Pathogenomic analysis of *Lichtheimia* species as a model for basal human pathogens of the order Mucorales

Dissertation

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One thing you can say for enemies; they make life more interesting.

The Traitor's Hand – Sandy Mitchell

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List of abbreviations

AS	Alternative splicing
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BPS	Bathophenanthrolinedisulfonic acid
cDNA	Complementary DNA
CFW	Calcofluor white
CO_2	Carbon dioxide
C / N - source	Carbon / nitrogen source
DAGAT	Diacylglycerol acyltransferase
DFO	Deferoxamine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
e.g.	Example given
ECM	Extracellular matrix
Ed(s)	Editor(s)
ER	Endoplasmatic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
et al	Et alii (lat. “and others”)
FAC	Ferric ammonium citrate
$FeCl_2$	Iron chloride
FLD	Farmer’s lung disease
GdA	Geldanamycin
GO	Gene Ontology
GPI	Glycophosphatidylinositol
h	Hour(s)
Hb	Haemoglobin

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	Human immunodeficiency virus
HOG	High osmolarity glycerol
HSF	Heat shock factor
HSP	Heat shock protein
H_2O_2	Hydrogen peroxide
i.e.	Id est (lat. “that is”)
ID	Identifier
IPS	Insect physiological saline
Kbp	Kilobase pairs
KCl	Potassium chloride
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KNO3	Potassium nitrate
max	Maximal
Mb	Megabases
MD	Menadione
min	Minute
MyA	Million years ago
NaCl	Sodium chloride
ncRNA	Non-coding RNA
NH_4SO_2	Ammonium sulphate
nt	Nucleotide
O_2	Oxygen
p (pp)	Page(s)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QC	Quality control
qPCR	Quantitative PCR
RNA	Ribonucleic acid

RNA-seq	RNA sequencing
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
sHSP	Small HSP
snoRNA	Small nucleolar RNA
SNP(s)	Single nucleotide polymorphism(s)
snRNA	Small nuclear RNA
sp. (spp.)	Species (singular / plural)
SRP RNA	Signal recognition particle RNA
TF	Transcription factor
tRNA	Transfer RNA
UPR	Unfolded protein response
vs.	Versus (lat. “against”)
WGD	Whole genome duplication
w/v	Weight to volume
YNB	Yeast nitrogen base

1 Introduction

1.1 Diversity of human pathogenic fungi

The fungal kingdom encompasses more than 100,000 described species and an estimated total number of 1.5 to 5 million species (de Hoog et al, 2010; Hibbett et al, 2011; Hawksworth, 2001; Blackwell, 2011). The majority of fungi are saprotrophic organisms that degrade organic matter in the environment. However, a variety of species are known to cause infections in plants or animals. Plant pathogenic fungi are of special interest as they affect agriculturally relevant plants and can cause dramatic losses (Fisher et al, 2012). Fungal infections in animals affect especially ectothermic groups such as amphibians and insects (Kilpatrick et al, 2010; Boomsma et al, 2013). In contrast, only a few hundred fungal species from different phyla have been described to cause infections in humans and other mammalian hosts (de Hoog et al, 2000).

1.1.1 Classification of mycoses

Fungal infections can be categorized by different concepts. One concept is the classification of the pathogen based on the immune status of the infected host. Primary pathogenic species can infect immunocompetent hosts but only a minority of fungi belongs to this group. In contrast, most fungal pathogens rely on an impaired immune system of the host in order to cause infections. This type of pathogens is called opportunistic pathogens and includes many well-known species such as *Candida* species, *Aspergillus fumigatus* and *Cryptococcus neoformans*. The second classification is based on the route of acquisition, which can be either exogenous or endogenous. Exogenous infections are caused by organisms from the environment, which are taken up via the respiratory tract, the skin or other ways. This group contains many important fungal pathogens including *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Fusarium* species and mucoralean fungi (Hajjeh and Warnock, 2001; Springer et al 2014; Richardson, 2009). In contrast, endogenous infections are caused by organisms which are already inside the body for example as part of the normal flora like *Candida* species. A third classification is based on the site of infection. Superficial infections involve only the outer layers of the body such

