D**. Number of genes with different alternative splicing modes at each salinity. Two intersecting circles show the number of genes with alternative splicing observed only at 10% NaCl, only at 30% NaCl, or at both (middle). **E**. Differences in expression of hydrophobins between the two salinities. Each bar represents a log_2 ratio of expression at each salinity for an individual hydrophobin. All values above and below the dashed grey lines were considered as significantly differentially expressed.

predicted at each salinity (Figure 6B). Twelve KEGG pathways had significantly higher proportions of differentially expressed genes than expected (Additional file 6: Table S5).

Alternative splicing was observed for 730 genes (15.0%), and for 51.6% of these, this occurred only at one of the limiting salinities. The majority of cases were identified as intron retentions, followed by alternative 3', and then 5', splicing (Figure 6D). Only one instance of exon skipping was detected. Such a distribution of alternative splicing modes is in agreement with observations from other fungi [23], where alternative splicing levels are generally much lower than in plant or human cells. While it is not possible to directly compare the values observed in other species [53], the around 700 events observed in *W. ichthyophaga* at each salinity was not outstanding (Additional file 1: Figure S4). Nevertheless, due to the small number of genes, the number of alternative splicing events per gene is close to the highest values observed in other species [23].

Transmembrane alkali-metal cation transporters are among the most important proteins in hypersaline environments [19]. Although hardly any studies have been performed on cells grown at constant salinities rather than those exposed to salinity shock, it has been noted that in *S. cerevisiae*, some groups of metal-cation transporters are expressed constitutively, while others have complex salt-responsive transcriptional regulation (e.g., P-type sodium ATPase Ena1 [19]). In the halotolerant ascomycetes *H. werneckii* and *Debaryomyces hansenii*, the expression of P-type H^+ and Na^+ ATPases is salt dependent, even when adapted to constant salinity [37,54,55]. It was therefore unexpected that the expression of most of the genes encoding metal-cation transporters was not affected by growth at 10% NaCl or 30% NaCl (w/v). Only three genes fulfilled the criteria for differential expression: [GenBank: EOR00619, GenBank:EOR04078, GenBank:EOR03958].

The first of these, EOR00619, was more expressed at high salinity ($\log_2$ ratio of expressions, 1.52), and it is a putative Na^+/ phosphate symporter (homologue of Pho89). Pho89 is the only Na^+-coupled secondary anion transport system in *S. cerevisiae*, and it is strongly induced at alkaline pH [56]. Since in such environment the use of a transmembrane proton gradient to drive secondary transporters is hindered, Na + gradient could serve as an alternative source of energy. The possible role of this transporter in hypersaline conditions was also noted in *H. werneckii*, which contains six copies of its gene [37]. Our results might indicate that *W. ichthyophaga* cells use this energy even under conditions of low pH, since the pH in the laboratory medium falls below 4 already in the exponential growth phase. The expression of [GenBank:EOR04078] (a putative P-type Na^+ ATPase), and [GenBank:EOR03958] (a P-type ATPase of unknown specificity, possibly an Acu K^+ importer), was higher at low salinity ($\log_2$ ratios, 1.00, 1.27, respectively). While the differences in expression are relatively small, these findings are difficult to explain.

All of the other transporters remained unaffected by the difference in salinity. Furthermore, their expression was relatively low. For example, when genes were ordered by their expression at high salinity, the two Na^+-exporting P-type ATPases were in positions 3812 and 4613 of the total of 4884 predicted proteins, while two Na^+/H^+ antiporters were at 2075 and 3927 (Additional file 5: Table S4). Among the first 400 of the most-expressed proteins, there were only various subunits of the P-type H^+-transporting ATPase, and a putative arsenite transporter [GenBank:EIM21938]. None of these were differentially expressed.

It has to be noted that the sequencing of the transcriptome will only reveal regulation at the level of transcription and mRNA stability. This cannot provide any indication about other possible modes of posttranscriptional control. Additionally, the functions of the genes analysed might be complemented by unconventional transporters that have not yet been identified. On the other hand, the apparent non-responsiveness of transporters might be just one of the aspects of the ecological strategy of *W. ichthyophaga*, a species that has evolved into a unique example of a narrowly specialised fungal halophile [9]. The observed absence of transcriptional response might explain the unusual inability of *W. ichthyophaga* to grow without salt.

Nevertheless, the transcription of some other genes responds to changes in salinity, among them also the above discussed hydrophobins. Half of these genes are differentially expressed (Figure 6E). It is interesting to note that some of these are more expressed at high salinity, and some at low salinity. Furthermore, there was no association between their expression profiles and isoelectric points, or the numbers of acidic amino acids. As was previously noted, the presence of multiple hydrophobin genes in an organism might be due to their different expression profiles at different developmental stages or under different environmental conditions, or to their different functional roles that are reflected in structural differences [26].

Among the most differentially expressed at low salinity, with $\log_2$ ratios of −9.19 and −3.34, were two expansin-like proteins of *W. ichthyophaga* [GenBank:EOR02784, GenBank:EOR03994]. These genes are distantly related to the plant expansins, through the presence of the double-psi beta-barrel domain and signal peptide [57]. Expansins loosen the cell wall by disrupting the non-covalent bonds between cellulose microfibrils and matrix polymers through a non-enzymatic mechanism [58]. This activity was recently shown also for a fungal expansin-like protein from *Bjerkandera adusta* [57]. As hydrophobins these proteins may also be linked to the cell wall and

possibly play a role in major changes in the cell wall of *W. ichthyophaga* at different salinities, which were mentioned earlier.

Thirty-four genes showed both high expression (RPKM >300 for at least one salinity, with RPKM indicating the number of reads which map per kilobase of the exon model per million mapped reads) and large differences in expression between the two salinities (absolute $\log_2$ ratio >2; Additional file 7: Table S6). Eight proteins with higher expression at high salinity included enzymes involved in the degradation of lipids ([GenBank: EOQ99895], a lipase) and their use in gluconeogenesis ([GenBank:EOR01390], an isocitrate lyase that enables synthesis of glucose from acetyl-CoA; and EOR04235, phosphoenolpyruvate carboxykinase, which catalyses the rate-limiting step of gluconeogenesis). In contrast at the lower salinity there was a higher expression of a phosphoglycerate mutase-like protein ([GenBank:EOR03927], involved in the eighth step of glycolysis), glycoside hydrolase [GenBank:EOQ98927], and a protein highly similar to fatty-acyl-CoA synthase from *W. sebi* [GenBank:EOQ99675]. This possibly indicates that at lower salinity, the metabolism is directed from carbohydrates to lipids, while the opposite is true at higher salinity. Among other genes of interest is an elevated expression of a stress-response protein, Rds1 [GenBank:EOR00712] at high salinty and of a tyrosinase-like protein [GenBank:EOR01004] at low salinity. The latter is one of three homologues in the genome (in addition to [GenBank:EOR00580, GenBank: EOR00979]). Tyrosinases are involved in melanin synthesis, a stress-protective pigment, which can also play a role in adaptation to hypersaline environment [59]. Nevertheless, it has to be noted that no melanin pigments have been reported to date in *Wallemia* spp., only a number of different pyrrolylpolyenes [60].

A list of all differentially expressed genes is available in Additional file 7: Table S6.

Conclusions

It is believed that low levels of adaptability and genetic recombination are important in the survival strategy of *W. ichthyophaga* [9]. The extreme specialisation of *W. ichthyophaga* indicates that it has adapted to a relatively stable hypersaline environment where competition from other species is scarce. This allows *W. ichthyophaga* to survive despite its relatively long generation times, its inability to grow without salt, and its lack of sexual reproduction. The characteristics of its genome and its transcriptomic response to salt confirm these findings. The genome and the number of predicted genes are among the smallest observed for fungi. Intriguingly, analysis of the genome also shows that it is possible to survive and grow in solutions saturated with NaCl with relatively small numbers of ion-transporter genes and although their transcription is relatively low and non-responsive to different salt concentrations.

A long-term survival strategy, where persistence is more important than rapid reproduction, good adaptability or competition for resources, will favour energy-efficient passive barriers against harmful effects of high salt concentrations. Previous morphological studies have reported the unusually thick cell wall of *W. ichthyophaga*, which could be one such mechanism [8]. Hydrophobins, proteins with multiple cellular functions, might also have important roles in fortifying the cell wall of *W. ichthyophaga* against the hostile environment, as indicated by genomic and transcriptomic analyses. This is especially so, as their unusually high proportion of acidic amino acids is a phenomenon that is a known characteristic of halophilic proteins from other organisms.

The peculiar lifestyle of *W. ichthyophaga* has had ample time to evolve. According to our estimates, 250 million years have passed since the ancestor of Wallemiomycetes separated from the ancestor of the contemporary Agaricomycotina, the closest known relative of *Wallemia* spp. The distinct characteristics of *W. ichthyophaga* make it a very interesting organism for studies of adaptation to hypersaline environments, which are different from those that have evolved in other species. The availability of its genomic sequence should be of significant help in such studies. At the same time, as shown with the findings of the unusual hydrophobins, it can also lead to the discovery of molecules that evolved along different evolutionary trajectory than their homologues from other species, and thus have novel traits that might be of interest in biotechnology and other fields.

Methods

Strain and DNA/RNA preparation

The *W. icththyophaga* (type strain EXF-994) used in this study was isolated from extremely saline water of Sečovlje solar saltern (Adriatic coast, Slovenia) and is preserved in the culture collections of the Department of Biology, Biotechnical Faculty, University of Ljubljana (EXF). The cells of *W. ichthyophaga* were grown at 24°C on a rotary shaker (180 rpm) in defined YNB medium (ForMedium, UK): 0.17% (w/v) yeast nitrogen base, 0.08% (w/v) complete supplement mixture (both Qbiogene), 0.5% (w/v) ammonium sulphate, 2.0% (w/v) glucose, in deionised water, with pH adjusted to 7.0 and supplemented with NaCl to 10%, 20% and 30% NaCl (w/v).

Growth was monitored by measuring the pH of the medium. The cells of mid-exponential cultures (pH 4.0) were harvested by centrifugation (4000× *g*; 10 min), frozen in liquid nitrogen, and homogenised using a pestle and mortar.

Highly purified fungal genomic DNA was isolated from mid-exponential cells grown in YNB media with 20% (w/v) NaCl, using the phenol/ chloroform/ isoamyl alcohol method, modified for DNA isolation from filamentous fungi, as described previously [61]. The quality and quantity of the DNA was evaluated on standard 1% agarose gel electrophoresis, as well as spectrophotometrically with NanoDrop 2000 (Thermo Fisher Scientific, USA).

The RNA of the *W. ichthyophaga* cells grown at 10% and 30% NaCl was isolated using the TRI Reagent (Sigma-Aldrich, Germany), according to the manufacturer instructions. Possible DNA contamination was degraded with DNAse I (Thermo Fisher Scientific - Fermentas, Lithuania), and the integrity and purity of the RNA was evaluated spectrophotometrically and by capillary electrophoresis (Agilent 2100 Bioanalyser; Agilent Technologies, USA).

Genome sequencing and assembly

From the genomic DNA of *W. ichthyophaga*, 500 bp and 2000 bp DNA sequencing libraries were constructed using 10 μg and 20 μg DNA, respectively. A total of 1.89 Gb and 1.80 Gb reads were generated by Illumina Hiseq™ 2000 at BGI-Shenzhen (Shenzhen, China). To ensure the accuracy of assembly, reads with 40 low-quality (≤Q2) bases, or 10% Ns, or 15 bp overlap between adapter and duplications were filtered. The short reads from the two libraries were assembled by *SOAPdenovo* 1.04 [62,63], with optimal assembly acquired with the key parameter K = 21.

Gene prediction and annotation

The prediction of the genes was made by determining the putative open reading frames with GeneMark-ES 2.3e [64]. Repeat sequences were identified by Repeat Masker version 3.3.0 with Repbase version 15.08, and the following parameters: -no_is, -norna, -engine, -s, -parallel = 1; and Repeat Protein Mask with parameters: -noLowSimple, -pvalue = 1e-4 [65]. Tandem repeats were found using the Tandem Repeat Finder software 4.04 [66]. Non-coding RNA was predicted by rRNAmmer 1.2, tRNAscan-SE 1.23, and Rfam 10.1. The protein-encoding genes were annotated through BLASTp searches in the KEGG (release: 55.1 2010-09-01) and COG (release: 20090331) databases, at the threshold e-value $\leq 1\times e^{-10}$, and the best hit was filtered using a 50% identity cut-off value.

cDNA Library construction, and sequencing

The cDNA library was constructed using 40 μg RNA for each of the two salinity samples. In short, beads with oligo(dT) were used to enrich poly(A) mRNA, which was then disrupted into short fragments of 200 nt to 700 nt. These were used as first-strand cDNA templates synthesised by using random hexamer primer. The second-strand cDNA was synthesised by adding buffer, dNTPs, RNaseH and DNA polymerase I. The cDNA library was purified using QiaQuick PCR extraction kits and resolved in elution buffer for end repair and adding poly(A). Finally, cDNA fragments were ligated with sequencing adaptors and fragments of 200 bp (±25 bp) were selected for the PCR amplification. The two constructed cDNA libraries were sequenced by Illumina Hiseq™ 2000 at BGI-Shenzhen (Shenzhen, China).

Trancriptome data processing, alternative splicing and novel transcript predictions

Raw data generated by the sequencer were converted to raw nucleotide reads with Illumina GA Pipeline 1.6. The clean reads were acquired by the removal of the adaptor and the low quality reads (Q ≤5), and they were mapped to the genome and gene sequences of *W. ichthyophaga*. This was done using the SOAPaligner/soap2 2.20 [67]. Up to five base mismatches were allowed.

The TopHat read-mapping algorithm [68] that does not rely on known splice sites was used to find splice junction sites of transcripts. This provided the information relating to the combinations of different exons of the individual transcripts, so that four basic types of alternative splicing events were distinguished (exon skipping, intron retention, alternative 5' splicing, and alternative 3' splicing). Candidates for novel transcripts were assigned all of the gene models in intergenic regions from 200 bp upstream or downstream from a gene with a length >150 bp and an average coverage >2.

Graphical representation of the genome and comparison with *Wallemia sebi*

Graphical representation of the genome was constructed with the Circos software version 0.62 [69]. The GC content was calculated in 1000 bp windows with gccount from Control-FREEC package 5.9 [70]. Repetitive sequences were identified with RepeatMasker 3.3.0 [71] with Fungi used as the model for analysis. Gene duplications were detected by aligning predicted proteins back to the genome with Exonerate 2.2.0 using the protein2genome model [72] and limiting the reported hits to those above the 50% maximal score obtainable for that query. The number of hits was counted for each query.

The whole genome alignment between *W. ichthyophaga* and the publicly available genome of *W. sebi* [4] was calculated with the promer algorithm as implemented in Mummer 3.23 and plotted with mummerplot utility [73]. All parameters were the same as described in [74] except that instead of discarding scaffolds less than 500 kbp only scaffolds less than 200 kbp were discarded.

The numbers of shared and unique proteins of *W. ichthyophaga* and *W. sebi* were determined by an all-against-all blast of their whole proteomes (e-value cut-off, 10^{-6}).

Phylogenetic analysis

A super alignment of the fungal proteomes was constructed with the Hal pipeline [75], allowing for no missing data. As well as *W. ichthyophaga* and *W. sebi* [4], several other publicly available proteomes were included. The following were obtained from the Broad Institute of MIT and Harvard (http://www.broad.mit.edu): *Coprinopsis cinerea* (*Coprinopsis cinerea* Sequencing Project); *Cryptococcus neoformans* (*Cryptococcus neoformans var. grubii* H99 Sequencing Project); *Ustilago maydis* (*Ustilago maydis* Sequencing Project); *Puccinia graminis* (*Puccinia* Group Sequencing Project); *Candida albicans* (*Candida* Sequencing Project); *Aspergillus nidulans* (*Aspergillus* Comparative Sequencing Project); *Neurospora crassa* (*Neurospora crassa* Sequencing Project); *Rhizopus oryzae* (*Rhizopus oryzae* Sequencing Project). The other proteomes were *Saccharomyces cerevisiae* (SGD project. http://www.yeastgenome.org/download-data/ (28.1.2013)); *Rhodotorula graminis* [76]; *Agaricus bisporus* [76]; and *Malassezia globosa* (Procter & Gamble: http://www.pgbeautygroomingscience.com/dandruff-genome.html/ (28.1.2013).

Conservative alignment (118776 bp) was used to estimate the best protein evolution model with ProtTest 3.2.1 [77]. The species tree was generated with the PhyML 3.0 software [78] with aLRT implementation, for the calculation of branch supports as Chi2 based support. The analysis was run using the LG model of evolution. The ProtTest estimate of the alpha parameter of the gamma distribution of six substitution rate categories (1.136) and the determined proportion of invariable sites (0.094) were used. The tree was then calibrated with r8s software [79], using four previously published calibration points [24]: *Rhizopus oryzae*–Dykaria split 495 mya, Ascomycota–Basidiomycota split 452 mya, Pezizomycotina crown 215 mya, Basidomycota crown 340 mya.

Protein sequences containing the Pfam domain PF00690 (P-type ATPase) were aligned using the L-INS-i method in the MAFFT software [80]. The gene tree was generated with the PhyML 3.0 software [78] with aLRT implementation, for the calculation of branch supports as Chi2 based support. The LG model of protein evolution was used, together with the alpha parameter of gamma distribution of six substitution rate categories (0.988) and the determined proportion of invariable sites (0.008), as estimated by ProtTest 3.2.1 [77]. A second tree (for comparison purposes, not shown) was generated by applying a maximum parsimony method as implemented in the Mega software version 5.05 [81].

Evolution of protein families

Analysis of protein family expansions and contractions was performed with the CAFE software [82]. Pfam domains of selected fungal proteomes were identified with a stand-alone Pfam scanner and a database downloaded on 30.1.2013 [83]. This was used to produce a Table of Pfam domains, which was used as input of CAFE, together with the chronogram constructed from the whole proteomes, as described above.

Proteins containing the hydrophobin Pfam domain (PF01185.13) were aligned using the L-INS-i method in the MAFFT software [80]. Sequence logos of amino-acid residues were drawn using the WebLogo 3 service [84], after removing the positions that were present in less than 75% of the proteins of a given species. The properties of the proteins were calculated with the pepstats utility included in the EMBOSS suite [85].

Searching for mating- and meiosis-related proteins and alkali-metal cation transporter homologues

To investigate the molecular evidence of sex in *W. ichthyophaga*, we searched for the presence of mating-type and meiotic-protein homologues in the proteome. Databases of mating-related proteins, homeodomain-containing proteins (PF00046), pheromone factor receptors (PF02076), pheromone response factors (Prf1), and pheromone precursors (PF08015) were created by retrieving sequences of selected basidiomycetes (*Agaricus bisporus, Coprinopsis cinerea, Cryptococcus neoformans, Cryptococcus heveanensis, Puccinia graminis, Ustilago maydis, and W. sebi*) and *S. cerevisiae* from UniProt Consortium (http://www.uniprot.org/). These were used as queries in BLASTp for the investigation of the mating-type gene homologues in *W. ichthyophaga*. The results were filtered according to the e-value cut-off 10^{-2} criteria, and compared against GenBank. Furthermore, the genome of *W. ichthyophaga* was investigated for the representative set of meiotic genes (i.e., a 'meiosis detection toolkit') [49], using protein homologues from *S. cerevisiae* and *C. cinerea* [86].

In the same way, the identification of all alkali-metal cation transporters encoded in the *W. ichthyophaga* genome was performed. In short, a database of *S. cerevisiae* transporters (Trk1, Trk2, Tok1, Pho89, Nha1, Ena and Pma P-type ATPases, Kha1, Nhx1, Vnx1, Pmc1, Mrs7) and transporters identified in other fungi (Acu1-4, Hak1-4) (*Ajellomyces capsulata, Ajellomyces dermatitidis, Candida albicans, Candida dubliniensis, Debaryomyces hansenii, Hordeum vulgare, Magnaporte oryzae, Millerozyma farinosa, Neosartorya fumigata, Neurospora tetrasperma, Physcomitrella patens, Pichia angusta, Sporisorium reilianum, Schwanniomyces occidentalis, Ustilago maydis, Ustilago hordei, and Wickerhamomyces ciferrii*) was constructed by collecting the protein sequences from UniProt Consortium.

BLASTp results were filtered according to the e-value cut-off 10^{-40}, and compared against GenBank.

Additional files

Additional file 1: Figure S1. Classification of the predicted genes into the KEGG database categories. **Figure S2.** Classification of the predicted genes into clusters of orthologous groups (COG database). **Figure S3.** Distribution of transcriptome gene coverage. **Figure S4.** Number of different alternative splicing events at each salinity.

Additional file 2: Table S1. Number of proteins of *W. ichthyophaga* with the given Pfam domain.

Additional file 3: Table S2. Evolution of protein families. Results of the analysis with the CAFE software. For each family, the P values for the family expansion/ contraction are shown for the whole tree, as well as for the branches leading to *W. ichthyophaga, W. sebi*, both *Wallemia* spp., and Agaricomycotina. Only families with a family-wide P-value lower than 0.01 are shown.

Additional file 4: Table S3. Alignment statistics of the transcriptome of *W. ichthyophaga* at 10% and 30% NaCl (w/v).

Additional file 5: Table S4. Expression of all of the genes of *W. ichthyophaga* at 10% and 30% NaCl (w/v).

Additional file 6: Table S5. KEGG database pathways with a significantly higher proportion of differentially expressed genes than expected (when comparing transcriptomes at 10% and 30% NaCl [w/v]).

Additional file 7: Table S6. Differentially expressed genes of *W. ichthyophaga* at 10% and 30% NaCl (w/v).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JZ isolated the DNA and RNA, analysed the mating-type and meiosis-related genes, genes encoding metal-cation transporters and hydrophobins, and the CAFE output, interpreted part of the transcriptomic data, and participated in writing the manuscript. YL submitted the data to GenBank and participated in writing the Methods section of the manuscript. WD, ZY and JH performed the sequencing, assembly and annotation of the genome and transcriptome, and participated in the project coordination. CG constructed the Circos images, performed the phylogenetic and CAFE analyses, interpreted part of the transcriptomic data, and participated in writing the manuscript. NGC conceived the study and participated in its coordination. All authors have read and approved the final manuscript.

Authors' information
Janja Zajc and Yongfeng Liu contributed equally as first authors.
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2.1.4 Morfološki odziv na visoko koncentracijo sladkorja se razlikuje od prilagajanja na visoke koncentracije soli pri kserofilnih glivah *Wallemia* spp.

Naslov v originalnem jeziku: Morphological responses to high sugar concentrations differ from adaptations to high salt concentrations in xerophilic fungi Wallemia spp.

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Izvleček:
Glive bazidiomicetnega rodu Wallemia so med najbolj kserofilnimi organizmi opisani do sedaj. Rod obsega vrste *Wallemia ichthyophaga*, *Wallemia muriae* in *Wallemia sebi*. Njihove morfološke prilagoditve pri visokih koncentracijah NaCl se kažejo v povečani debelini celične stene in velikosti celičnih agregatov. Cilj raziskave je bil preučiti njihovo rast in opredeliti celično morfologijo vključno s spremembami ultrastrukture celične stene teh gliv rastočih v gojiščih z nizko in visoko koncentracijo glukoze in medu. Analizirali smo rastne parametre in morfološke značilnosti s svetlobno mikroskopijo ter s presevno in vrstično elektronsko mikroskopijo. *Wallemia ichthyophaga* je rasla počasi v vseh gojiščih s sladkornimi topljenci, medtem ko sta *W. muriae* in *W. sebi* pokazali boljšo rast. Prva se je na visoko koncentracijo glukoze in medu univerzalno prilagodila s tvorbo večjih celičnih agregatov, medtem ko se je debelina celične stene povečala le pri visoki koncentraciji glukoze. Pri vrstah *Wallemia muriae* in *W. sebi* so bili agregati hif manjši pri visoki koncentraciji glukoze, opažene pa so bile drugačne in manj eksplicitne spremembe v debelini celične stene. Prilagoditveni odzivi kažejo, da je filogenetsko bolj oddaljena *W. ichthyophaga* bolje prilagojena na visoke koncentracije soli, vrsti *W. muriae* in *W. sebi* pa se lažje spopadata z visoko koncentracijo sladkorja v okolju.

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Morphological responses to high sugar concentrations differ from adaptation to high salt concentrations in the xerophilic fungi *Wallemia* spp.

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Osmotic stress

A B S T R A C T

Fungi from the food-borne basidiomycetous genus *Wallemia*, which comprises *Wallemia ichthyophaga*, *Wallemia muriae* and *Wallemia sebi*, are among the most xerophilic organisms described. Their morphological adaptations to life at high NaCl concentrations are reflected in increased cell-wall thickness and size of cellular aggregates. The objectives of this study were to examine their growth and to define cell morphology and any ultrastructural cell-wall changes when these fungi are grown in low and high glucose and honey concentrations, as environmental osmolytes. We analysed their growth parameters and morphological characteristics by light microscopy and transmission and scanning electron microscopy. *Wallemia ichthyophaga* grew slowly in all of the sugar-based media, while *W. muriae* and *W. sebi* demonstrated better growth. *Wallemia ichthyophaga* adapted to the high glucose and honey concentrations with formation of larger cellular aggregates, while cell-wall thickness was increased only at the high glucose concentration. *Wallemia muriae* and *W. sebi* demonstrated particularly smaller sizes of hyphal aggregates at the high glucose concentration, and different and less explicit changes in cell-wall thickness. Adaptive responses show that the phylogenetically more distant *W. ichthyophaga* is better adapted to high salt conditions, whereas *W. muriae* and *W. sebi* cope better with a high sugar environment.

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Introduction

Food represents a far more nutritious habitat for potential spoilage microorganisms than their natural systems, such as soil and water (Pitt & Hocking 2009). Glucose is a common component in many types of foods, such as fruit, jam, maple syrup, sweets, and jelly. Honey is often used as sweetener or is eaten directly, and it is mainly composed of a complex mixture of carbohydrates and other minor substances (White 1975). Fructose predominates in almost all honey types, followed by glucose. These two sugars account for 85 %–95 % of honey carbohydrates (Finola *et al.* 2007). To prevent

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pathogen growth and spoilage of foods, reduction of the biologically available water using drying or freezing, or by adding solutes such as salt or sugar, has been used for centuries.

Low amounts of biologically available water represent one of the most pervasive stresses for biological systems. By lowering the water activity (a_w) of a food to below 0.90, the growth of bacterial pathogens is restricted to a few highly resistant representatives (Brewer 1999), while many xerotolerant and xerophilic fungi can thrive. Xerophilic fungi, as defined by Pitt (1975), can grow at $a_w < 0.85$, which corresponds to 17 % NaCl or 50 % glucose added to growth medium (de Hoog *et al.* 2005), and these are typical spoilage organisms on sugary foods (Brown 1976; Snowdon & Cliver 1996; Samson *et al.* 2002; Grant 2004; Pitt & Hocking 2009). The importance of sugar-tolerant fungi is especially emphasised in the food industry, in which they frequently appear as spoilage organisms and agents of undesirable fermentation (Brown 1976).

It was originally a general belief in mycology that xerophilic fungi do not have any preference according to the chemical nature of a solute that lowers the a_w of a medium (Scott 1957). However, over the last decade, it has been demonstrated that some species of fungi have a preference for salt, such as the halophilic *Polypaecilum pisce* and *Basipetospora halophila* (Wheeler *et al.* 1988), while some have a preference exclusively for sugar, like *Xeromyces bisporus* (Pitt & Hocking 1977). Indeed, the majority of fungi have a general xerophilic phenotype (Northolt *et al.* 1995).

Most fungi that can tolerate low a_w belong to the division *Ascomycota* (de Hoog *et al.* 2005), while xerophily is rare in the *Basidiomycota*. Interestingly, physiological tests of strains of the basidiomycetous genus *Wallemia* have shown that it represents one of the most xerophilic fungal taxa (Zalar *et al.* 2005). Food-borne *Wallemia* is the only genus in the order *Wallemiales* (Wallemiomycetes, Basidiomycota), and these have often been isolated from dried, salty, and sweet foods (Samson *et al.* 2002), and also from the air, soil, sea salt, and hypersaline water of salterns on different continents (Wollenzien *et al.* 1995; Zalar *et al.* 1999a, 1999b, 2005; Wasser *et al.* 2003). Based on morphological and genetic differences, the genus *Wallemia* contains three species: *Wallemia ichthyophaga*, *Wallemia muriae* and *Wallemia sebi*. Phylogenetic trees based on ITS rDNA sequences supported the relatedness of *W. sebi* and *W. muriae* and have shown that *W. ichthyophaga* is genetically distant from these two species by numerous molecular steps (Zalar *et al.* 2005). As all three of these species show optimal growth in media with lowered a_w, they are considered xerophilic. *Wallemia sebi*, which was considered for a long time to be the only representative of the genus *Wallemia*, can grow over a wide range of a_w (0.99–0.69) in glucose/fructose media (Pitt & Hocking 1977), while in media with NaCl as the major solute, its lowest a_w for growth has been reported as 0.80 (Pitt & Hocking 1977; Zalar *et al.* 2005). *Wallemia muriae* and *W. ichthyophaga* require media with low a_w either *via* NaCl or sugar addition, to a_w ranges of 0.98–0.81 and 0.96–0.77, respectively. Furthermore, *W. ichthyophaga* can thrive in media saturated with NaCl.

Microorganisms that thrive in an environment of high solute concentrations are exposed to low a_w, which results in turgor-related stress that affects the cellular systems. As the cytoplasmic membrane is freely permeable to water, this situation leads to dehydration and cessation of growth, unless the organism has the means to adapt physiologically and morphologically to such environments (Galinski 1995). To date, relatively well-studied adaptations to high concentrations of NaCl have revealed a number of such adaptations, particularly in the extremely halotolerant black yeast *Hortaea werneckii*. These adaptations have included: intracellular accumulation of compatible solute mixtures and extrusion of toxic sodium ions from the cytoplasm (Prista *et al.* 1997; Kogej *et al.* 2005, 2007; Plemenitaš *et al.* 2008), changes in fungal colony morphology, plasma-membrane composition (Turk *et al.* 2004), and cell-wall structure (Kralj Kunčič *et al.* 2010), and meristematic growth, presence of protective extracellular polymeric substances (EPSs), and pigmentation (Kogej *et al.* 2006). In contrast, potential adaptations of fungi to low a_w induced by high concentrations of sugar have received little attention to date (Koh 1975; Tomita *et al.* 1996; Membre & Kubaczka 2000).

The three xerophilic species of the genus *Wallemia* represent suitable model organisms for the study of adaptations to both high salt and high sugar environments. Morphological adaptations to moderate and high NaCl concentrations of *Wallemia* spp. have been studied recently (Kralj Kunčič *et al.* 2010), and have revealed species-specific unique morphological adaptations. In the study of Kralj Kunčič *et al.* (2010), it was shown that response to stressful environments are not only reflected in a thickened cell wall, but also in changes in the morphology of the cells, including: multiple spherical cells of *W. ichthyophaga* joined into cellular aggregates (also referred as multicellular clumps), and hyphae of *W. muriae* and *W. sebi* that grow in the form of large and dense aggregates (also referred as mycelial pellets) (Kralj Kunčič *et al.* 2010). All of these morphological adaptations have been shown to be crucial under hypersaline conditions.

The aim of the present study was to extend the investigations of growth characteristics, cell morphology, and cell-wall ultrastructure to environments where a_w is reduced using high sugar concentrations (as high glucose and honey concentrations), with analyses by light microscopy and transmission and scanning electron microscopy. We studied whether the adaptations to high glucose and honey concentrations are different to those of high salt concentrations, for all three *Wallemia* spp.

Materials and methods

Media, strains, and growth conditions

The fungal strains *Wallemia ichthyophaga* EXF-994 (CBS 113033) and *Wallemia muriae* EXF-951 (CBS 116628) were originally isolated from hypersaline waters of the Sečovlje Adriatic solar salterns in Slovenia. *Wallemia sebi* EXF-958 (CBS 818.96) was isolated from sunflower seeds in Sweden (Zalar *et al.* 2005). All three species are maintained in the ExF Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Slovenia) and in the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). These strains were cultivated in standard yeast nitrogen base (YNB; Qbiogene, Heidelberg, Germany), and chemically defined liquid and solid medium supplemented with different

concentrations of glucose and honey: *W. ichthyophaga*, 55 % and 70 % glucose, 70 % and 90 % honey; *W. muriae* and *W. sebi*, 20 % and 65 % glucose, 20 % and 90 % honey (all w/v). Liquid cultures were grown at 28 °C, with constant shaking at 180 rpm, and plate cultures were incubated at 24 °C.

Determination of growth curves

For each of these *Wallemia* spp., their growth was followed at the low and high glucose and honey concentrations. The fungi were precultured on YNB media with the same glucose or honey concentration added as used later in the experiments. Inoculi for the cultures were prepared as cell suspensions in sterile spore-suspension solutions (0.05 % Tween 80, 0.05 % agar, distilled water) supplemented with the same glucose and honey concentrations as in the growth media. Drops of the cell suspensions were inoculated onto solid media, in triplicate.

Determination of dry weight

For determination of the final dry biomass yield, 1200 ml samples of the fungal cultures grown in liquid YNB media supplemented with the low and high glucose and honey concentrations were filtered through nitrocellulose filters (1.2-μm pore size) and dried at 100 °C to constant weight.

Sample preparation for light microscopy

Cells from cultures in mid-exponential growth phase were analysed under an Olympus BX51 light microscope (Olymus Imaging America Inc., Center Valley, PA, United States) equipped with an Olympus DP12 digital camera. The sizes of cells, and their hyphal compartments and multicellular clumps, were measured using the DP-Soft 3.2 software (Olympus).

Sample preparation for transmission electron microscopy

Samples of *Wallemia* spp. cells were prepared as previously described (Kralj Kunčič *et al.* 2010). Briefly, cultures grown to mid-exponential growth phase were filtered through nitrocellulose filters (1.2-μm pore size) and fixed for 2 h at room temperature in 2.5 % glutaraldehyde and 4 % paraformaldehyde in 0.1 M Na-phosphate buffer (pH 7.2). NaCl was added into the fixative to keep the same osmolarity as in the growth media. After fixing the cells, they were rinsed three times with 0.1 M Na-phosphate buffer with decreasing concentrations of NaCl, to prevent osmotic shock and the consequent collapse of the cell structure.

Due to the low amount of cell mass, embedding in 3 % agarose was used after fixing, to facilitate the manipulation of the samples and to enhance the penetration of chemicals and embedding medium into the fungal cells. Post-fixing was performed in 1 % OsO_4 in distilled water with a drop of 0.1 M Na-phosphate buffer, for 24 h at 4 °C. After washing three times in distilled water, the samples were dehydrated in a series of graded ethanol solutions (v/v): 30 % (10 min), 50 % (2 × 10 min), 70 % (2 × 10 min), 80 % (2 × 10 min), 90 % (2 × 10 min), absolute ethanol (3 × 10 min). Agar 100 resin (Agar Scientific) was used for embedding. Sections (70–90 nm) were cut with an ultramicrotome (Ultracut, Reichert) and contrasted with uranyl acetate and lead citrate. The sections were examined with a PHILIPS CM 100 transmission electron microscope (Royal Philips Electronics, Amsterdam, The Netherlands)

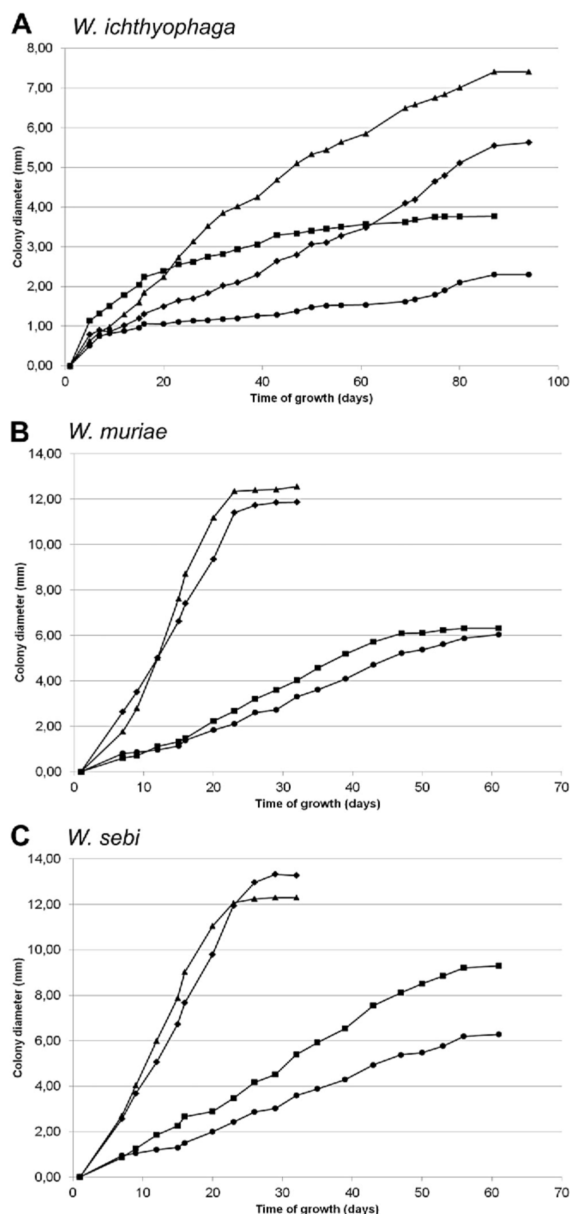

Fig 1 – Growth of *Wallemia* spp. in the low and high glucose and honey concentrations. Growth curves of *W. ichthyophaga* (A), *W. muriae* (B), and *W. sebi* (C) on solid YNB media with the low (◆) and high (■) glucose concentrations and the low (▲) and high (●) honey concentrations. For growth curve determination, the diameter of fungal cultures grown on the solid media was measured every 2–5 d over 3 m, and a mean of two lines perpendicular to each other was taken. Three replicate plates of each fungal strain and media with glucose or honey were used in each experiment.

(80 kV) with a Gatan Bioscan Camera 792 digital camera, using the Digital Micrograph 3.3.1 software.

Sample preparation for scanning electron microscopy

Cells from cultures grown to mid-exponential growth phase were transferred onto a polycarbonate filter that was placed into a polypropylene filter holder. The cells were fixed and washed using a plastic syringe (Agar Scientific). All of the preparation steps, including the fixing and dehydration, were similar for both the transmission and scanning electron microscopy. For scanning electron microscopy, after dehydration, the samples were dried in 1,1,1,3,3,3-hexamethyldisilazan for 45 min at room temperature. The cells were then mounted on aluminium stubs and coated with gold by magnetron sputtering (Bal-Tec SCD 050 sputter coater). The samples were analysed using a JEOL JSM-6500 F scanning electron microscope (JEOL ltd., Tokyo, Japan).