as the skin or nails and are common fungal infections affecting up to 20-25 % of the global population (Havlickova et al, 2008; Brown et al, 2012). These infections are primarily caused by dermatophyte fungi, which are specialised to the growth on these substrates and are able to degrade keratin (Havlickova et al, 2008; Burmester et al, 2011). Infections can also occur frequently on the mucosa of the oral or genital tract and are mainly caused by different *Candida* species (Brown et al, 2012). While these superficial infections are widely distributed among humans, they are in general not life-threatening and mostly curable. Some fungi, such as *Sporothrix schenckii,* are able to cause cutaneous or subcutaneous infections, which involve the invasion of deeper parts of the skin (Pang et al, 2004). The most severe types of infections are systemic mycoses, which can affect single internal organs or become disseminating infections when the pathogen invades into the blood vessels and spreads to other organs. This type of infection is caused by a variety of fungi such as *Candida albicans*, *Aspergillus fumigatus*, *Histoplasma capsulatum* and *Cryptococcus neoformans* (Brown et al, 2012). While systemic mycoses occur less frequently, they are in general associated with a high mortality. Due to the difficult diagnosis, the limitation in therapeutic options and the consequent high mortality rates, more than 1.5 million people die from fungal infections per year (Brown et al, 2012).

1.1.2 Taxonomy of fungal pathogens

Fungal pathogens belong to different taxonomical groups within the fungal kingdom (Figure 1.1). Especially the derived fungal phyla, collectively described as Dikarya, are well-known for their pathogenic potential. The phylum Ascomycota encompasses a total of 68,230 species (personal communication by Paul M. Kirk, Royal Botanic Gardens Kew, UK; based on www.Indexfungorum.org as of October 2015) and 325 are described as human pathogens (de Hoog et al, 2010). Important examples are the opportunistic commensal *Candida albicans* but also the usually saprophytic species *Aspergillus fumigatus*, which cause more than 400,000 and 200,000 life-threatening infections per year, respectively (Brown et al, 2012). The phylum Basidiomycota encompasses 45,528 species (personal communication by Paul Kirk based on

www.Indexfungorum.org as of October 2015) and 37 species are known to cause infections (de Hoog et al, 2010) including *Cryptococcus neoformans*, which is involved in more than 1,000,000 cases per year (Brown et al, 2012).

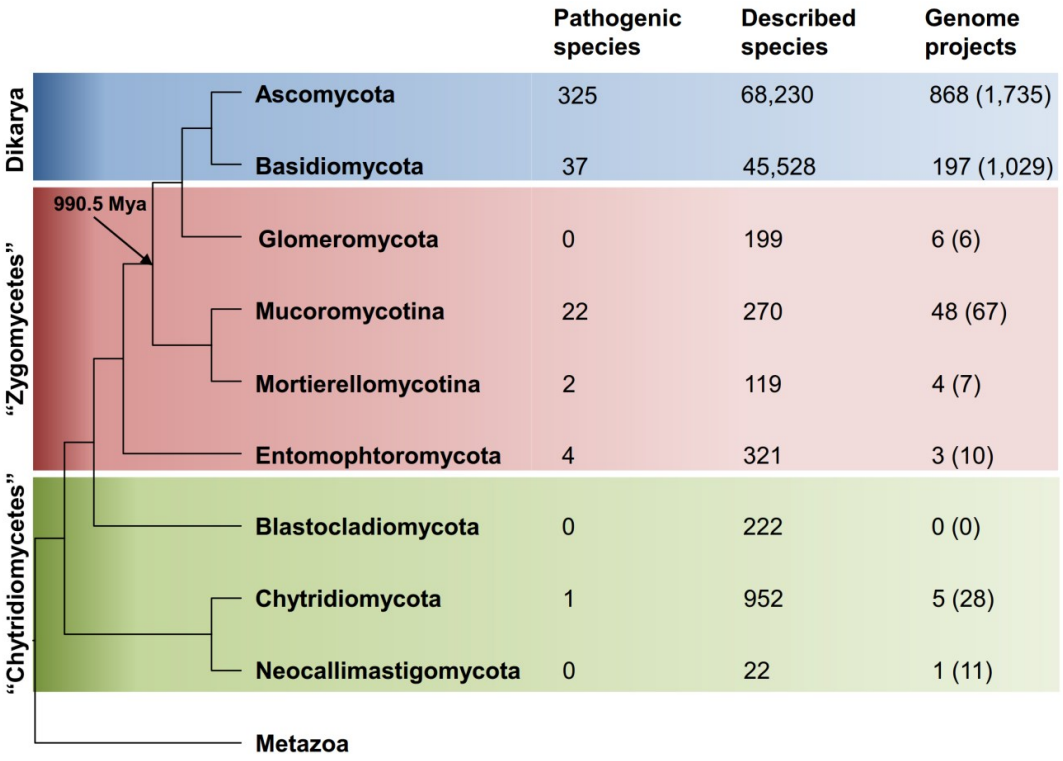

Figure 1.1: Phylogenetic distribution of fungal pathogens. Schematic phylogenetic tree of the fungal groups (simplified after James et al, 2006). Numbers of species are based on Indexfungorum (as of October 2015); numbers of clinically relevant species are based on the Atlas of Clinical Fungi (de Hoog et al, 2010). Numbers of whole genome sequencing projects are based on published sequences in Genbank (http://www.ncbi.nlm.nih.gov/genome as of October 2015) or registered projects in the GOLD database (Reddy et al, 2015) (indicated in brackets, only whole genome sequencing projects, as of October 2015).

However, also basal fungal groups show pathogenicity towards humans. The basal fungal lineages were classically divided into the aquatic Chytridiomycota and the land-living Zygomycota. These lineages separated from the higher fungi around 990 million years ago, which is similar to the divergence time of mammals and sponges (Hedges et al, 2006; Hedges et al, 2015). Both groups were found to be paraphyletic and several new systematic groups were established (Hibbett et al, 2007). Of the basal fungal lineages only three orders, which were previously included in the Zygomycota, contain pathogenic species, namely the Mortierellomycotina, Entomophthoromycotina

and Mucoromycotina. Previously, the term zygomycosis was used for all infections with organisms of the former Zygomycota. However, depending on the causative agents they were separated into entomophthoromycosis (also entomophthoramycosis) and mucormycosis (also mucoralomycosis). Mortierello- and Entomophthoromycotina contain only a small number of pathogens with 2 and 4 species, respectively (de Hoog et al, 2010). Since infections with Mortierellomycotina occur only very rarely, no specific term is established. *Mortierella* infections are usually found in cattle and infection in humans was only reported once (de Hoog et al, 2010, Layios et al, 2014).

In contrast, 22 human pathogenic species of the Mucoromycotina are known and represent the most common cause of infections with basal lineage fungi. The disease caused by members of the Mucoromycotina is called mucormycosis independent of the fungal species involved.

1.2 Mucormycosis

Fungi of the order Mucorales are known to be pathogens of warm-blooded animals since the 19th century but were believed to be only saprotrophic organisms on already damaged tissue in humans. The first case of mucormycosis in humans was reported in 1885 and *L. corymbifera* (formerly named *Mucor corymbifer*) was identified as the fungal species involved (Platauf, 1885). While mucormycosis does not occur frequently, the rapid progress of the disease and the difficult diagnosis lead to high mortality rates. Moreover, mucoralean fungi are resistant to several antifungals and treatment often involves massive debridement of infected tissue. Mucormycosis occurs worldwide in patients with different risk factors and possess a wide range of clinical manifestations.

1.2.1 Epidemiology and risk factors

Mucormycoses are rare fungal infections causing an estimated number of 10,000 life-threatening infections worldwide per year (Brown et al, 2012). The incidence of mucormycosis worldwide ranges between 0.4 to 2 cases per million, which is about ten-fold lower than invasive aspergillosis (Beardsley et al, 2015; Klimko et al, 2015; Rodriguez-Tudela et al, 2015). However, higher rates have been observed in certain geographical regions such as Qatar (Taj-Aldeen et al, 2015). While the number of mucormycosis infections is still low, there appears to be an increase in the clinical cases over the last decades (Roden et al, 2005; Bitar et al 2009). This might be caused by the increasing number of risk-group patients such as patients undergoing transplantations, corticosteroid treatments, suffering from diabetes or malignancies and the prolonged survival of such patients. However, also the increasing prophylactic use of voriconazole in patients may play a role in the increasing number of mucormycosis cases (Pongas et al, 2009). Finally, also improved diagnosis and increased awareness of physicians may be a possible explanation for the increase of diagnosed mucormycosis.