Statistical analyses

The cell diameter of the *Wallemia ichthyophaga* spherical cells was measured. For *Wallemia muriae* and *Wallemia sebi* hyphae diameter and length was measured. The size of the cellular aggregates (clumps and pellets) was measured in several dimensions (the longest and at least one approximately perpendicular to the axis of the first measurement), and the median was calculated. The number of samples analysed is shown in the legends to Figs 4–6 and 8. The differences among the medians of the cell diameters and cluster sizes, the hyphal compartment lengths, and the cell-wall thicknesses of all three of these *Wallemia* spp. at the low and high glucose and honey concentrations were compared using Mann–Whitney U-tests. All of the calculations were carried out using the STAT-GRAPHICS Plus 4.0 statistics software for Windows. Statistical differences between the two different solute concentrations were categorised into three groups, as: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

Growth characteristics of Wallemia spp. in the low and high glucose and honey concentrations

According to previously described growth ranges in media supplemented with NaCl (Zalar et al. 2005; Kralj Kunčič et al. 2010), we selected two glucose and two honey concentrations for each of the *Wallemia* spp., such that the high concentrations lowered a_w of the medium to approximately the same values as with NaCl in our previous studies. Therefore, all of the present experiments were carried out under non-stress conditions and under stress. The low glucose and low honey concentrations represented the non-stress conditions, as: *Wallemia ichthyophaga* at 55 % glucose and 70 % honey (w/v; a_w 0.88); and *Wallemia muriae* and *Wallemia sebi* at 20 % glucose and 20 % honey (w/v; a_w 0.96). The stress responses were triggered at the high glucose and high honey concentrations, as: *W. ichthyophaga* at 70 % glucose and 90 % honey (w/v; a_w 0.80);

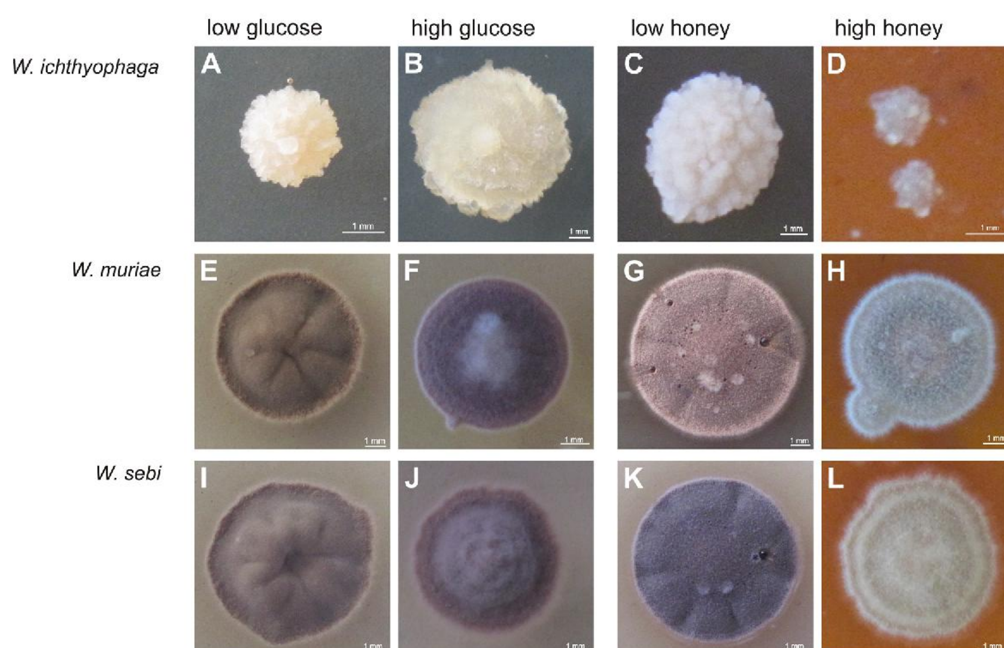

Fig 2 – Effects of glucose and honey on colony morphology of *Wallemia* spp. The colonies of *W. ichthyophaga* (A–D), *W. muriae* (E–H), and *W. sebi* (I–L) on the solid YNB media with the low and high glucose (*W. ichthyophaga*: 55 % glucose and 70 % glucose; *W. muriae* and *W. sebi*: 20 % glucose and 65 % glucose) and honey (*W. ichthyophaga*: 70 % honey and 90 % honey; *W. muriae* and *W. sebi*: 20 % honey and 90 % honey) concentrations. Scale bars as indicated.

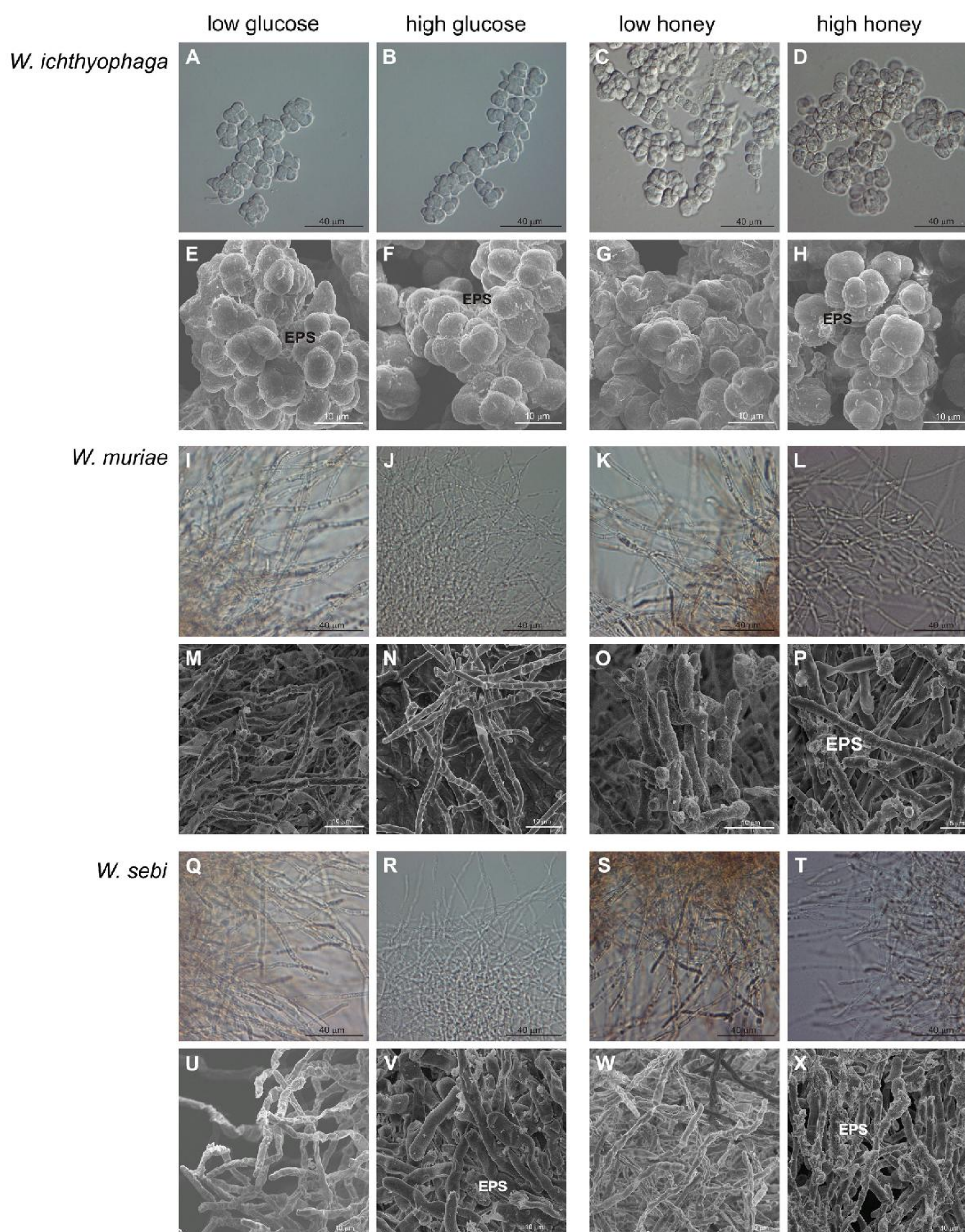

Fig 3 – Effects of glucose and honey on cell morphology of *Wallemia* spp. Light micrographs and scanning electron micrographs showing the cell morphology of *W. ichthyophaga* (A–H), *W. muriae* (I–P), and *W. sebi* (Q–X) grown in the liquid YNB media with the low and high glucose (*W. ichthyophaga*: 55 % glucose and 70 % glucose; *W. muriae* and *W. sebi*: 20 % glucose and 65 % glucose) and the low and high honey (*W. ichthyophaga*: 70 % honey and 90 % honey; *W. muriae* and *W. sebi*: 20 % honey and 90 % honey) concentrations. Scale bars as indicated. EPS – extracellular polymeric substances.

and *W. muriae* and *W. sebi* at 65 % glucose and 90 % honey (w/v; a_w 0.85).

The growth of all of the three species was slower on the media with the high glucose and high honey, as compared to the low concentrations (Table 1). The slowest growth rates on solid media for the low glucose and low honey were for *W. ichthyophaga* (0.15 mm week^{-1} and 0.18 mm week^{-1}, respectively) (Table 1; Fig 1A). The specific growth rates on media with the low glucose and low honey for *W. muriae* were 0.68 mm week^{-1} and 0.88 mm week^{-1}, respectively, and for

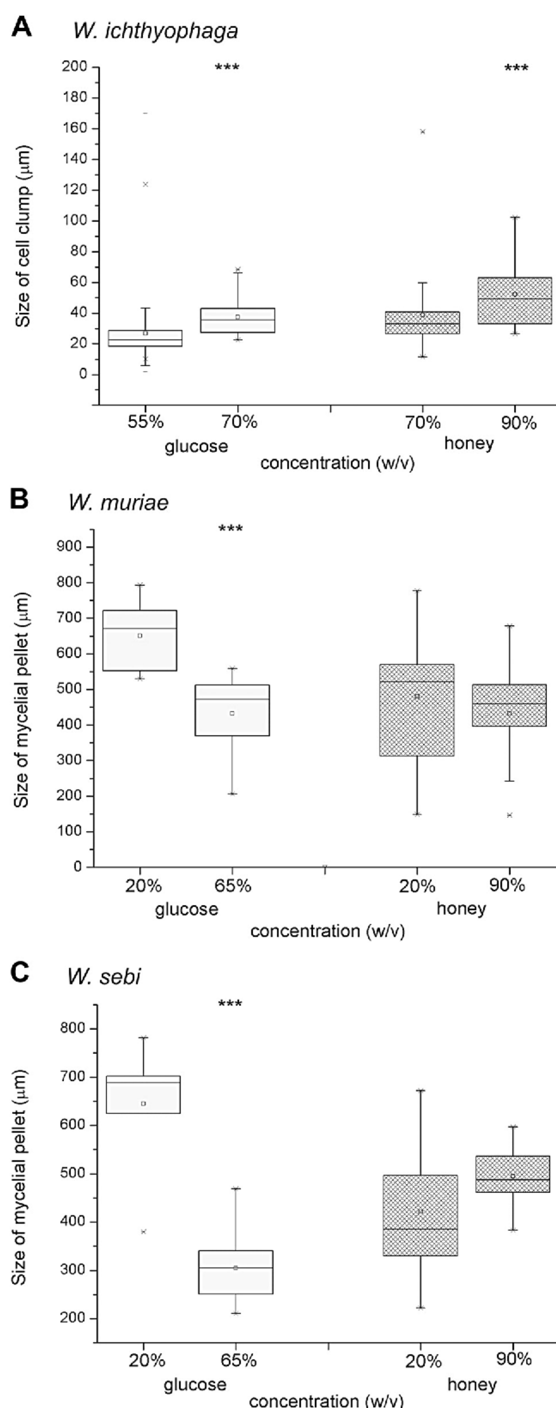

Fig 4 – Morphological characteristics of cell aggregates morphology of *Wallemia* spp. at the low and high glucose and honey concentrations. Box plots showing changes in the sizes of the cell clumps of *W. ichthyophaga* (A), and mycelial pellets of *W. muriae* (B) and *W. sebi* (C) grown in the liquid YNB media with the low and high glucose (*W. ichthyophaga*: 55 % glucose and 70 % glucose; *W. muriae* and *W. sebi*: 20 % glucose and 65 % glucose) and the low and high honey (*W. ichthyophaga*: 70 % honey and 90 % honey; *W. muriae* and *W. sebi*: 20 % honey and 90 % honey) concentrations. Data are medians from, respectively, 329, 44, 63, 27 cell clump (A), 18, 38, 36, 21 mycelial pellet of *W. muriae* (B), and 7, 48, 24, 9 mycelial pellet of *W. sebi* (C). *P < 0.05, **P < 0.01, ***P < 0.001.

W. sebi, 0.59 mm week^{-1} and 0.66 mm week^{-1}, respectively (Table 1; Fig 1B and C). *Wallemia muriae* and *W. sebi* showed comparable reduced specific growth rates on media with the high glucose and high honey, as 0.32 mm week^{-1} and 0.30 mm week^{-1} for *W. muriae*, and 0.27 mm week^{-1} and 0.29 mm week^{-1} for *W. sebi*, respectively (Table 1; Fig 1B and C). The greatest colony diameters for *W. muriae* were measured after 32 days with the low honey (12.55 mm), and for *W. sebi*, after 29 d with the low glucose (13.32 mm). The greatest colony diameter of *W. ichthyophaga* for grown on medium with the low honey was reached after 87 d (7.41 mm). The growth rate of *W. ichthyophaga* was lower at all of the tested glucose and honey concentrations compared to the other two species, which had similar growth characteristics (Table 1). The colonies of *W. ichthyophaga*, *W. muriae*, and *W. sebi* differed in diameter and colour on the solid media with respect to the low and high glucose and honey concentrations (Fig 2; Table 2).

The final fungal biomass yields (Table 3) were obtained from dry-weight determinations of early stationary cultures. Compared to the low glucose and low honey, in the media with the high glucose and high honey, the final biomass yields of all of the three species were reduced. *Wallemia ichthyophaga* produced higher biomass with the low and high glucose (131.8 mg/100 ml and 108.3 mg/100 ml medium, respectively), as compared to the media containing the low and high honey (112.2 mg/100 ml and 6.7 mg/100 ml medium, respectively). *Wallemia muriae* and *W. sebi* produced comparable amounts of dry biomass in all of these tested media. They both showed the highest biomass yields in the media with the low honey as 616.7 mg/100 ml and 548.1 mg/100 ml medium, respectively. The dry biomass yields of *W. ichthyophaga* were also always lower compared to those of *W. muriae* and *W. sebi*, in all of the tested media.

Changes in cell morphology of Wallemia spp. in the low and high glucose and honey concentrations

When grown in liquid medium, all three of these species formed distinctive cellular aggregates. *Wallemia ichthyophaga* (Fig 3A–H) formed compact and irregular multicellular clumps, while the filamentous *Wallemia muriae* and *Wallemia sebi* formed globular hyphal aggregates in the submerged cultures, known as mycelial pellets (Fig 3I–L and Q–T).

The multicellular clumps of *W. ichthyophaga* (Fig 3A–D) were significantly larger with the high glucose and high honey, at 36.1 μm and 49.2 μm, respectively, when compared to those in the low glucose and low honey, with 22.3 μm and 32.8 μm, respectively (as median sizes; Fig 4A). The diameters of the individual cells of *W. ichthyophaga* at the low and high glucose concentrations were not significantly different

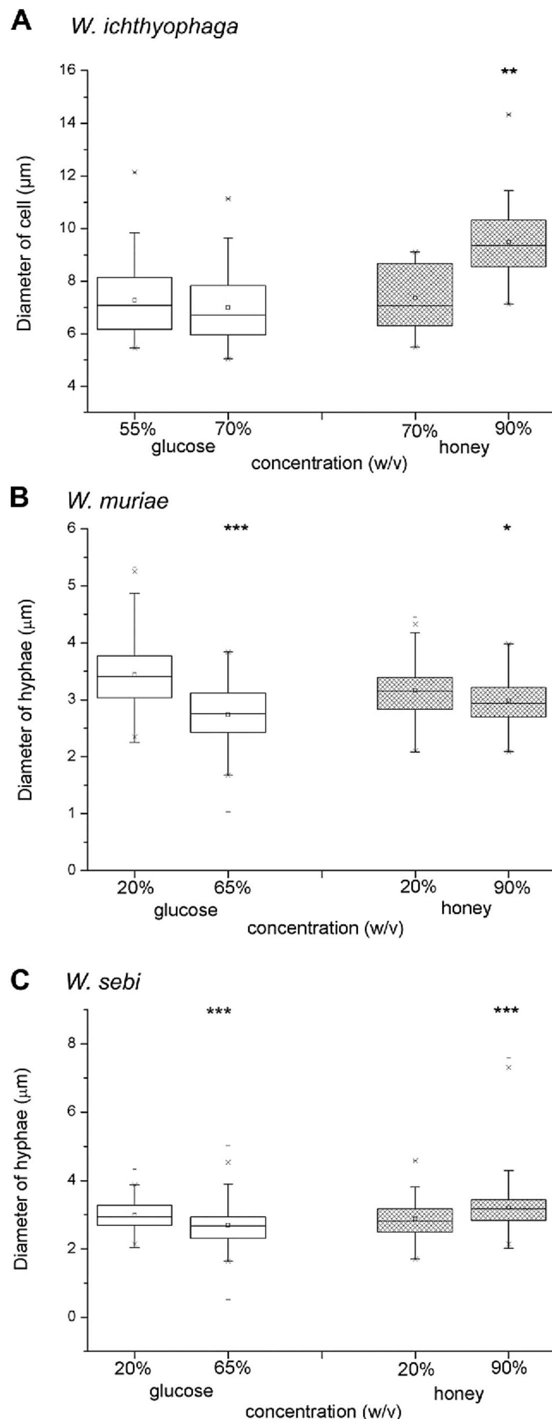

Fig 5 – Morphological characteristics of cells of *Wallemia* spp. at the low and high glucose and honey concentrations. Box plots showing changes in the sizes of the cells of *W. ichthyophaga* (A), *W. muriae* (B), and *W. sebi* (C) grown in the liquid YNB media with the low and high glucose (*W. ichthyophaga*: 55 % glucose and 70 % glucose; *W. muriae* and *W. sebi*: 20 % glucose and 65 % glucose) and the low and high honey (*W. ichthyophaga*: 70 % honey and 90 % honey; *W. muriae* and *W. sebi*: 20 % honey and 90 % honey) concentrations. Data are medians from, respectively, 78, 43, 9, 21 cell (A), 144, 147, 104, 57 for hyphal diameter of *W. muriae* (B), and 101, 208, 94, 134 for hyphal diameter of *W. sebi* (C). *P < 0.05, **P < 0.01, ***P < 0.001.

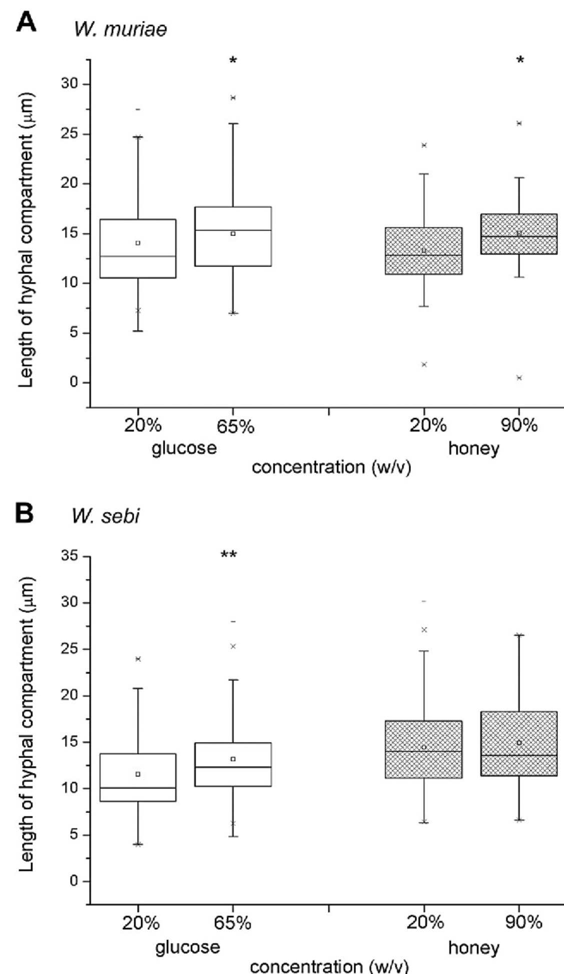

Fig 6 – Morphological characteristics of hyphal compartment length of *W. muriae* and *W. sebi* at the low and high glucose and honey concentrations. Box plots showing changes in the sizes of the hyphal compartment length of *W. muriae* (A) and *W. sebi* (B) grown in the liquid YNB media with the low and high glucose (20 % glucose and 65 % glucose) and the low and high honey (20 % honey and 90 % honey) concentrations. Data are medians from, respectively, 124, 86, 70, 31 for *W. muriae* (A), and (B) 60, 104, 117, 50 for *W. sebi*. *P < 0.05, **P < 0.01, ***P < 0.001.

(median diameter: low glucose, 7.1 µm; high glucose, 6.7 µm; Fig 5A). However, the high honey in the medium resulted in significant increases in cell diameter for *W. ichthyophaga*: from 7.1 µm at the low honey, to 9.3 µm when high (Fig 5A).

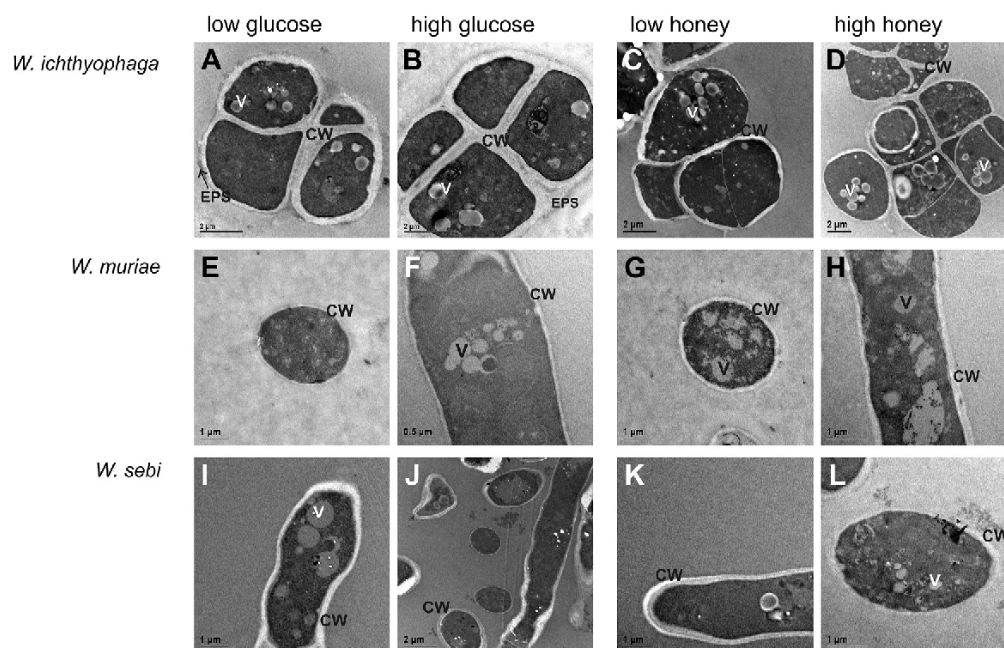

Fig 7 – Effects of glucose and honey on cell-wall ultrastructure of *Wallemia* spp. Transmission electron micrographs showing the ultrastructure of cell wall of *W. ichthyophaga* (A–D), *W. muriae* (E–H) and *W. sebi* (I–L) grown in the liquid YNB media with the low and high glucose (*W. ichthyophaga*: 55 % glucose and 70 % glucose; *W. muriae* and *W. sebi*: 20 % glucose and 65 % glucose) and the low and high honey (*W. ichthyophaga*: 70 % honey and 90 % honey; *W. muriae* and *W. sebi*: 20 % honey and 90 % honey) concentrations. Scale bars as indicated. V – vacuoles, CW – cell wall, EPS – extracellular polymeric substances.

Hyphae extended from some individual cells, in particular with the low glucose and low honey (Fig 3A–D). Scanning electron microscopy of *W. ichthyophaga* revealed a relatively smooth surface appearance of the multicellular clumps, independent of the type and concentration of solute (Fig 3E–H).

The mycelial pellets of *W. muriae* and *W. sebi* were darker and more compact and variable in shape in the media with the low glucose and low honey. In contrast, at the high glucose and high honey, the irregularly shaped mycelial pellets were lighter in colour, hyaline in appearance, and had longer hyphal tips at the outer parts (Fig 3I–L and Q–T). At the high solute concentrations, their mycelial pellets were compact only at the centre, where the hyphae were more densely packed.

The sizes of the mycelial pellets of *W. muriae* differed significantly with the low and high glucose, as 672.7 μm and 475.7 μm, respectively (median sizes, Fig 4B). Instead, in the media with the low and high honey concentrations, the sizes did not change significantly (528.6 μm, 458.9 μm, respectively). Similarly, the sizes of the mycelial pellets of *W. sebi* were significantly larger with the low glucose (688.0 μm) than with the high glucose (306.3 μm) (Fig 4C), while with honey, they did not change significantly (low and high honey, 404.7 μm and 487.1 μm, respectively). The high glucose and high honey concentrations resulted in significant decreases in the hyphal diameter of *W. muriae* (low and high glucose, 3.4 μm and 2.8 μm, respectively; low and high honey, 3.1 μm and 2.9 μm, respectively; Fig 5B). At the high concentrations of the sugars, the length of the hyphal compartment of *W. muriae* was significantly greater than for the low concentrations (low and high glucose, 12.7 μm and 15.3 μm; low and high honey, 12.9 μm and 14.7 μm; Fig 6A). Pronounced hyphal branching was not seen. Scanning electron micrographs of the *W. muriae* mycelial pellets showed relatively smooth and regular hyphal surfaces with the low and high glucose and honey concentrations in the growth media (Fig 3M–P).

The hyphal diameters of *W. sebi* were significantly higher with the low glucose as compared to the high glucose (2.9 μm and 2.6 μm, respectively). This was in contrast to the significantly lower hyphal diameters of *W. sebi* with the low honey concentration, compared to the high honey (2.8 μm and 3.2 μm, respectively; Fig 5C). Furthermore, the hyphae of *W. sebi* were significantly shorter with the low glucose (10.1 μm) compared to the high glucose (12.3 μm), while with the honey, the changes in the hyphal length were not significant (low and high honey, 14.1 μm and 13.5 μm, respectively; Fig 6B).

The scanning electron micrographs of *W. sebi* showed smooth and regular hyphal surfaces with both the low and high glucose and honey concentrations (Fig 3U–X).

Changes in the ultrastructure of the cell wall of Wallemia spp. in the low and high glucose and honey concentrations

The cell walls of *Wallemia ichthyophaga* were clearly structured in two distinct layers in all of the media: a thinner, electron-dense, outer layer, and a thicker, electron-translucent, inner

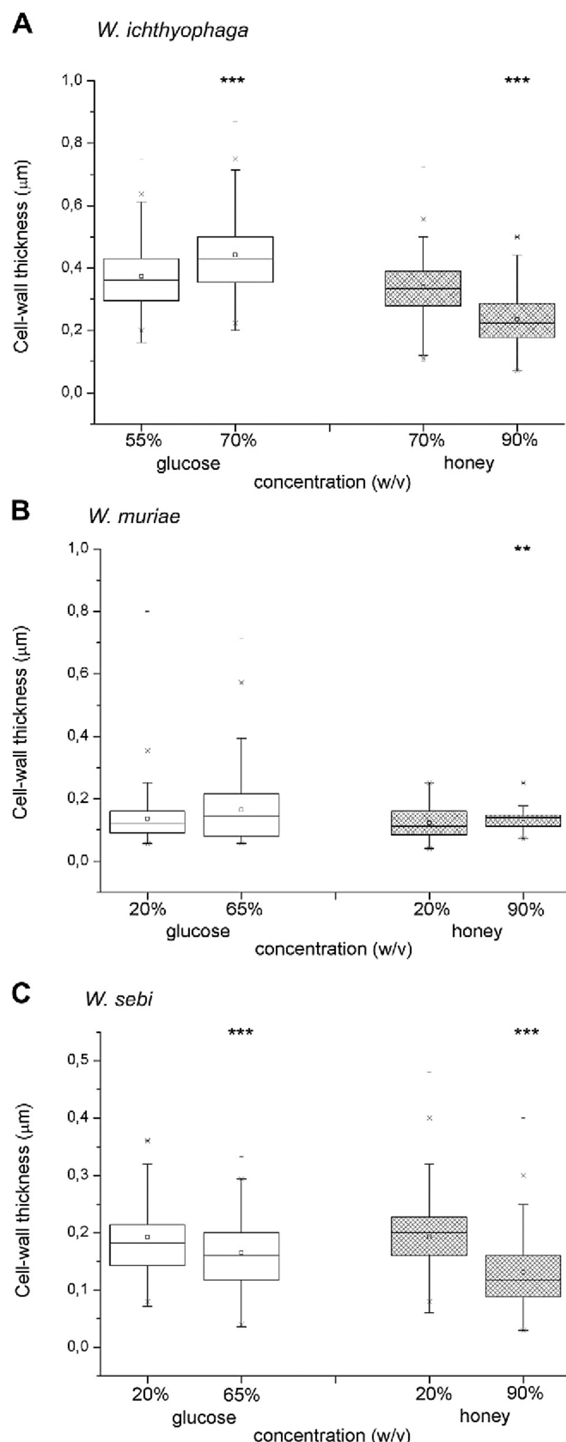

Fig 8 – Morphological characteristics of cell wall of *Wallemia* spp. at the low and high glucose and honey concentrations. Box plots showing changes in the cell-wall thicknesses of *W. ichthyophaga* (A), *W. muriae* (B), and *W. sebi* (C) grown in the liquid YNB media with the low and high glucose (*W. ichthyophaga*: 55 % glucose and 70 % glucose; *W. muriae* and *W. sebi*: 20 % glucose and 65 % glucose) and the low and high honey (*W. ichthyophaga*: 70 % honey and 90 % honey; *W. muriae* and *W. sebi*: 20 % honey and 90 % honey) concentrations. Data are medians from, respectively, 228, 122, 143, 211 cells of *W. ichthyophaga* (A), 127, 136, 131, 96 cells of *W. muriae* (B), and 170, 190, 169, 117 for cells of *W. sebi* (C). *P < 0.05, **P < 0.01, ***P < 0.001.

layer (Fig 7A–D). Transmission electron microscopy also revealed a higher content of EPS covering the outer cell-wall layer of *W. ichthyophaga* at the low glucose and low honey concentrations, compared to that at the high concentrations (not visible at magnifications shown in Fig 7). Compared to the glucose concentrations and the low honey concentration, vacuoles were present in greater numbers at the high honey concentration (Fig 7D). No cell-wall indentations were observed at any of the glucose and honey concentrations. The median cell-wall thicknesses with the low glucose and low honey concentrations were 0.37 µm and 0.34 µm, respectively, for *W. ichthyophaga*. However, in media with the high glucose, the median cell-wall thickness was significantly increased (0.44 µm; Fig 8A), whereas in media with the high honey concentrations, it was significantly decreased (0.23 µm).

As for *W. ichthyophaga*, transmission electron microscopy analysis of *Wallemia muriae* and *Wallemia sebi* hyphae showed similar cell-wall structures: the cell walls were two-layered, although less clearly structured (Fig 7E–L). The outer layer was thinner and electron dense, while the inner was thicker and electron-translucent. The cell walls were covered with low levels of EPS, which were independent of the glucose and honey concentrations. Vacuoles were more visible and were larger with the media with low glucose and low honey, compared to the high concentrations (data not shown). Transmission electron microscopy analyses of *W. muriae* hyphae showed that at the low glucose and low honey, the median cell-wall thicknesses were 0.14 µm and 0.12 µm, respectively, while with high glucose and high honey, these increased a little, by 0.02 µm (Fig 8B).

The analysis of the *W. sebi* cell walls by transmission electron microscopy showed that the median cell-wall thicknesses with the low glucose and low honey concentrations were both 0.19 µm. With the high concentrations of glucose and honey, these significantly decreased by 0.03 µm and 0.06 µm, respectively (Fig 8C).

Discussion

We investigated the three *Wallemia* species, *Wallemia ichthyophaga*, *Wallemia muriae*, and *Wallemia sebi*, when grown in media containing low and high concentrations of glucose and honey. These analyses include the different growth characteristics and morphology of the cellular aggregates, and in individual cells, the cell-wall ultrastructure, which we examined using light microscopy and transmission and scanning electron microscopy. We have demonstrated here that compared to the low glucose and low honey concentrations, high glucose and high honey as low a_w stress conditions trigger distinct responses in growth and morphological features across all three

Table 1 – Specific growth rate of *Wallemia* spp., when grown on solid YNB media with low and high concentration of glucose and honey.

	Specific growth rate (mm × week^{-1})			
	Low glucose concentration[a]	High glucose concentration[b]	Low honey concentration[c]	High honey concentration[d]
W. ichthyophaga	0.15	0.12	0.18	0.08
W. muriae	0.68	0.32	0.88	0.30
W. sebi	0.59	0.27	0.66	0.29

a *W. ichthyophaga*, 55 %; *W. muriae*, *W. sebi* 20 %.
b *W. ichthyophaga*, 70 %; *W. muriae*, *W. sebi*, 65 %.
c *W. ichthyophaga*, 70 %; *W. muriae*, *W. sebi*, 20 %.
d *W. ichthyophaga*, *W. muriae*, *W. sebi*, 90 %.

Wallemia spp. These can thus be compared to our previous study of the influence of high NaCl concentrations (Kralj Kunčič *et al.* 2010).

These high glucose and high honey concentrations restricted the growth of all three *Wallemia* spp. (smaller colony diameter, lower biomass yield), similar to the biomass reduction at high salinity (Kralj Kunčič *et al.* 2010). This reflects the high-energy cost of life under high osmolarity conditions (Oren 1999). In the present study, with *W. sebi* and *W. muriae* grown on media with the same glucose and honey concentrations, they both showed similar growth curve slopes and formed colonies of almost the same diameter. In contrast, across both of the glucose and honey concentrations, *W. ichthyophaga* grew more slowly, formed smaller colonies, and had lower biomass yield. Indeed, *W. ichthyophaga* showed by far the highest biomass yield in media supplemented with high concentrations of NaCl (Kralj Kunčič *et al.* 2010). Furthermore, comparisons of growth of all three *Wallemia* spp. on media containing either glucose or honey (present study) or NaCl (for details, see Kralj Kunčič *et al.* 2010) show that only the growth of *W. ichthyophaga* is stimulated by increased NaCl, thus revealing its exceptional halophilic character. It can also be noted that among the xerophilic fungi for which solute tolerances have been defined, their growth is either not influenced by solutes or is more vigorous on media containing high concentrations of sugars, rather than polyols or NaCl (Pitt & Hocking 1977; Hocking & Pitt 1979; Luard & Griffin 1981).

In submerged culture, *W. ichthyophaga* formed multicellular clumps, while both *W. muriae* and *W. sebi* formed mycelial pellets. These *W. ichthyophaga* multicellular clumps were significantly larger at the high glucose and high honey concentrations, as also observed in our previous study for high

Table 2 – Morphological characteristics of the colonies of *Wallemia* spp. on solid media with glucose and honey.

Wallemia spp.	Addition to growth medium	Colony morphology on solid media
W. ichthyophaga	55 % glucose	White to light yellow; punctiform; heaped; soft; moist; irregular surface and margin; not spreading deeply into agar; reduced area in contact with agar, light yellow reverse (Fig 2A)
	70 % glucose	Creamy yellow; heaped in centre; soft; moist; irregular surface and margin; did not spread deeply into agar; reduced area in contact with agar; light yellow reverse (Fig 2B)
	70 % honey	White; soft; punctiform; heaped; moist; irregular surface and margin; short aerial hyphae; not spreading deeply into agar; light yellow reverse (Fig 2C)
	90 % honey	White; soft; very moist; punctiform; heaped; irregular surface and margin; short aerial hyphae; not spreading deeply into agar; light yellow reverse (Fig 2D)
W. muriae	20 % glucose	Brown; round shape with light margin; powdery (high number of spores), dry; heaped and concave in darker centre; brown reverse (Fig 2E)
	65 % glucose	Brown to light brown; white pronounced aerial hyphae in centre; dry; powdery (high number of spores); variable shape with light beige shaggy margin; heaped; reduced area in contact with agar; brown reverse (Fig 2F)
	20 % honey	Brown; round shape marginal surface; less heaped; less powdery and dry; lighter in the centre, with white aerial hyphae; large area in contact with agar; brown reverse (Fig 2G)
	90 % honey	White to light brown; round shape with light marginal surface; powdery (high number of spores), dry; heaped in the light centre; with short aerial hyphae; brown reverse (Fig 2H)
W. sebi	20 % glucose	Brown; round shape with light brown margin; powdery (high number of spores), dry; centre heaped and concave; white aerial hyphae, brown reverse (Fig 2I)
	65 % glucose	Brown with light margin; powdery; dry; heaped in the centre; white aerial hyphae in the centre; reduced area in contact with agar, brown reverse (Fig 2J)
	20 % honey	Brown; round shape with light marginal surface; less heaped; less powdery and dry; lighter in the centre, with light tan hyphae; brown reverse (Fig 2K)
	90 % honey	White to light brown; round shape with light marginal surface; powdery (high number of spores), dry; heaped in the yellowish centre; with short aerial hyphae; brown reverse (Fig 2L)

Table 3 – Final biomass yields of *Wallemia* spp. at the low and high glucose and honey concentrations.

Wallemia spp.	Final biomass yield (mg/100 ml medium)			
	Low glucose concentration[a]	High glucose concentration[b]	Low honey concentration[c]	High honey concentration[d]
W. ichthyophaga	131.8	108.3	112.2	6.7
W. muriae	210.7	172.0	616.7	95.8
W. sebi	254.7	181.6	548.1	279.2

a *W. ichthyophaga*, 55 %; *W. muriae*, *W. sebi* 20 %.
b *W. ichthyophaga*, 70 %; *W. muriae*, *W. sebi*, 65 %.
c *W. ichthyophaga*, 70 %; *W. muriae*, *W. sebi*, 20 %.
d *W. ichthyophaga*, *W. muriae*, *W. sebi*, 90 %.

NaCl concentrations (Kralj Kunčič *et al.* 2010). It appears that the increase in size of these multicellular clumps is a universal adaptation strategy of *W. ichthyophaga* to both high ionic (NaCl) and non-ionic (glucose) solute concentrations in its surroundings. Interestingly, the mycelial pellets of *W. muriae* decreased in size when cultured in the media with the high glucose and high honey concentrations, as compared to the low concentrations of both of these solutes. With *W. sebi*, for the same comparison, the mycelial pellets showed different responses to these solutes: a decrease at the high glucose concentration, and a non-significant increase at the high honey concentration. However, the dense mycelial pellets of both *W. muriae* and *W. sebi* became less pigmented at the high glucose and high honey concentrations, as occurred also at high concentrations of NaCl (Kralj Kunčič *et al.* 2010). It appears that it is not the toxicity of the ions, but the high-energy cost of the osmotic stress that causes this less intense synthesis of pyrrolylpolyene pigments (Ahmed & Toube 1983, 1984; Ahmed *et al.* 1984; Oren 1999).

In general, a comparison of the median sizes of these *Wallemia* spp. cellular aggregates (multicellular clumps of *W. ichthyophaga*; mycelial pellets of *W. muriae* and *W. sebi*) in media with high NaCl (Kralj Kunčič *et al.* 2010) and high sugar concentrations shows that they are all larger in the media containing NaCl. This might indicate a more pronounced stress response in *Wallemia* spp. due to the ionic solute as compared to the non-ionic solute, and probably due, in particular, to the toxicity of Na^+ ions (Prista *et al.* 1997). In contrast, high glucose concentrations will provoke only turgor-related stress.

The ability of these *Wallemia* spp. to organise into cellular aggregates or 'multicellular communities' as a mechanism to enhance survival in environments with low a_w or with changed environmental conditions has been proposed for yeast populations (Palkova 2004; Palkova & Vachova 2006). Multicellular clumps have a decreased cell-surface area that needs to be exposed to the hyperosmotic environment. According to the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Hermansson 1999), high concentrations of solutes in the environment enhance the aggregation of cells. This adherence depends on the interplay of van der Waals forces, which enhance aggregation, and electrostatic 'double-layer' forces, which cause repulsion (Israelachvili & Mcguiggan 1988). Thus, the adhesion of cells changes according to the ionic strength, which is higher in media with high NaCl concentrations. According to the DLVO theory, these clumps in the high-saline media will be larger mainly due to the physical laws in these environments, and so they will not necessarily reflect an adaptation strategy of the organism.