Patients suffering from diabetes are the main risk group for the development of mucormycosis worldwide. More than one third of mucormycosis cases were associated with diabetes in the most comprehensive global study to date (Roden et al, 2005). However, there seem to be differences in the incidence of diabetes-associated mucormycosis between geographical regions. While a study from India found an even higher association of diabetes and mucormycosis (>70 %), recent European studies showed a lower association (9-23 %) (Chakrabarti et al, 2006; Skiada et al, 2011; Lanternier et al, 2012a). Possible explanations for this observation are differences in the diagnosis and treatment of diabetes between the countries and the incidence of uncontrolled diabetes (Meis and Chakrabarti, 2009; Taj-Aldeen et al, 2015). Diabetic ketoacidosis was confirmed as an important risk factor in a mouse infection model for *Rhizopus arrhizus* (= *R. oryzae*) and macrophages of diabetic mice were less able to clear spores of the fungus (Ibrahim et al, 2010a; Liu et al, 2010; Waldorf, 1984a; Waldorf et al, 1984b). Recently, the glucose-regulated endothelial receptor GRP78 was found to be involved in the adherence and invasion of *R. arrhizus* into endothelial cells (Liu et al, 2010). The higher expression of GRP78 in diabetic conditions could be

directly linked to the pathogenicity of mucoralean pathogens supporting the link between diabetes and mucormycosis (Liu et al, 2010).

Untreated diabetes is relatively uncommon in European countries and immunosuppression represents the most important risk factor for the development of mucormycosis. This includes mostly patients suffering from haematological malignancies but also patients undergoing solid organ or bone marrow transplantations. Recent studies from Europe identified haematological malignancy as major risk factor representing 44-50 % of the reviewed cases (Skiada et al, 2011; Lanternier et al, 2012a). In addition, the increased incidence of mucormycosis could be correlated to the increasing numbers of patients suffering from haematological malignancies in a Belgian hospital (Saegeman et al, 2010). In contrast, malignancy was only found as the second-most common predisposition for mucormycosis in a global study and played almost no role in a study from India (Roden et al, 2005; Chakrabarti et al, 2006). Contrary to the situation in other fungal infections, mucormycosis plays only a minor role in HIV patients (Roden et al, 2005; Skiada et al 2011; Lanternier et al, 2012a).

The increasing use of the antifungal voriconazole has also been suggested as a risk factor for mucormycosis and cultivation of *R. arrhizus* on voricozole-containing medium increased the virulence in animal infections (Pongas et al, 2009; Lamaris et al, 2009). However, the mechanism for the increase of virulence is still unclear and recent data show that *in vivo* treatment with voriconazole without pre-exposure of the fungus does not alter virulence (Lewis et al, 2011). In addition, mucormycosis was found in dialysis patients undergoing therapy with the iron chelator deferoxamine. Mucoralean fungi can use deferoxamine as xenosiderophore *in vitro* and deferoxamine treatment increased mortality in a murine model of mucormycosis (Boelaert et al, 1993; Ibrahim et al, 2007). In contrast, application of other iron chelators like deferasirox protect mice from mucormycosis indicating that this chelator cannot be used as an iron source (Ibrahim et al, 2007). Mucormycosis can also occur in healthy individuals after penetrating trauma caused by accidents or following surgeries and account for 17-25 % of mucormycosis cases (Roden et al, 2005; Skiada et al, 2011; Lanternier et al, 2012a).

1.2.2 Clinical presentation

Clinical presentations of mucormycosis include cutaneous, rhino-orbito-cerebral, pulmonary, gastrointestinal and disseminating infections (Mantadakis and Samonis, 2009). The main route of infection is the inhalation of fungal spores and infections of the respiratory tract are the most common clinical presentations in adult patients accounting for more than 50 % of mucormycosis cases (Roden et al, 2005; Skiada et al, 2011; Lanternier et al, 2012a). Pulmonary infections are mainly associated with malignancies, while sinus infections are typical for diabetic patients (Roden et al, 2005; Skiada et al, 2011; Lanternier et al, 2012a).