The size of the cells of *W. ichthyophaga* did not significantly change when grown in the media with the low and high glucose concentrations. In contrast, a significant increase in cell size was seen when *W. ichthyophaga* was grown with high honey concentrations. Interestingly, the cells of *W. ichthyophaga* are smaller with the high sugar (6.7 μm) than in the media with high NaCl (8.4 μm; Kralj Kunčič *et al.* 2010). For the other two *Wallemia* spp., *W. muriae* and *W. sebi*, when compared to the hyphal morphology in saline media (hyphal diameter, 3.3–3.6 μm; hyphal compartment, 11.5–11.6 μm; Kralj Kunčič *et al.* 2010), they show shorter hyphal diameters (2.6–3.2 μm) and longer hyphal compartments (12.3–15.3 μm) with the high glucose and honey concentrations in the present study.

EPS biosynthesis is one of the most common protective adaptive mechanisms of extremophiles to compensate for the deleterious effects of extreme conditions (Nicolaus *et al.* 2010). EPSs have a protective role in Antarctic endolithic microcolonial fungi (Selbmann *et al.* 2005), in the halophilic alga *Dunaliella salina* (Mishra & Jha 2009), and abundantly in *Wallemia* spp. in extremely saline environments (Kralj Kunčič *et al.* 2010). In comparison, in these media with glucose and honey, the cell walls of all three *Wallemia* spp. were covered with relatively low levels of EPS. Indeed, on the cell surface of *W. ichthyophaga*, more EPSs were seen at the low glucose and honey concentrations, while on the cell surfaces of *W. muriae* and *W. sebi*, EPSs were present, but independent of the type and concentration of solute.

The cell walls of the *Wallemia* spp. in the media with glucose and honey were clearly structured in two distinct layers: a thinner, electron-dense, outer layer; and a thicker, electron-translucent, inner layer. In adapting to high salinity, as we have previously reported (Kralj Kunčič *et al.* 2010), all three *Wallemia* spp. responded with increases in cell-wall thickness. Interestingly, at the high glucose and honey concentrations in the present study, significant changes in cell-wall thickness were not always observed. In *W. ichthyophaga*, the cell-wall thickness at the high honey concentration even decreased, and although there was a significant increase with the high glucose, the increase in cell-wall thickness was by far the most apparent at high NaCl (at 25 % NaCl, the cell-wall thicknesses increased to 1.6 μm; Kralj Kunčič *et al.* 2010). In *W. muriae* the cell-wall thickness was moderately increased with the high concentrations of both of the sugar-based

media, while in *W. sebi*, it actually decreased in both of the sugar-based media at the high solute concentrations. Changes in the thickness of the cell wall can be a consequence of its changed molecular composition, such as elevated or lowered β-1-3 glucan or chitin levels (Kapteyn *et al.* 1999). A study of the stress-related consequences of sorbitol on the cell-wall composition of *Trichoderma reesei* showed that with the increased stress, there was limited synthesis of cell-wall glucans and chitin, and an overall inhibition of protein glycosylation (Górka-Niec *et al.* 2010). Also, in the case of *Neurospora crassa* cultivated with sorbitol, the cell-wall components were synthesised in limited amounts (Górka-Niec *et al.* 2010). The osmophilic yeast *Zygosaccharomyces rouxii* has a higher content of cell-wall chitin in media with high sugar concentrations, while in media with increased NaCl concentrations, this chitin content decreases dramatically (from an initial 15 %, to 1 %–2 %) (Tokuoka 1993; Tomita *et al.* 1996). Similarly, this has been observed in the case of NaCl stress for *Aspergillus flavus* and *Penicillium roquefortii* (Abu-Seidah 2007). Apparently osmotic stress can cause a reduction in cell-wall biogenesis in osmosensitive and osmophilic species. Thus, in the present study, the thinner cell wall observed in *W. ichthyophaga* at the high honey concentration, and also in *W. sebi* at the high glucose and honey concentrations, might be a consequence of impaired cell-wall biogenesis. In these media with excessive glucose, the impaired cell-wall biosynthesis reflected by the thinner cell wall is not likely to be affected by the consumption of the glucose, with the intensive synthesis of intracellular glycerol, or to be due to other metabolic demands. Therefore, the reason for the thinner cell wall in these media containing sugar at an equivalent a_w as reached with NaCl remains unresolved. Apparently, salinity triggers different responses for cell-wall morphology compared to high osmolarity caused by sugars. According to our data, we believe that the cell-wall thickness in the media containing the high glucose concentration is not the main protective response, in contrast to what appears to be the case in the media with high NaCl concentrations (Górka-Niec *et al.* 2010).

All of the morphological responses of these three *Wallemia* spp. to environments with high sugar concentrations are in agreement with their phylogenetic relatedness. It has been previously shown that *W. muriae* and *W. sebi* are phylogenetically closer to each other, and that they share the common morphology of individual hyphae, mycelial pellets, and colonies (Zalar *et al.* 2005). In contrast to *W. muriae* and *W. sebi*, *W. ichthyophaga* has a unique colony morphology, as it can form multicellular clumps that are composed of densely packed cells (Zalar *et al.* 2005) with thick cell walls (Kralj Kunčič *et al.* 2010). However, in our present and our previous study (Kralj Kunčič *et al.* 2010), we have shown that a common feature of the adaptation strategies to high solute concentrations of all three *Wallemia* spp. is an increase in the size of the multicellular clumps (of *W. ichthyophaga*) or the mycelial pellets (for *W. muriae* and *W. sebi*). *Wallemia ichthyophaga* is unique in its response to high osmolarity in terms of the thickening of the cell wall, which is particularly pronounced at high NaCl concentrations (Kralj Kunčič *et al.* 2010), and less apparent at the high glucose concentration of the present study. The growth characteristics of *W. sebi* and *W. muriae* on the media with the added glucose and honey were different from *W. ichthyophaga*, as *W. ichthyophaga* grew slowly and formed smaller colonies.

Thus, overall, these data show differences in the adaptation to osmotic conditions between these three *Wallemia* spp., demonstrating the general xerophilic character of *W. muriae* and *W. sebi*, and the pronounced halophilic character of *W. ichthyophaga*.

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2.1.5 Kserofilni glivni rod Wallemia - biološko aktivni prebivalci solarnih solin in slane hrane

Naslov v originalnem jeziku: Xerophilic fungal genus Wallemia - bioactive inhabitants of marine solar salterns and salty food.

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Izvleček:
Wallemia je rod svetovno razširjenih kserofilnih gliv, ki so pogosto vpletene v kvarjenje sladke, slane in posušene hrane. Do nedavnega je bila v rodu poznana le ena vrsta, *Wallemia sebi*. Analiza fizioloških, morfoloških in molekularnih lastnosti velike skupine sevov osamljenih iz solin ter drugih habitatov po vsem svetu, je v kraljestvo glive dodala nov bazidiomicetni razred Wallemiomycetes z redom Wallemiales in s tremi vrstami rodu Wallemia: *W. ichthyophaga*, *W. sebi* in *W. muriae*. Vrsta *W. ichthyophaga* bila spoznana za najbolj halofilni evkariontiski organizem, zato predstavlja možen model za poglobljene študije prilagoditev na slane pogoje. Naše predhodne raziskave so pokazale, da vse tri vrste rodu Wallemia sintetizirajo še neopisano hemolitično spojino. Presenetljivo je to, da je sinteza te spojine še posebej poudarjena pri pogojih nizkih vodne aktivnosti. Zaradi nedavno opredeljenega taksonomskega statusa, do sedaj še niso poročali o proizvodnji kakršnih koli bioaktivnih spojin pri vrstah znotraj tega rodu. V članku predstavljamo taksonomijo, ekologijo, fiziologijo in doslej opisane molekularne mehanizme prilagajanja na nizko vodno aktivnost, kot tudi bioaktivne potenciale rodu *Wallemia*, filogenetsko starega in osamelega taksona znotraj debla Basidiomycota.

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XEROPHILIC FUNGAL GENUS WALLEMIA – BIOACTIVE INHABITANTS OF MARINE SOLAR SALTERNS AND SALTY FOOD

ABSTRACT: *Wallemia* is a genus of cosmopolitan xerophilic fungi, frequently involved in food spoilage of particularly sweet, salty, and dried food. Until recently, only a single species, *Wallemia sebi,* was recognized in the genus. When a large group of strains globally collected in salterns and other different ecological niches was analyzed on the level of physiological, morphological and molecular characteristics, a new basidiomycetous class, Wallemiomycetes, covering an order of Wallemiales was proposed and three *Wallemia* species were recognized: *W. ichthyophaga*, *W. sebi* and *W. muriae*. *Wallemia ichthyophaga* was recognized as the most halophilic eukaryote known, thus representing an appropriate eukaryotic model for in depth studies of adaptation to hypersaline conditions. Our preliminary studies indicated that all three *Wallemia* species synthesized a yet undescribed haemolytic compound under, surprisingly, low water activity conditions. Due to the taxonomic status w hich was unrevealed only recently, there were so far no reports on the production of any bioactive compounds by the three newly described species. The article aims to present the taxonomy, ecology, physiology and so far described molecular mechanisms of adaptations to life at low water activity, as well as bioactive potential of the genus *Wallemia*, a phylogenetically ancient taxon and a taxonomic maverick within Basidiomycota.

KEY WORDS: fungi, *Wallemia* spp., taxonomy, low water activity, xerophiles, halophiles, osmoadaptation, secondary metabolites

ABBREVIATIONS

AAS, atomic absorption spectroscopy; a_w, water activity; EPS, extracellular polysaccharides; GPD1, glycerol-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; ITS rDNA, internal transcribed spacer regions ribosomal deoxyribonucleic acid; NMR, nuclear magnetic res-

onance; PTS2, peroxisomal targeting sequence; SSU rDNA, small subunit ribosomal deoxyribonucleic acid; UV, ultraviolet.

INTRODUCTION

Water, with its central role in biological processes, is the key life-limiting parameter. Therefore, low amount of biologically available water (low water activity [a_w]) represents one of the most pervasive stresses for biological systems, as only specially adapted organisms can thrive at such conditions. Tolerance of low water-activity is apparent in only ten out of 140 known orders of fungi, most of them belonging to the Ascomycota (de Hoog, Zalar et al., 2005), while xerotolerance is rare in the phylum Basidiomycota. Xerophilic fungi are capable of growth at water activity below 0.85, corresponding to 17% NaCl, or 50% glucose added to the growth medium (Gunde-Cimerman, Oren et al., 2005). Fungi are not only being capable of growing at low a_w, but they also show preferences for certain chemical nature of the solute lowering the a_w (de Hoog, Zalar et al., 2005; Gunde-Cimerman and Plemenitaš, 2006), as xerophilic fungi are able to live in the environments rich in sugar, while halophilic live in the environments rich in salt. Halotolerance and extreme halotolerance describe the salt-adaptable fungi that do not necessarily require salt (NaCl) for viability, but are able to sustain a range of different salt concentrations, even across the whole salinity range – from fresh water to NaCl saturation (Gunde-Cimerman and Plemenitaš 2006). An obligate halophilic character is possessed by only few fungal species that exhibit superior growth on media with NaCl as controlling solute (Wheeler, Hocking et al., 1988; Zalar, de Hoog et al., 2005; Kralj Kunčič, Kogej et al., 2010).

Xerophilic fungi were first only known as domestic extremophiles that contaminate food preserved by the reduction of biologically available water by means of drying, freezing or adding solutes (Pitt and Hocking 1977; Pitt and Hockering, 2009). Natural saline and hypersaline environments, where high concentrations of NaCl are prevailing, were believed to be populated almost exclusively by bacteria, archaea and eukaryotic alga *Dunaliella salina* (Rodriguez-Valera, Ruiz-Berraquero et al., 1981; Schiewer, 1991; Oren, 2005), until fungi as active inhabitants of solar salterns were first reported (Gunde-Cimerman, Zalar et al., 2000).

Later numerous halotolerant and extremely halotolerant fungi (Zalar, de Hoog et al., 1999; Gunde-Cimerman, Zalar et al., 2000; Butinar, Santos et al., 2005; Zalar, de Hoog et al., 2007; Zalar, Frisvad et al., 2008; Zalar, Gostincar et al., 2008), and only two halophilic representatives, both from genus *Wallemia* (Zalar, de Hoog et al., 2005) were isolated from hypersaline water of the Sečovlje solar salterns (Adriatic coast, Slovenia). Since the first discovery of fungi in salterns, numerous fungal species thriving in extremely saline environments around the

globe have been described, amongst them xerophilic and halophilic species of the genus *Wallemia*, and are in the focus of the above review.

Ecology of the *Wallemia* spp.

Fungi from genus *Wallemia* are frequently involved in food spoilage of particularly sweet, salty, and dried food (Samson, Hoekstra et al., 2002), and are also often isolated from indoor or outdoor air (Takahashi, 1997), soil (Domsch, Gams et al., 1990) and sea salt (Das Sarma, Klebahn et al., 2010). Until recently (Zalar, de Hoog et al., 2005), a single cosmopolitan species, *W. sebi,* that was isolated from jams, dates, bread, cakes, salted beans, maize flour, crystalline sugar, fish, bacon, fruits, soil, hay and textiles around the globe (Eduard, Lacey et al., 1990; Hanhela, Louhelainen et al., 1995; Zeng, Westermark et al., 2004; Zalar, de Hoog et al., 2005), have been recognized in the genus. *Wallemia sebi* commonly causes allergological problems resulting in farmer's lung disease (Lappalainen, Pasanen et al., 1998; Roussel, Reboux et al., 2004) and has been proved to be, although rarely, the causative agent of cutaneous or subcutaneous infections in humans (de Hoog and Guarro, 1996).

In order to clarify unresolved phylogenetical position of the genus *Wallemia* within the fungal kingdom (Wu, Tsumura et al., 2003), as well as its taxonomy and ecology, a large group of strains collected globally from food preserved with low a_w (e.g. peanuts, cakes, dried fish), different extreme ecological niches (hypersaline waters of the Dead Sea and salterns of the Red Sea, Dominican Republic and Slovenia), and from some medically relevant samples (e.g. chronic ulcerative skin lesion of human and hay sample associated with livestock toxicosis) were studied. Morphological and physiological characteristics were analyzed, as well as the sequence data of ribosomal DNA internal transcribed spacer regions 1 and 2 (ITS1 and ITS2 rDNA) including the 5.8S ribosomal DNA (5.8S rDNA). Based on the unique morphology, evolution and xerotolerance, a new basidiomycetous class, Wallemiomycetes, covering an order Wallemiales was proposed (Zalar et al., 2005). In addition, molecular data from six nuclear genes (18S, 25S, and 5.8S rDNA and genes coding *rpb1, rpb2* and *tef1* nuclear proteins) reinforced the isolated position of *Wallemia* in the Basidiomycota and suggested that class Wallemiomycetes is an early diverging lineage of Basidiomycota as it occupies a basal position near the Entorrhizomycetidae (Matheny, Gossmann et al., 2006). Based on differences in conidial size, xerotolerance, and sequence data of the ITS rDNA, three *Wallemia* species were segregated, named *W. ichthyophaga, W. sebi* and *W. muriae*. Since the *W. ichthyophaga* differs in numerous nucleotides of the small subunit (SSU) rDNA and ITS rDNA from the other two species, existence of at least two cryptic genera not distinguishable by morphological characteristics were indicated (Zalar, de Hoog et al., 2005). Despite considerable molecular distances among *Wallemia* species,

they all exhibit unique conidiogenesis, including basauic development of fertile hyphae, segregation of conidial units more or less basipetally, and disarticulation of conidial units into mostly four arthrospore-like conidia (Zalar, de Hoog et al., 2005).

Interestingly, tests of xerotolerance of the *Wallemia* spp. have shown that it represents one of the most xerophilic fungal taxa (Zalar, de Hoog et al., 2005). The two halophilic *Wallemia* species, *W. muriae* and *W. ichthyophaga*, necessarily require media with low a_w, while *W. sebi* also shows growth on media without additional solutes. The narrow a_w ranges for the growths of *W. muriae* and *W. ichthyophaga* are 0.984 – 0.805 and 0.959 – 0.771, respectively. Moreover, *W. ichthyophaga* shows preferences to certain solutes for lowering a_w as it exhibited poor growth by means of prolonged growth phases and smaller colonies on the media with high concentrations of glucose compared to media with presence of high concentrations of salt (Zalar, de Hoog et al., 2005; Kralj Kunčič, Kogej et al., 2010). The fact that it can only thrive in the media with NaCl above 1.7 M and up to the saturation (5.3 M NaCl) makes it one of the most halophilic fungi known today. *Wallemia sebi* is capable of growth over a wider range of a_w (0.997 – 0.69) in glucose/fructose media (Pitt and Hocking, 1977), but in media with NaCl as the major solute, the lowest a_w for growth was reported to be 0.80 (Pitt and Hocking, 1977; Zalar, de Hoog et al., 2005), corresponding to 4.5M NaCl.

Adaptations of the *Wallemia* spp. to the life at high concentrations of salt

Life in the environment with high concentrations of NaCl is stressful not only due to high osmotic pressure but also due to the toxicity of the sodium ions. As the cytoplasmatic membrane is freely permeable to water, this situation leads to subsequent dehydration and cessation of growth unless the organism has the means to adapt physiologically and morphologically to such an environment (Galinski, 1995). Mechanisms of salt-tolerance in fungi have been mostly studied in salt-sensitive *Saccharomyces cerevisiae* (Blomberg and Adler, 1992; Blomberg, 2000; Hohmann, 2002), halotolerant yeast *Debaryomyces hansenii* (Larsson and Gustafsson, 1987; Andre, Nilsson et al., 1988; Larsson, Morales et al., 1990; Larsson and Gustafsson, 1993; Prista, Almagro et al., 1997; Almagro, Prista et al., 2000) and in extremely halotolerant black yeast *Hortaea werneckii* (Kogej, Ramos et al., 2005; Kogej, Gorbushina et al., 2006; Kogej, Gostinčar et al., 2006; Kogej, Stein et al., 2007; Plemenitaš, Vaupotič et al., 2008). The latter is currently being a model organism for eukaryotic halophily studies. The response of eukaryotic cells to environmental stress involves complex alterations in gene expression which leads to metabolic changes and subsequent adaptation to the new conditions (Yale and Bohnert, 2001; Petrovič, Gunde-Cimerman et al.,

2002; Vaupotič and Plemenitaš, 2007). An important level of adaptation is balancing the osmotic pressure of the medium by accumulating and/or synthesyzing organic compatible solutes and maintaing low salt concentration within cytoplasm (Oren, 1999). Additional adaptations at the levels of plasma membrane composition (Petrovič, Gunde-Cimerman et al., 1999; Turk, Mejanelle et al., 2004; Gostincar, Turk et al., 2008; Gostincar, Turk et al. 2009) and cell wall structure (Kralj Kunčič, Kogej et al., 2010) are required in order to prevent the damage of cells in such environments. Numerous morphological adaptations reflected in the extremophilic ecotype, characterized by meristematic growth, pigmentation and changes in colony morphology, have also been observed (Kogej, Gorbushina et al., 2006). Research of physiological and molecular adaptations of the genus *Wallemia*, especially halophilic representative *W. ichthyophaga*, to the environments with high salinity are only at an early stage.

Morphological adaptations to moderate and high NaCl concentrations of *Wallemia* spp. have been only recently studied (Kralj Kunčič, Kogej et al., 2010). The combination of light, focused-ion-beam/ scanning and transmission electron microscopy revealed an impact of high concentrations of NaCl on the cell morphology of *Wallemia* spp. Hyphal compartments of *W. sebi* and *W. muriae* were thicker and shorter and mycelial pellets were larger at high salinity.

Wallemia ichthyophaga differs from the other two *Wallemia* spp. not only from the molecular aspect, but also from the aspect of morphology, since it forms sarcina-like multicellular clumps composed of compactly packed spherical cells. The size of the cells did not respond to increased salinity, whereas multicellular clumps became significantly larger. The ability to grow meristematically, or in the form of multicellular clumps, is hypothesized to greatly enhance the survival in stressful environment (Wollenzien, de Hoog et al., 1995; Palkova and Vachova, 2006). The presence of extracellular polysaccharides (EPS) observed in all three *Wallemia* spp. is involved in the protection against desiccation in rock-inhabiting fungi (Selbmann, de Hoog et al., 2005) and might also have a protective function at high salinities. An increase in the thickness of the multilayered cell wall at higher salinities occurred in all three, but it was especially pronounced in *W. ichthyophaga*, which had extremely thick cell wall compared to *W. sebi* and *W. muriae*. The thickened cell wall of the *W. ichthyophaga* is rather an exception in the so-far-known fungal responses to extremely saline conditions (Kralj Kunčič, Kogej et al., 2010). The unique morphological adaptations of *Wallemia* spp. to high NaCl, such as increase in cell wall thickness and size of multicellular clumps or mycelial pellets, which were pronounced at high NaCl concentration (Kralj Kunčič, Kogej et al., 2010), were interestingly less apparent at high glucose concentrations (our unpublished data). To conclude, morphological phenomena observed in the above studies are believed to have an important role for successful growth in extremely saline conditions (Kralj Kunčič, Kogej et al., 2010).

As we realized from our preliminary results obtained by NMR and HPLC measurements, all three *Wallemia* spp. accumulate polyols, among which glycerol is the most significant, in response to increased salinity in the environment (our unpublished data). This is a common strategy of osmoadaptation of other fungi, as well as extremely halotolerant black yeast *H. werneckii* (Petrovič, Gunde-Cimerman et al., 2002; Kogej, Stein et al., 2007). As we have discovered from atomic absorption spectroscopy (AAS), cells of *Wallemia* spp. keep intracellular cationic (Na^+ and K^+) concentrations low in the environments with high concentrations of salt, and are, therefore, considered as Na^+-excluders (our unpublished data). So far, only halotolerant yeast *D. hansenii* has shown to maintain relatively high internal concentrations of sodium when coping with salt stress together with production and intracellular retention of compatible solutes, particularly glycerol (Prista, Almagro et al., 1997). In addition to that, a key enzyme for glycerol biosynthesis and redox balancing, Gpd1 (glycerol-3-phosphate dehydrogenase) in *W. ichthyophaga* was identified and characterized. Similarly to *S. cerevisiae* (Ansell, Granath et al., 1997), levels of mRNA of *WiGPD1* in cells adapted to different salinities showed a gradual increase in the transcript at higher salinities, with the maximum at 4.5 M (25% w/v) NaCl and responded to saline stress. Comparison of WiGpd1 and Gpd1 from *S. cerevisiae* revealed high overall amino-acid similarity, but more importantly, the N-terminal PTS2 sequence which was important for peroxisome localization (Jung, Marelli et al., 2010) was found to be lacking in the case of *W. ichthyophaga* (and *H. werneckii*) homologue. Constant cytosolic localization of the Gpd1 has appeared to be beneficial for the organisms living in extremely saline environments due to its function in osmotic stress (Lenassi, Zajc et. al., 2011, paper in press).

Bioactive potential of the genus *Wallemia*

Research of the production of biologically active compounds has been focused on mostly cosmopolitan fungi, while halotolerant and halophilic fungi, such as *W. muriae* and *W. ichthyophaga*, have been so far ignored. Toxicity of the culture filtrate of cosmopolitan *W. sebi* for HeLa cell lineage, (Saito, Ohtsubo et al., 1971) and in other biological tests was observed earlier (Wood, 1984). Two related tricyclic dihydroxysesquiterpenes were reported to be isolated from *W. sebi*, designated walleminol A and walleminol B, or walleminone that were toxic to certain cell lineages, protozoa and brine shrimps. The minimum inhibitory dose of walleminol A in the bioassays was approximately 50 micrograms/ml, which was comparable with a number of mycotoxins, such as citrinin and penicillic acid (Wood, Mann et al., 1990). Additionally, two components were identified in *W. sebi,* namely azasteroides UCA 1064-B (Chamberlin, Chaney et al., 1974) and UCA 1064-B (Takahashi, Maruta et al., 1993), that both exhibited antibacterial and antimycotic activity, while only UCA 1064-B showed anti-tumor activity.

Our recent study on screening of fungi from extreme environments, including all three *Wallemia* species for the production of haemolytic and antibacterial activities, indicated that selected halotolerant and halophilic species synthesized specific bioactive metabolites under stressful conditions. They were cultivated under controlled conditions (low concentrations of NaCl, glucose and optimal growth temperature) and under conditions with lowered a_w, high concentrations of NaCl or glucose, and at low temperature. Water and organic (acetone and methanol) extractions of the biomass were evaporated and dissolved in the appropriate solvent, water or ethanol, and used for biological assays.

Water extracts showed no biological activity, regardless of the growth conditions, suggesting that these organisms do not synthesize proteins or other polar molecules with haemolytic or antibacterial activity (Sepcic, Zalar et al., 2011). The organic extracts showed haemolytic and antibacterial potential that was considerably higher if the fungi were exposed to stressful growth conditions. *W. ichthyophaga* showed higher haemolytic activity when organic extracts were obtained from the cultures grown at high concentrations of glucose, and higher antibacterial activity at high concentrations of NaCl and glucose. Interestingly the haemolytic potential of the organic extracts of *W. muriae* and *W. sebi* was considerably higher if they were exposed to low temperature (10 °C). Enhancement of this kind was not observed for antibacterial activity as it occurred at low temperature and high concentrations of glucose for *W. muriae* and for *W. sebi,* even in the case of cultivation under controlled conditions. All the active extracts exclusively inhibited growth of Gram-positive bacterium *Bacillus subtilis*, while growth of Gram-negative *Escherichia coli* remained unaffected. Taken together, low a_w induced the production of bioactive metabolites in xerophilic Wallemia spp., what may have, besides contribution to territorial competition, a yet-to-be described protective role in the adaptation to the environments with low a_w (Sepcic, Zalar et al., 2011).

A novel model organism for eukaryotic halophily studies

The species of the genus *Wallemia* are able to thrive at a_w lower than most of the known fungi, and the obligative halophilic character of *W. ichthyophaga* is exceptional not only in the phylum Basidiomycota but in the whole fungal kingdom. Our studies have so far revealed some unique morphological adaptations, especially regarding the cell wall, which enable *Wallemia* spp. to thrive in extremely saline environments. Thus, it represents the potential model organism for studies of eukaryotic halophily. Additional research on the adaptations to different stress, on a molecular level, will presumably give rise to new biotechnological applications for designing salt tolerant yeasts and plants. Nevertheless, *Wallemia* spp. are also interesting due to their production of bioactive metabolites with broad spectrum of activities (antibacterial, antifungal, antitumor and haemolytic). *Wallemia* spp. are commonly involved

in the spoilage of foods with low a_w, and the presence of walleminol A has already been found in the food contaminated by *W. sebi* (Mitchell, Godfree et al., 1999). Therefore, their bioactive potential should also be considered in the food quality control.

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ХЕТЕРОФИЛНЕ ГЉИВЕ РОДА *WALLEMIA* – БИОАКТИВНИ СТАНОВНИЦИ ПРИМОРСКИХ СОЛАНА И СЛАНЕ ХРАНЕ

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Резиме

Wallemia је род космополитских, хетерофилних гљива, често укључених у разлагање посебно слатког, сланог и сувог дрвета. Доскора је из овог рода била описана само врста *Wallemia sebi*. Када је велика група узорака, сакупљених у соланама и другим еколошким нишама, анализирана на нивоу физиолошких, морфолошких и молекуларних разлика, раздвојена је нова класа базидиомицета Wallemiomycetes, која покрива ред Wallemiales и индентификовано је три врсте рода *Wallemia*: *W. ichthyophaga, W. sebi* and *W. muriae*. *W. ichthyophaga* је призната као најхалофилнији еукариот откривен до данас и као таква представља еукариотски модел за стручније анализе адаптације на хиперсалинске услове. Наше прелиминарне студије указују да све три врсте синтетишу за сада недетерминисано хемолитичко једињење, у условима изненађујуће ниске влажности. Због доскора неоткривене таксономске ситуације, до сада није било налаза о продукцији било ког биоактивног једињења код три новоописане врсте. Циљ овог чланка је да представи таксономију, екологију, физиологију и до сада описане молекуларне механизме адаптације на живот под ниским условима водне активности, као и биоактивни потенцијал рода *Wallemia,* физиолошко-генетски старог и усамљеног таксона у оквиру Basidiomycota.

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2.1.6 Mikobiota solin

Naslov v originalnem jeziku: The mycobiota of the salterns.

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Izvleček:
Solarne soline so zgrajene kot sistem plitvih bazenov za proizvodnjo halita z izhlapevanjem morske vode. Glavna značilnost teh solin je nekontinuiran gradient slanosti, ki ponuja številne dobro določene habitate z naraščajočimi slanostmi, od zmerne do ekstremne. Ti habitati so eno izmed najbolj ekstremnih okolji, zaradi nizke stopnje biološko razpoložljive vode in toksičnih koncentracij ionov. Do leta 2000 so znanstveniki menili, da so ta okolja poseljena skoraj izključno s prokariotskimi mikroorganizmi, nato so bile glive prepoznane kot aktivni prebivalci solin. Od tedaj so bile v izjemno slani vodi po vsem svetu opisane številne glivne vrste. Mikobioto solin predstavljajo različne vrste rodu *Cladosporium* in sorodnih meristematskih melaniziranih črnih kvasovk, nemelanizirane kvasovke, filamentozni predstavniki rodov *Penicillium* in *Aspergillus* in njihove teleomorfne oblike (rodova *Eurotium* in *Emericella*), in tudi bazidiomicetni rod Wallemia. Med vsemi temi glivami sta dve vrsti postali nova modelna organizma za preučevanje mehanizmov skrajne tolerance na sol: izjemno halotolerantna askomicetna črna kvasovka *Hortaea werneckii* in obligatno halofilna bazidiomicetna gliva *Wallemia ichthyophaga*.

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Chapter 7
The Mycobiota of the Salterns

Janja Zajc, Polona Zalar, Ana Plemenitaš, and Nina Gunde-Cimerman

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Abstract Solar salterns are constructed as shallow multi-pond systems for the production of halite through evaporation of seawater. The main feature of salterns is the discontinuous salinity gradient that provides a range of well-defined habitats with increasing salinities, from moderate to hypersaline. These present one of the most extreme environments, because of the low levels of biologically available water and the toxic concentrations of ions. Up to the year 2000, hypersaline environments were considered to be populated almost exclusively by prokaryotic

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microorganisms till fungi were reported to be active inhabitants of solar salterns. Since then, numerous fungal species have been described in hypersaline waters around the world. The mycobiota of salterns is represented by different species of the genus *Cladosporium* and the related meristematic melanized black yeasts, of non-melanized yeasts, of the filamentous genera *Penicillium* and *Aspergillus* and their teleomorphic forms (*Eurotium* and *Emericella*), and of the basidiomycetous genus *Wallemia*. Among these, two species became new model organisms for studying the mechanisms of extreme salt tolerance: the extremely halotolerant ascomycetous black yeast *Hortaea werneckii* and the obligate halophilic basidiomycete *Wallemia ichthyophaga*.

7.1 Introduction

7.1.1 The Saltern Ecosystem

Thalassohaline hypersaline environments are generally considered to be those originating by evaporation of sea water and with halite (NaCl) concentrations of greater than 10% (m/w) (Oren 2002). These provide some of the most extreme habitats in the World. They are common all around the globe, and include, for example, marine ponds and salt marshes that are subjected to evaporation, salt or soda lakes, and sea-salt and man-made salterns (Trüper and Galinski 1986). Solar salterns, the focus of this chapter, are composed of multiple shallow ponds that are located in tropical, subtropical, and temperate parts of the World. In these ponds, the NaCl is gradually concentrated as the seawater evaporates.

Salt production in salterns usually begins with seawater as the initial source of brine, which is evaporated through a series of ponds, to the final pond where the NaCl and other salts precipitate out of the saturated brine. The bittern that remains after this crystallization of the halite is rich in magnesium chloride and provides a special ecological habitat within the salterns. Variable water activities (a_w) because of the increasing concentrations of NaCl, as well as the low oxygen concentrations and high light intensity, present life-limiting parameters in salterns (Brock 1979).

The Sečovlje solar salterns (Fig. 7.1a) were established in the ninth century as a man-made system of ponds, and they are located in the northern Adriatic Sea, in the Gulf of Trieste in Slovenia. The sub-Mediterranean climate and unstable weather conditions allow the production of salt only during the summer, when climate is arid. The strong local winds enhance evaporation of water and keep the temperatures of the water in brines moderate (18–32°C). When the water reaches a suitable concentration of salt, it is directed over the wooden barriers to the next of the 16 evaporative ponds separated by canals. These are the only salterns along the eastern Adriatic coast where salt is produced according to the traditional procedures, with the daily gathering of the saturated brine on the cultivated microbial mat covering the salterns, known as the petola. The role of the petola is dual; it prevents the mixing of the crystallized halite with the mud at the bottom of

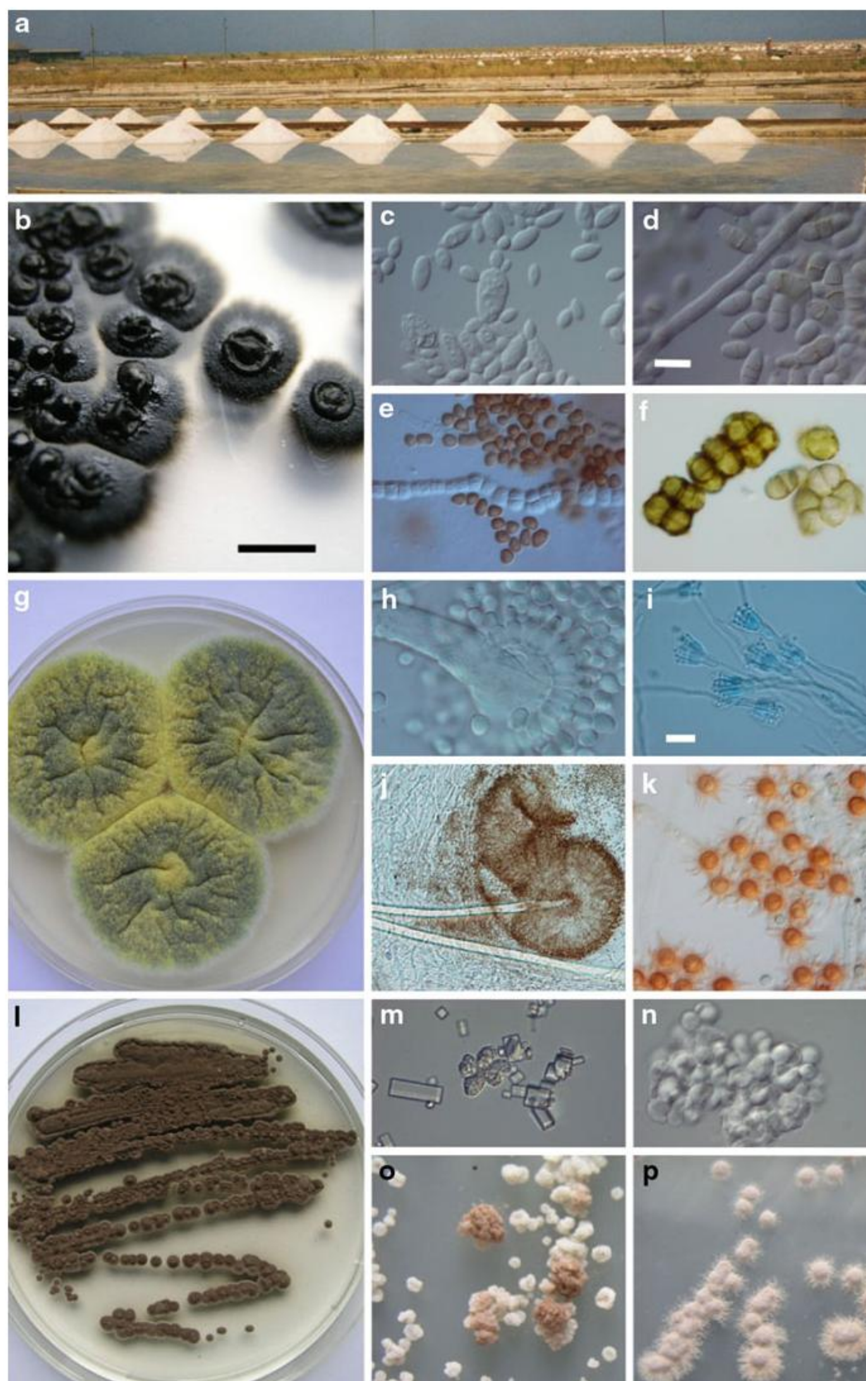

Fig. 7.1 Representatives of the saltern mycobiota. (**a**) salterns Sečovlje; (**b**) *Hortaea werneckii* colonies; (**c**) *Aureobasidium pullulans* budding cells; (**d**) *Hortaea werneckii* budding cells;

the ponds, and it prevents the incorporation of undesired ions of iron and manganese into the halite crystals (Pahor and Poberaj 1963).

The solar salterns Sečovlje with their successive evaporation ponds, the salt-pan mud, and the wooden fences provide a relatively simple ecosystem that is popular for studies of halotolerant and halophilic microorganisms.

These hypersaline waters in salterns were at first believed to be populated almost exclusively by archaea, bacteria, and the eukaryotic alga *Dunaliella salina* (Rodriguez-Valera et al. 1981; Javor 1989; Oren 2005). However, the high diversity of eukaryotic microorganisms in hypersaline waters became evident soon after fungi were first reported as active inhabitants of solar salterns (Gunde-Cimerman et al. 2000). Numerous halotolerant and halophilic fungi were initially isolated from the Sečovlje solar salterns (Gunde-Cimerman et al. 2000), and later also from salterns around the World: La Trinidad in the Ebro River Delta and Santa Pola on the Mediterranean coast of Spain, Camargue in France, and the salterns on the Atlantic coast in Portugal, and in Namibia, the Dominican Republic and Puerto Rico (Butinar et al. 2005a, b, c; Cantrell et al. 2006).

Numerous halotolerant and extremely halotolerant fungi (Zalar et al. 1999a, b, c; Gunde-Cimerman et al. 2000; Butinar et al. 2005a, b, c; Zalar et al. 2005b, 2007, 2008a, b; Butinar et al. 2011) and a few halophilic representatives (Zalar et al. 2005a) have been isolated from these hypersaline waters.

The xerophilic fungi that can grow at low a_w were previously known only from domestic environments, as they can contaminate food preserved through the reduction of the biologically available water, by means of drying, freezing, or solute addition (Pitt and Hocking 1977, 1997). It was assumed that these food-borne fungi that can grow at low a_w reflect a general xerophilic phenotype, and that they would therefore not populate natural hypersaline environments.

7.1.2 The Definition of Halophily in Fungi

Over the years, several definitions for the description of the diverse abilities of fungi to adapt to a wide range of salt concentrations have been proposed. For these fungi that prefer reduced a_w, the most commonly used adjectives were xerophilic and osmophilic, as these fungi can grow at a_w below 0.85; this corresponds to 17% NaCl or 50% glucose added to a growth medium (Gunde-Cimerman et al. 2005). However, these fungi are not only able to grow at low a_w; some also show preferences

Fig. 7.1 (continued) (**e**) *Phaeotheca triangularis* conidia and hyphae; (**f**) *Trimmatostroma salinum* meristematic clumps; (**g**) *Eurotium amstelodami* in culture; (**h**) *Eurotium chevalieri* anamorph; (**i**) *Penicillium crustosum* conidiophores; (**j**) *Aspergillus niger* conidiophores; (**k**) *Emericella stella-maris* ascospores; (**l**) *Wallemia muriae* in culture; (**m**, **n**) *W. ichthyophaga meristematic clumps;* (**o**) *W. ichthyophaga* colonies; (**p**) *W. sebi* colonies. Scalebar indicated in (**b**) is 10 mm, and also applies for (**o**, **p**); scalebar on (**d**) indicates 10 μm, and is also valid for (**c**–**h**, **k**, **n**); scalebar on (**i**) indicates 20 μm and is valid for (**i**, **j**, **m**)

for certain chemical natures of the solutes that lower the a_w (de Hoog et al. 2005; Gunde-Cimerman and Plemenitaš 2006). Hence these osmotolerant/osmophilic fungi can live in environments that are rich in sugar, whereas those that are halotolerant/halophilic can live in environments that are rich in salt. In contrast to obligate halophilic archaea and bacteria, in the fungal kingdom, no evidence of obligate requirements for salt was reported until only recently. When fungi were found to constitute active communities in the hypersaline water of solar salterns, they were considered halophilic if they were regularly isolated from water at 17–32% NaCl, primarily on saline selective media, and if they were able to grow *in vitro* on 17% NaCl (Gunde-Cimerman et al. 2000). These fungi were then shown to sustain a range of different salt concentrations, right across the whole salinity range. Later, a few fungal species that show superior growth on media with NaCl as the controlling solute and necessarily require lowered a_w for growth were first reported (Zalar et al. 2005a); hence the term halophilic fungi was challenged again. Thus, halotolerant and extreme halotolerant are now the terms used to describe fungi that can grow across a range of different salt concentrations, even from fresh water to NaCl saturation (Gunde-Cimerman and Plemenitaš 2006), and the term halophily remains reserved for those that require salt for growth.

7.2 The Mycobiota of the Salterns

7.2.1 Methods for the Isolation of the Fungi from the Salterns

To avoid selection of certain mycobiota that are favored by a particular method, four different methods of isolation have often been used in the studies of fungal diversity in hypersaline brines: (a) filtration of the saline and placing the membrane filter on different selective media; (b) agar baits in dialysis tubing and glass tubes that were left for 5 months in crystallization ponds and then placed on media; (c) enrichment of saline with glucose and yeast extract; and (d) spreading of biofilms on selective media (Gunde-Cimerman et al. 2000).

The selective media have low a_w (at least 0.89, and lower) because of the supplements of either salt or sugar. In the salt-based selective media, 10–32% NaCl is added to malt-extract yeast agar media, and for the sugar-selective media, these are supplemented with 50–70% glucose, or with 18% glycerol (Gunde-Cimerman et al. 2000; Butinar et al. 2005a). The enumeration of the colony-forming units (CFU) per 100 ml hypersaline water can be performed with a general-purpose medium (dichloran rose bengal chloramphenicol [DRBC]; a_w 1.0) (King et al. 1979) and a medium for the detection of moderate xerophiles (Hocking and Pitt 1980). Bacterial overgrowth is prevented by adding different antibiotics to all of the selective and enumeration media, like chloramphenicol (100 mg l^{-1}). The cultures are incubated for 1–10 weeks at 25°C, and examined every few days (Gunde-Cimerman et al. 2000).

7.2.2 Diversity of the Saltern Mycobiota

After a decade of research into the fungal diversity in salterns, together with new taxa, a number of fungal genera with high diversities of halotolerant and halophilic species have been described. The melanized fungi isolated are represented by meristematic black yeast-like hypomycetes of the following species: *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, *Aureobasidium pullulans*, and the phylogenetically related genus *Cladosporium* (Gunde-Cimerman et al. 2000).

A number of nonmelanized yeast species have been described for the different salterns and salt lakes worldwide. The most abundant among these are *Pichia guilliermondii*, *Debaryomyces hansenii*, and *Candida parapsilosis*, to name but a few (Butinar et al. 2005a). Among the filamentous fungi that appear with the highest frequencies there are different species of the genera *Aspergillus* and *Penicillium* (Butinar et al. 2011). Representatives of the newly described basidiomycetous class Wallemiomycetes cover the order *Wallemiales* with the genus *Wallemia*, and these were recently isolated from hypersaline waters of man-made salterns on different continents in Europe, Asia, Africa, and North America. Interestingly, the genus *Wallemia* represents one of the most xerophilic and halophilic fungal taxa known to date (Zalar et al. 2005a). Morphology and growth features of some of these fungi are shown in Fig. 7.1b–p. Their geographical distribution and salinity range for growth are listed in Table 7.1.

7.2.3 The Black Yeasts

Black yeasts are a group of dark pigmented (melanized) polymorphic hyphomycetes that besides filamentous have also the ability of yeast-like growth. Their slowly expanding melanized colonies have been detected in different extreme environments, such as on rock surfaces in arid, semi-arid, hot, and cold desserts that were previously considered to lack eukaryotic extremophiles (Gorbushina et al. 1996; Sterflinger and Krumbein 1997; Sterflinger et al. 1999).

These black yeasts are characterized by thick, melanized cell walls, with slow, often meristematic, growth and proliferation with endoconidiation. A similar morphology, which is regarded as an “extremophilic ecotype,” was observed also with fungi isolated from the hypersaline waters of salterns (Gunde-Cimerman et al. 2000). Because of their morphological plasticity and polymorphic anamorphic stages, their certain identification can only be correctly performed by complementing morphological criteria with molecular methods and physiological tests of carbon and nitrogen metabolism without or with high NaCl concentrations. The morphological types of black yeasts isolated from the Sečovlje salterns were speculated to be different species, as they showed different growth patterns in the presence of salt. This was confirmed by additional molecular data that were obtained by sequencing the internal

Table 7.1 The most frequent fungal species isolated from hypersaline water of salterns across the globe

Group/class	Order	Species	Salinity range (NaCl) (%)		Geographical distribution: Slovenia	Spain		France	Portugal	Namibia	Dominican Republic		Puerto Rico	Israel	Bosnia and Herzegovina	USA (Utah)
					Sečovlje	La Trinidad	Santa Pola	Camargue	Samouco	Skeleton coast	Salterns	Lake Enriquillio	Cabo Rojo	Eilat	Ston	Great Salt Lake
Ascomycota	Capnodiales	*Hortaea werneckii*	0–32	L	X	X	X	X	X	X		X	X			
		Phaeotheca triangularis	0–26	L	X	X	X	X	X	X		X	X			
	Helotiales	*Trimmatostroma salinum*	0–24	L	X											
	Dothideales	*Aureobasidium pullulans*	0–18	L	X	X	X	X	X	X		X	X			
	Capnodiales	*Cladosporium cladosporioides*	0–17[a]	S	X								X		X	
		Cladosporium herbarum	nd													
		Cladosporium oxysporum	nd								X	X	X			
		Cladosporium sphaerospermum	0–20	S	X						X		X	X		
		Cladosporium halotolerans	0–20	S	X		X			X		X			X	
		Cladosporium dominicanum	0–20	S							X	X				
		Cladosporium velox	0–20	S	X											
		Cladosporium psychrotolerans	0–17	S	X						X					
		Cladosporium spinulosum	0–17	S	X											
		Cladosporium salinae	0–17	S	X		X									
		Cladosporium fusiforme	0–17	S	X											
		Cladosporium ramotenellum	nd		X											
		Cladosporium subinflatum	nd		X											
		Cladosporium tenuissimum	nd		X					X						
		Cladosporium tenellum	nd											X		
		Cladosporium herbaroides	nd											X		
		Cladosporium macrocarpum	nd		X											
		Cladosporium subtilissimum	nd		X											
		Cladosporium bruhnei	nd		X		X									
	Saccharomycetales	*Candida parapsilosis*	0–17[a]	S	X				X							
		Debaryomyces hansenii	0–17[a]	S						X						X
		Metschnikowia bicuspidata	nd													X
		Pichia guilliermondii	0–17[a]	S	X			X								
		Yarrowia lipolytica	nd											X		

(continued)

Table 7.1 (continued)

Group/class	Order	Species	Salinity range (NaCl) (%)		Geographical distribution											
					Slovenia	Spain		France	Portugal	Namibia	Dominican Republic		Puerto Rico	Israel	Bosnia and Herzegovina	USA (Utah)
					Sečovlje	La Trinidad	Santa Pola	Camargue	Samouco	Skeleton coast	Salterns	Lake Enriquillio	Cabo Rojo	Eilat	Ston	Great Salt Lake
	Eurotiales	*Eurotium amstelodami*	0–27.5	S	X	X	X			X	X	X		X		X
		Eurotium chevalieri	0–22.5	S	X	X	X									
		Eurotium herbariorum	0–25	S	X	X								X		
		Eurotium rubrum	0–20	S	X											
		Eurotium repens	0–25	S	X	X										
		Eurotium halotolerans	0–27.5	S												
		Emericella filifera	0–17	S	X											
		Emericella stella-maris	0–17	S	X											
		Aspergillus niger	0–17[a]	S	X	X	X	X			X		X			
		Aspergillus flocculosus	0–17[a]	S	X						X	X				
		Aspergillus caesiellus	0–17[a]	S	X											
		Aspergillus ochraceus	0–17[a]	S	X	X	X						X			
		Aspergillus flavus	0–17[a]	S	X	X							X			X
		Aspergillus roseoglobulosus	0–17[a]	S							X					
		Aspergillus tubingensis	0–17[a]	S	X									X		
		Aspergillus melleus	0–17[a]	S									X			
		Aspergillus sclerotium	0–17[a]	S	X											
		Aspergillus versicolor	0–17[a]	S	X									X		
		Aspergillus sydowii	0–17[a]	S	X					X	X			X		
		Aspergillus wentii	0–17[a]	S	X	X					X					
		Aspergillus flavipes	0–17[a]	S	X								X			
		Aspergillus terreus	0–17[a]	S	X											
		Aspergillus candidus	0–17[a]	S	X	X							X			
		Aspergillus penicillioides	0–17[a]	S	X								X			
		Aspergillus restrictus	0–17[a]	S	X											
		Aspergillus proliferans	0–17[a]	S	X											
		Aspergillus nidulans	0–17[a]	S									X			
		Aspergillus fumigates	0–17[a]	S	X											
		Penicillium chrysogenum	0–17[a]	S	X	X	X							X		

		Penicillium brevicompactum	0–17^{a}	S								
		Penicillium citrinum	0–17^{a}	S	X				X		X	
		Penicillium oxalicum	0–17^{a}	S						X		
		Penicillium steckii	0–17^{a}	S	X							X
		Penicillium sizovae	0–17^{a}	S	X							X
		Penicillium westlingii	0–17^{a}	S	X							
		Penicillium freii	0–17^{a}	S	X							
		Penicillium cyclopium	0–17^{a}	S	X							
		Penicillium solitum	0–17^{a}	S	X							
		Penicillium albocoremium	0–17^{a}	S	X							
		Penicillium crustosum	0–17^{a}	S		X						
Basidiomycota	Wallemiales	*Wallemia sebi*	0–27	L	X		X		X		X	
		Wallemia muriae	0–25	L	X					X		X
		Wallemia ichthyophaga	9–32	L	X				X			
	Tremellales	*Trichosporon mucoides*	nd									X
	Sporidiobolales	*Rhodosporidium babjevae*	0–13^{a}	S	X			X				
		Rhodosporidium sphaerocarpum	0–17^{a}	S	X			X				

Data were adopted from Gunde-Cimerman et al. (2000); Butinar et al. 2005a, b, c, 2011; Diaz-Munoz and Montalvo-Rodriguez 2005; Zalar et al. 2005a, 2007, 2008a; Cantrell et al. 2006; Schubert et al. 2007, and unpublished data (Gostinčar, personal communication)

nd no data; *X* the presence of the species in the salterns; *L* salinity range determined in the liquid media; *S* salinity range determined in solid media

[a]Maximum tolerated NaCl concentration not determined

transcribed spacer (ITS) rDNA and by restriction fragment length polymorphism (RFLP) of small-subunit (SSU) and ITS rDNA. The representatives of the black yeasts identified include: *H. werneckii* (Zalar et al. 1999c) and *P. triangularis* (Zalar et al. 1999a; c), both comprise the order *Capnodiales*; *A. pullulans* (Zalar et al. 1999c), from the order *Dothideales*; and *T. salinum*, a newly described species from the order *Helotiales* (Zalar et al. 1999b).

Initially, these black yeasts were isolated from the Adriatic salterns, but they were also later identified in hypersaline waters of six salterns on three continents. These were La Trinidad in the Ebro River Delta and Santa Pola on the Mediterranean coast of Spain, Camargue in France, the Atlantic coast in Portugal, and in Namibia and the Dominican Republic. Exceptionally, *T. salinum* was detected only in the Adriatic salterns (Zalar et al. 1999b). *H. werneckii* represents 70–80% of all of the isolates, followed in abundance by *T. salinum* and *P. triangularis*, while *A. pullulans* appears mainly at lower salinities (Butinar et al. 2005b). The extremely halotolerant nature of the black yeasts was first noted when they were isolated from the saline media in the highest numbers according to two peaks, one at the beginning of the sampling season (May), and the other and during the crystallization (August) (Gunde-Cimerman et al. 2000); this was later confirmed by *in vitro* studies.

Hortaea werneckii has also been named as *Cladosporium werneckii, Exophiala werneckii, Dematium werneckii, Pullularia werneckii, Aureobasidium werneckii, A. mansonii, Sarcinomyces crustaceous*, and *Pheoannellomyces werneckii* in the past, but has been because of special conidiogenesis described in a new genus (Nishimura and Miyaji 1983). It was long known as the primary etiological agent of a human surface skin problem called tinea nigra, a superficial fungal infection of the human hand that is frequent in warmer areas of the world. The ecology of *H. werneckii* was linked to the presence of salt, as it was isolated from seawater (Iwatsu and Udagawa 1988), marine fish (Todaro et al. 1983), salted freshwater fish (Mok et al. 1981), and beach soil (de Hoog and Guého 2010). Its ecological niche has been suggested to be intermittently drying salty pools (de Hoog and Gerrits van den Ende 1992) or highly saline water of the crystallization ponds in the solar salterns (Gunde-Cimerman et al. 2000). Among all of the melanized fungi, *H. werneckii* is dominant in the hypersaline waters of the Adriatic salterns and of all of the other salterns that have environmental salinities above 20% (Gunde-Cimerman et al. 2000; Butinar et al. 2005b; Diaz-Munoz and Montalvo-Rodriguez 2005; Cantrell et al. 2006). *H. werneckii* is most abundant (1,400 CFU l^{-1}) at 23% salinity, and remains present with low CFU also after the end of the salt production season. *H. werneckii* is the only black yeast that can grow across the whole range of NaCl concentrations, from 0% to NaCl saturation, with a broad optimum from 6 to 14% NaCl (Butinar et al. 2005b), and it is thus considered to be the most halotolerant fungus so far described.

Trimmatostroma salinum is the only black yeast-like fungus from hypersaline waters that occurs only in the Adriatic salterns, and almost exclusively in one pond, where it was discovered growing on the wooden fence immersed in hypersaline

water (Zalar et al. 1999b, 2005b). *T. salinum* peaks at 25% salinity (700 CFU l^{-1}) and it can appear within broad environmental salinities, as 8–25% NaCl (Butinar et al. 2005b). The broad salinity range for in-vitro growth is from 0 to 24% NaCl, with the optimal values at 2–8% NaCl.

Phaeotheca triangularis was detected in all of the ponds of the Adriatic salterns throughout the year, as well as in crystallizer ponds of other salterns that have been sampled (Butinar et al. 2005b). It shows high adaptability to saline conditions, as it appeared most abundantly (100–340 CFU l^{-1}) in the range of 18–25% NaCl; at lower salinities, its CFUs decrease to less than 50 CFU l^{-1}. In contrast to *H. werneckii*, *P. triangularis* cannot grow *in vitro* on 32% salt, with 24% (or at times also 26%) NaCl reported as its maximum. Its optimal concentration is much narrower, as 6–12% NaCl. *P. triangularis* has been most often isolated from storage ponds with relatively constant salinity. It has also been considered to be an oligotroph, as it can grow on nutritionally poor agar media, and it has been most frequently isolated from low-nutrient storage ponds (Butinar et al. 2005b). *P. triangularis* can form biofilms on solid and liquid saline media, and it frequently appears in sampled microbial biofilms (Gunde-Cimerman et al. 2000; Butinar et al. 2005b). *P. triangularis* is thus an extremely halotolerant species with a narrow ecological amplitude (Gunde-Cimerman et al. 2000).

Aureobasidium pullulans is a cosmopolitan species that can be found in different environments that have fluctuating water activities, such as the phyllosphere (Andrews et al. 1994), foods, feedstuff, bathrooms (Samson et al. 2002), polluted water (Vadkertiova and Slavikova 1995), and others. It has also been found in osmotically stressed environments, such as on rocks and monuments (Urzi et al. 1999), on surface sediments and detritus in salt marshes (Torzilli et al. 1985), and in the hypersaline waters of the Adriatic salterns (Gunde-Cimerman et al. 2000; Butinar et al. 2005b). According to multilocus molecular analysis, the strains of *A. pullulans* from the hypersaline water of the Sečovlje salterns have segregated mostly to the globally ubiquitous variety (var.) *pullulans* (Zalar et al. 2008b). In all of the ponds of the Adriatic salterns and of other sampled salterns, *A. pullulans* occurs with low CFU (up to 50 CFU l^{-1}), and generally at environmental salinities below 8% NaCl. The counts are the highest (800 CFU l^{-1}) at 5% salinity, before or after the salt production season. *A. pullulans* is regarded as a halotolerant species, rather than halophilic, as it can tolerate up to 18% NaCl in vitro and grows optimally on medium without NaCl (Butinar et al. 2005b).

The sampling and inspection of wooden boards that support the walls of the crystallization ponds that are immersed in the hypersaline waters of the active solar salterns of Sečovlje have demonstrated active lignicolous saprobic roles of the halophilic fungi in the hypersaline water. Melanized hyphae in the black-stained parts of the wood from these walls were recognized as belonging to *H. werneckii* and *T. salinum*. These show xylanolytic and lignolytic activities under hypersaline and nonsaline conditions; and *T. salinum* alone shows cellulolytic activity. This

suggests a complementary role of these two black yeasts for the invasion of wood in hypersaline environments (Zalar et al. 2005b).

7.2.4 The Genus Cladosporium

The cosmopolitan genus *Cladosporium* is currently in revision process (Crous et al. 2007; Bensch et al. 2010), but is accommodating over 772 different species names (Dugan et al. 2004). It was phylogenetically placed into the order Capnodiales, after the discovery of a teleomorph stage *Davidiella* (Braun et al. 2003). A few of these species, namely *C. herbarum*, *C. cladosporioides*, *C. sphaerospermum*, *C. tenuissimum*, and *C. oxysporum*, have often been isolated from habitats characterized by low a_w, such as sugary and salty foods (Samson et al. 2002), Egyptian salt marshes, the rhizosphere of halophytic plants, and the phylloplane of Mediterranean plants (Abdel-Hafez et al. 1978). The minimal a_w for growth are 0.82, 0.85, and 0.86 for *C. sphaerospermum*, *C. herbarum*, and *C. cladosporioides*, respectively. In the hypersaline waters of the Sečovlje solar salterns, *Cladosporium* strains are among the most frequently found and are the most abundant of the melanized fungi (Gunde-Cimerman et al. 2000; Butinar et al. 2005b). They have the broadest occurrence among all of the melanized fungi throughout the year in the ponds of the Adriatic salterns, as well as in other hypersaline environments such as Cabo Rojo solar salterns in Puerto Rico (Cantrell et al. 2006). Interestingly, they occur with the highest CFU (1,000–3,600 CFU l^{-1}) between 15 and 25% NaCl, and as the NaCl concentrations increase, they seem to be gradually replaced by different species of black yeasts. As these were mainly obtained from sugar-based media, they are considered as xerotolerant or xerophilic. First, *C. herbarum*, *C. cladosporioides*, *C. sphaerospermum*, and *C. oxysporum* were identified (Butinar et al. 2005b; Cantrell et al. 2006). Then with the different taxonomic methods that were performed later, *C. ramotenellum*, *C. tenellum*, *C. subinflatum*, and *C. herbaroides* were also shown to be part of the hypersaline mycobiota (Schubert et al. 2007).

Strains at that time identified as *C. sphaerospermum*, which was later recognized as a species complex of cosmopolitan as well as specialized air-borne species, have been reported from a wide range of habitats, including osmotically non-stressed niches, and they show pronounced osmotolerant behavior as they can grow at very low a_w (0.82) (Hocking et al. 1994).

In a wide ecological and phylogenetical study of isolates from the hypersaline waters from Mediterranean salterns, from different coastal areas along the Atlantic Ocean, and from the Red Sea, the Dead Sea, and the salt Lake Enriquillio (Dominican Republic), seven more species of the *Cladosporium* genus were newly described for these hypersaline water: *C. halotolerans*, *C. dominicanum*, *C. velox*, *C. psychrotolerans*, *C. spinulosum*, *C. salinae*, and *C. fusiforme*. *C. psychrotolerans* and *C. spinulosum* are currently known only from hypersaline water. Interestingly, a pathogen on the human skin, *Cladosporium langeronii* (= *Hormodendrum langeronii*), is halotolerant, although it has not yet been recorded from hypersaline environments. The maximum NaCl concentration in the growth media for the

development of colonies of these various halotolerant *Cladosporium* representatives has been reported to be 17–20% (Zalar et al. 2007).

7.2.5 *The Nonmelanized Yeasts*

Only a few reports on the existence of the osmophilic yeasts in natural hypersaline waters have appeared in the literature, although these yeasts have been recognized for their tolerance to high concentrations of sugars or salt. A few species, such as *Torulopsis famata, Rhodotorula rubra, Pichia etchelsii, C. parapsilosis*, and *D. hansenii*, are known to be able to grow above 10–15% NaCl (Samson et al. 2002). Most strains of *Metschnikowia bicuspidata* var. *bicuspidata* have been found to be associated with diseased brine shrimps (*Artemia salina*) from salt lakes and ponds with 10% NaCl, and they appear to require 2% NaCl to grow in vitro (Blackwell 2001). Lahav et al. (2002) described two yeasts, *Pichia guilliermondii* and *Rhodotorula mucilaginosa*, that can survive the extremely high salinity (between 3% and saturation) and pH (2.0–10.0) fluctuations.

Until 2005, there was no evidence that natural hypersaline brines contain nonmelanized yeast populations. Then, yeast diversity was reported for hypersaline waters of eight different salterns and three salt lakes worldwide. These were the Dead Sea, Enriquillo Lake in the Dominican Republic, and the Great Salt Lake in Utah, USA (Butinar et al. 2005a). This diversity included *P. guilliermondii, D. hansenii, Yarrowia lipolytica, M. bicuspidata, C. parapsilosis, Rhodosporidium sphaerocarpum, Rhodosporidium babjevae*, and *Trichosporon mucoides*. In bittern (water rich in magnesium chloride) from the La Trinidad salterns (Spain), two new species were discovered and provisionally named as *Candida atmosphaerica*-like and *Pichia philogaea*-like. Among the isolates obtained, *P. guilliermondii, D. hansenii, Y. lipolytica*, and *C. parapsilosis* were already known contaminants of low a_w food, whereas *R. sphaerocarpum, R. babjevae*, and *T. mucoides* were identified for the first time in hypersaline habitats. Moreover, the ascomycetous yeast *M. bicuspidata*, which is known to be a parasite of the brine shrimp, was isolated as a free-living form from Great Salt Lake brine. The frequency of its occurrence was low, as the counts only occasionally reached several hundred cells per liter across all of sampled salterns (on average, between 0 and 300 CFU l^{-1}). The most frequently occurring species in the Adriatic salterns were identified as *P. guilliermondii* (up to 270 CFU l^{-1}) and *C. parapsilosis*. They have also been sporadically isolated in other salterns (Butinar et al. 2005a).

In contrast to the melanized fungi that appear in the highest numbers during the crystallization in the solar salterns (Gunde-Cimerman et al. 2000; Butinar et al. 2005b), nonmelanized yeasts were isolated primarily outside the salt production season (Adriatic salterns) or in waters with NaCl concentrations below 20% (Butinar et al. 2005a). In addition, these yeasts were never isolated on 32% NaCl medium, but mainly on medium with 10% NaCl, and to a lesser extent on media with 17–25% NaCl (Butinar et al. 2005a).

7.2.6 The Order Eurotiales

The group of filamentous fungi that have been isolated from different salterns around the World is mainly represented by the order *Eurotiales*, or more precisely, by the teleomorphic genera *Eurotium* and *Emericella* and the anamorphic *Aspergillus*, and *Penicillium*. Tolerance for high salt concentrations has for a long time been recognized for many species of the ubiquitous food-borne genera *Aspergillus* and *Penicillium* (Tresner and Hayes 1971), but only recently have their biodiversity, together with their teleomophic forms, been investigated in the hypersaline waters of salterns from different geographical locations (Cantrell et al. 2006; Butinar et al. 2011).

The mycotoxin-producing genus *Eurotium* was for a long time known to comprise species that are contaminants of foods and feedstuffs that are preserved by high concentrations of sugar or NaCl. They can grow at a_w as low as 0.7, and are therefore considered xerophilic (Pitt and Hockering 1997). Occasionally, different *Eurotium* species have been isolated from natural habitats with low a_w, including saline soils and waters (Abdel-Hafez et al. 1978) and the Dead Sea (Kis-Papo et al. 2001). In a study of the mycodiversity of hypersaline water of different salterns from Europe, Asia, Africa, and North America, six different *Eurotium* species were identified, namely *E. amstelodami*, *E. chevalieri*, *E. herbariorum*, *E. rubrum*, *E. repens*, and *Eurotium* sp. The last one is probably a new species, and it has tentatively been named "*E. halotolerans*." Most (74%) of the 208 *Eurotium* spp. isolates obtained belonged to *E. amstelodami*, followed by *E. repens* and *E. herbariorum* (10% of the isolates). These three species are probably part of the indigenous mycobiota of salterns, while other *Eurotium* species are believed to be only temporal inhabitants or occasional air-borne contaminants of low-salinity brines. During the salt production season, two pronounced peaks of occurrence appear: first, at the 10–15% NaCl range, with counts up to 5,000 CFU l^{-1}, and then in the 18–25% NaCl range, with the highest counts (up to 30,000 CFU l^{-1}); outside the season of salt production, the counts for all of the *Eurotium* species remain below 100 CFU l^{-1}. The salinity growth ranges determined in vitro are broad, ranging from 0 up to 27.5% NaCl, and the spores and mycelia of these species can survive long-term exposure to solutions of up to 30% NaCl, with growth stimulated up to 10% NaCl for *E. rubrum*, *E. chevalieri*, and *E. amstelodami* and up to 12.5% NaCl for "*E. halotolerans*," *E. repens*, and *E. herbariorum*. *E. amstelodami* and "*E. halotolerans*" have the broadest salinity range, growing up to 27.5% NaCl, followed by *E. repens* and *E. herbariorum* (up to 25%), and *E. chevalieri* (22.5%) and *E. rubrum* up to 20% (Butinar et al. 2005c). Some of the *Erotium* species (e.g., *E. amstelodami* and *E. rubrum*) prefer media with sugar to media with NaCl, indicating that they have a xerophilic, rather than a halophilic, character (Wheeler and Hocking 1988).

The representatives of genus *Emericella*, which are recognizable by hülle cells in the chleistothecial walls and ornamented ascospore, have frequently been isolated from dry substrata in hot and arid areas worldwide. These appear to be well adapted to dry and warm climates (Samson and Mouchacca 1974) and low a_w (Zalar et al. 2008a). The soil representative *E. nidulans* was isolated also from desert saline soil

(Samson and Mouchacca 1974), while from the hypersaline water of the Sečovlje salterns in Slovenia, two newly described halotolerant species, *E. filifera* and *E. stella-maris*, were reported. The ascospores of *E. filifera* form long appendages that emerge radially from narrow stellate crests, and the ascospores of *E. stella-maris* have star-shaped equatorial crests (Zalar et al. 2008a). *E. striata* was obtained from Lake Enriquillo in Dominican Republic (Butinar et al. 2011).

The genus *Aspergillus* inhabits hypersaline waters that cover the greatest global diversity. *A. niger* and *A. caesiellus* contribute to the stable fungal communities in natural hypersaline waters, while *A. ochraceus*, *A. flavus*, *A. roseoglobulosus*, and *A. tubingensis* are primarily or exclusively present in hypersaline localities at higher environmental temperatures. *A. melleus*, *A. sclerotiorum*, and holomorphic species *Petromyces alliaceus* have been recognized within these taxonomic groups, although they have appeared only locally. *A. versicolor* and *A. sydowii* are both common in marine environments and in dry foods, and these have also been identified as part of the fungal communities in the hypersaline environments. *A. wentii*, *A. flavipes*, *A. terreus*, and particularly *A. candidus* have been repeatedly isolated from Adriatic salterns, whereas *A. penicillioides*, *A. proliferans*, and *A. restrictus* have been found only sporadically at salinities below 10% NaCl. *A. fumigates* is common in arid environments (deserts) at high temperatures, and has been found consistently in solar salterns, although it is also most abundant at salinities below 10% NaCl (Butinar et al. 2011).

Although many species in the subgenus *Penicillium* grow well in salted foods, only five species have been recognized as part of the indigenous fungal communities here. *P. chrysogenum* is a common, widely distributed species, which appears regularly in saline lakes and salterns worldwide, while *P. brevicompactum* is particularly common in Adriatic salterns. The other three species, two of which are most probably new, are seen consistently at low counts or at low salinities, or appear in hypersaline waters only sporadically. A series of soil-borne *Penicillium* species has also been identified as part of the hypersaline mycobiota, namely *P. citrinum*, *P. oxalicum*, and *P. steckii*. Although *P. sizovae* and *P. westlingii* are also common, their counts decrease with increased salinity and they are therefore considered as temporal inhabitants. Four Penicillia isolates identified as *P. nordicum* were isolated from salt used for human consumption and from the salting of foods, at counts of 5 CFU g^{-1} salt crystals (Butinar et al. 2011).

To conclude, in hypersaline environments, the pan-global stable mycobiota of the order *Eurotiales* are represented by *A. niger*, *E. amstelodami*, and *P. chrysogenum*, and possibly also by *A. sydowii*, *A. candidus*, and *E. herbariorum*, which are also quite abundant, although more locally distributed (Butinar et al. 2011).

7.2.7 The Genus Wallemia

Fungi from the genus *Wallemia* are xerophilic food- and air-borne fungi (Samson et al. 2002) that have also been isolated from soil (Domsch et al. 1990), sea salt (DasSarma et al. 2010; Butinar et al. 2011), and hypersaline water (Zalar et al. 2005a).

Until recently (Zalar et al. 2005a), a single cosmopolitan species *Wallemia sebi* was recognized in the genus which was known as the causative agent of allergological problems arising in farmer's lung disease (Lappalainen et al. 1998; Roussel et al. 2004). Its unresolved phylogenetic position was worked out when a large group of strains were collected globally from environments with low a_w (e.g., foods, hypersaline waters from different part of the World) and the sequence data of their different genes were analyzed (Zalar et al. 2005a; Matheny et al. 2006). On the basis of their unique morphology, xerotolerance, and isolated phylogenetic position, a new basidiomycetous class of Wallemiomycetes and the order *Wallemiales* were proposed for the genus *Wallemia*.

The genus *Wallemia* is an early diverging lineage of Basidiomycota and it segregates into at least three species that have been identified so far: *W. ichthyophaga*, *W. sebi*, and *W. muriae*. These species have been isolated consistently, although with low counts (maximally, 40 CFU l^{-1}), from hypersaline waters of salterns in Slovenia, Spain, the Dominican Republic, Israel, and Namibia. This is particularly extraordinary, because basidiomycetous fungi rarely show xerophilic or halophilic characteristics, as they generally appear outstandingly intolerant to NaCl (Tresner and Hayes 1971). Also within the Ascomycota there are only a few known fungi, such as *Basipetospora halophila*, *Polypaecilum pisce* (Wheeler et al. 1988), and *H. werneckii* (Gunde-Cimerman and Plemenitaš 2006), which are stimulated by NaCl, although with no obligate requirements.

Interestingly, tests on xerotolerance of the *Wallemia* spp. have shown that it represents one of the most xerophilic fungal taxa (Zalar et al. 2005a). The two halophilic *Wallemia* species, *W. muriae* and *W. ichthyophaga*, necessarily require media with low a_w, whereas *W. sebi* grows also on media without additional solutes. The narrow a_w ranges for growth of *W. muriae* and *W. ichthyophaga* are 0.984–0.805 and 0.959–0.771, respectively. Moreover, *W. ichthyophaga* shows preference for certain solutes for the lowered a_w, as it shows poor growth that is characterized by prolonged growth phases and smaller colonies on media with high concentrations of glucose, compared with media with high concentrations of salt (Zalar et al. 2005a; Kralj Kunčič et al. 2010). As it can thrive in media with NaCl necessarily above 1.7 M and up to saturation (5.3 M NaCl), this makes it the most halophilic fungi known to date. *W. sebi* can grow over a wider range of a_w (0.997–0.690) in glucose/fructose media (Pitt and Hocking 1977), but in media with NaCl as the major solute, its lowest a_w for growth has been reported to be 0.80 (Pitt and Hocking 1977; Zalar et al. 2005a), corresponding to 4.5 M NaCl.

7.3 Special Adaptations and Properties of the Fungi in the Salterns

The responses of eukaryotic cells to low a_w and high concentrations of toxic ions involves complex alterations in gene expression that lead to metabolic changes and subsequent adaptation to the new conditions (Yale and Bohnert 2001; Petrovič et al. 2002; Vaupotič and Plemenitaš 2007). The mechanisms of salt tolerance in fungi have

been mostly studied in salt-sensitive *Saccharomyces cerevisiae* (Blomberg and Adler 1992; Blomberg 2000; Hohmann 2002), the halotolerant yeast *D. hansenii* (Larsson and Gustafsson 1987; Andre et al. 1988; Larsson et al. 1990; Larsson and Gustafsson 1993; Prista et al. 1997; Almagro et al. 2000) and the extremely halotolerant black yeast *H. werneckii* (Turk et al. 2004, 2007a; Kogej et al. 2005, 2006a, b, 2007; Plemenitaš et al. 2008). The last species has been proposed as a model organism for eukaryotic halophily studies. Physiological and molecular adaptations of the true halophilic representative known so far, *W. ichthyophaga*, are only at an early stage.

The pathway for the sensing of osmolarity changes is of vital importance for the survival of the cell. In *S. cerevisiae*, this pathway is known as the high-osmolarity glycerol (HOG) signaling pathway, which is also part of one of the best understood mitogen-activated protein kinase (MAPK) cascades (Hohmann 2002). The existence of a similar signaling pathway has been demonstrated and extensively studied in *H. werneckii* (Turk and Plemenitaš 2002; Plemenitaš et al. 2003; Lenassi and Plemenitaš 2005, 2007; Plemenitaš et al. 2008; Fettich et al. 2011). An important level of adaptation to hypersaline environment is the need to balance the osmotic pressure of the medium by accumulating and/or synthesizing compatible organic solutes (Oren 1999) and maintaining low salt concentrations within the cytoplasm. Additional adaptations at the level of the plasma membrane composition (Petrovič et al. 1999; Turk et al. 2004; Gostinčar et al. 2008, 2009) and the cell wall structure (Kralj Kunčič et al. 2010), which represent the first line of defense against environmental stress (Mager and Siderius 2002), are also required to prevent damage of the cells in such environments.

Numerous morphological adaptations that are reflected in the extremophilic ecotype characterized by meristematic growth, pigmentation, and changes in colony morphology are believed to have important roles for successful growth under extremely saline conditions (Kogej et al. 2006a; Kralj Kunčič et al. 2010). The morphology of black yeasts uniquely depends on the environment, as isodiametrical cells are typical in water and hyphal growth is shown only on solid media. This optimization of the volume-to-surface ratio, together with meristematic growth, thick melanized cell walls, extracellular polysaccharides, and adhesion and propagation with endoconidiation, is interpreted as a response to stress factors such as low a_w, low nutrition, and high temperatures (Sterflinger et al. 1999). A study of the morphological adaptations to moderate and high NaCl concentrations of *Wallemia* spp. using different microscopy approaches revealed an impact of high concentrations of NaCl on the cell morphology of the *Wallemia* spp. (Kralj Kunčič et al. 2010). Hyphal compartments of *W. sebi* and *W. muriae* are thicker and shorter, and their mycelial pellets are larger at high salinity. The phylogenetically distinct *W. ichthyophaga* differs from the other two *Wallemia* spp. at the level of its morphology also, as it forms sarcina-like multicellular clumps that are composed of compactly packed spherical cells. The size of the cells does not respond to increased salinity, whereas these multicellular clumps become significantly large. Clustered growth allows the sheltering of the cells in the interior and minimizes the number of cells that are directly in contact with the hostile environment. Therefore, the ability to grow meristematically or in the form of multicellular clumps is

believed to greatly enhance their survival in stressful environments (Wollenzien et al. 1995; Palkova and Vachova 2006).

The presence of extracellular polysaccharides is part of the protection against desiccation in rock-inhabiting fungi (Selbmann et al. 2005), and it might have a protective function at high sanities, as a pronounced extracellular polysaccharide layer has been observed in all the three *Wallemia* spp. (Kralj Kunčič et al. 2010) and for *T. salinum* (Kogej et al. 2006a). Furthermore, an increase in the thickness of the multilayered cell wall occurs at higher salinities in all the three *Wallemia* spp., although it is especially pronounced in *W. ichthyophaga*. The extremely thick cell wall of *W. ichthyophaga* is however an exception in the fungal responses to extremely saline conditions known to date (Kralj Kunčič et al. 2010).

A common strategy of osmoadaptation in response to increased salinity in the environment in most eukaryotic microorganisms, as well as in halotolerant and halophilic fungi, is based on the synthesis and cytoplasmic accumulation of a mixture of polyols as compatible solutes. Among these, glycerol is the most significant, and it has been measured in the highest amounts in the extremely halotolerant black yeast *H. werneckii* (Petrovič et al. 2002; Kogej et al. 2007) as well as in the halophilic *W. ichthyophaga* (our unpublished data). Besides glycerol, *H. werneckii* accumulates erythritol, arabitol, and mannitol (Kogej et al. 2007), and interestingly, mycosporine-glutaminol-glucoside also, as complementary compatible solutes (Kogej et al. 2006b). *H. werneckii* and *A. pullulans* keep their intracellular concentrations of sodium and potassium cations low in environments with high concentrations of salt, and they are therefore considered as Na^+-excluders (Kogej et al. 2005). So far, only the halotolerant yeasts *D. hansenii* (Prista et al. 1997) and *P. guilliermondii* (Lahav et al. 2002) have been shown to maintain relatively high internal concentrations of sodium when coping with salt stress, together with the production and intracellular retention of compatible solutes, particularly glycerol (Prista et al. 1997; Lahav et al. 2002).

Glycerol can easily pass through lipid bilayers because of its small molecular mass. Therefore, eukaryotic cells using glycerol as a compatible solute either accumulate lost glycerol using energetically costly transport systems or change their membrane structure by an increased sterol content or reduced membrane fluidity (Oren 1999). Interestingly, in the black yeasts *H. werneckii* and *P. triangularis*, the total sterol content remains mainly unchanged with increased salinity (Turk et al. 2004), while the plasma membrane is significantly more fluid over a wide range of salinities, in comparison with the membranes of salt-sensitive and halotolerant fungi (Turk et al. 2004, 2007a). Higher plasma-membrane fluidity results from an increase in the unsaturated fatty acid content and length, because of the salt stress (Turk et al. 2004, 2007a). One of the mechanisms that allows the precise regulation of membrane fluidity in *H. werneckii* and *A. pullulans* is the change in the expression of fatty-acid-modifying enzymes, such as desaturases and elongase (Gostinčar et al. 2008, 2009). Similar to halophilic/halotolerant black yeasts, the membrane fluidity in the osmotolerant *D. hansenii* is not significantly affected at high levels of NaCl, although the sterol-to-phospholipid ratio and the

fatty acid unsaturation increase (Turk et al. 2007b). As high salt tolerance correlates well with higher membrane fluidity, this is of crucial importance for tolerance to salt stress.

Hortaea werneckii can grow at very high salinities, which require large amounts of intracellular glycerol, while at the same time it maintains a very fluid membrane and a constant sterol content. Instead of modifying its membrane properties *H. werneckii* uses its melanized cell wall to reduce glycerol leakage from cells at the optimal salinities (Kogej et al. 2007). The outer part of the melanized cell wall has a continuous layer of melanin granules that minimizes glycerol loss from the cells, as this layer creates a mechanical permeability barrier for glycerol by reducing the size of the pores in the cell wall (Jacobson and Ikeda 2005). At higher salinities, melanization is diminished, and glycerol retention is less effective; therefore, the growth rates and biomass yields of *H. werneckii* are reduced (Kogej et al. 2007).

7.4 The Ecological Types of Fungi in the Salterns

Fungal species can be classified into three ecological groups according to their abilities to inhabit extreme environments. First, there are the mesophiles, which predominantly inhabit environments without extreme conditions. The second group is represented by the generalists, which tolerate a variety of moderately stressful conditions, but not the extreme ones, and they have their growth optimum under moderate conditions. As a result of their limited competition with mesophiles and their inability to survive the most extreme conditions, they are often predominantly found under moderately stressful conditions. In hypersaline environments, *A. pullulans* is a good example of a generalist, as it can tolerate up to 18% NaCl, but not in saturated NaCl solution, and it grows optimally without NaCl. The third group comprises the specialists, which are extremely halo tolerant or even halophilic, and their growth optima are shifted toward extreme conditions. They predominantly inhabit extreme habitats, as they cannot compete with species in moderate environments, or are simply not able to survive moderate conditions. Examples of the specialists among the hypersaline mycobiota are *H. werneckii*, which grows under extreme (up to saturation) salt conditions as well as without salt, and *W. ichthyophaga*, which necessarily requires 10% NaCl and also grows in saturated NaCl solution. Therefore, adaptive extremophiles with a broad ecological amplitude (e.g., *H. werneckii*) and obligate extremophiles with a narrow ecological amplitude (e.g., *W. ichthyophaga*) have been distinguished. Both *H. werneckii* and *W. ichthyophaga* are found in hypersaline environments, and they are characterized by growth optima at extreme salinity. This narrow-amplitude strategy is more of an exception among the fungal kingdom, because *W. ichthyophaga* is so far the only representative of halophilic fungi with an obligate salt requirement (Gostinčar et al. 2010).

7.5 The Importance of the Saltern Mycobiota

Over the last few years, fungi thriving under conditions that are extreme from an anthropocentric point of view, and which thus live at the so-called ecological periphery, have deserved and received increasing scientific attention. Some halotolerant and halophilic fungi have possible important biotechnological applications. These will arise from studies of their basic characteristics and adaptation mechanisms, at the level of their secondary metabolites, cell membranes, intracellular and extracellular enzymes, genetic transfer systems and intracellular osmolytes, and especially of their compatible solutes, which have a wide range of applications because of their ability to stabilize proteins and nucleic acids (Arakawa and Timasheff 1985; Kurz 2008). The salt-tolerant black yeast *A. pullulans* produces exopolymer pullulan that has a broad spectrum of use in the food and pharmaceutical industry (Leathers 2003; Singh and Saini 2008), and the extremely halotolerant black yeasts *T. salinum* and *H. werneckii* have been shown to produce extracellular hydrolytic enzymes that are active at high salt concentrations and that could therefore have important roles in different industries (Zalar et al. 2005b). *H. werneckii* also produces antibiotic compounds that remain to be commercially exploited (Brauers et al. 2001). Indeed, many halophilic and halotolerant fungi synthesize specific bioactive metabolites under stress conditions, and particularly at increased salt concentrations, when higher hemolytic activities have been seen (Sepčić et al. 2011).

Progressive salinization represents a serious agricultural problem worldwide, as 10 million hectares of arable land is lost in this way annually. Genetic manipulations of crops that have increased salt tolerance have still not yielded satisfactory results. The use of halophilic and halotolerant fungal genetic sources might provide the desired improvement in the breeding of such crops in the future. The halotolerant *H. werneckii* has been shown to be a promising source of salt-tolerant transgenes for agriculture. In yeast, as well as in plants, Hal2 is a sodium- and lithium-sensitive 3′-phosphoadenosine-5′-phosphatase, which is an important determinant for halotolerance (Gläser et al. 1993). Thus, overexpression of novel isoenzymes or of Hal2-like proteins from *H. werneckii* can remarkably increase the halotolerance of *S. cerevisiae* (Vaupotič et al. 2007).

Microorganisms and their metabolites can affect the salt production in the evaporating ponds of salterns, as they can physically affect the evaporation process and as their by-products can chemically modify or bind with the dissolved ions (Javor 2002). Moreover, the biological systems in the salterns can also “contaminate” the salt that is used for food preservation. It has been known for a long time that haloarchaea can be introduced into food via salt and can spoil heavily salted proteinaceous products (Norton and Grant 1988; Grant 2004). Recently, different fungi have been isolated from salt used for human consumption and for food salting (Butinar et al. 2011).

As many fungi from salt and hypersaline water produce the same mycotoxins as those found on the salted meat products, the salt is most probably the contamination source for some toxinogenic fungi (Andersen 1995; Larsen et al. 2001; Butinar

et al. 2011). Precautionary measures should therefore be considered seriously, such as heat treatment of salt prior to adding it to the meat products, to kill the fungal conidia (Butinar et al. 2011).

7.6 Conclusion

The taxonomic and physiological characterization of halotolerant/halophilic fungi isolated from different salterns and hypersaline lakes on three continents have shown a surprising diversity of fungi. The reports of existance of fungi along with other complex microbial communities in the salterns has improved our understanding of interlinked microbial and chemical processes. As evaporation and mineral precipitation are intimately linked to microbial communities and their products, microbial activity, including fungal, can affect both the quality and quantity of salt. The mycobiota can thus be "imported" in natural hypersaline environments through air, but it can also be "exported" as living cells bounded to salt crystals, and in this way contaminating our foods and homes.

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2.2 OSTALO POVEZOVALNO DELO

2.2.1 Rod Wallemia

Naslov v originalnem jeziku: *Wallemia*

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Izvleček:
Glivni rod Wallemia je edini rod v redu Wallemiales (Wallemiomycetes, Basidiomycota). Do leta 2005 je rod Wallemia vseboval le vrsto *W. sebi*, sedaj pa je znano, da so v rodu tri vrste: *W. ichthyophaga*, *W. sebi* in *W. muriae*. Imajo edinstveno morfologijo z značilnim bazavskalnim načinom konidiogeneze in bazipetalnim ločevanjem konidialnih enot ter disartikulacijo na večinoma štiri artrosporam podobne konidije. Vse tri vrste imajo skupno fiziologijo, saj so vse kserofilne in kažejo optimalno rast pri nizki vodni aktivnosti. Poleg velikosti konidijev in prisotnosti oziroma odsotnosti sarcinam podobnih struktur, je glavni kriterij za njihovo razlikovanje stopnja kserofilije. Vrsta *W. sebi* lahko raste pri vodni aktivnostjo 1.0, medtem ko *W. muriae* in *W. ichthyophaga* nujno potrebujeta gojišče z znižano vodno aktivnostjo. Vrste rodu Wallemia so pogosto vključene v kvarjenja hrane, še posebej za suhe, sladke in slane, mogoče jih je najti tudi v zraku zaprtih prostorov in na prostem, v kmetijskih aerosolih in senu, na različnih žitaricah (riž, pšenica, ječmen, koruza), v cvetnem prahu, zemlji, morski sol, v morskih sedimentih, na morskih organizmih in v solinah po vsem svetu. *Wallemia* lahko povzroči alergološke težave, kot je bolezen kmetovih pljuč, in tudi čeprav redko kožne in podkožne okužbe pri ljudeh. Pred letom 2005, ko je rod Wallemia predstavljala le vrsta *W. sebi*, sta bila iz seva, ki je okuževal tortno pecivo osamljena dva sorodna mikotoksina -waleminol A in waleminol B ali waleminone. Prisotnost teh toksinov še ni bila določena v kasneje opisanih vrstah *Wallemia*, čeprav je bila v izjemno slanih pogojih povečana hemolitična aktivnost pri vseh treh vrstah. Kakorkoli, uživanje hrane in krme, kateri se podaljševanje roka trajanja dosega

z nizko vodno aktivnostjo in je kontaminirana z vrstami rodu *Wallemia,* predstavlja spregledano zdravstveno tveganje.

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WALLEMIA

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ABSTRACT

The cosmopolitan fungal genus *Wallemia* is the only genus in the order *Wallemiales* (Wallemiomycetes, Basidiomycota), and it has a worldwide distribution. Until 2005, the genus *Wallemia* contained only one species, *W. sebi*; now it is known to be comprised of three species: *W. ichthyophaga, W. sebi* and *W. muriae*. These all have unique morphologies, including a basauxic mode of conidiogenesis, and basipetal segregation of conidial units and their disarticulation into mostly four arthrospore-like conidia. All three of these species have a common physiology, as they are all xerophilic and show growth optima at low water activity. As well as the conidial size and the presence/ absence of sarcina-like structures, the major criterion for their distinction is their degree of xerophily. *W. sebi* can grow at a water activity 1.0, while *W. muriae* and *W. ichthyophaga* necessarily require media with lower water activities. The *Wallemia* spp. are frequently involved in food spoilage, particularly of dried, sweet and salty food, and they can be found also in indoor and outdoor air, agriculture aerosols and hay, different cereals (rice, wheat, barley, corn), pollen, soil, sea salt, sea sediments, sea organisms and hypersaline water of salterns around the globe. *Wallemia* can cause allergological problems, such as farmer's lung disease, and can, albeit rarely, cause cutaneous or subcutaneous infections in humans.

Before 2005, when the genus *Wallemia* comprised only *W. sebi*, two related mycotoxins, walleminol A and walleminol B or walleminone, were isolated from a strain that had contaminated a cake. The presence of these toxins has not yet been determined for the later-described *Wallemia* spp., although enhanced haemolytic activity has been shown for all three of these species when grown under highly saline conditions. In conclusion, consumption of food and feed that has been preserved with low water activity and contaminated with *Wallemia* might represent a largely overlooked health risk.

Keywords: *Wallemia*, xerophile, walleminol, walleminon, haemolytic activity, subcutaneous infection.

Abbreviations:

a_w, water activity; GPD1, glycerol-3-phosphate dehydrogenase; ITS rDNA, internal transcribed spacer region ribosomal deoxyribonucleic acid; MEA, malt extract agar, MY50G, malt extract yeast extract 50 % glucose agar; PTS2, peroxisomal targeting sequence; SSU rDNA, small subunit ribosomal deoxyribonucleic acid; UV, ultraviolet.

INTRODUCTION

Food is an ecosystem that includes higher levels of nutrients for growth of potential spoilage microorganisms than their natural environments such as soil and water [1]. The spoilage of fresh, living food, particularly fresh fruits, vegetables, and also grains and nuts before harvest is limited to those microorganisms that can overcome defence mechanisms, while the spoilage of processed, dormant and non-living food depends on physical and chemical factors [1]. Prevention of food spoilage traditionally depends on a reduction of the biologically available water, using drying or freezing, or by adding different solutes.

As the central molecule for biological processes, low amounts of biologically available water (i.e., a low water activity [a_w]) represents one of the most pervasive of stresses for biological systems and only specially adapted organisms can thrive under such conditions. Food with a a_w below 0.9 will only support the growth of a few highly resistant bacterial pathogens [2], while relatively few xerotolerant and xerophilic fungal species can grow. Indeed, tolerance to low a_w is apparent in only 10 of the 140 known orders of fungi, with most of them belonging to the Ascomycota [3], and xerotolerance is extremely rare in the division Basidiomycota. Therefore, the genus *Wallemia* represents a rare genus of cosmopolitan, xerotolerant and xerophilic basidiomycetous fungi that are frequently involved in food spoilage of, in particular, sweet, salty and dried food, and that have mycotoxigenic and mycotic potential.

PHYLOGENY AND IDENTIFICATION OF THE GENUS WALLEMIA

Based on the analysis of the nuclear small subunit ribosomal RNA gene (SSU rRNA) and ultrastructural characteristics (e.g., dolipore septum), *Wallemia* is included in the phylum Basidiomycota. The unique phylogenetic position of *Wallemia*, accompanied by the rarely encountered conidiogenesis and extreme xerotolerance, resulted in the description of the new class of Wallemiomycetes, and the new order of Wallemiales [4]. This taxonomic placement was later supported by molecular analyses of three ribosomal RNA genes (18S, 25S, 5.8S) and three nuclear protein-coding genes (rpb1, rpb2, tef1). The class Wallemiomycetes was described as an early diverging lineage of Basidiomycota. It has a basal position near the Entorrhizomycetidae and might be a sister group of the Agaricomycotina and Ustilaginomycotina [5].

The hypothesis that *Wallemia* is closely related to Agaricomycotina [6] was confirmed in a detailed genome study of *Wallemia sebi* [7]. This phylogenetic analyses of a 71-protein dataset supported the position of *Wallemia* as the earliest diverging lineage of Agaricomycotina, which was confirmed also by the septal-pore ultrastructure, which showed the septal pore apparatus as a variant of the *Tremella*-type. The relationships between the three subphyla of Basidiomycota have been difficult to resolve. Recent data support Ustilaginomycotina as the sister group to the branch that unites Wallemiomycetes and the remaining Agaricomycotina, and Pucciniomycotina as the sister group to the rest of Basidiomycota [7].

Based on differences in conidial size, on their xerotolerance, and on sequence data of the internal transcribed spacer region (ITS) rDNA, three *Wallemia* species have been identified: *W. ichthyophaga, W. sebi* and *W. muriae*. *W. ichthyophaga* differs from the other two species in many of the nucleotides of the SSU rDNA and the ITS rDNA [4]. In addition, based on molecular analyses of rpb2 and hal2, at least two new species are indicated. One of these is closely related to *W. sebi*, while the second (with only two known isolates) represents a new species and a putative link between *W. ichthyophaga* and the other species of this genus (Jančič, Gunde-Cimerman et al., unpublished data).

On standard mycological media, the *Wallemia* spp. can be recognised by small, walnut-brown colonies. Despite the considerable molecular distances between the individual *Wallemia* spp., they all show a unique form of conidiogenesis, which is seen as the basauxic development of fertile hyphae, and the basipetal segregation of the conidial units and their disarticulation into mostly four arthrospore-like conidia (Fig. 1. e, f). Nevertheless, the different species within the genus *Wallemia* can be distinguished by morphological and physiological characteristics, such as the size range of the conidia (Fig. 1. e), the presence of sarcina-like structures (Fig. 1. g) in *W. ichthyophaga*, and the differences in their degrees of xerophily [4]. *W. sebi* is currently the only recognised *Wallemia* spp. that can grow on MEA without the addition of NaCl or sugar, and it has smaller conidia than the other two species.

ECOLOGY OF THE GENUS WALLEMIA

The *Wallemia* spp. are frequently involved in food spoilage, and particularly of dried, salty and sweet foods like chocolate (Fig. 1. c) [8]. They have often been isolated from indoor [9]

and outdoor [10] air in urban and agricultural environments [11,12], and from cereal grains, like rice [13], wheat, barley and corn. The *Wallemia* spp. have also been detected in hay, pollen and soil, bound to sea-salt crystals (Jančič, Gunde-Cimerman et al., unpublished data), and in sea sediments [14], sea-water organisms [15], and hypersaline water of man-made salterns (Fig. 1. a) on different continents . Salters are therefore proposed as a natural ecological niche for *Wallemia* spp [4]. The *Wallemia* spp. are also agents of degradation of cultural heritage objects [16].

To date, only the species *W. sebi* has been described as the common cause of allergological problems, which are better known as farmer's lung disease [17,18], and although rarely, it has been shown to be the causative agent of cutaneous and subcutaneous infections in humans [19]. However, it is worth noting that none of the isolates involved in the above-listed studies were identified using molecular methods, and therefore their designation as *W. sebi* must remain questionable.

XEROPHILY OF THE GENUS WALLEMIA

The genus *Wallemia* is one of the best known low-a_w-tolerant groups of fungi, and it is distributed in well-defined ecological niches [20]. Out of 140 known orders of fungi, only 10 include species that tolerate low a_w, with most of these belonging to the Ascomycota [21]. Xerotolerance, and even more xerophily, is relatively rare in Basidiomycota, and therefore it is surprising that the *Wallemia* spp. represent one of the most xerophilic fungal taxa.

Two out of three of the described *Wallemia* spp., *W. muriae* and *W. ichthyophaga*, require media with low a_w, and thus these can be considered as obligate xerophiles. *W. sebi* can also thrive on media without additional solutes [4], and it can grow over a wider range of a_w (0.997 - 0.690) in glucose/ fructose media [22]. However, in media with added NaCl as the major solute, the lowest a_w for the growth of *W. sebi* was reported to be 0.80 [4,22], which corresponds to 4.5 M NaCl. The a_w growth ranges of *W. muriae* and *W. ichthyophaga* are 0.984 - 0.805 and 0.959 - 0.771, respectively [23]. *W. muriae* can tolerate 0.7 M to 4.3 M NaCl, while *W. ichthyophaga* can thrive in media with NaCl above 1.7 M, and up to NaCl saturation (5.3 M). Due to its obligate requirement of at least 10 % NaCl in the medium, *W. ichthyophaga* is the most halophilic fungus known to date. The halophilic *versus* xerophilic nature of *W. ichthyophaga* was further demonstrated by its considerably more

rapid and more abundant growth on media with added NaCl as the solute, thus lowering the a_w, in comparison with media with high concentrations of either glucose or honey [4,24,25]. Interestingly, all of the *Wallemia* spp. show optimal growth in media with low a_w, with all three showing their greatest colony diameters at lowered a_w: *W. sebi* and *W. muriae* at 0.96, and *W. ichthyophaga* at 0.88 [4].

MOLECULAR AND PHYSIOLOGICAL ADAPTATIONS OF THE WALLEMIA SPP. TO LIFE AT LOW WATER ACTIVITY

Microbial survival in different environments depends on the ability of an organism to sense and to respond to environmental factors. Such responses involve complex alterations in gene expression, which can lead to metabolic changes and the subsequent adaptation to the new conditions [26-28].

In environments with low a_w, organisms are exposed to turgor-related stress, due to high concentrations of solutes and to the toxicities of certain ions. The adaptations of fungi to high concentrations of NaCl have been well studied, while osmotic stress induced by high concentrations of sugar have received little attention. The mechanisms of salt tolerance in fungi have been mostly studied in the salt-sensitive *Saccharomyces cerevisiae* [29-31], the halotolerant yeast *Debaryomyces hansenii* [32-37], and the extremely halotolerant black yeast *Hortaea werneckii* [38-42]. Only more recently have the physiological and molecular adaptations of the genus *Wallemia* become the focus of studies, and especially of the most halophilic representative, *W. ichthyophaga*.

The pathway for the sensing of osmolarity changes in fungi (e.g., *S. cerevisiae* and *Hortaea werneckii*) and for the facilitation of adaptation of cells to increased osmolarity of the environment is known as the high-osmolarity glycerol (HOG) signalling pathway. This is one of the best understood of the mitogen-activated protein kinase (MAPK) cascades [31]. Homology searches in the genome of *W. sebi* have shown that the HOG pathway is mostly conserved [7], although it lacks some of the genes: (a) two Hog1 (MAPK) homologues that appear to function in osmotolerance; (b) putative homologues of genes that encode various proteins that are involved in the activation of the upstream HOG pathway (e.g., Ste11p, Cla4p) [7]; and (c) the downstream target orthologues Rck2p [43] and Sgd1p [44]. Genes involved in the ability to live under osmotic stress have also been investigated using *in-silico* analyses, which identified 93 putative osmotic-stress proteins, including the two

Hog1-like genes [7]. Finally, the HOG pathway of the halophilic *W. ichthyophaga*, is under investigation.

As with other halophilic and halotolerant microorganisms, most of the halotolerant and halophilic fungi prevent the loss of internal water and achieve osmotic balance in hypersaline environments by the synthesis and/or accumulation of small organic molecules that are known as compatible solutes. At the same time, they maintain low concentration of salt in their cell cytoplasm [45].

Increased production and accumulation of glycerol in environments with high concentrations of NaCl is a common strategy of osmoadaptation in different halotolerant fungi, for example, *H. werneckii* [28,38]. This appears to be the preferred strategy among eukaryotes as the production of glycerol requires low amounts of energy [45]. NAD-dependent glycerol-3-phosphate dehydrogenase (Gpd) is the key enzyme in the synthesis of glycerol from the glycolytic intermediate dihydroxyacetone phosphate[46,47]. Recently, Gpd1 was identified and characterized for the first time in the halophilic fungus *W. ichthyophaga* (WiGpd1). This Gpd1 was shown to be salt inducible, and therefore to contribute to osmoadaptation, as the transcript numbers of the mRNA from Wi*GPD1* in salt-adapted cells showed a gradual increase at increasing salinities, as a response to saline stress [48]. Furthermore, although comparison of Gpd1 from the salt-sensitive *S. cerevisiae* to WiGpd1 showed high overall amino-acid similarity, a significant difference was also revealed. The N-terminal PTS2 sequence that is important for peroxisome localisation [49] is lacking in the WiGpd1 *W. ichthyophaga* homologue which appears to be because of its function in osmotic stress: the constant cytosolic localisation of Gpd1 is beneficial for to organisms that live in extremely saline environments [48].

Morphological adaptations of the *Wallemia* spp. to moderate and high NaCl concentrations have been studied using combinations of light microscopy and focused-ion-beam/ scanning and transmission electron microscopy[24]. *W. sebi* and *W. muriae* have thicker and shorter hyphal compartments and larger mycelial pellets at high salinity, while *W. ichthyophaga* forms sarcina-like multicellular clumps that are composed of compactly-packed spherical cells, with no hyphae. The sizes of these cells did not correlate with the increased salinity, whereas their multicellular clumps become significantly larger. Meristematic growth in the form of multicellular clumps and additional cover with extracellular polysaccharides appear to greatly enhance the survival of the *Wallemia* spp.

in stress environments [50-53]. The cells of all three of the *Wallemia* spp. are covered with extracellular polysaccharides, which while protecting these cells against desiccation, might also protect at high salinity and high sugar concentrations [24,25].

To prevent damage to these cells, additional adaptations at the levels of the plasma-membrane composition [54-57] and the cell wall structure [24] are also required. An interesting and unique phenomenon has been seen at the level of the ultrastructure of the cell wall of *Wallemia* spp. at higher salinity, with the thickness of multilayered cell walls increasing. The cell wall thickness of *W. sebi* and *W. muriae* at low salinity is approximately 0.2 µm, and of *W. ichthyophaga*, 0.6 µm. At high salinity, the cell wall thickness of both *W. sebi* and *W. muriae* increase only slightly, while in *W. ichthyophaga*, the thicknesses increases up to 1.6 µm, thus resulting in a 1.67-fold increase in thickness. Such significant cell wall thickening as this described for *W. ichthyophaga* as a response to extremely saline conditions is a unique, and previously undescribed fungal response. It appears that the different morphological phenomena in the study of Kralj Kunčič et al. [24] have important roles for the successful growth of *Wallemia* spp. at extreme salinity.

BIOACTIVE METABOLITES PRODUCED BY THE WALLEMIA SPP.

The search for the production of biologically active compounds, including mycotoxins, has been mostly focused on cosmopolitan mesophilic fungi, with the more rare halotolerant and halophilic fungi being overlooked. As fungi adapted to grow at low a_w can contaminate food preserved with high concentrations of salt or sugar, or by dessication, as shown for *Wallemia* spp., these fungi might represent a serious threat to human and animal safety.

Our knowledge of the mycotoxigenic potential of *Wallemia* spp. is at present limited, as the presence of mycotoxins has been studied exclusively in *W. sebi*, which until 2005 [4], represented the only known species of this genus. The toxicity of *W. sebi* culture filtrates has been shown on the HeLa cell lineage, in mice [58], and in other biological assays [59]. In 1990, two related tricyclic dihydroxysesquiterpenes that are known as walleminol A and walleminol B or walleminone were isolated from *W. sebi* from a contaminated a cake. Both of these are toxic to certain cell lines, protozoa and brine shrimps. The minimum inhibitory dose of walleminol A in the bioassays is comparable to a number of mycotoxins, such as citrinin and penicillic acid (approximately 50 µg/ml) [60]. As well as mycotoxins, two other bioactive components have been identified in *W. sebi*: the azasteroids UCA

1064-B [61] and UCA 1064-B [62]. They both exhibit antibacterial and antimycotic activities, while only UCA 1064-B also shows antitumour activity.

Another new bioactive compound, cyclopentanopyridine alkaloid (3-hydroxy-5-methyl-5,6-dihydro-7H-cyclopenta[b]pyridin-7-one), was isolated when *W. sebi* were grown in a medium with 10 % NaCl. This showed antimicrobial activity against *Enterobacter aerogenes*, with a minimum inhibition concentration of 76.7 μM. Also, 11 known aromatic secondary metabolites were detected under saline conditions [63].

All three of the *Wallemia* spp. have been investigated recently for the production of haemolytic and antibacterial compounds, with the screening of fungi isolated from different extreme environments. Organic extracts of the three species have shown high haemolytic and moderate antibacterial activities against Gram-positive *Bacillus subtilis*, particularly under stress conditions [64]. In *W. ichthyophaga*, there was a higher haemolytic activity in extracts from cultures grown with high concentrations of glucose, while the haemolytic potential of the organic extracts of *W. muriae* and *W. sebi* considerably increased when the cultures were exposed to low temperatures (10 °C) [64].

Gas chromatography–mass spectrometry analysis of a *W. sebi* ethanol extract revealed a complex mixture of twenty-one sterols and fatty acids. The most intense chromatographic peaks corresponded to palmitic acid (C16:0), a mixture of linoleic (C18:2) and oleic (C18:1) acids, and ergosterol. Unsaturated fatty acids were responsible for haemolytic activities towards red blood cells and artificial small lipid vesicles with various lipid compositions. The study showed concentration-dependent haemolysis and preference for lipid membranes with higher fluidity [65].

In summary, low a_w conditions have been shown to induce the production of bioactive metabolites in the xerophilic *Wallemia* spp.. These might have protective roles in adaptation to these environments [64]. As has been shown for free fatty acids, some bioactive compounds might contribute to territorial competition in aquatic ecosystems, by affecting the growth of phytoplankton, algae and cyanobacteria [66].

It is also of note that the *Wallemia* spp. that are commonly involved in food spoilage of low a_w foods can synthesise mycotoxins, such as walleminol A, which can be excreted into food contaminated by *W. sebi* [60]. Thus, the bioactive and mycotoxigenic potential of all three of the *Wallemia* spp. should be considered in food quality control, as food and feed contaminated with *W. sebi* might represent a health risk [65].

PATHOGENESIS, CLINICAL FEATURES AND DIAGNOSIS OF *Wallemia sebi*

Similar to the production of bioactive compounds, our knowledge of the involvement of *Wallemia* spp. in human infections and pathogenesis has so far been limited to only *W. sebi*, which until 2005 [4] was the only known species of this genus.

W. sebi is a ubiquitous mold that can thrive in low a_w foods and feed, as well as in other dry environments, such as dust. *W. sebi* is commonly reported as the cause of respiratory allergies, such as bronchial asthma[17,67] and farmer's lung disease (FLD)[17,68], and albeit rarely, cutaneous and subcutaneous infections in humans [19].

W. sebi was also reported to be a causative agent in atopic diseases as some asthmatic individuals showed immediate type hypersensitivity to *W. sebi*. The skin prick tests of its extract elicited positive reactions in 5.4 % asthmatic patients and radioallergosorbent test showed positive results in 18.9 % [69,70].

Farmer's lung disease (FLD) is a form of occupational hypersensitivity pneumonitis (extrinsic allergic alveolitis) caused by chronic inhalation of microorganisms (antigens) from mouldy hay, straw, or grain[71]. Its clinical expression is characterized by symptoms of dyspnea, cough, tiredness, headaches, occasional fever/night sweats and general feeling of sickness. Any one or all of the symptoms may be apparent depending on the severity of FLD: acute, sub-acute or chronic [18,68,72-74]. *W. sebi,* together with *Eurotium amstelodami* and *Absidia corymbifera* are likely to be the main causes of FLD [18,68] in eastern France. Fungi involved in FLD reached a peak in January and February, which corresponded to the period when the number of FLD cases in the region was the highest. The main factor of their proliferation in hay is bad harvest conditions (rain during harvest, soil in the hay) [68].

Two cases of cutaneous and subcutaneous infections described in 1909 and another one in 1950 were named as 'hemisporiosis', after the synonymous species *Hemispora stellata.* No clinical features were given. Other infections caused by *W. sebi* were described more than 50 years later, in only 2008 [19]. The reason for these rare reports might be the extremely slow growth of the fungus, which on mesophilic media can be quickly overgrown by contaminants, and/or the misidentification of cultures. A rapid and precise alternative to conventional morphological and biochemical detection methods of *W. sebi* is PCR amplification and sequence analysis of the ITS rDNA [4].

Infections by *Wallemia* spp. are either infrequent or under-diagnosed, and therefore there is little information on their clinical features and treatments. In a study by Guarro et al. [19], a case of subcutaneous phaeohyphomycosis, of a 43 year old woman in northern India, was described as a non-healing ulcer on the dorsum of the foot. The erythematous lesion, which started as an itchy papule, lasted 8 months and gradually increased in size. The patient could not recall any prior injury. The diagnosis was based on the histological demonstration of septate hyphae and the recovery of the fungus in culture. The patient was treated with itraconazole, but did not return for evaluation. It should be highlighted that the infected patient was immunocompetent and diabetes and HIV free. This case report added the genus *Wallemia* to the relatively short list of basidiomycetous fungi that have been reported to be the causative agents of infections in humans.

METHODS

Cultivation

For the isolation of all three Wallemia species selective media with sugar (10 % glucose–12 % NaCl; MY10–12, a_w = 0.916) are used [1]. Cultures of *W. ichthyophaga* grow well on solid malt extract medium (2 % malt extract) with at least 10 % NaCl and those of *W. muriae* and *W. sebi* on malt extract, yeast extract, and 50 % glucose agar (MY50G; 2 % malt extract, 0.5 % yeast extract)[1]. *Wallemia* species can also be cultivated in liquid and on solid yeast nitrogen base (YNB) medium [1.7 g of YNB, 5 g of $(NH_4)_2SO_4$ per liter, 0.8 g of complete supplement mixture per liter, 20 g of glucose per liter, (pH 7.0)] supplemented with NaCl [24]. For the cultivation of *W. sebi* also dichloran–18 % glycerol (DG18) agar (Fig. 1. b) is used [12]. On these media, the *Wallemia* spp. can be recognised by small, walnut-brown colonies (Fig. 1. b, c, d). A biopsy sample of subcutaneous infection can be cultured on media supplemented with antibiotic such as Sabouraud dextrose agar (SDA; Difco) containing chloramphenicol (0.05 mg/mL) and SDA with chloramphenicol and cycloheximide (0.5 mg/mL) [19]. The cultivation should last for 14 days at room temperature (22-24 °C) [4]. Cultures in liquid media are incubated in the dark at 28 °C with constant shaking at 180 rpm [24].

Identification of Wallemia spp.

For the identification of the *Wallemia* spp., the following dichotomous key that is based on phenotypic characteristics has been proposed [4]:

(1a) Colonies grow well at 24 °C on MEA, and reach 3 mm to 6 mm in diameter in 14 days; conidia are short and cylindrical, 1.5 µm to 2.5 µm in diameter, with no sarcina-like structures…………………………………………………………………………*W.sebi*

(1b) Colonies only grow on MEA with additional solutes (NaCl, glucose); conidia are larger than 2.5 µm in diameter, with or without sarcina-like structures ……………………………………………………………………………………2

(2a) Colonies grown on malt extract yeast extract with 50 % glucose (MY50G) agar are dark brown, with a cerebriform surface; conidia 3.5 µm to 5.0 µm in diameter, with sarcina-like structures…………………………………..…………………*W. ichthyophaga*

(2b) Colonies grown on MY50G agar are walnut brown, with a powdery surface; conidia 2.5 µm to 3.0 µm in diameter, without sarcina-like structures……………………………………………………………………..*W. muriae*

However, for the precise identification to the level of species, the above-mentioned morphological and physiological characteristics do not suffice, and they need to be complemented by the ITS** rDNA and SSU* rDNA sequence analysis, and compared to the type strains or other reference strains: *W. sebi* (AY328915**, AY741379*), *W. muriae* (AY302534**, AY741381*), *W. ichthyophaga* (AY302523**, AY741382*) [4]. DNA is extracted from ca. 1 cm^2 of 14 days old cultures by mechanical lysis [75]. Amplification of ITS rDNA is performed by using primers V9G (TTAAGTCCCTGCCCTTTGTA) [76] and LS266 (GCATTCCCAAACAACTCGACTC) [77]. Amplification of SSU rDNA is performed by using primers NS1 (GTAGTCATATGCTTGTCT) [78] and NS24 (AAACCTTGTTACGACTTTTA) [79]. PCR is performed in a 50 µl reaction volume containing 10 to 100 ng of nuclear DNA, 50 pmol of each primer, 0.5-2 U of *Taq* DNA polymerase, 200 µM each deoxynucleoside triphosphate. PCR amplification is performed as follows: 2-5 min of initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 45-60°C, and 1-2 min at 72°C. Finally, 2-5 min at 72°C of extension is performed. The annealing temperature depends on the primer combination used [80]. PCR fragments are purified using the commercial kit and sequence reactions are analyzed on an ABI Prism

3700 (Applied Biosystems). Sequences are assembled and edited using SeqMan 3.61 (DNAStar, Inc., Madison, USA) [4].

None of the ITS rDNA sequence of any *Wallemia* strains could be compared with any fungal sequence published so far. ITS rDNA sequences of *W. sebi* and *W. muriae* strains are well alignable, while sequences of *W. ichthyophaga* are 63 – 78 bp longer with multiple gaps at various positions and consequently hardly alignable to those of *W. sebi* and *W. muriae* strains. ITS rRNA sequences of *W. ichthyophaga* appear unrelated to those of other *Wallemia* taxa, therefore it is possible that the genus *Wallemia* comprises a complex of phylogeneticaly remote genera and that taxa in between the extant *Wallemia* species have become extinct or have not yet been isolated and identified [4]

Detection of *W. sebi* by conventional and real-time PCR

For the rapid detection and quantification of *W. sebi* in environmental samples two sets of PCR primers specific to *W. sebi* were designed*:* Wall-SYB4 (5'-GTAGTGAACTATATTGAAGAA-3') and Wall-SYB6 (5'-ATGAGTCAATAATATAACGTC-3') (Wall-SYB4/6) and Wall-SYB7 (5'-GATTGGATGACGTTATATTAT-3') and Wall-SYB8 (5'-ACAACAAAATGTCGTACCG-3') (Wall-SYB7/8). Primer pair Wall-SYB4/6 cover nucleotide positions 621 to 991 in the *W. sebi* 18S rDNA sequence (GenBank accession number AF548107), and the pair Wall-SYB7/8 cover nucleotides 963 to 1290. These pairs can be applied in either conventional PCR or real-time PCR, both PCR systems proved to be highly specific and sensitive for the detection of *W. sebi* even in high background of other fungal DNAs.

Conventional PCR is performed in a 25 µl reaction volume containing 1 to 5 ng of template DNA, 10 pmol of each primer, 0.75 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, Calif.), 200 µM each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Uppsala, Sweden), and 1.5 mM $MgCl_2$. The thermal profile of the reaction is as follows: 3 min of initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C. At the end 3 min at 72°C of final extension step is performed.

Real-time PCR is performed in 25 µl of a reaction volume consisting of 1 to 5 ng of template DNA, 10 pmol of each primer and 12.5 µl of iQ SYBR Green Supermix (Bio-

Rad) with an iCycler iQ real-time PCR detection system (Bio-Rad). The thermal profile of the reaction is as follows: 3 min at 95°C and 40 cycles consisting of 10 s at 95°C and 60 s at 60°C, followed by a dissociation curve. After amplification, the melt-curve analysis is run for 60 s at 95°C, 60 s at 60°C, and a slow rise in temperature to 95°C at a rate of 0.5°C/10 s with continuous acquisition of fluorescence decline. The real-time PCR conditions for the two primer pairs are the same except that the annealing and extension times at 60°C were 60 and 30 s for Wall-SYB4/6 and Wall-SYB7/8, respectively. Each DNA sample, including the negative control, is analyzed by three replicate assays.
Assuming one fungal genome is ca. 4.0 × 10−5 ng of DNA, the conventional PCR could potentially detect 5–10 fungal spores in a reaction, while the real-time PCR system described in this study can potentially detect one spore in a PCR. Similar to the conventional PCR, the detection limit of the real-time PCR system is not affected by the presence of nontarget DNAs. In conclusion, these analytical methods facilitate the rapid detection and quantification of *W. sebi* in environmental samples, thus providing information about its distribution and ecology [12].

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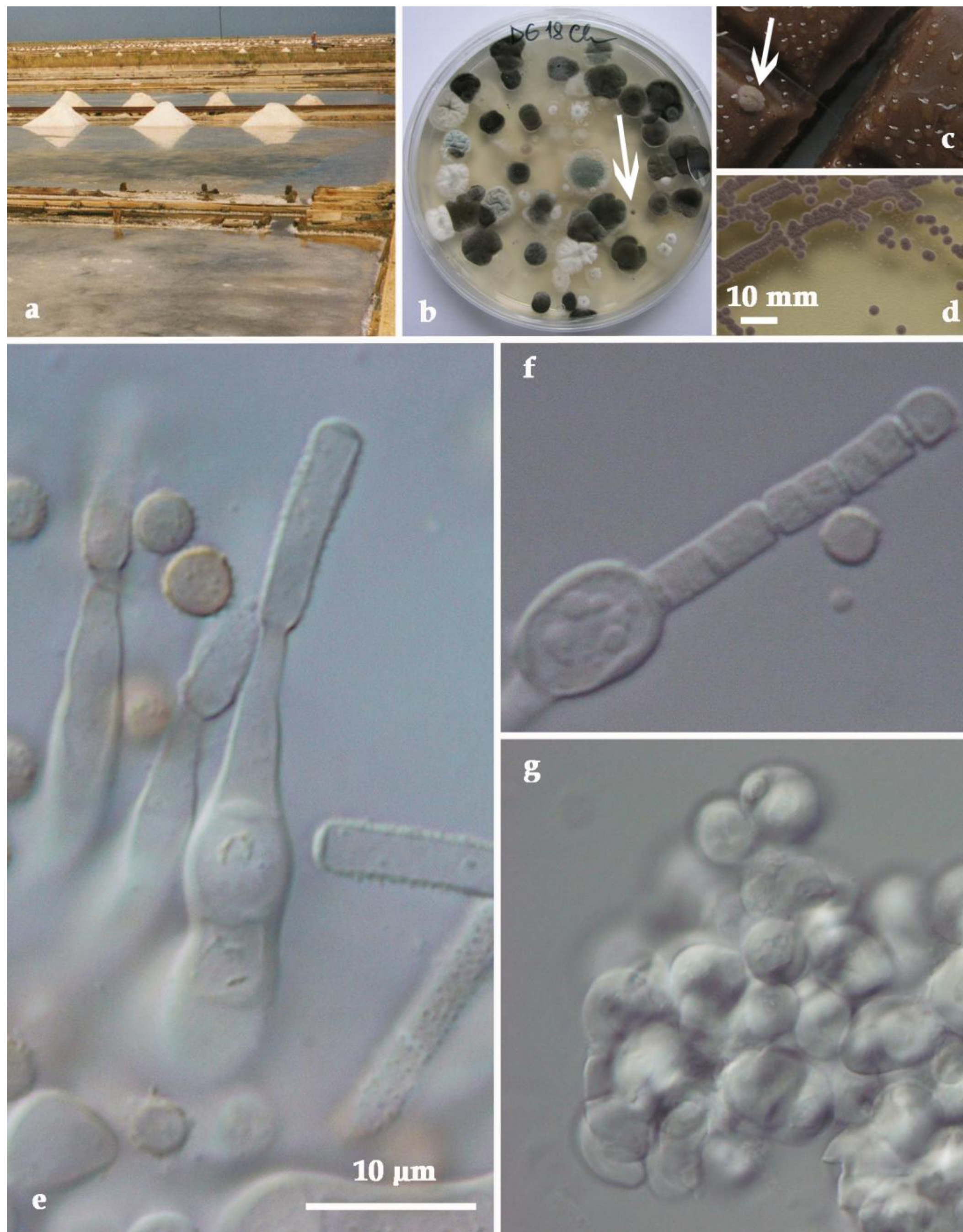

Figure 1. **(a)** Salterns as a natural ecological habitat for *Wallemia* spp.. **(b)** DG18 medium after outdoor air filtration, with small *Wallemia* colony indicated by an arrow. **(c)** *Wallemia* colony growing on a piece of chocolate (see arrow). **(d)** Culture of *W. sebi* growing on MY30G culture medium. **(e, f)** *W. ichthyophaga* conidiogenous aparatus. (g) Meristemytic clumps of *W. ichthyophaga*. Scale bar in panel (e) also applies to (f) and (g).

3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 *Wallemia ichthyophaga* ima optimalno rast pri visokih slanostih

Običajno je, da glive rastejo pri nižjih vodnih aktivnostih (a_w), če je le-ta znižana z glukozo in fruktozo ne pa s solmi, kot sta NaCl in $MgCl_2$ (Pitt in Hocking, 2009). Večina gliv ima torej splošen kserotoleranten (redkeje tudi kserofilen) fenotip, le redke vrste pa kažejo preferenco po ionskih (soli) in ne po organskih topljencih za zniževanje vodne aktivnosti. Vrste s preferenco po NaCl so *Polypaecilum pisci* in *Scopulariopsis halophilica* (Wheeler in sod., 1988) ter bazidiomicetna gliva *Wallemia ichtyhophaga* (Zalar in sod., 2005a).

Opisano je bilo, da gliva *W. ichtyhophaga* raste v slanostnem območju od 10 % do 30 % NaCl, da imajo kolonije največjo velikost pri 15% NaCl (Zalar in sod., 2005a) ter da ima večjo specifično hitrost rasti, večji prirast biomase in krajšo lag rastno fazo pri 15 % kot pri 25 % NaCl (Kralj Kunčič in sod., 2010). V naši raziskavi smo določili rastne karakteristike pri različnih slanostih tako na trdnem kot tudi v tekočem gojišču ter določili rastni optimum znotraj njenega slanostnega območja rasti.

Rast *W. ichtyhophaga* na trdnih definiranih gojiščih je izjemno počasna, končna velikost kolonij po vsaj 19-ih dneh gojenja pa relativno majhna. Z višanjem slanosti gojišča, se je povečevala tudi velikost kolonij (od 1,8 pri 10 % NaCl do 3,5 mm pri 25 % NaCl) (slika 1 A, stran 26), kar kaže na halofilen značaj *W. ichtyhophaga.* Kolonije nekaterih halotolerantnih vrst (npr. *Trimmatostroma salinum* in *T. abietis*) pri višjih slanostih zmanjšajo površino, ki je v stiku s slanim okoljem (Kogej in sod., 2006).

Počasna rast in majhne kolonije otežujejo osamitev sevov *W. ichtyhophaga* iz okoljskih vzorcev. Gojišča (agarne plošče) je treba inkubirati dlje časa (vsaj 14 dni) ter uporabiti visoke koncentracije soli (nad 15 % NaCl), saj kolonije *W. ichtyhophaga* pri nižjih slanostih hitro prerastejo mezofilne ali generalistične glive. To in pa dejstvo, da je vrsta visoko specializirana in poseljuje le nekaj omejenih okolij z ekstremnimi parametri, sta

glavna razloga za precej nizko število do sedaj opisanih izolatov *W. ichtyhophaga* (Zajc in sod., 2014).

Rastne krivulje v tekočem definiranem gojišču s soljo imajo jasno razločne štiri faze rasti (lag, log, faza upadanja in stacionarna faza), katerih dolžina se je spreminjala glede na slanost (slika 1 B, stran 26). Najdaljši fazi prilagajanja (lag) sta bili opazni pri najnižji (10 % NaCl) ter pri najvišji (25 % NaCl) slanosti. Rastni parametri tako v tekočem gojišču (trajanje lag faze, tvorba biomase in specifična hitrost rasti) kot tudi na trdnih gojiščih (oblika rastne krivulje in vrednosti radialne hitrosti rasti) so bili praktično nerazločljivi pri 15 % in 17 % NaCl in primerljivi s tistimi pri 20 % NaCl. Območje slanosti med 15 % in 20 % NaCl je torej optimalno za rast *W. ichtyhophaga*, saj je bila lag faza tu najkrajša, specifična hitrost rasti in tvorba biomase pa najvišji (Zajc in sod., 2014). Rastni optimum med 15 % in 20 % NaCl (Zajc in sod., 2014) je najvišji slanostni optimum kadarkoli opisan med glivami (Tresner in Hayes, 1971; Wheeler in sod., 1988; Gunde-Cimerman in sod., 2009; Pitt in Hocking, 2009).

Primerjava dolžine podvojitvenih časov *W. ichtyhophaga, H. werneckii* in *A. pullulans* je pokazala, da ima *W. ichtyhophaga* pri 10 % NaCl najdaljši podvojitveni čas, pri 17 in 25 % NaCl pa najkrajšega (slika 5 A, stran 29). Podvojitveni čas *W. ichtyhophaga* pri 25 % NaCl je zgolj polovica podvojitvenega časa *H. werneckii* pri enakih pogojih. Podobno je tudi pri tvorbi biomase; *W. ichtyhophaga* stvori najmanj biomase pri 10 % NaCl, pri 17 % NaCl je količina njene biomase med količinama *H. werneckii* in *A. pullulans*, pri 25 % NaCl pa je le-ta najvišja pri *W. ichtyhophaga* (slika 5 B, stran 29) (Zajc in sod., 2014). Ti rezultati kažejo na visoko uspešnost rasti glive *W. ichtyhophaga* pri izjemno visokih slanostih.

Ker *W. ichtyhophaga* raste v mnogoceličnih kompaktnih skupkih, je spremljanje rasti tekoče kulture z merjenjem optične gostote in uporabo Beer-Lamberovega zakona praktično nemogoče. Rast smo zato spremljali z določanjem proizvedene biomase pri različnih slanostih v časovnih intervalih, istočasno s tem pa smo merili tudi pH spremembe gojišča (slika 1 C, stran 26). Ugotovili smo, da se gojišče najhitreje zakisa pri tistih slanostih, kjer je tudi rast najhitrejša (primerjava slike 1 B in C, stran 26) (Zajc in sod., 2014). Poleg tega pa pH gojišča doseže najnižjo vrednost (2,5) pri optimalni slanosti,

nekoliko višji pH (3,0) je ob koncu rasti pri 25 % NaCl, še višji (4,0) pa pri 10 % NaCl, kjer je rast najslabša. Tako lahko napredovanje rasti *W. ichtyhophaga* v šaržni kulturi definiranega YNB gojišča z NaCl spremljamo ne le z določanjem suhe biomase, temveč s hitrejšim in bolj priročnim merjenjem pH gojišča (Zajc in sod., 2014). Narava zakisanja gojišča ni bila eksperimentalno raziskana. Zelo verjetno je, da do znižanja pH pride zaradi izločanja organskih kislin, ki so stranski produkt metabolizma glukoze (Lapathiatis 1998). Razlago za povezavo med zakisanjem in slanostjo gojišča je mogoče najti tudi v dejstvu, da je zakisanje lahko posledica aktivnosti membranske H^+-ATPaze, ki generira elektrokemijski gradient preko plazemske membrane. Ta predstavlja gonilno silo za sekundarne simporterje in antiporterje, ki so nujni za vzdrževanje homeostaze znotrajceličnih koncentracij Na^+ in K^+ (Zajc in sod., 2014).

Opravili smo tudi primerjalno študijo, s katero smo ugotavljali nekatere rastne parametre in tudi morfološke odzive gliv rodu *Wallemia* v gojišču, kjer je bila vodna aktivnost znižana z glukozo, v primerjavi s slanimi gojišči (Kralj Kunčič in sod., 2013). Primerjava rasti *W. ichtyhophaga* pri pogojih znižane a_w z NaCl in z glukozo je najbolj smiselna v tekočem gojišču, saj je tu rast hitrejša, razlika v rastnih parametrih med 15 % in 25 % koncentracijo NaCl pa dovolj očitna. Poleg tega so vse celice v tekoči kulturi (vsaj teoretično) izpostavljene enakih osmotskim pogojem, ne samo spodnja plast celic kolonije na trdnem gojišču, ki je v neposrednem stiku s slanim okoljem. Primerjamo lahko količine proizvedene biomase v začetni stacionarni fazi kultur *W. ichtyhophaga*, ki so rastle pri a_w 0,88 (kar ustreza 15% NaCl ali 55% glukozi v gojišču) in pri 0,80 (25% NaCl ali 70% glukoza). V obeh primerih je *W. ichtyhophaga* v gojišču z NaCl pridelala več biomase kot v gojišču z glukozo. Ko je bila a_w znižana s 15 % NaCl, je *W. ichtyhophaga* pridelala kar 3,8-krat več biomase (504 mg/100 ml) kot pa, če je s 55 % glukozo (132 mg/100 ml) (primerjava slike 1 na strani 26 in tabele 3 na strani 79). Še višjo razliko v biomasi da primerjava med 25 % NaCl in 70 % glukozo - proizvodnja biomase je bila 4,8-krat večja v slanem gojišču (514 mg/100 ml v primerjavi s 108 mg/100 ml) (Kralj Kunčič in sod., 2010; Kralj Kunčič in sod., 2013; Zajc in sod., 2014). To jasno kaže, da gliva *W. ichtyhophaga* preferenčno raste v okolju, kjer je a_w znižana z NaCl, ne pa z organskimi topljenci, na primer z glukozo, čeprav jo uporablja kot vir ogljika.

Najbolj običajna sol v izjemno slanih okoljih je NaCl, vendar veliko evaporitskih depozitov in slanic vsebuje velike količine drugih soli, tudi $MgCl_2$. Poleg tega, da soli znižajo a_w v okolju, imajo na biološke sisteme tudi vrsto drugih aktivnosti, na primer delujejo kozmotropno ali kaotropno. Kaotropne soli, kakršna je tudi $MgCl_2$, oslabijo elektrostatične interakcije in destabilizirajo biološke makromolekule s podiranjem njihove tridimenzionalne strukture, medtem ko kozmotropi (na primer NaCl) okrepijo elektrostatične interakcije in stabilizirajo makromolekule. Čeprav je magnezij ključnega pomena za biokemične procese v vseh vrstah celic, je njihova rast ustavljena že pri nizkih koncentracijah $MgCl_2$, ki le malo presegajo ozko optimalno območje. V okolju z visokimi koncentracijami $MgCl_2$ so mikroorganizmi podvrženi tako vodnemu kot tudi kaotropnemu stresu. Ker je topnost $MgCl_2$ v vodi zelo visoka, lahko zniža a_w do izjemno nizkih vrednosti (do 0,4 pri 5 M $MgCl_2$) (Hallsworth in sod., 2007). Hallsworth in sodelavci so že leta 2007 objavili, da je teoretična zgornja meja aktivnega življenja pri 2,3 M $MgCl_2$ in najavili iskanje predstavnikov nove skupine ekstremofilnih mikrobov, ki uspevajo v kaotropnih razmerah, t.i kaofilov (Hallsworth in sod., 2007; Williams in Hallsworth, 2009). Kljub napovedim, tovrstnih mikrobnih predstavnikov do zdaj še niso opisali.

Halofilne glive, med njimi *W. ichtyhophaga*, predstavljajo potencialne kandidate za rast pri visokih koncentracijah $MgCl_2$. Pri preliminarni izolaciji gliv iz grenčice (z $MgCl_2$ bogat ostanek slanice po kristalizaciji NaCl v solarnih solinah), iz katere so bile izolirane le redke bakterije (Baati in sod., 2011), je bilo izoliranih nekaj sevov *W. ichtyhophaga* (Sonjak, Jančič in Gunde-Cimerman, neobjavljeni podatki). To nas je vodilo do presejalne raziskave tolerance izbranih gliv iz ekstremnih okolij na različne vrste soli, ki so pokazale, da je vrsta *W. ichthyophaga* med njimi absolutna rekorderka, saj je sposobna rasti pri najvišjih koncentracijah $MgCl_2$ (2.0 M $MgCl_2$), ki so blizu zgornje preživetvene meje. Poleg tega pa v gojiščih z enako vodno aktivnostjo znižano z 10 % in 21 % (1,7 M in 3,6 M) NaCl ali z 9,5 % in 19 % (1 M in 2 M) $MgCl_2$ *W. ichthyophaga* raste primerljivo hitro in stvori primerljivo količino biomase (Zajc in Gunde-Cimerman, neobjavljeni podatki). Poleg tega, da je *W. ichthyophaga* edina obligatno halofilna gliva, iz preliminarnih rezultatov tolerance na magnezijeve soli sklepamo, da je tudi eden izmed najbolj kaofilnih mikroorganizmov.

3.1.2 Genom *Wallemia ichthyophaga*

Z namenom raziskovanja genomske osnove tega izjemnega fenotipa, smo *de-novo* pridobili genomsko zaporedje tipskega seva vrste *W. ichtyhophaga* EXF-994 in analizirali transkriptomska odziva na slanosti, ki predstavljata njeno spodnjo (10 % NaCl) in zgornjo (30 % NaCl) mejo aktivnega življenja. Projekt sekvenciranja genomskega zaporedja ter obeh transkriptomov vrste *W. ichthyophaga* je potekal v sodelovanju s Pekinškim genomskim inštitutom BGI (Shenzen, Kitajska). Uporabljena tehnologija sekvenciranja s sintezo (Illumina), je ena izmed najuspešnejših platform sekvenciranja naslednje generacije. Analizirali smo osnovne lastnosti genoma (velikost, število modelov genov, odstotek GC parov, odstotek ponavljajočih se zaporedij) ter ga primerjali z njeno najbližjo sorodnico z znanim genomskim zaporedjem *W. sebi* (Padamsee in sod., 2012). Preučili smo, katere družine proteinov so statistično značilno obogatene in/ali osiromašene glede na zadnjega skupnega prednika vrst *W. ichthyophaga* in *W. sebi* po metodi CAFE (De Bie in sod., 2006) in izbrani dve družini – družino ATPaz tipa P in družino proteinov celične stene (t.i. hidrofobinov), podrobneje bioinformatsko pregledali. Poleg tega smo naredili tudi filogenomsko analizo rodu *Wallemia* ter poiskali gene, ki so vključeni v paritev (angl. *mating*). V luči halofilnega značaja *W. ichthyophaga* smo pregledali tudi gene, ki nosijo zapis za homologe kationskih transporterjev, ki so glede na podatke iz literature izjemno pomembni za preživetje v slanem okolju. Genomski podatki so deponirani v javno dostopni bazi DDBJ/EMBL/GenBank pod identifikacijsko številko GenBank: APLC00000000 in na portalu inštituta JGI - Mycosm (Grigoriev in sod., 2011; Grigoriev in sod., 2012).

3.1.2.1 Genom *Wallemia ichthyophaga* je izredno majhen in kompakten

Genomsko zaporedje *W. ichthyophaga* je dolgo le 9,6 mega baznih parov (Mb) in je sestavljeno v 101 kontigu (soseskah) oziroma v 82 superkontigih (ogrodjih) (tabela 1, stran 51; slika 2, stran 52). Takšna velikost genoma je med najmanjšimi med vsemi znanimi haploidnimi bazidiomicetnimi glivami, ki imajo od dvakrat pa tudi do nekaj desetkrat večji genom (Gregory in sod., 2007). Celo genom sorodne *W. sebi* je nekoliko večji (9.82 Mb) (Padamsee in sod., 2012). Število predvidenih proteinov je nizko tako za *W. ichthyophaga*

(4884) kot tudi za *W. sebi* (5284), saj je med bazidiomicetnimi glivami običajnih preko 10 000 predvidenih proteinov. Za boljšo predstavo zgolj omenjam dejstvo, da je število proteinov glive *W. ichthyophaga* primerljivo s številom proteinov bakterije *Escherichia coli* (Lukjancenko in sod., 2013). V skladu z majhnostjo genoma je tudi njegova izjemna kompaktnost; vsebuje le 1,67 % ponavljajočih se sekvenc, skoraj 75 % genoma pokrivajo kodirajoča zaporedja, gostota genov pa je izjemno visoka (pri *W. ichthyophaga* 514 genov/Mb, pri *W. sebi* pa celo 538 genov/ Mb). V povprečju je predvideni gen vseboval 2,42 61 bp dolgih intronov, kar je skladno s podatki iz literature (od 1,0 introna/kb kodirajoče sekvence pri *Schizosaccharomyces pombe* do 5.0 intronov/ kb cds pri *Cryptococcus neoformans*) (McGuire in sod., 2008).

3.1.2.2 Primerjava genomskega zaporedja *Wallemia ichthyophaga* in *W. sebi*

Poravnava celotnega zaporedja genoma *W. ichthyophaga* in *W. sebi* je pokazala razmeroma dolge regije skupne sintenije (slika 3 A, stran 53). Glede na analizo BLASTp (mejna E-vrednost 10^{-6}) je kar 93,9 % proteoma *W. ichthyophaga* homolognega *W. sebi* (slika 3 B, stran 53). To seveda ni presenetljivo, glede na njuno filogenetsko bližino (Zalar in sod., 2005a). Vrsti sta bili do leta 2005 obravnavani zgolj kot *W. sebi*, ko se je rod razdelil na tri do sedaj opisane vrste. Med proteini, ki so v *W. ichthyophaga* edinstveni v primerjavi z *W. sebi*, je največ tistih, ki so vključeni v popravilo in procesiranje DNA, pa tudi encima vključena v nehomologno rekombinacijo. Glede na to, da je *W. ichthyophaga* izgubila sposobnost spolnega razmnoževanja (več v poglavju 3.1.1.4), bi lahko ta nehomologna rekombinacija predstavlja način genske rekombinacije (Zajc in sod., 2013).

3.1.2.3 Filogenomska analiza rodu *Wallemia*

Rod Wallemia je bil skozi raziskave umeščen na različna mesta v glivnem filogenetskem drevesu. Sprva je bil postavljen na bazo bazidiomicetnih gliv (Zalar in sod., 2005a), nato kot sestrska skupina z Agaricomycotina in Ustilaginomycotina (Matheny in sod., 2006) ali je bil celo neumeščen - *incertae sedis* (Hibbett, 2006). Z objavo genomskega zaporedja *W. sebi* so s filogenetsko analizo 71-proteinov in s pregledom ultrastrukture septalnih por umestili rod Wallemia kot najzgodnješo divergentno linijo Agaricomycotina (Padamsee in sod., 2012). Glede na razpoložljivost celotnih genomov, smo v naši študiji naredili

filogenomsko analizo, ki temelji na največjem možnem naboru podatkov, kar na 14 celotnih proteomih obravnavanih gliv. Pokazali smo, da je razred Wallemiomycetes sestrska skupina Agaricomycotina (Zajc in sod., 2013), kar je v skladu s filogenetsko umestitvijo *W. sebi* (Padamsee in sod., 2012), poleg tega pa naša filogenomska analiza podpira tudi Pucciniomycotina kot najzgodnejšo odcepljeno linijo v Basidiomycota (slika 1, stran 51). Če upoštevamo predhodno objavljene kalibracijske točke razvejitev (npr. razcep *Rhizopus oryzae*–Dykaria pred 495 milijoni let, razcep Ascomycota–Basidiomycota pred 452 milijoni let, itn.) (Taylor in Berbee, 2006), lahko grobo ocenimo, da se je razcepitev med *W. ichthyophaga* in *W. sebi* zgodila pred 11,9 milijoni let, med Wallemiomycetes in Agaricomycotina pa pred 250 milijoni let (Zajc in sod., 2013).

3.1.2.4 Geni mejoze in paritve pri *Wallemia ichthyophaga*

Spolno razmnoževanje gliv rodu Wallemia je nerazjasnjeno, saj teleomorfnih oblik ali plodilnih telesc še ni bilo opisanih ali opaženih. Dostopnost genomskega zaporedja zato ponuja odlično priložnost za ugotavljanje reproduktivnih sposobnosti z iskanjem homologov genov za paritev (angl. mating) in mejozo (Malik in sod., 2008; Schurko in sod., 2009). Pri *W. sebi* so našli en genski lokus paritvenega tipa *MAT* in skoraj popoln set mejotskih genov. To nakazuje na sposobnost spolnega razmnoževanja *W. sebi*, čeprav le-ta ostaja kriptična (Padamsee in sod., 2012). Iskanje paritvenih genov pri *W. ichthyophaga* je odkrilo prisotnost gena z zapisom za transkripcijski faktor z domeno HD (t.i. homeodomena), ki je povezana s paritvijo bazidiomicetni gliv. Gen je podoben paritvenemu Sxi1D alfa *Cryptococcus neoformans var. neoformans*, ki sodeluje pri začetni tvorbi dikariona. Ostalih genov povezanih s paritvenim tipom (receptorjev feromonov ali prekurzorjev feromonov) nismo našli. Pri *W. ichthyophaga* ni očitnega lokusa paritvenega tipa *MAT* (Zajc in sod., 2013). Po prisotnosti mejotskih genov lahko sklepamo o sposobnosti mejoze in zato tudi o spolnem razmnoževanju (Malik in sod., 2008). Po enakih kriterijih iskanja (BLASTp, mejna vrednost E $<10^{-40}$) kot so to predhodno storili za *W. sebi* (Padamsee in sod., 2012), smo v genomu *W. ichthyophaga* našli le tri izmed osmih genov specifičnih za mejozo (Zajc in sod., 2013), medtem ko vrsti *W. sebi* manjka le en homolog iz tega repertoarja (Padamsee in sod., 2012).

Vrsta *W. ichthyophaga*, za razliko od bližnje sorodnice *W. sebi*, nima očitnega lokusa paritvenega tipa *MAT* ter ima le majhno število genov specifičnih za mejozo, zato najverjetneje ni sposobna spolnega razmnoževanja. V ekstremnem okolju so nekatere pomembne prednosti nespolnega razmnoževanja: varčevanje z energijo in ohranjanje okolju prilagojene genomske konfiguracije oziroma preprečevanje redčenja uspešnega genotipa (Sun in Heitman, 2011). Slabost nespolnega razmnoževanja pa je nesposobnost hitrega prilagajanja na spreminjajoče se biotske in abiotske pogoje v okolju (Van Doninck in sod., 2003). Zato so nespolni mikroorganizmi, kakšrna je tudi *W. ichthyophaga*, omejeni na stalna (ekstremna) okolja.

3.1.2.5 Razširjene in skrčene družine proteinov v proteomu *Wallemia ichthyophaga*

Proteini imajo običajno eno ali več funkcionalnih regij oziroma domen, ki se pojavljajo v različnih kombinacijah. Identifikacija teh domen v proteinih omogoča vpogled v njihovo biološko funkcijo. Več kot 80 % (3924) predvidenih proteinov *W. ichthyophaga* vsebuje vsaj eno izmed vseh domen Pfam (Zajc in sod., 2013) glede na podatkovno bazo Pfam, ki vključuje 14831 proteinskih družin (Punta in sod., 2012). Nabor vseh domen Pfam *W. ichthyophaga* in ostalih gliv iz filogenetskega drevesa skupaj s kronogramom (Zajc in sod., 2013), smo uporabili za vhodne podatke za statistično analizo evolucije velikosti genskih družin (CAFE) (De Bie in sod., 2006). Med družinami z največjim številom proteinov so številne v povezavi s transportnimi funkcijami celice, na primer družina MFS (major facilitator family), mitohodrijski prenašalci, transporterji ABC in drugi transporterji (priloga B). V predvidenem skupnem predniku *W. ichthyophaga* in *W. sebi* je 17 družin proteinov signifikantno spremenjenih, v vrsti *W. ichthyophaga* pa 26; od tega je le 7 družin signifikantno obogatenih, ostalih 19 pa osiromašenih (priloga C). Med obogatenimi družinami sta dve, za kateri smatramo, da imata pomembno vlogo v haloadaptaciji glive *W. ichthyophaga* na izjemno slane pogoje; družina hidrofobinov (PF01185; slika 4 A, stran 55) in družina ATPaz tipa P (PF00690; slika 4 B, stran 55) (Zajc in sod., 2013). Obe proteinski družini bosta v nadaljevanju diskusije podrobneje obravnavani.

3.1.3 Primerjava dveh trankriptomov *Wallemia ichthyophaga*

Pripravili smo cDNA knjižnico *W. ichthyophaga*, jo presejali v *S. cerevisiae* in iskali transformante z izboljšano toleranco na sol. Zaporedja vstavljenih genov smo določili s sekvenciranjem. Kandidatne gene, ki bi izboljšali toleranco na stres, smo nameravali klonirati, izraziti v kvasovki *S. cerevisiae* in jih tudi biokemijsko okarakterizirati. Poskuse smo izvajali v mnogih ponovitvah, vendar nismo dobili zadovoljivih rezultatov. V transformantah smo v največjem številu primerov določili zgolj dele genov ribosomalnih in hipotetičnih proteinov, ne pa tudi genov, ki bi kodirali zapis za specifične membranske črpalke, kanalčke ali za katere koli (metabolne) encime, katere bi lahko dalje preučevali. Prav tako s to visoko-zmogljivo metodo pregledovanja biotehnološko zanimivih genov v knjižnicah cDNA, ki se je izkazala za zelo uporabno pri nekaterih drugih ekstremofilnih vrstah (Gostinčar in sod., 2012), nismo našli genov, ki bi nosili zapis za proteine vključene bodisi v sintezo bodisi v strukturo celične stene. Zato smo sekvenirali in primerjali transkriptoma celic *W. ichthyophaga,* ki so rastle pri najnižji (10 %) in najvišji (30 %) koncentraciji NaCl, kjer vrsta še aktivno raste (Zajc in sod., 2013).

Spreminjajoča se slanost izzove številne, predvsem prehodne, spremembe v transkriptomu kvasovke *S. cerevisiae*. Takšna nihanja v izražanju genov, ki se kmalu vrnejo na bazalni nivo, niso vključena v dolgoročno preživetje pri visokih slanostih (Gasch in sod., 2000). Namen naše preiskave transkriptoma *W. ichthyophaga* ni bilo ugotavljanje prehodnih transkripcijskih motenj, temveč transkriptomskih razlik celic, ki so rastle pri stalnih pogojih, in sicer pri spodnji (10% NaCl) in zgornji (30 % NaCl) rastni meji.

Od 13,1 % (639) diferencialno izraženih genov, sta bili dve tretjini (425) bolj izraženi pri nižji slanosti kot pri višji (214) (slika 6 A, stran 60). Ena izmed možnih razlag za večje število diferenčno izraženih genov pri nižji slanosti, je lahko moteno delovanje celice. Meritev fluidnosti membrane namreč kažejo, da se pri 10% NaCl le-ta hiperfluidizira, kar kaže na moteno delovanje celičnih procesov (Turk in Gunde-Cimerman, neobjavljeni podatki). Dvanajst poti KEGG je imelo signifikantno višji delež diferenčno izraženih genov (priloga D). Pri obeh slanostih smo predvideli okrog 200 novih transkriptov (slika 6 B, stran 60). Za 15 % (730) genov smo določili štiri načine alternativnega spajanja (AS)

eksonov (zadrževanje intronov, 3' in 5' spajanje ter preskakovanje eksonov). Število genov s posameznim načinom AS je bilo primerljivo pri obeh slanostih in skladno s primeri opisanimi v literaturi (McGuire in sod., 2008). Zanimivo pa je to, da je razmerje med številom dogodkov AS in številom genov *W. ichthyophaga* (Zajc in sod., 2013) blizu najvišjih vrednosti opisanih doslej (McGuire in sod., 2008).

Kriteriju visokega nivoja izražanja (vrednost RPKM >300 pri vsaj eni slanosti) kot tudi visoke razlike v izražanju med obema slanostima (absolutno razmerje $\log_2 > 2$) je ustrezalo 34 genov. Nekatere ugotovitve primerjav transkriptomov so obravnanev nadaljevanju diskusije pri posameznih sklopih raziskav.

3.1.4 *Wallemia ichthyophaga* kopiči glicerol in arabitol

V celičnih ekstraktih smo določili mešanico treh poliolov; največje količine glicerola, manj arabitola, manitol pa le v sledeh. Gene, ki so vpleteni v sintezo teh poliolov smo našli tako v genomu *W. ichtyhophaga* kot tudi pri *W. sebi* (Zajc in sod., 2013).

Glavni kompatibilni topljenec v celicah *W. ichtyhophaga* je glicerol, saj so bile njegove količine najvišje izmed vseh treh poliolov, poleg tega pa se je njegova vsebnost v celicah statistično značilno večala z naraščajočo slanostjo in se zmanjšala s hipoosmotskim šokom (Zajc in sod., 2014). Podobno je bilo ugotovljeno za *H. werneckii,* ki poleg glicerola (Petrovič in sod., 2002) kopiči še eritritol, arabitol in manitol (Kogej in sod., 2007). Količina arabitola pri *W. ichtyhophaga* ni korelirala ne s slanostjo ne z rastno fazo. Njegova najvišja količina je bila izmerjena v logaritemski fazi, in sicer pri obeh mejhnih slanostih (pri 10 % in 25 % NaCl). Količina arabitola se je sicer zmanjšala po hipoosmotskem šoku, vendar neodvisno od jakosti šoka. Pri *D. hansenii* so za glavna tipa poliolov določili glicerol in arabitol, a le produkcija glicerola je bila inducirana s slanostjo (Jovall in sod., 1990). Tudi pri *A. pullulans* so ugotovili, da slanostni stres sproži kopičenje glicerola in manitola (Managbanag in Torzilli, 2002), vendar je glicerol najbolj zastopan topljenec v odzivu na slanostni stres (Kogej in Gunde-Cimerman, neobjavljeni podatki). Sestava koktajla poliolov v celicah *W. ichtyhophaga* je bila enaka tako v logaritemski kot tudi v stacionarni fazi, kjer se je znižala le njegova količina. V stacionarni fazi korelacija

med količino poliolov in slanostjo ni bila tako očitna kot v logaritemski fazi, kar je verjetno posledica porabe substrata in/ali umiranja celic. Razlog znižanja vsebnosti poliolov ni kompenzacijsko kopičenje kationov, saj so naše preliminarne raziskave pokazale nizko vsebnost kationov v stacionarni fazi rasti (neobjavljeni podatki). Izgleda, da kot večina halofilnih bakterij in halotolerantnih evkariontov, tudi gliva *W. ichtyhophaga* uporablja strategijo kopičenja in sinteze kompatibilnih topljencev (poliolov) (Zajc in sod., 2014).

Kopičenje glicerola je pri glivah pogosto posledica tako njegove biosinteze s povišano encimsko aktivnostjo Gpd1 kot tudi aktivnega privzema. V genomskem zaporedju *W. ichtyhophaga* smo našli štiri možne homologe simporterjev glicerola in H^+ (homologi Stl1) (Zajc in sod., 2013), ki pri *S. cerevisiae* dokazano privzemajo glicerol proti koncentracijskemu gradientu z uporabo protonske gibalne sile (Ferreira in sod., 2005). Tudi v genomu *H. werneckii* so prisotni Stl1 homologi (vsaj trije, najverjetneje več) (Gostinčar, neobjavljeni podatki). Glede na prisotnost Stl1 homologov v genomu *W. ichtyhophaga,* je povsem mogoče, da privzem glicerola izvaja tudi *W. ichtyhophaga.* Kadar je *W. ichtyhophaga* izpostavljena razredčenju slanega vodnega okolja (hipoosmotskem šoku) se vsebnost glicerola dokazano zmanjša. Pri kvasovki imajo pri tem pomembno vlogo akvagliceroporinski kanalčki (Fps1), skozi katere poteka olajšana difuzija glicerola (Luyten in sod., 1995). Tudi pri *W. ichtyhophaga* in *W. sebi* zmanjšanje vsebnosti glicerola verjetno poteka na tak način, saj smo v obeh genomih identificirali po tri možne homologe akvagliceroporinskih kanalčkov (Zajc in sod., 2013).

3.1.5 Adaptacija encima Gpd1 na visoko slanost pri *Wallemia ichthyophaga* je drugačna kot pri *Hortaea werneckii*

V sintezo glicerola sta vključena dva encima; najprej od (NAD)- odvisna glicerol-3-fosfat dehidrogenaza (Gpd1) pretvori dihidroksiaceton fosfat v glicerol-3-fosfat, od katerega glicerol-3-fosfataza (Gpp) odcepi fosfat in nastane glicerol (Albertyn in sod., 1994; Norbeck in sod., 1996). Kljub temu, da ima glicerol številne vloge v celičnem metabolizmu, je pri kvasovki *S. cerevisiae* najbolje preučena njegova vloga v osmoadaptaciji. Znotrajcelično kopičenje kot odgovor na osmotski stres, ki ga vodi pot

HOG, je posledica *de novo* izražanja gena *GPD1*, posledičnega povišanja encimske aktivnosti ter sinteze glicerola in/ali pa je posledica privzema glicerola preko membranskih kanalčkov (Hohmann, 2002). Glicerol je glavni kompatibilni topljenec pri glivah, tako pri *H. werneckii* kot tudi pri *W. ichtyhophaga.* V primerjalni študiji smo kot prvi identificirali homologe genov *GPD1*, ki nosijo zapis za ključen encim za sintezo glicerola (Gpd1) pri izjemno halotolerantni *H. werneckii* in halofilni *W. ichtyhophaga*. Preučili smo njihovo od soli odvisno izražanje in funkcijo v *gpd1* in *gpd1/gpd2* mutiranih sevih *S. cerevisiae*.

Homologa *HwGPD1A* in *HwGPD1B* imata kar 98 % identičnih aminokislin ter podoben vzorec izražanja. Njuno izražanje se je petkrat povišalo pri 1,0 M NaCl za *HwGPD1B* in pri 1.8 M NaCl za *HwGPD1A*, pri visoki slanosti (4,5 M NaCl) pa je bil nivo izražanja obeh genov več kot 10-krat višji kot v gojišču brez soli (slika 3 A, stran 39). Tudi izražanje *WiGPD1* je naraščalo z višanjem slanosti gojišča. Pri 4,5 M NaCl je bilo izražanje *WiGPD1* kar 3,7-krat višje od izražanja pri 1,8 M NaCl (slika 3 B, stran 39) (Lenassi in sod., 2013). Tudi pri *S. cerevisiae* je nivo mRNA *GPD1* naraščal postopno z višanjem koncentracije soli (Ansell in sod., 1997). Pri hiperslanostnem stresu (prenos celic iz 1,8 M na 4,5 M NaCl) je bil odziv *W. ichthyophaga* na nivoju izražanja gena *GPD1* nižji in počasnejši kot pri *H. werneckii*. Izražanje je 3-krat narastlo šele po petih urah po izpostavitvi šoku, medtem ko je bila pri *H. werneckii* lag faza odziva 40 oziroma 60 minut, indukcija pa tudi do 7-kratna (slika 3, stran 39) (Lenassi in sod., 2013). Podoben odziv kot pri *H. werneckii* je bil opisan tudi pri *S. cerevisiae* (Rep in sod., 1999). Izpostavitev hipoosmotskemu stresu (s prenosom celic iz 4,5 M na 1,8 M NaCl pri *W. ichthyophaga* in iz 3,0 na 0 M NaCl pri *H. werneckii*) je povzročila podoben vzorec izražanja *GPD1* homologov; takojšnje prehodno povišanje izražanja, ki se je po določenem času (90 min za *W. ichthyophaga* in 60 min za *H. werneckii*) uravnalo na nivo izražanja pred šokom.

Homologi Gpd1 *W. ichthyophaga* in *H. werneckii* imajo dobro ohranjeno aminokislinsko zaporedje NAD(P)H/NAD(P)(+) Rossmann vezavne domene na N-koncu in katalitične domene (NAD_Gly3P_dh_C) na C- koncu. V Rossmann-ovi domeni je skupno zaporedje glicin–serin–glicin–asparagin–triptofan–glicin (GSGNWG) popolnoma ohranjeno v vseh obravnavanih homologih, medtem ko imata le ortologa HwGpd1 v tej domeni dodatno 17 aminokislin dolgo regijo (slika 2, stran 38). Presenetljivo je to, da nobeden od Gpd1

homologov ni imel tipa 2 zaporedja za usmerjanje v peroksisom (angl.: **p**eroxisomal **t**argeting **s**equence type **2**; PTS2) na N-koncu proteinskega zaporedja (slika 2, stran 38). Fiziološka vloga Gpd1 v peroksisomu je verjetno povezano z regeneracijo NAD^+ iz NADH proizvedenega pri β-oksidaciji maščobnih kislin (Valadi in sod., 2004; Jung in sod., 2010), medtem ko je njegova funkcija v osmotskem stresu odvisna od citosolne in jedrne frakcije Gpd1 (Jung in sod., 2010). Zato sklepamo, da je stalna citosolna lokalizacija Gpd1 homologov očitno adaptacija organizmov, ki živijo v izjemno slanih okoljih (Lenassi in sod., 2011).

Funkcijo homologov *WiGPD1* ter *HwGPD1A/B* smo določili s funkcionalno komplementacijo *gpd* mutant *S. cerevisiae* in meritvami glicerola. Glede na visoko ohranjenost aminokislinskega zaporedja je presenetljivo, da sta funkcijo Gpd1 v *gpd1* mutantah *S. cerevisiae* komplementirala le *WiGPD1* in *HwGPD1B*, osmotoleranco dvojne mutante *gpd1gpd2* pa celo samo *WiGPD1* (slika 4, stran 41). Razlika v funkciji homologov Gpd1 je verjetno povezana z razliko v dodatnem 17-aminokislin dolgem segmentu paralogov *H. werneckii*, čeprav vloga tega segmenta ni poznana. Iz naših rezultatov lahko sklepamo le, da je za povrnitev tolerance kvasovke na sol s HwGpd1B nujen funkcionalen Gpd2, ki verjetno dimerizira z HwGpd1B in zakrije njegove morebitne pomanjkljivosti, tako da lahko uspešno deluje v *S. cerevisae*. Podoben mehanizem povrnitve funkcije mutiranega Gpd1 z dimerizacijo z nativnim homologom pri *S. cerevisiae* je pokazal že Jung s sodelavci (2010).

Število kopij genov smo določili s hibridizacijo označene sonde za gen *GPD1* z razrezano genomsko DNA, ki smo jo na membrano odtisnili po Southernu, saj genomsko zaporedje tedaj še ni bilo znano. Ugotovili smo, da ima *H. werneckii* dve kopiji *GPD1* gena (*HwGPD1A* in *B*), *W. ichthyophaga* pa le eno (*WiGPD1*) (slika 1, stran 37) (Lenassi in sod., 2011). Z novejšo analizo genoma, je bilo ugotovljeno, da je večina genov v genomu *H. werneckii* podvojenih, med njimi tudi tudi *GPD1* (Lenassi in sod., 2013). Tudi v genomu *W. ichthyophaga*, ki sicer ni dupliciran, smo s pregledovanjem genov našli še en paralog *GPD1* gena (v nadaljevanju *WiGPD1B*) (Zajc in sod., 2013). Glede na to, da smo s supresijsko subtrakcijsko hibridizacijo med populacijama cDNA nižje (1,8 M NaCl) in višje (4,5 M NaCl) slanosti zaznali le en gen *GPD1*, se očitno druga kopija tega gena ni

izražala diferencialno in verjetno nima posebne vloge v osmoadaptaciji. Poleg tega sta si gena le v 58 % nukleotidov identična, proteina pa le v 22,2 %. Pomembno je tudi to, da protein *WiGPD1B* nima ohranjenega motiva GSGNWG v Rossman-ovi domeni, čeprav je le-ta izjemno ohranjen pri homologih Gpd1 od kvasovke do človeka. To verjetno kaže na nefunkcionalnost paraloga *WiGPD1B*.

Raziskava je pokazala, da imata glivi, ki sta bili osamljeni iz enakega okolja (Sečoveljske soline), drugačne prilagoditve in odzive na stalno slanost in na nihanja slanosti na nivoju gena *GPD1*, ki pa so v skladu z njuno ekofiziologijo. Izjemno halotolerantna *H. werneckii* kaže hitrejše odzive in večje spremembe v izražanju *GPD1*, medtem ko je halofilna *W. ichthyophaga* v tem odzivu bolj toga- indukcija nivoja mRNA gena *GPD1* je manjša in počasnejša (Lenassi in sod., 2011). Odzivi na nivoju izražanja *GPD1* so v skladu z meritvami vsebnosti glicerola, vsebnost katerega narašča do 4.5 M slanosti pri celicah adaptiranih na stalno slanost ter pade po hipoosmotskem šoku (Zajc in sod., 2014).

3.1.6 *Wallemia ichthyophaga* vzdržuje nizko vsebnost kationov znotraj celice

Obligatna potreba po soli je običajna lastnost ekstremofilnih prokariontov, med evkarionti pa je izjemno redka. Glede na to, da je *W. ichtyhophaga* edina gliva, ki po svojih halofilnih lastnostih spominja na arheje; ima obligatno zahtevo po soli v gojišču in optimalno raste pri izjemno visokih slanostih; nismo izključili možnosti kopičenja visoke koncentracije kationov znotraj celice, čeprav med evkarionti te strategije še niso opisali.

Z metodo atomske absorpcijske spektroskopije smo izmerili vsebnost kationov v celicah halofilne *W. ichthyophaga,* podobno kot je bilo že predhodno opravljeno na izjemno halotolerantni *H. werneckii,* halotolerantni (oziroma poliekstremotolerantni) *A. pullulans* in halotolerantni *D. hansenii.* Tako *H. werneckii* kot *A. pullulans* tudi pri višjih koncentracijah soli v okolju vzdržujeta nizke znotrajcelične koncentracije kationov (Kogej in sod., 2005). Nasprotno pa so v celicah morske kvasovke *D. hansenii* izmerili visoko vsebnost kationov (Larsson in sod., 1990; Kogej in sod., 2005). Slednja velja za edino glivo, ki se poslužuje kombinirane strategije - tako vnosa kationov kot tudi sinteze kompatibilnih topljencev v odgovor na osmotski stres (Prista in sod., 1997).

Celice *W. ichthyophaga* vzdržujejo zelo nizko vsebnost kationov v primerjavi z ostalimi tremi glivnimi vrstami, na katerih je bil opravljen enak eksperiment. Vendar je primerjava vsebnosti kationov pri glivah, ki imajo popolnoma drugačno pojavno obliko (kvasne celice v primerjavi z veliki meristematski skupki) zavajajoča in je zato potrebna skrajna previdnost pri razlagi tega rezultata. Primerjali bi lahko znotrajcelično koncentracijo kationov, za kar bi morali poznati prostornine celic pri vsaki koncentraciji soli. Pri *W. ichthyophaga* bi se takšen preračun dodatno zapletel, saj rastejo v večjih ali manjših skupkih tesno povezanih celic. To pomeni, da ne moremo enostavno ugotoviti števila celic, ki ustreza določeni masi, niti ne vemo ali/in koliko celic je dejansko živih v tem skupku in ali so vse celice vključene v osmoregulacijo. Zelo verjetno je, da so direktno v stiku s slanim okoljem le tiste celice, ki predstavljajo zunanjo plast skupka. To pomeni, da je možno, da podcenjujemo vsebnost osmolitov ali kationov, ki so vključeni v osmoregulacijo, zato se v nadaljevanju diskusije osredotočamo le na primerjavo znotrajceličnega razmerja K^+/Na^+ pri vseh štirih vrstah gliv.

Z višanjem slanosti pada razmerje med znotrajceličnim razmerjem K^+/Na^+ tako zaradi zmanjševanja vsebnosti K^+ kot tudi zaradi povečevanja vsebnosti Na^+ pri vseh do sedaj obravnavanih halotolerantnih in halofilnih glivah (*H. werneckii*, *A. pullulans in D. hansenii* (Kogej in sod., 2005), ter pri *W. ichthyophaga* (Kogej in sod., 2005; Zajc in sod., 2014). Pri *H. werneckii* in *A. pullulans* je padec zelo strm, medtem ko je pri *D. hansenii* nekoliko manj. Pri halofilni *W. ichthyophaga* so nihanja vsebnosti kationov razmerova majhna v primerjavi z ostalimi glivami (slika 5 C, stran 29). Poleg tega je potrebno poudariti, da *W. ichthyophaga* vzdržuje K^+/Na^+ višje od ostalih gliv pri vseh merjenih slanostih. Najnižje razmerje K^+/Na^+ so imele celice, ki so rastle pri 20 % NaCl, kjer je bila tudi specifična hitrost rasti blizu najvišje vrednosti in proizvodnja biomase najvišja. Po drugi strani pa je bilo najvišje razmerje med kationoma določeno pri celicah *W. ichthyophaga* gojenih na 10 % NaCl, kjer je bila rast najpočasnejša, proizvodnja biomase pa najnižja (Zajc in sod., 2014). To je v nasprotju s podatki iz literature za *S. cerevisiae*, pa tudi za halotolerantni *H. werneckii* in *A. pullulans* (Kogej in sod., 2005) in kaže na dejstvo, da nizko razmerje K^+/Na^+ ni toksično za rast *W. ichthyophaga* (Zajc in sod., 2014), podobno kot ne za *D. hansenii* (Prista in sod., 1997; Prista in sod., 2005). Pri pregledu več kot 40 bazidiomicetnih kvasovk so ugotovili, da imajo celice gojene pri 7,2 % NaCl nižje

razmerje med K^+/Na^+ predvsem na račun povišanja vsebnosti Na^+, nikakor pa rast kvasovk ni bila stimulirana s soljo (Tekolo in sod., 2010).

Ko so bile pri 15 % NaCl rastoče celice *W. ichthyophaga* nenadoma izpostavljene 25 % NaCl, se je vsebnost obeh kationov močno povečala in izmerjene vrednosti so daleč presegale tiste, ki so bile izmerjene v celicah, ki so rastle pri stalnih 25 % NaCl. Razmerje med K^+/Na^+ ob koncu hiperosmotskega šoka pa je bilo skoraj enako kot pri celicah, ki niso bile osmotsko šokirane (Zajc in sod., 2014).

3.1.7 Kationski transporterji *Wallemia ichthyophaga* – genomski in transkriptomski vidik

Z analizo evolucije proteinskih družin smo ugotovili, da so v genomu *W. ichthyophaga* kationske prenašalne ATPaze tipa P (PF00690) signifikantno obogatene; saj naj bi jih imel predvideni skupni prednik gliv rodu *Wallemia* šest, *W. sebi* le štiri, *W. ichthyophaga* pa kar osem. ATPaze tipa P pri *W. ichthyophaga* predstavljajo tri H^+ (WiPma), dve Na^+ (WiEna) ATPazi v plazmatski membrani, po ena Ca^{2+} ATPaza tipa P v vakuoli (WiPmc1) in Golgijevem aparatu (WiPmr1), ter ena ATPaza z neznano specifičnostjo (slika 5 A, stran 56; tabela 2, stran 57). Te črpalke lahko vsekakor pomembno prispevajo k halofilnem ekotipu te glive (Zajc in sod., 2013).

Črpalke Pma so med najštevilčnejši proteini v plazmatski membrani in porabijo vsaj 20 % celične energije v obliki ATP za vzpostavljanje elektrokemijskega gradienta protonov preko membrane. Ta je nujen kot gonilna sila za vse sekundarne aktivne simporterje ali antiporterje (Ariño in sod., 2010). Gliva *W. ichthyophaga* nosi v genomu zapis za tri H^+-črpalke Pma, ki so v 91 % identične z obema črpalkama Pma *W. sebi*, v 50 % pa so identične Pma1 *S. cerevisiae* (Zajc in sod., 2013). ATPaze Ena so pri *S. cerevisiae* glavna determinatna tolerance na sol (povzeto v Ariño in sod., 2010). Dve ATPazi Ena *W. ichthyophaga* sta si identični v 92 % aminokislin, a vendar sta zelo različni od ScEna1 (le 36 % in 37 % identičnosti) (Zajc in sod., 2013). Pri halotolerantni *D. hansenii* (Almagro in sod., 2001) ter pri izjemno halotolerantni *H. werneckii* (Gorjan in Plemenitaš, 2006) so identificirali dve Ena črpalki, izjemno halotolerantna *W. sebi* pa ima eno kopijo (Zajc in

sod., 2013). Nedavno so z analizo genoma *H. werneckii* našli celo štiri Ena ATPaze, kot posledice duplikacije (Lenassi in sod., 2013).

Poleg zgoraj omenjenih črpalk, pa so za vzdrževanje ionske homeostaze, ki je posebej problematično v izjemno slanem okolju, potrebni še številni drugi sekundarni trasnporterji. V genomu *W. ichthyophaga* smo poleg homologov Ena in Pma črpalk našli še druge homologe transporterjev plazmatske membrane (Nha1, Trk1 in Pho89) ter homologe znotrajceličnih transporterjev (Kha1, Pmr1, Nhx1, Vnx1, Vma1, Pmc1, Mrs7/Mdm37). Za razliko od obogatene družine ATPaz tipa P, pa število genov za druge transporterje, ni različno od nehalofilne sorodnice *W. sebi*, niti posebno visoko (Zajc in sod., 2013). V genomu *H. werneckii* so odkrili znatno višje število kationskih transporterjev, na primer osem K^+ transporterjev Trk, štiri K^+ kanalčke Tok, osem Na^+ (K^+)/H^+ antiporterjev Nha in šest Na^+/P_i simporterjev Pho89 (Lenassi in sod., 2013), v primerjavi z enim Trk, nič Tok, dvema Nha in enim homologom Pho89 pri *W. ichthyophaga* (Zajc in sod., 2013). Halofilna *W. ichthyophaga* očitno uporablja drugačno strategijo življenja pri visokih slanostih kot halotolerantna *H. wernecki.*

Poleg transporterjev znanih za *S. cerevisiae*, smo v genomu *W. ichthyophaga* iskali še gene vpletene v aktivno privzemanje K^+, to sta $K^+(Na^+)$-ATPaza Acu (angl. **a**lkali **c**ation **u**ptake) in K^+-H^+ simporter (Hak), ki so jih našli pri nekaterih nekonvencionalnih kvasovkah (Rodriguez-Navarro in Benito, 2010; Ramos in sod., 2011; Benito in sod., 2012; Gostinčar in sod., članek v pripravi) in ki bi lahko bili pomembni v izjemno slanem okolju. Homologov Hak nismo našli, predvideli pa smo dva možna homologa ATPaze Acu, katerih biološko funkcijo bi bilo treba v nadaljnih poskusih preveriti (Zajc in sod., 2013).

Čeprav je bilo razmeroma malo študij opravljenih na celicah, ki so rastle pri stalnih slanostih in niso bile izpostavlje različnim slanostnim šokom, je jasno, da so nekateri transporterji pri *S. cerevisiae* konstitutivno izraženi, medtem ko imajo drugi kompleksno transkripcijsko regulacijo odvisno od soli, npr. ATPaza Ena1 (Ariño in sod., 2010). Homologa črpalk Ena imata, tako pri *D. hansenii* (Almagro in sod., 2001) kot pri *H. werneckii* (Gorjan in Plemenitaš, 2006), podoben vzorec izražanja. Le po eden izmed

homologov pri obeh vrstah (DhEna1 in HwEna2) je odziven na sol, drugi pa se izraža pri visokih pH (Almagro in sod., 2001; Gorjan in Plemenitaš, 2006).

Tako je na prvi pogled precej nepričakovano, da na izražanje večine genov, ki kodirajo kationske transporterje pri *W. ichthyophaga*, ni vplivala koncentracija soli v okolju (10 in 30 % NaCl). Zgolj trije geni z zapisom za kationske transporterje so izpolnili kriterij dvakratnega diferenčnega izražanja (razmerje log_2>1): homolog Na^+/fosfatnega simporterja WiPho89 (log_2 1,52), eden izmed homologov ATPaze WiEna (log_2 -1,00) in K^+(Na^+)-ATPaza WiAcu (log_2 -1,27) (Zajc in sod., 2013). Transportni sistem Pho89 (Na^+/fosfatni simporter) je pri *S. cerevisiae* edini, ki je sklopljen z Na^+ in je induciran pri visokih pH (Persson in sod., 1999). Ker je pri visokih pH uporaba transmembranskega protonskega gradient ovirana, bi lahko gradient Na^+ služil kot alternativni vir energije. Takšna možna vloga Pho89 transporterja je bila predlagana že pri *H. werneckii*, ki ima 6 kopij tega gena (Lenassi in sod., 2013), vendar o njihovih vzorcih izražanja ni še nič znanega. Pri *W. ichthyophaga* je možno, da celice uporabljajo ta vir energije tudi pri nižjih pH, saj se gojišče že med aktivno fazo rasti, ko smo vzorčili celice za sekvenciranje transkriptoma, zakisa. Razlika v izražanju ostalih dveh črpalk (homolog ATPaze Ena in možne Acu) je bila manjša, izražanje pa je bilo višje pri 10 % NaCl, kar je težko razložiti (Zajc in sod., 2013).

Poleg transkripcijske neodzivnosti na sol, je potrebno poudariti tudi to, da je bil nivo izražanja kationskih transporterjev relativno nizek, kar lahko predstavimo na naslednji način: ureditev genov po padajoči ekspresiji pri višji slanosti, je postavila homologa WiEna ATPaz na 3812. in 4613, homologa Na^+/H^+ antiportejev pa na 2075. in 3927. mesto od skupno 4884 genov. Med prvimi 400 najbolj izraženimi geni so bile različne podenote Pma in domnevni transporter arzenita, ki pa niso bile diferenčno izražene (Zajc in sod., 2013).

Nizko število kationskih transporterjev v proteomu *W. ichthyophaga* in odsotnost nekaterih izmed njih, je verjetno povezano z življenjem pri stalnih (čeprav visokih) slanostih. Odsotnost kanalčka Tok za izpust K^+, je verjetno neškodljivo v okolju, v katerem ni nenadnih hipoosmotskih šokov in posledične potrebe po hitrem izpustu viška K^+ ionov

(Zajc in sod., 2013). Nasprotno pa velja za *H. werneckii*, ki vsebuje številne kanalčke tako za vnos kot tudi izhod K^+ in se lahko učinkovito prilagodi celi vrsti slanosti (Lenassi in sod., 2013). Poleg tega izražanje transporterjev pri *W. ichthyophaga*, razen treh zgoraj omenjenih, ni bilo odvisno od soli. Kljub temu, da imajo kationski transporterji pomembno vlogo pri toleranci na sol, pri najbolj halofilni glivi med njimi nismo našli izjemnosti v njihovi raznovrstnosti, niti v številu genov, niti v njihovem izražanju (Zajc in sod., 2013). Vsekakor pa so proteini in ne geni tisti, ki vplivajo na fenotip organizma, zato na nivoju sekveniranja genoma in transkriptomov zgodbe ne moremo dokončati. Potrebne bi bile namreč nadaljne študije posttranslacijskih modificikacij in proteomov, skupaj z ugotavljanjem morebitne vloge celične stene pri oviranju prehoda ionov v celico (potencialna vloga hidrofobinov).

3.1.8 Prilagoditve *Wallemia ichthyophaga* na nivoju celične stene

3.1.8.1 Debelina celične stene *Wallemia ichthyophaga*

Znano je, da so morfološke spremembe tako na nivoju skupkov celic kot tudi debeline celične stene pomembne za prilagoditev *W. ichthyophaga* na izjemno slane pogoje (Kralj Kunčič in sod., 2010). V gojiščih z nižjo (55 %) in višjo (70 %) koncentracijo glukoze, ki je a_w znižala do enake vrednosti kot 15 % in 25 % NaCl (do 0,88 ter 0,80), smo izmerili debelino celične stene na mikrografijah pridobljenih s presevnim elektronskim mikroskopom. Celična stena *W. ichthyophaga* je bila v gojišču z glukozo precej tanjša kot v gojišču z NaCl. V gojišču s 55 % glukozo je povrečna debelina celične stene dosegla 67,2 % debeline celične stene izmerjene pri 15 % NaCl; v gojišču s 70 % glukozo pa le 55,7 % debeline stene celic zrastlih pri 25 % NaCl (primerjava med Kralj Kunčič in sod. 2010 in Kralj Kunčič in sod. 2013). Debelina celične stene *W. ichthyophaga* se pri 25 % NaCl v primerjavi s 15 % NaCl, statistično značilno odebeli - v povprečju 1,4-, največ pa tudi 2,7-kratno (Kralj Kunčič in sod., 2010). Podoben odziv, vendar z manjšo spremembo v debelini (največ 1,2-kratna odebelitev), smo pokazali tudi v gojišču z nižjo (55 %) in višjo (70 %) koncentracijo glukoze (Kralj Kunčič in sod., 2013).

W. ichthyophaga raste v tekočem gojišču v obliki zelo kompaktnih skupkov celic, ki so podobni sarcinam. Predhodno je bilo ugotovljeno, da je pomemben fenomen prilagoditve *W. ichthyophaga* na visoko slanost, poleg odebelitve celične stene, tudi povečanje velikosti skupkov. Ti večcelični skupki so bili statistično značilno večji (štirikrat in več) pri visoki slanosti (Kralj Kunčič in sod., 2010). Tudi pri visoki koncentraciji glukoze (Kralj Kunčič in sod., 2013) se velikost skupkov poveča, iz povprečno 22,3 na 36,1 μm (povprečno torej 1,6-krat). Vsekakor pa je zanimivo, da so skupki *W. ichthyophaga* v gojišču s soljo mnogo večji kot v gojišču z glukozo: na primer pri 25 % NaCl so veliki tudi do 482 (±18,2) μm, pri 70 % glukozi pa dosežejo le 68,4 (±10,8) μm (Kralj Kunčič in sod., 2013). Skupki gliv *Wallemia* nekoliko spominjajo na sklerocije, ki so strukture z debelim (melaniziranim) ovojem in služijo predvsem premostitvi ekstremnih razmer (Engelberg in sod., 1998; Scherz in sod., 2001). Celične stene zunanje plasti celic skupkov *W. ichthyophaga* so debelejše kot tiste, ki niso direktno izpostavljene slanemu okolju (celice v notranjosti skupka) (Kralj Kunčič, 2010). Ta »sistem trdnjave« kaže na to, da so notranje celice na nek način zaščitene. Zmožnost organizacije v vecčeline skupke močno poveča možnost preživetja v stresnem okolju, kot so pokazali tudi za populacije kvasovk (Palkova, 2004; Palkova in Vachova, 2006).

Odziv *W. ichthyophaga* na pogoje visoke osmolarnosti je edinstven, njena stena se odebeli v gojišču, v katerem je glavni osmotsko aktivni topljenec bodisi NaCl bodisi glukoza. V nasprotju z *W. ichthyophaga,* pa signifikantnih sprememb v debelini celične stene nismo ugotovili ne za *W. sebi* ne za *W. muriae*, ki sta njeni najbližnji sorodnici (Kralj Kunčič in sod., 2013). Odebelitev stene namreč ni splošen odziv gliv na visoko slanost, temveč kakor lahko sklepamo iz naših rezultatov in iz podatkov v literaturi, je ta sposobnost omejena na izjemne glive, ki so prilagojene na stalne visoke koncentracije soli v svojem okolju. Takšna je vsakakor halofilna *W. ichthyophaga* in tudi izjemno halotolerantna vrsta *T. salinum* (Kogej in sod., 2006). Pri halotolerantni vrsti *A. pullulans* se stena celo stanjša, iz 0,20 μm v gojišču brez soli na 0,15 μm pri 17 % NaCl (Kogej in Gunde-Cimerman, neobjavljeni podatki). Podobno se zgodi tudi pri *Aspergillus flavus* in *Penicillium roqueforti* (Abu-Seidah, 2007). Te raziskave potrjujejo predvidevanja o pomembni vlogi celične stene, ki z odebelitvijo ohranja celično integriteto in posledično zagotavlja preživetje halofilnih in izjemno halotolerantnih, ne pa tudi mezofilnih do zmerno

halotolerantnih gliv, v okoljih z nizko vodno aktivnostjo (Kralj Kunčič in sod., 2010; Kralj Kunčič in sod., 2013).

Poleg meritev debeline celične stene, smo določili tudi delež mase celične stene glede na celotno suho biomaso celic *W. ichthyophaga*, ki so rastle pri različnih koncentracijah NaCl. Rezultati so bili presenetljivi; pri 10 % NaCl je bil delež mase celične stene glede na celotno suho biomaso 26 %, kar je v skladu s podatki iz literature za kvasovko *S. cerevisiae,* pri kateri celična stena predstavlja od 15 do največ 30 % suhe celične mase (Orlean, 1997). Pri slanostih nad 10 %, pa se je delež celične stene v suhi biomasi *W. ichthyophaga* povišal na preko 50 %, ter pri 25 % NaCl dosegel 58 %, kar je izjemno visoko. Podoben trend so opazili tudi pri *D. hansenii*, a z nižjimi deleži celične stene v suhi masi celic kot pri *W. ichthyophaga* (Jose Ramos, neobjavljeni podatki). V izjemno slanem okolju celice dolgoročno vzdržujejo višji turgorski tlak, zato ojačanje celične stene z odebelitvijo ni popolnoma presenetljivo (De Martinez in sod., 1996; Smith in sod., 2000; Klis in sod., 2002).

Preprosta povezava rastnih parametrov (specifična hitrost rasti) z deležem celične stene v biomasi, nam pokaže, da je pri najnižji slanosti (10 % NaCl) rast najpočasnejša, tvorba biomase in delež celične stene v biomasi pa najnižja. Nasprotno velja za optimalne slanosti (od 15 do 20 % NaCl), kjer je rast najhitrejša, tvorba biomase in delež celične stene pa visok (Zajc in sod., 2014). Glede na debelino celične stene pri optimalni (15 % NaCl) in suboptimalni (25 % NaCl) slanosti (Kralj Kunčič in sod., 2013) ter glede na rastne parametre in deleže celične stene v biomasi *W. ichthyophaga* preko celotnega slanostnega območja rasti (10-25 % NaCl) (Zajc in sod., 2014) lahko sklepamo, da je ojačanje celične stene povezano z uspešno rastjo *W. ichthyophaga* pri visokih slanostih.

3.1.8.2 Proteini, vključeni v celično steno *Wallemia ichthyophaga*

Izjemno pomembna vloga celične stene v hiperslanih pogojih se je pokazala tudi z analizo genoma in transkriptomov *W. ichthyophaga* (poglavje 3.1.2). Izmed vseh evolucijskih sprememb v proteinskih družinah v genomu *W. ichthyophaga* je bila obogatitev hidrofobinov najbolj signifikantna. V predvidenem skupnem predniku *W. ichthyophaga* in *W. sebi* je bilo ocenjenih 15 hidrofobinov. Pri *W. ichthyophaga* se je zgodila statistično

značila obogatitev na 26 predstavnikov, nasprotno pa se je pri *W. sebi* zgodila redukcija števila na 12 predstavnikov hidrofobinov (slika 4 A, stran 55). Običajno je, da je v genomih filamentoznih gliv več zapisov za hidrofobine, zaradi njihove različne funkcionalne vloge ali diferencialnega izražanja. Hidrofobini so majhni (≤20 kDa) sekretorni proteini celične stene, ki jih proizvajo številne filamentozne glive in imajo pomembno vlogo v različnih procesih (Wösten, 2001). Karakterizira jih njihova amfipatska narava (imajo hidrofilno in hidrofobno domeno) (Linder in sod., 2005). Hidrofobini različnih glivnih vrst imajo malo podobna primarna zaporedja, najpomembnejši je le karakteristični vzorec osmih cisteinskih ostankov, ki tvorijo štiri disulfidne mostičke (Hektor in Scholtmeijer, 2005; Linder in sod., 2005). Ta vzorec je ohranjen tudi pri hidrofobinih *W. ichthyophaga* in *W. sebi*. Hidrofobini obeh vrst so si podobni, saj imajo velik delež ohranjenih aminokislinskih mest (slika 5 B, spodaj, stran 56), poleg tega vsebujejo visok delež kislih aminokislin. Glede na to, da so glivnih hidrofobini direktno izpostavljeni slanemu okolju, visoka vsebnost kislih aminokislin kot prilagoditev na slanost, ki je sicer značilna za halofilne proteine (Madern in sod., 2000; Siglioccolo in sod., 2011), ni presenetljiva. Zanimivo je, da so za halofilne proteine pokazali, da imajo nizko hidrofobnost in nizko zastopanost cisteinov (Paul in sod., 2008; Siglioccolo in sod., 2011), medtem ko so hidrofobini *W. ichthyophaga* in *W. sebi* na eni strani hidrofobni in bogati s cisteini, po drugi strani pa kažejo na slanost prilagojeno kislo aminokislinsko sestavo (Zajc in sod., 2013).

Hidrofobini se spontano sestavljajo v amfipatske monosloje na hidrofobnih in hidrofilnih stikih, kar narekuje njihove številne funkcije pri rasti in razvoju gliv – od tega da omogočajo prehod hif iz vode v zrak, pritrditev celic na hidrofobne površine, vplivajo tudi na prehodnost celične stene za topljence, in dajejo celični steni jakost in rigidnost (Wösten, 2001; Bayry in sod., 2012). Glede na to, da *W. ichthyophaga* raste v okolju izjemno visokih slanosti, kjer toksični kationi neprestano vdirajo v celico, glicerol pa izhaja iz celice, imajo hidrofobini verjetno pomembno vlogo v modulaciji permeabilnosti celične stene in vplivu na njeno jakost in togost (Zajc in sod., 2013). Znano je, da so nekateri hidrofobini vpleteni v povezovanje celic, kot pri glivi *Fusarium verticillioides*, kjer sta dva specifična hidrofobina ključna za tvorbo mikrokonidijskih verižic (Fuchs in sod., 2004). Takšno vlogo bi lahko imeli tudi hidrofobini pri *W. ichthyophaga*, saj so njene celice kot

tudi celice drugih halotolerantnih gliv (Gostinčar in sod., 2010), v tekočem slanem gojišču povezane v kompaktne meristematske skupke (Zajc in sod., 2013).

Preiskava in primerjava transkriptoma celic vzgojenih pri 10 % in 30 % NaCl je pokazala, da so hidrofobini dejansko med redkimi geni, ki so se transkripcijsko odzivali na višjo in nižjo slanost. Polovica hidrofobinskih genov je bilo namreč diferenčno izraženih - nekateri so bili povišano izraženi pri višji (razmerje $\log_2$ tudi do 6,0), drugi pa pri nižji slanosti (razmerje $\log_2$ tudi do -10,0), in sicer neodvisno od njihovega deleža kislih aminokislin ali od izoelektrične točke (Zajc in sod., 2013).

Transkripcijska odzivnost hidrofobinskih genov v sicer transkripcijsko neodzivnem halofilnem specialistu, kaže na njihovo pomembno vlogo pri prilagoditvi na slanost, bodisi z modulacijo celične stene, bodisi s povezovanjem celic. Odkritje halofilnih hidrofobinov je izjemno pomembno tudi z biotehnološkega stališča. Zaradi svoje sposobnosti zamenjave hidrofilnega in hidrofobnega značaja površine ali njihove detergentske sposobnosti, imajo hidrofobini številne možnosti uporab: so surfaktanti, emulgatorji v prehrambni industriji, premazi proti obraščanju (antifouling), inertne prevleke biomaterialov, uporabni so v imobilizaciji različnih snovi (Hektor in Scholtmeijer, 2005; Linder in sod., 2005). Razlike v aminokislinski sestavi (posebej visok delež kislih aminokislin) »halofilnih hidrofobinov« *W. ichthyophaga* in *W. sebi* verjetno dajejo tem proteinom edinstvene lastnosti, ki bi lahko razširile paleto možnosti za njihovo uporabo (Zajc in sod., 2013).

V primerjavi transkriptomov celic *W. ichthyophaga*, ki so rastle pri dveh skrajnih koncentracijah soli (10 % in 30 % NaCl) sta bila med najbolj diferenčno izraženimi geni (razmerje $\log_2$ −9,19 in −3,34) pri nižji slanosti gena z zapisom za ekspanzinom podobna proteina. Rastlinski ekspanzini rahljajo celično steno z ne-encimatskim prekinjanjem ne-kovalentnih vezi med celuloznimi mikrovlakni in polimeri matriksa (McQueen-Mason in Cosgrove, 1994). Takšno aktivnost pa so pokazali tudi pri ekspanzinu podobnem proteinu glive *Bjerkandera adusta* (Quiroz-Castaneda in sod., 2011). Ta dva proteina, ki se pri *W. ichthyophaga* povišano izražata pri nižji slanosti, bi lahko imela vlogo v prilaganju (debeline) celične stene *W. ichthyophaga* na slane pogoje (Zajc in sod., 2013). Pri nižji slanosti se je povišano izražal tudi gen, ki kodira protein podoben tirozinazi. V genomu

W. ichthyophaga so sicer trije homologi tirozinazi podobnih proteinov, ki so običajno vpleteni v sintezo melanina, za katerega vemo, da ima pomembno vlogo pri prilagoditvi na slano okolje nekaterih gliv (Kogej in sod., 2004; Kogej in sod., 2006). Kolonije *W. ichthyophaga* so pri nižji slanosti obarvane svetlo rjavo do olivno zeleno, medtem ko so pri visokih slanostih (nad 25% NaCl) svetlejše rjave ali kremno bele (Kralj Kunčič in sod., 2010). Melaninskih pigmentov sicer še pri nobeni izmed vrst rodu Wallemia niso opisali, pokazali so druge pigmente kot so pirolilpolieni (Ahmed in sod., 1984), za katere pa ni znano ali pri njihovi sintezi sodeluje tudi tirozinaza.

Glede na to, da si običajno hidrofobini med različnimi vrstami gliv niso podobni, sklepamo, da je imel skupni prednik *W. ichthyophaga* in *W. sebi* takšne halofilne hidrofobine, ki jih evolucijska divergenca v nastanku dveh novih vrst ni bistveno spreminjala, verjetno zaradi njihove pomembne vloge. Da imajo očitno res pomembno vlogo pri rasti *W. ichthyophaga* pri različnih slanostih, je pokazala tudi primerjava transkriptomov. Razmerje log_2 izražanja hidrofobinov je najvišje od vseh diferenčno izraženih genov transkripcijsko precej neodzivne *W. ichthyophaga*. Zgolj za primerjavo naj še enkrat omenim, da je imel edini kationski transporter Pho89 log_2 razmerje 1,52, kar je skoraj štirikrat manj kot pri hidrofobinu z najvišjo spremembno pri 30 % NaCl. Vsekakor pa o bioloških funkcijah hidrofobinov pri *W. ichtyhophaga* ne moremo biti prepričani, dokler ne opravimo nadaljnih laboratorijskih poskusov. Tudi diferencialno izražanje ekspanzinom-podobnih genov kaže na vlogo celične stene v odzivu na slanost (Zajc in sod., 2013).

Tako vloga celične stene, kot tudi tvorbe (večjih) meristematskih skupkov je že opisana prilagoditev *W. ichtyhophaga* na izjemno slanost (Kralj Kunčič in sod., 2010). Glede na odebelitve celične stene, povečane investicije biomase v izgradnjo celične stene pri višji slanosti, pa tudi glede na posebnosti v številu in povišanem izražanju nekaterih proteinov (predvsem hidrofobinov) povezanih s celično steno, ugotavljamo, da je odziv *W. ichtyhophaga* na različne slanosti pasiven in v veliki meri odvisen od prilagoditev na nivoju celične stene. To je verjetno razlog, da z visoko-zmogljivo metodo pregledovanja biotehnološko zanimivih genov s knjižnico cDNA, ki se je izkazala za zelo uporabno pri nekaterih drugih ekstremofilnih vrstah (Gostinčar in sod., 2012), nismo uspeli najti genov,

ki bi izboljšali toleranco kvasovke na sol. Naše raziskave so standardizirale pogoje gojenja *W. ichtyhophaga* in razkrile strategijo osmoadaptacije tako na nivoju meritev vsebnosti kationov in organskih topljencev, kot tudi na nivoju izražanja gena ključnega encima pri biosintezi glicerola, analiza genoma in transkriptomov pa je odkrila nekatere posebnosti v genski osnovi tega izjemnega fenotipa. Zaporedje genoma, ki je sedaj javno dostopno, bo bistveno olajšalo vse nadaljne študije tega organizma, ki uspešno živi pri pogojih, ki so smrtni za večino ostalih evkariontov.

3.2 SKLEPI

a) Rastni optimum *Wallemia ichtyhophaga*

- Rastni optimum glive *W. ichtyhophaga* je med 15 % in 20 % NaCl in je najvišji slanostni optimum kadarkoli opisan med glivami.
- Napredovanje rasti *W. ichtyhophaga* v definiranem YNB gojišču z NaCl lahko spremljamo ne le z določanjem suhe biomase, temveč s hitrejšim in bolj priročnim merjenjem pH gojišča.
- *W. ichtyhophaga* ima preferenco po NaCl, ne pa po glukozi, za znižajanje a_w gojišča, saj v gojišču s soljo stvori bistveno več biomase.

b) Vsebnost kompatibilnih topljencev

- *W. ichthyophaga* sintetizira mešanico predvsem glicerola in arabitola, manitola je le v sledeh. Glavni kompatibilni topljenec je glicerol, saj so bile njegove količine najvišje izmed vseh treh poliolov, poleg tega pa je njegova vsebnost v celicah statistično značilno korelirala z naraščajočo slanostjo in s hipoosmotskim šokom.
- *W. ichthyophaga* ima drugačne prilagoditve in odzive na stalno slanost in na nihanja slanosti na nivoju gena *GPD1*, kot pa izjemno halotolerantna *Hortaea werneckii*, v skladu z njuno ekofiziologijo. Izjemno halotolerantna *H. werneckii* se hitreje odziva in kaže večje spremembe v izražanju *GPD1*, medtem ko je halofilna *W. ichthyophaga* v odzivu na slanost in na nihanja slanosti bolj toga- indukcija nivoja mRNA gena *GPD1* je manjša in počasnejša.

c) Vsebnost kationov

- Vsebnosti kationov (podane kot koncentracije K^+ in Na^+ na mg suhe biomase) so pri *W. ichthyophaga* zelo nizke v primerjavi z nekaterimi ostalimi glivami iz podobnih okoljih (*H. werneckii, A. pullulans* in *D. hansenii*).
- Razmerje K^+/Na^+ v celicah *W. ichthyophaga* je višje kot pri ostalih halotolerantnih glivah pri vseh merjenih koncentracijah NaCl.
- Rast *W. ichthyophaga* je boljša, ko je vsebnost Na^+ višja od K^+. V tem je *W. ichthyophaga* edinstvena.

d) Značilnosti genoma in primerjava transkriptomov

- Genom *W. ichthyophaga* je majhen (9,6 Mb) in kompakten (1,67 % ponavljajočih se zaporedij) in ima predvidenih 4884 protein-kodirajočih genov, ki pokrivajo skoraj tri četrtine celotnega zaporedja. Od 639 diferencialno izraženih genov, sta dve tretjini bolj izraženi pri nižji slanosti.
- Filogenomska analiza 14 proteomov gliv, je pokazala da je razred Wallemiomycetes 250 miljonov let sestrska skupina Agaricomycotina.
- Vrsta *W. ichthyophaga* je najverjetneje izgubila sposobnost spolnega razmnoževanja, saj nima določenega lokusa *MAT* in ima le majhen delež za mejozo specifičnih genov.
- Več proteinskih družin je značilno razširjenih ali skrčenih v genomu *W. ichthyophaga*. Med razširjenimi družinami so ATPazni kationski prenašalci tipa P in družina proteinov celične stene t.i. hidrofobini.
- V genomskem zaporedju *W. ichthyophaga* nismo opazili nenavadnih lastnosti v običajnih mehanizmih tolerance na sol, na primer v transportu anorganskih ionov ali sintezi kompatibilnih topljencev.
- *W. ichthyophaga* je ekstremofilni specialist, ki kaže le nizko raven prilagodljivosti in genetske rekombinacije. To se odraža tako v značilnostih genoma, kot tudi v transkriptomskem odzivu na sol. Izražanje kationskih transporterjev je razmeroma nizko in, razen treh, neodvisno od soli.

e) Celična stena *Wallemia ichtyhophaga*

- Celična stena *W. ichtyhophaga* je tanjša v gojišču z glukozo kot v gojišču z NaCl.
- Celična stena *W. ichtyhophaga* se odebeli v gojišču z višjo koncentracijo glukoze v primerjavi z nižjo, vendar odebelitev ni tako povdarjena kot v slanih gojiščih.
- Delež mase celične stene glede na celotno biomaso je izjemno visok pri slanostih nad 10 % NaCl.
- Hidrofobini so med najbolj diferenčno izraženimi geni pri *W. ichtyhophaga.*
- Različni podatki kot so debelina celične stene, delež celične stene v biomasi, pa tudi visoko število in transkripcijska odzivnost proteinov celične stene, hidrofobinov, kažejo na pomembno vlogo celične stene *W. ichthyophaga* v odgovoru na sol.

4 POVZETEK

4.1 POVZETEK

Ekstremofilni mikroorganizmi, kakršne so tudi halofilne glive, predstavljajo pomemben vir znanja za razumevanje odzivov na stres ter za razvoj novih biotehnoloških aplikacij. Predhodne raziskave so razkrile veliko biološko pestrost gliv v ekstremnih okoljih. Osamljene so bile številne vrste izjemno halotolerantnih gliv, med katerimi prevladujejo črne kvasovke (Gunde-Cimerman in sod., 2000), ter vrste rodu *Wallemia*, znotraj katerega je najbolj halofilna vrsta *Wallemia ichthyophaga*. Za rast *in vitro* potrebuje minimalno 10 % (m/V) NaCl v gojišču in je metabolno aktivna tudi pri 32 % NaCl. Rod Wallemia je zanimiv tudi s filogenetskega stališča, saj predstavlja staro, ločeno linijo znotraj debla *Basidiomycota*, med katerimi tako rekoč ni opisanih kserofilnih gliv in je komajda nekaj kserotolerantnih (Zalar in sod., 2005a).

Večina gliv, ki rastejo v slanih okoljih, kaže splošen kserofilni fenotip; za zniževanje vodne aktivnosti okolja ne potrebujejo nujno soli. Vodna aktivnost je do iste vrednosti lahko znižana tudi s povišanimi koncentracijami sladkorja ali drugih ustreznih topljencev. Opisane so le redke »prave« halofilne/kserofilne glive, torej tiste, ki za rast nujno potrebujejo povišane koncentracije soli, in so skorajda neproučene. Okolja z visokimi koncentracijami NaCl predstavljajo organizmom težavo zaradi izgubljanja vode preko citoplazmatske membrane in vdora toksičnih anorganskih ionov. Halofilni organizmi uporabljajo dve osnovni strategiji prilagoditve na visoko slanost v zunanjem okolju. Pri prvi strategiji celice vzdržujejo visoko znotrajcelično koncentracijo soli (strategija vnosa soli), ki je osmotsko najmanj ekvivalentna koncentraciji v zunanjosti, kar zahteva prilagoditev vseh znotrajceličnih sistemov. Pri drugi strategiji celice vzdržujejo nizko koncentracijo soli v citoplazmi, osmotski tlak medija pa uravnotežijo z organskimi kompatibilnimi topljenci v citoplazmi (strategija kompatibilnih topljencev) (Oren, 1999). Poleg tega se celice prilagodijo tudi na nivoju sestave in lastnosti celične stene in membrane ter same morfologije celic.

Prilagoditve na slana okolja so pri glivah proučene pri kvasovki *S. cerevisiae*, ki je občutljiva na sol in raste bolje brez nje, pri zmerni halotolerantni kvasovki *D. hansenii*, ki ima optimalno rast pri nizkih slanostih ter pri izjemno halotolerantni črni kvasovki *H. werneckii,* ki optimalno raste pri 10% NaCl (obe vrsti povzeti v Gunde-Cimerman in sod., 2009; Gostinčar in sod., 2011). Namen naših raziskav je bil preučiti rastne značilnosti, strategijo osmoadaptacije in prilagoditve na nivoju celične stene glive *W. ichthyophaga*, ki ima obligatno zahtevo po soli, raste le nad 10% NaCl in uspeva tudi v s soljo nasičenih raztopinah.

Rast *W. ichtyhophaga* na trdnem gojišču je počasna, njene kolonije pa so majhne. To je treba upoštevati pri osamitvi gliv *W. ichtyhophaga* iz okoljskih vzorcev. Predlagamo vsaj 14-dnevno inkubacijo in uporabo gojišč z visoko koncentracijo soli (nad 15 % NaCl), saj drobne in počasi rastoče kolonije *W. ichtyhophaga* pri nižjih slanostih hitro prerastejo mezofilne ali generalistične glive, kar je verjetno eden izmed glavnih razlogov za nizko število do sedaj opisanih izolatov *W. ichtyhophaga*. V tekočih šaržnih kulturah *W. ichthyophaga* se je proizvodnja biomase in specifična hitrost rasti znatno povečevala pri slanostih višjih od 10 %. Le pri najvišjih merjenih slanostih (25 % NaCl) je *W. ichtyhophaga* tvorila največ biomase izmed preučevanih halotolerantnih gliv (*Hortaea werneckii* in *Aureobasidium pullulans*) iz istega okolja. Rastni optimum med 15 % in 20 % NaCl, tako na trdnih kot tudi v tekočih gojiščih, je najvišji slanostni optimum kadarkoli opisan med glivami. Napredovanje rasti *W. ichtyhophaga* v šaržni kulturi z NaCl lahko spremljamo ne le z določanjem suhe biomase, temveč s hitrejšim in bolj priročnim merjenjem pH gojišča, saj je krivulja zniževanja pH gojišča obratno proporcionalna biomasni rastni krivulji. Gliva *W. ichtyhophaga* je preferenčno rastla v gojišču, kjer je a_w znižana z NaCl, ne pa z organskimi topljenci, na primer z glukozo, saj je v gojišču s soljo pridelala skoraj do petkrat več biomase kot v gojišču z glukozo. Ugotovili smo tudi, da je *W. ichthyophaga* sposobna rasti pri izjemno visokih koncentracijah $MgCl_2$ in je torej tudi kaofilna.

Sekvencirali in analizirali smo genomsko zaporedje tega izjemnega fenotipa ter preučili transkriptomska odziva na slanosti, ki predstavljata njeno spodnjo (10 % NaCl) in zgornjo (30 % NaCl) mejo življenja. Genom je nenavadno majhen (9,6 Mb) in kompakten (vsebuje

le 1,67 % ponavljajočih se zaporedij). Predvidenih je zgolj 4884 protein-kodirajočih genov, ki pokrivajo skoraj tri četrtine celotnega zaporedja. Dve tretjini genov od 639 diferencialno izraženih ima povišano izražanje pri nižji slanosti. Filogenomska analiza, ki je temeljila na celotnih proteomih gliv, je potrdila uvrstitev razreda Wallemiomycetes kot sestrsko skupino Agaricomycotina. Ocenili smo, da se je ločitev zgodila pred 250 milijoni let, vrsti *W. ichthyophaga* in *W. sebi* pa sta se ločili pred 11,9 miljoni let. V nasprotju s tesno sorodno vrsto *Wallemia sebi*, je *W. ichthyophaga* najverjetneje aseksualna, saj nima niti opredeljenega lokusa *MAT* povezanega s paritvijo niti popolnega nabora genov specifičnih za mejozo.

V genomu *W. ichthyophaga* je večina proteinskih družin značilno skrčenih (19 od skupno 26 signifikantno spremenjenih), sedem pa je signifikantno obogatenih. Med obogatenimi družinami so ATPazni kationski transporterji tipa P, ki imajo pri halofilnih evkariontih izjemen pomen za uspešno rast pri slanih pogojih; ter družina hidrofobinov, ki pa so proteini celične stene z več biološkimi funkcijami. Za družino ATPaz tipa P pri *W. ichthyophaga* nismo ugotovili povišanega transkripcijskega odziva na sol ter razmeroma nizko raven izražanja, medtem ko je kar polovica izmed 26 hidrofobinov diferenčno izraženih in imajo visok nivo izražanja. Večina hidrofobinov kaže prilagoditev na visoko slanost na nivoju primarne aminokislinske sekvence, saj vsebujejo nenavadno visoko število kislih aminokislin. To odkritje je še posebej zanimivo zaradi široke biotehnološke uporabnosti hidrofobinov gliv v industriji, farmaciji in medicini.

Strategija prilagoditve halofilne *W. ichthyophaga* je kopičenje kompatibilnih topljencev, ki so, kot pri večini gliv, polioli. Glavni osmotsko uravnavan poliol je glicerol, saj se je njegova vsebnost povečevala z naraščajočo slanostjo ter se je zmanjšala po hipoosmotskem šoku. Poleg glicerola, smo odkrili tudi manjše količine arabitola in sledove manitola.

V obligatno halofilni *W. ichthyophaga* smo preučili *WiGPD1* paralog, katerega prepisovanje je odvisno od soli. V primerjavi s homologoma *H. werneckii* je transkripcijski *WiGPD1* odziv na hiperosmotski šok počasnejši. Heterologno izražanje *WiGPD1* v *S. cerevisiae* povrne osmotoleranco tako *gpd1* kot tudi *gpd1gpd2* mutiranih sevov, kar je verjetno posledica visoke splošne aminokislinske podobnosti *GPD1* proteinov pri

W. ichthyophaga in *S. cerevisiae*. Proučevani Gpd1 proteini (HwGpd1A in HwGpd1*B* ter WiGpd1 in ScGpd1) se razlikujejo po tem, da imata le ortologa *H. werneckii* v katalitični Rossmann-ovi domeni dodatno 17 aminokislin dolgo regijo. Nobeden od Gpd1 homologov *H. werneckii* in *W. ichthyophaga* pa ni imel zaporedja za usmerjanje v peroksisom tipa 2 (angl.: **p**eroxisomal **t**argeting **s**equence type **2**; PTS2) na N-koncu proteinskega zaporedja. Ker je funkcija Gpd1 pri osmotskem stresu odvisna od njegove citosolne in jedrne frakcije, sklepamo, da bi bila posledična stalna citosolna lokalizacija Gpd1 homologov adaptacija organizmov na izjemno slana okolja.

Skladno s strategijo kompatibilnih topljencev je *W. ichthyophaga* ohranjala relativno nizke vsebnosti kalija in natrija pri pogojih stalne slanosti, med hiperosmotskih šokom pa so se vsebnosti obeh kationov znatno povečale. Razmerje med kationoma je padalo, zaradi naraščanja vsebnosti Na^+ in padanja vsebnosti K^+ z naraščajočo slanostjo. Rast je bila najboljša pri tisti slanosti, kjer je razmerje med K^+ in Na^+ najnižje, zato sklepamo da za halofilno *W. ichthyophaga* Na^+ ioni niso toksični. Podatki iz genoma kažejo, da je v predvidenem proteomu *W. ichthyophaga* moč najti le nizko število kationskih transporterjev, nekateri izmed njih pa celo manjkajo, razen obogatene družine ATPaz tipa P. Izražanje transporterjev, razen treh izjem, je bilo nizko in neodvisno od soli. To je verjetno povezano z življenjem pri stalnih slanostih, čeprav so le-te izjemno visoke.

Morfološke študije gliv rodu *Wallemia* so pokazale, da so spremembe tako na nivoju skupkov celic in kot tudi debeline celične stene pomembne za prilagoditev *W. ichthyophaga* na izjemno slane pogoje (Kralj Kunčič in sod., 2010). V gojiščih z glukozo je celična stena *W. ichthyophaga* precej tanjša kot v gojišču z NaCl in se je, podobno kot v slanih gojiščih, odebelila pri višji koncentraciji glukoze. Sprememba v debelini celične stene je bila manjša - največ 1,2-kratna, v primerjavi s skoraj trikratno odebelitvijo v gojišču z NaCl. Pri slanostih višjih od 10 %, se je delež celične stene v suhi biomasi *W. ichthyophaga* povišal na preko 50 %, kar je izjemno visoko. V ekstremno slanem okolju je ojačenje celične stene z odebelitvijo zelo koristno, saj celice dolgoročno vzdržujejo višji turgorski tlak.

Tudi analiza genoma in transkriptoma nazakuje na pomembno vlogo celične stene pri prilagoditvi *W. ichthyophaga* na visoko slanost. Proteini celične stene, hidrofobini, imajo pri glivah različne vloge, med drugim vplivajo na prehodnost celične stene za topljence, in dajejo celični steni jakost in rigidnost (Wösten, 2001; Bayry in sod., 2012). V genomu *W. ichthyophaga* je kar 26 zapisov za tako imenovane halofilne hidrofobine, ki so med relativno redkimi geni, ki so diferenčno izraženi. Transkripcijska odzivnost hidrofobinskih genov v sicer transkripcijsko neodzivnem halofilnem specialistu, kaže na njihovo pomembno vlogo pri prilagoditvi na slanost, bodisi z modulacijo celične stene, bodisi s povezovanjem celic.

W. ichthyophaga je ekstremofilni specialist, ki kaže le nizko raven prilagodljivosti in genetske rekombinacije. To se odraža tako v značilnostih genoma, kot tudi v transkriptomskem odzivu na sol. Kljub temu, da imajo kationski transporterji pomembno vlogo pri toleranci na sol, pri halofilni *W. ichthyophaga* med njimi na nivoju preiskave genoma in transkriptoma nismo našli posebnosti. Namesto tega se je na več nivojih pokazala pomembna vloga celične stene v prilagoditvi *W. ichtyhophaga* na izjemno visoko slanost. Glede na odebelitve celične stene, povečane investicije biomase v izgradnjo celične stene pri višji slanosti, pa tudi glede na posebnosti v številu in izražanju nekaterih proteinov (predvsem hidrofobinov) povezanih s celično steno, ugotavljamo, da je odziv *W. ichtyhophaga* na različne slanosti pasiven in v veliki meri odvisen od prilagoditev na nivoju celične stene.

Pričakujemo, da bodo naše raziskave od optimizacije gojenja in spremljanja rasti ter razpoložljivost genomskega zaporedja olajšale nadaljnje raziskave te edinstvene vrste in omogočile še boljši pogled na prilagoditve, ki *W. ichthyophaga* omogočajo uspevanje v pogojih, ki so smrtni za večino drugih evkariontov.

4.2 SUMMARY

Extremophilic microorganisms, such as the halophilic fungi, are an important source of knowledge for understanding responses to stress and for the development of new biotechnological applications. Previous studies have revealed a high biodiversity of fungi in extreme environments. Many types of extremely halotolerant fungi were isolated, among which predominate black yeasts (Gunde-Cimerman et al., 2000), and the genus *Wallemia*, which comprises the most halophilic species known, *Wallemia ichthyophaga*. It needs a minimum 10 % (w/v) NaCl in the medium for *in vitro* growth and it is metabolically active even at 32 % NaCl. The genus *Wallemia* is also phylogenetically interesting as it is an old and isolated lineage within the phylum Basidiomycota, which comprise only few xerotolerant fungi and practically no xerophilic (Zalar et al., 2005a).

Most fungi from saline environments show general xerophilic phenotype as they do not necessarily require salt for lowering the water activity of the medium. Water activity can be reduced either with higher concentrations of sugar or other relevant solutes. There are only few "real" halophilic/xerophilic fungi described, *i.e.* those that necessary need elevated salt concentrations for growth, and are almost unstudied. Environments with high concentrations of salts present a problem for organisms due to the loss of water through the cytoplasmic membrane and toxicity of inorganic ions. Halophilic organisms use two basic strategies to adapt to high salinity in the external environment. Employing the salt-in strategy, the cells maintain a high intracellular concentration of salt, which is at least equivalent to the osmotic concentration of the exterior. This requires adjustment of all of the intracellular systems to high concentrations of salt. In the second strategy, the osmotic pressure of the medium is balanced with organic compatible solutes in the cytoplasm (the compatible solutes strategy), while low intracellular concentration of salt is maintained (Oren 1999). In addition, the cells also need to adapt at the level of the composition and properties of the cell-walls and membranes, as well as the morphology of the cells themselves.

Fungal adaptations to the saline environments were so far examined in salt sensitive yeast *S. cerevisiae*, which grows best without NaCl; in halotolerant yeast *D. hansenii*, which grows optimally at low salinities and the extremely halotolerant black yeast *H. werneckii*, which grows optimally at 10% NaCl (both summarized in Gunde-Cimerman et al., 2009; Gostinčar et al., 2011). The goal of our research was to examine the growth characteristics, osmoadaptation strategy and the role of the cell wall in the only obligate halophile *W. ichthyophaga*, which only grows at salitinies higher than 10% NaCl and thrives also in saturated salt solutions.

Growth of *W. ichtyhophaga* on solid medium was slow and its colonies were small. This should be considered in isolation of strains of *W. ichtyhophaga* from environmental samples. We suggest at least 14 days of incubation using the culture medium with a high salt concentration (above 15 % NaCl), as small and slow-growing colonies of *W. ichtyhophaga* are quickly overgrown by the mesophilic or generalistic fungi at lower salinity. This is probably one of the main reasons for the low number of isolates of *W. ichtyhophaga* described so far. In the liquid batch cultures the specific growth rate of *W. ichthyophaga* was higher and the biomass production significantly increased in salinities higher than 10 % NaCl. Only at the highest measured salinity (25 % NaCl), *W. ichtyhophaga* produced more biomass than halotolerant *Hortaea werneckii* and *Aureobasidium pullulans*¸ two black yeasts from the same environment. The determined growth optimum between 15 % and 20 % NaCl, on solid as well as in liquid culture media, for *W. ichtyhophaga* is the highest salinity optimum ever described among fungi. The decrease in the pH of the growth medium was inversely related to the increase in the biomass. Therefore, progression of growth of *W. ichtyhophaga* in liquid batch culture with NaCl can be monitored not only by determination of dry biomass, but with faster and more convenient measurement of the pH of the growth medium. The fungus *W. ichtyhophaga* preferentially grows in the medium, wherein a_w is reduced with NaCl, but not with organic solutes such as glucose, as it produced almost up to five times more biomass in saline media than in the media with glucose. We have also found that *W. ichthyophaga* is able to grow at extremely high concentrations of $MgCl_2$ and should therefore also be considered as chaophile.

We have sequenced and analyzed the genome of *W. ichtyhophaga* and examined its transcriptomic response to salinities, which represent its lower (10 % NaCl) and upper (30 % NaCl) limit of growth in order to study the genetic bases of this remarkable phenotype. Genome is unusually small (9.6 Mb) and compact (containing only 1.67 % of repetitive sequences). There are only 4884 predicted protein - coding genes, which cover almost three-quarters of the entire sequence. Two-thirds of the 639 differentially expressed genes are more expressed at lower salinity. Phylogenomic analysis based on the total proteomes of fungi confirmed the position of Wallemiomycetes as a sister group Agaricomycotina. We have estimated that this separation occurred 250 million years ago, whereas *W. ichthyophaga* and *W. sebi* separated around 11.9 million years ago. In contrast to the closely related *W. sebi*, *W. ichthyophaga* is probably asexual as it has no discernible *MAT* locus and only few of the genes specific to meiosis.

In line with the small genome of *W. ichthyophaga* the majority of protein families are contracted (19 out of 26 significantly changed) and seven significantly enriched. Among the extended families there are P-type ATPase cation transporters, which are highly important for the successful growth in saline conditions, and hydrophobins, which are cell wall proteins with multiple biological functions. P-type ATPases have a relatively low level of expression and no increased transcriptional response to salt, whereas hydrophobins have a high level of expression and half of them are differentially expressed. Most of hydrophobins show adaptation to high salinity at the level of primary amino acid sequences, as they contain an unusually high number of acidic amino acids. This finding is particularly interesting because of the wide biotechnological applicability of fungal hydrophobins in the industry, pharmacy and medicine.

Strategy of osmoadaptation of the halophilic *W. ichthyophaga* is the accumulation of compatible solutes, which are, as in the majority of fungi, polyols. The main osmotically regulated polyol is glycerol, as its level is increased with increasing salinity and decreased after hipoosmotic shock. In addition to glycerol, we have discovered smaller amounts of arabitol, and traces of mannitol. We have studied one *GPD1* paralogue with salt-induced transcription in obligately halophilic *W. ichthyophaga*. As compared with the homologues of *H. werneckii* the response of *WiGPD1* to hyperosmotic shock is slower. Heterologous

expression of *WiGPD1* in S. *cerevisiae* reimbursed the osmotolerance of both *gpd1* and *gpd1gpd2* mutant strains, which is probably due to the high overall amino acid similarity of Gpd1 proteins in *W. ichthyophaga* and *S. cerevisiae*. Only orthologs from *H. werneckii* have an additional 17 amino acids long region in the catalytic Rossmann domain of the enzyme. Interestingly, no N-terminal type 2 peroxisomal targeting sequence (PTS2) was found in the Gpd1 homologues of the halotolerant/halophilic fungi studied. Since the function of Gpd1 in osmotic stress depends on its cytosolic and nuclear fractions, we conclude that the consequent permanent cytosolic localization of Gpd1 homologues is an adaptation to extremely saline environment.

In line with the strategy of compatible solutes *W. ichthyophaga* maintains relatively low levels of potassium and sodium at constant salinities, however when experiencing hyperosmotic shock the levels of both cations increase significantly. The ratio between the cations decreased with increasing salinity, due to the rising levels of Na^+ and lowering of the K^+. Given that the growth of *W. ichthyophaga* is the best when the K^+/Na^+ is minimal, it can be assumed that the Na^+ ions are not toxic for the halophilic *W. ichthyophaga*. Data from the genome and predicted proteome show that there are only a low number of cation transporters, except for the enriched protein family P type ATPases. The expression of the cation transporters, except for the three exceptions, was low and independent of the salt. This is probably associated with life of *W. ichthyophaga* at constant salinities, although these are extremely high.

Previously the morphological studies of Wallemia spp. have shown that the changes at the level of cell-aggregates and cell wall thickness are crucial for the adjustment of *W. ichthyophaga* to extremely saline conditions (Kralj Kunčič et al., 2010). In the medium supplemented with glucose the cell wall *W. ichthyophaga* was thinner than in the medium with NaCl and, as also seen before, became thicker at higher glucose concentrations. The change in the cell wall thickness was however lower - a maximum 1.2-times, compared to almost three times thicker cell wall in media with NaCl. At salinities higher than 10 %, the proportion of the cell-walls in dry biomass *W. ichthyophaga* increased to over 50 %, which is extremely high. The reinforcement of the cell wall in extremely saline environments is however beneficial as the cells maintain higher turgor pressure on long-term.

Also the analysis of the genome and transcriptome indicates the important role of cell wall in adaptation of *W. ichthyophaga* to high salinity. The cell wall proteins, hydrophobins have different roles in fungi. Among other things they affect the permeability of the cell wall for solutes, give the cell wall strength and rigidity and are sometimes responsible for connecting the cells. In the genome of the *W. ichthyophaga* there are 26 genes coding for the so-called halophilic hydrophobins that are among the relatively few genes that are differentially expressed. The transcriptional response of hydrophobin-coding genes shows their important role in adaptation to salinity, either by modulation of the cell walls or by aggregating the cells into multicellular clumps.

W. ichthyophaga is the extremophilic specialist, which shows only a low level of adaptability and genetic recombination. This is reflected in the characteristics of its genome, as well as in the transcriptomic response to the salt. Despite the important role of cation transporters in the salt tolerance, we have not found any unusual features when investigating the genome and transcriptome of the halophilic *W. ichthyophaga*. Instead the role of the cell wall of *W. ichtyhophaga* as the adaptation to the extreme salinity was confirmed on different levels. Given the cell wall thickening, increased investment of the biomass in the construction of cell walls at higher salinities, as well as the particularities of the number and expression of some proteins (especially hydrophobins) associated with the cell wall, we state that the response of *W. ichtyhophaga* to different salinity is passive and largely depends on the adaptation of the cell wall.

We expect that our optimization of the cultivation and growth monitoring and mainly the availability of genomic sequence will facilitate further research of this unique species that will give an even better view of the adjustments that allow *W. ichthyophaga* to thrive in conditions that are lethal to most other eukaryotes.

5 VIRI

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Najlepša hvala staršem za podporo in neprecenljivo pomoč pri varstvu hčerke pogosto do večernih ur med končevanjem doktorata. Hvala tudi bratu Alešu z družino za varstvo hčerke. Neizmerna hvala mojemu partnerju Milanu, da me je prenašal v najbolj obremenilnih zadnjih mesecih in mi večkrat omogočil jutranji spanec. Hvala tudi moji hčerki Pii, ki mi z otroško prisrčnostjo razsvetljuje dneve in daje mojemu življenju višji smisel. In hvala vsem prijateljem, da to so.

Doktorsko disertacijo iz srca posvečam pokojnemu dedku Dragu Zajcu, ki mi je za 6. rojstni dan kupil mikroskop in me kot entuziastičen naravoslovec navdušil za mikrosvet.

PRILOGE

Priloge vsebuje dodatno gradivo (Additional files) k poglavju X oziroma članku Zajc in sod. (2013). Gradivo je dostopno tudi na spodnji povezavi:

http://www.biomedcentral.com/1471-2164/14/617/additional (13. sep. 2013)

PRILOGA A

Slika S1: Klasifikacija predvidenih proteinov po kategoriji baze KEGG

Slika S1: Klasifikacija predvidenih proteinov v gruče ortolognih skupin (baza COG)

Additional file 1:

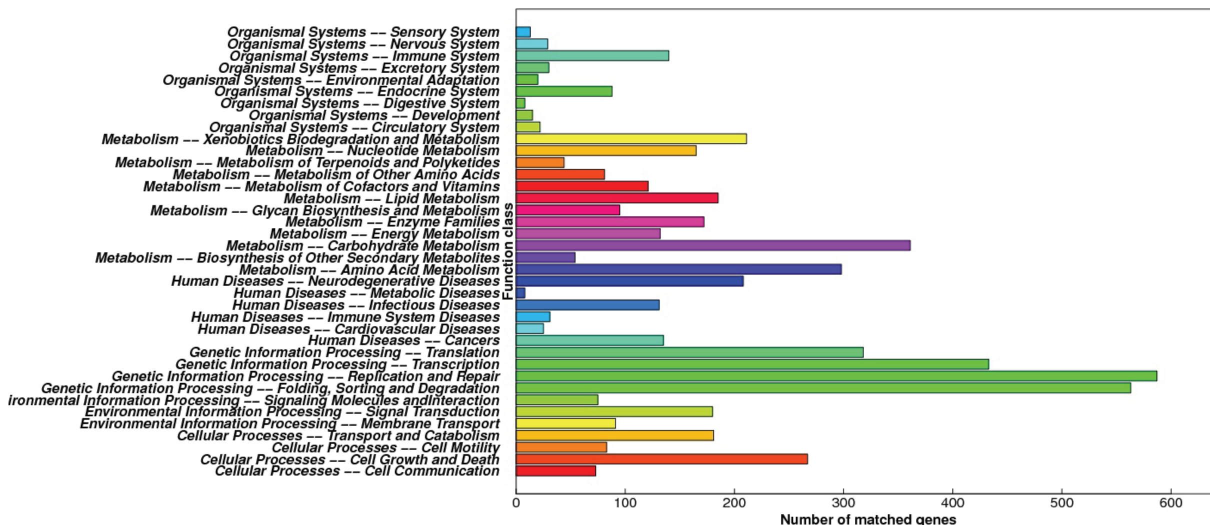

Figure S1: Classification of the predicted genes into the KEGG database categories.

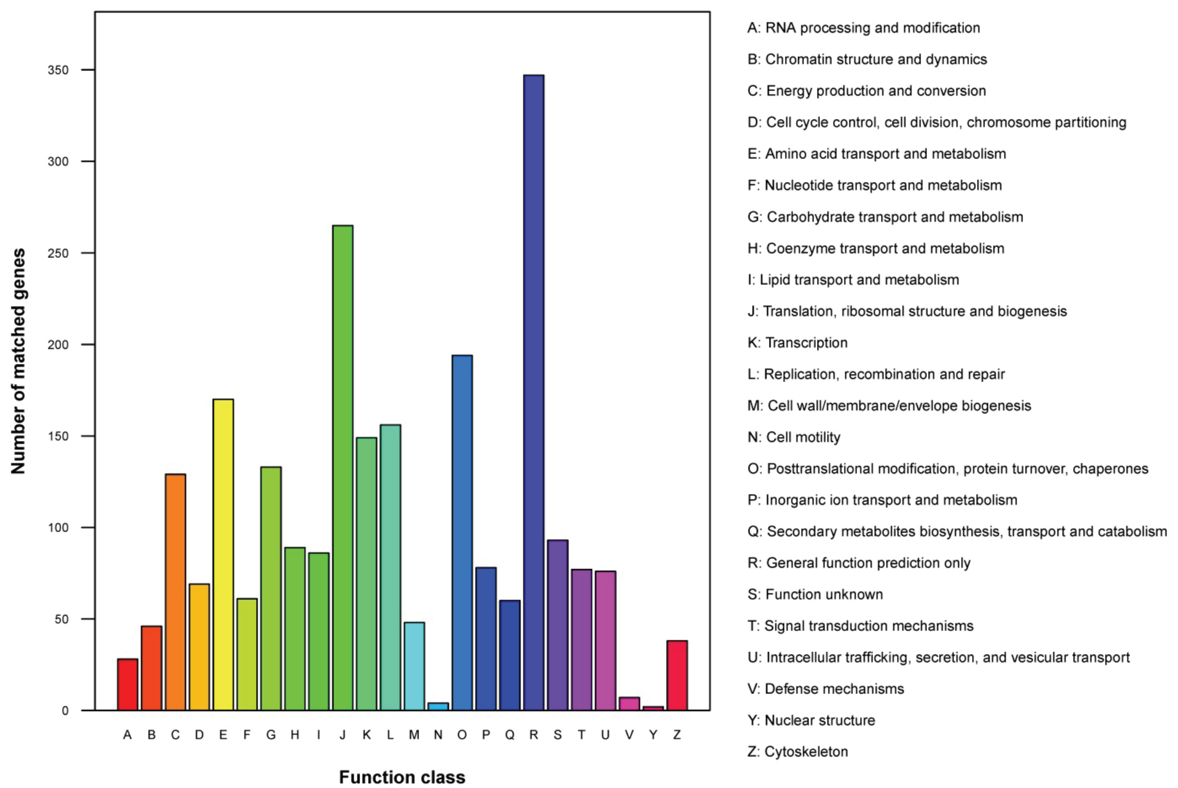

Figure S2. Classification of the predicted genes into clusters of orthologous groups (COG database).

Slika S3: Razporeditev pokritosti transkriptoma.

Slika S4: Število različnih dogodkov alternativnega spajanja pri obeh slanostih.

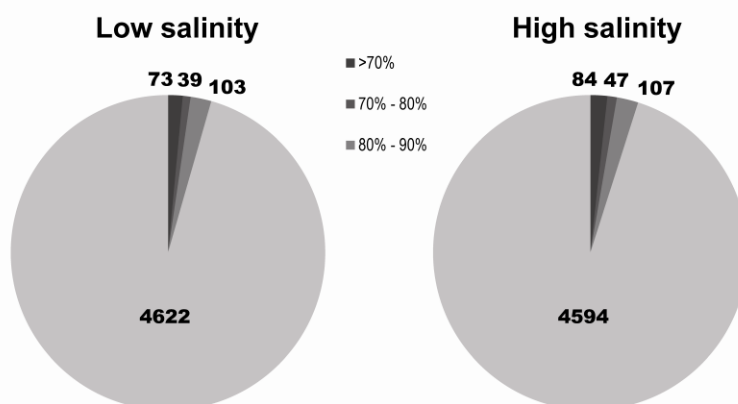

Figure S3 Distribution of transcriptome gene coverage. The pie charts show the distributions of gene coverage (the percentage of a gene covered by reads) of *Wallemia ichthyophaga* grown at 10% NaCl (low salinity) and 30% NaCl (high salinity).

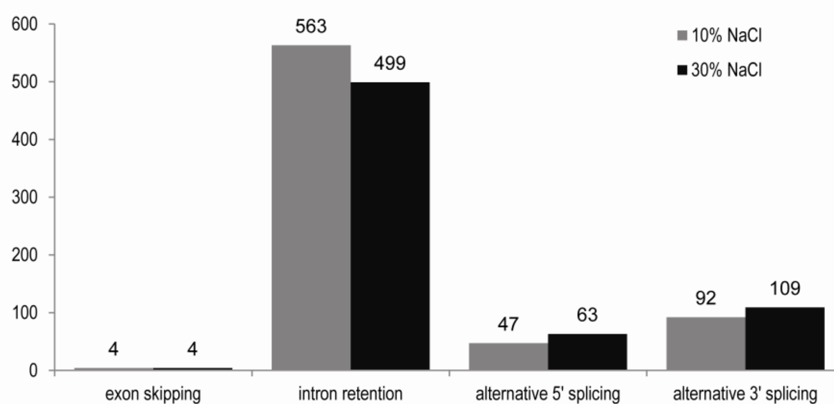

Figure S4 Number of different alternative splicing events at each salinity.

PRILOGA B

Seznam proteinov *Wallemia ichthyophaga*, ki imajo 10 ali več predstavnikov določene domene Pfam.

Additional file 2: Table S1: Number of proteins of *W. ichthyophaga* with the given Pfam domain.

PFAM	Number of Proteins	Short Description	Name
PF00400.27	93	WD40	WD40 repeat
PF00069.20	81	Pkinase	Protein kinase domain
PF00271.26	60	Helicase_C	Helicase conserved C-terminal domain
PF00076.17	57	RRM_1	RNA recognition motif
PF07690.11	51	MFS_1	Major facilitator family
PF00270.24	46	DEAD	DEAD/DEAH box helicase
PF00153.22	32	Mito_carr	Mitochondrial carrier
PF00005.22	30	ABC_tran	ATP-binding domain of ABC transporters
PF00172.13	29	Zn_clus	Zinc finger
PF00004.24	29	AAA	AAA proteins
PF13414.1	27	TPR_11	TPR repeat
PF04082.13	26	Fungal_trans	Fungal specific transcription factor domain
PF00226.26	26	DnaJ	Chaperone DnaJ
PF01185.13	26	Hydrophobin	Hydrophobin
PF00106.20	25	adh_short	Short-chain dehydrogenase
PF00125.19	22	Histone	Histone
PF00071.17	21	Ras	Ras subfamily
PF00083.19	19	Sugar_tr	Sugar (and other) transporter
PF00018.23	19	SH3_1	SH3 domain
PF00009.22	17	GTP_EFTU	GTP-binding elongation factor family, EF-Tu/EF-1A subfamily
PF00179.21	16	UQ_con	Ubiquitin-conjugating enzyme
PF00173.23	16	Cyt-b5	Cytochrome b5
PF00107.21	16	ADH_zinc_N	Zinc-binding dehydrogenase
PF00501.23	15	AMP-binding	AMP-binding enzyme
PF00248.16	15	Aldo_ket_red	Aldo-keto reductase
PF00664.18	15	ABC_membrane	Transmembrane domain of ABC transporters
PF00149.23	15	Metallophos	Calcineurin-like phosphoesterase
PF12697.2	15	Abhydrolase_6	Alpha/beta hydrolase fold
PF00227.21	14	Proteasome	Proteasome subunit
PF03144.20	14	GTP_EFTU_D2	GTP-binding elongation factor family, EF-Tu/EF-1A subfamily
PF00122.15	14	E1-E2_ATPase	Proton ATPase
PF00176.18	14	SNF2_N	SNF2 family N-terminal domain

Se nadaljuje…

Nadaljevanje priloge B. Seznam proteinov *Wallemia ichthyophaga*, ki imajo 10 ali več predstavnikov določene domene Pfam.

PF08240.7	13	ADH_N	Alcohol dehydrogenase GroES-like domain
PF13465.1	13	zf-H2C2_2	Zinc-finger double domain
PF00134.18	12	Cyclin_N	Cyclin
PF01926.18	12	MMR_HSR1	50S ribosome-binding GTPase
PF00787.19	12	PX	PX domain
PF00621.15	12	RhoGEF	RhoGEF domain
PF00856.23	12	SET	SET domain
PF00515.23	12	TPR_1	Tetratricopeptide
PF00169.24	12	PH	Pleckstrin homology domain
PF00443.24	12	UCH	Ubiquitin carboxyl-terminal hydrolase
PF01399.22	12	PCI	PCI domain
PF12796.2	12	Ank_2	Ankyrin repeat
PF03372.18	11	Exo_endo_phos	Endonuclease/Exonuclease/phosphatase family
PF03443.9	11	Glyco_hydro_61	Glycoside hydrolase family 61
PF01423.17	11	LSM	LSm
PF00702.21	11	Hydrolase	Haloacid dehalogenase-like hydrolase
PF00439.20	11	Bromodomain	Bromodomain
PF00293.23	10	NUDIX	Nudix family
PF00155.16	10	Aminotran_1_2	Aminotransferase class I and II
PF00171.17	10	Aldedh	Aldehyde dehydrogenase family
PF00096.21	10	zf-C2H2	Zinc finger
PF00160.16	10	Pro_isomerase	Cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD

PRILOGA C

Evolucija proteinskih družin. Rezultati analize po metodi CAFE. Za vsako družino je prikazana vrednost P njene obogatitve ali krčenja za celotno drevo, kot tudi za razvejitev, ki vodijo k *W. ichthyophaga*, *W. sebi*, in k *Wallemia* spp., ter Agaricomycotina. Predstavljene so zgolj družine z vrednostjo P manjšo od 0,01.

Additional file 3: Table S2:

Evolution of protein families. Results of the analysis with the CAFE software. For each family, the P values for the family expansion/ contraction are shown for the whole tree, as well as for the branches leading to *W. ichthyophaga*, *W. sebi*, both *Wallemia* spp., and Agaricomycotina. Only families with a family-wide P-value lower than 0.01 are shown.

		Viterbi P-values for specific nodes					
PFAM domain	**Family-wide P-value**	**Agaricomycotina**	**and**	**Wallemiomycetes**	***W. ichthyophaga***	**and**	***W. sebi***
PF01185.13	0	**0,579676**	**and**	**0,000115**	**0**	**and**	**0,000362**
PF07690.11	0	**0,083393**	**and**	**0,002657**	**0**	**and**	**0,00001**
PF04082.13	0	**0,253097**	**and**	**0,118608**	**0,000001**	**and**	**0,007802**
PF13371.1	0	**0,528973**	**and**	**0,764375**	**0,000003**	**and**	**0,03508**
PF00075.19	0	**0,555452**	**and**	**0,341929**	**0,00001**	**and**	**0,000001**
PF00005.22	0	**0,28712**	**and**	**0,017243**	**0,000028**	**and**	**0,073854**
PF13813.1	0	**0,542509**	**and**	**0,07204**	**0,000044**	**and**	**0,083689**
PF01061.19	0	**0,631721**	**and**	**0,225772**	**0,00005**	**and**	**0,005878**
PF07719.12	0,002	**0,622192**	**and**	**0,724129**	**0,000073**	**and**	**0,007343**
PF00394.17	0	**0,579676**	**and**	**0,06076**	**0,000228**	**and**	**0,534459**
PF00560.28	0	**0,542509**	**and**	**0,813748**	**0,000228**	**and**	**0,017957**
PF01535.15	0,001	**0,579676**	**and**	**0,289571**	**0,000449**	**and**	**0,023932**

Se nadaljuje…

Nadaljevanje priloge C. Evolucija proteinskih družin.

PF13516.1	0	**0,567832**	**and**	**0,607634**	**0,000449**	**and**	**0,023932**
PF06422.7	0	**0,567832**	**and**	**0,607634**	**0,000449**	**and**	**0,545143**
PF02656.10	0,005	**0,567832**	**and**	**0,607634**	**0,000449**	**and**	**0,545143**
PF00664.18	0	**0,725376**	**and**	**0,066545**	**0,000624**	**and**	**0,105067**
PF13520.1	0	**0,601859**	**and**	**0,157357**	**0,000738**	**and**	**0,029895**
PF13460.1	0	**0,579676**	**and**	**0,642152**	**0,000738**	**and**	**0,00236**
PF00098.18	0	**0,649601**	**and**	**0,002774**	**0,001095**	**and**	**0,565399**
PF00010.21	0	**0,601859**	**and**	**0,359639**	**0,001095**	**and**	**0,035843**
PF02668.11	0	**0,59101**	**and**	**0,669135**	**0,001095**	**and**	**0,035843**
PF00690.21	0	**0,59101**	**and**	**0,669135**	**0,003378**	**and**	**0,001095**
PF13639.1	0	**0,666035**	**and**	**0,160115**	**0,003821**	**and**	**0,610202**
PF07883.6	0,001	**0,514814**	**and**	**0,67503**	**0,005987**	**and**	**0,017821**
PF02170.17	0,002	**0,514814**	**and**	**0,67503**	**0,005987**	**and**	**0,017821**
PF06325.8	0,01	**0,514814**	**and**	**0,67503**	**0,005987**	**and**	**0,017821**

PRILOGA D

Dvanajst poti po KEGG bazi s signifikantno višjim deležem diferenčno izraženih genov kot pričakovano v primerjavi transkriptomov pri 10 % in 30 % NaCl (m/v).

Additional file 6: Table S5:

KEGG database pathways with a significantly higher proportion of differentially expressed genes than expected (when comparing transcriptomes at 10% and 30% NaCl [w/v]).

	Pathway	DEGs with pathway annotation (341)	All genes with pathway annotation (2951)	Pvalue	Qvalue	Pathway ID
1	**Starch and sucrose metabolism**	16 (4.69%)	44 (1.49%)	**1,42E+01**	0.001302875	ko00500
2	**Peroxisome**	17 (4.99%)	55 (1.86%)	**8,54E+01**	0.002491536	ko04146
3	**Biosynthesis of secondary metabolites**	51 (14.96%)	269 (9.12%)	**0.0001295401**	0.002491536	ko01110
4	**Fructose and mannose metabolism**	18 (5.28%)	63 (2.13%)	**0.0001650053**	0.002491536	ko00051
5	**Butanoate metabolism**	17 (4.99%)	58 (1.97%)	**0.0001779237**	0.002491536	ko00650
6	**Pentose and glucuronate interconversions**	16 (4.69%)	53 (1.8%)	**0.0001884938**	0.002491536	ko00040
7	**Linoleic acid metabolism**	13 (3.81%)	38 (1.29%)	**0.0001895734**	0.002491536	ko00591
8	**Pyruvate metabolism**	19 (5.57%)	72 (2.44%)	**0.0003417571**	0.003930207	ko00620
9	**Metabolic pathways**	104 (30.5%)	699 (23.69%)	**0.001301264**	0.011371986	ko01100
10	**Limonene and pinene degradation**	14 (4.11%)	51 (1.73%)	**0.001359694**	0.011371986	ko00903
11	**Galactose metabolism**	14 (4.11%)	51 (1.73%)	**0.001359694**	0.011371986	ko00052
12	**Methane metabolism**	10 (2.93%)	31 (1.05%)	**0.001765991**	0.013539264	ko00680

PRILOGA E

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