Cutaneous and subcutaneous infections are reported frequently representing about 20 % of the cases (Roden et al, 2005; Skiada et al, 2011; Lanternier et al, 2012a). While these infections occur also in patients of the typical risk groups for mucormycosis, they are mainly associated with patients without underlying diseases following trauma (Lelievre et al, 2014; Roden et al, 2005). Direct inoculation of fungal material into the wounds is the most likely route of infection in these cases. Besides burns and wounds caused by major traffic accidents also smaller wounds have been implicated in cutaneous mucormycosis such as needle stick injuries or injuries caused by plants and animals (Ribes et al, 2000; Kobayashi et al, 2001; Roden et al, 2005; Skiada and Petrikkos, 2009; Skiada et al, 2011). However, cutaneous infections have also been reported as nosocomial infections following surgery and are often associated with the use of non-sterile medical devices and bandages (Roden et al, 2005; Skiada and Petrikkos, 2009; Skiada et al, 2011; Rammaert et al, 2012; Lanternier et al, 2012a; Duffy et al, 2014). Extension of the infection into deeper tissues is a frequently observed complication of cutaneous infections (Roden et al, 2005; Skiada and Petrikkos, 2009; Mantadakis and Samonis, 2009).

Gastrointestinal infections occur mainly in premature neonates and children, while they are less common in adults (Roilides et al, 2009).

Dissemination of the infection is reported frequently independent of the original site of infection especially in patients suffering from malignancies (Roden et al, 2005; Skiada and Petrikkos, 2009; Skiada et al, 2011; Lanternier et al, 2012a).

All forms of mucormycosis are associated with high mortality ranging between 30 % and 90 % depending on the site of infection (Brown et al, 2012). While mortality rates are lower in cutaneous infections (~30 %), pulmonary and rhino-orbito-cerebral infections result in higher mortality rates between 50 % and 80 % (Roden et al, 2005; Skiada and Petrikkos, 2009; Skiada et al, 2011; Lanternier et al, 2012a).

1.2.3 Species involved in mucormycosis

To date, all mucoralean pathogens can be classified as exogenous and opportunistic species. More than 20 mucoralean species, belonging to different clades within the Mucorales, are known to be involved in human infections. Three genera account for the majority of all mucormycosis, namely *Rhizopus*, *Mucor* and *Lichtheimia* (Figure 1.2; Gomes et al, 2011). The most common causes of mucormycosis are members of the family Rhizopodiaceae with *R. arrhizus* (= *R. oryzae*) as most prominent species worldwide (Figure 1.2; Roden et al, 2005; Alvarez et al, 2009; Skiada et al, 2011; Lanternier et al, 2012a). A second important group of mucoralean pathogens are *Mucor* species belonging to the family Mucoraceae with *M. circinelloides* as most common causative agent (Roden et al, 2005; Alvarez et al, 2009; Skiada et al, 2011; Lanternier et al, 2012a). Both families represent derived groups of the Mucorales and are quite closely related to each other compared to other mucoralean pathogens (Figure 1.2). In contrast, the family Lichtheimiaceae represents one of the most basal groups of mucoralean fungi and encompasses human-pathogenic fungi of the genera *Lichtheimia* and also *Rhizomucor* species depending on the family concept applied (Hoffmann et al, 2013). Infections with *Lichtheimia* species differ in frequency depending on the geographical region. Global and regional studies from the USA showed only small involvement of *Lichtheimia* species in mucormycosis, accounting for only 5 % of infections (Roden et al, 2005; Alvarez et al, 2009). In contrast, they were the second-most common causative agents in recent European studies (Lanternier et al, 2012a; Skiada et al, 2011). In addition, *Lichtheimia* species were found as the most common pathogens in a study from Egypt (Zaki et al, 2014). Other mucoralean pathogens are members of the Cunninghamellaceae, Syncephalastraceae and

Saksanaeaceae including pathogens like *Cunninghamella* spp., *Syncephalastrum racemosum*, *Apophysomyces* spp., *Saksenaea* spp., which are reported less frequently (Figure 1.2; Roden et al, 2005; Alvarez et al, 2009; Gomes et al, 2011; Skiada et al, 2011; Lanternier et al, 2012a).

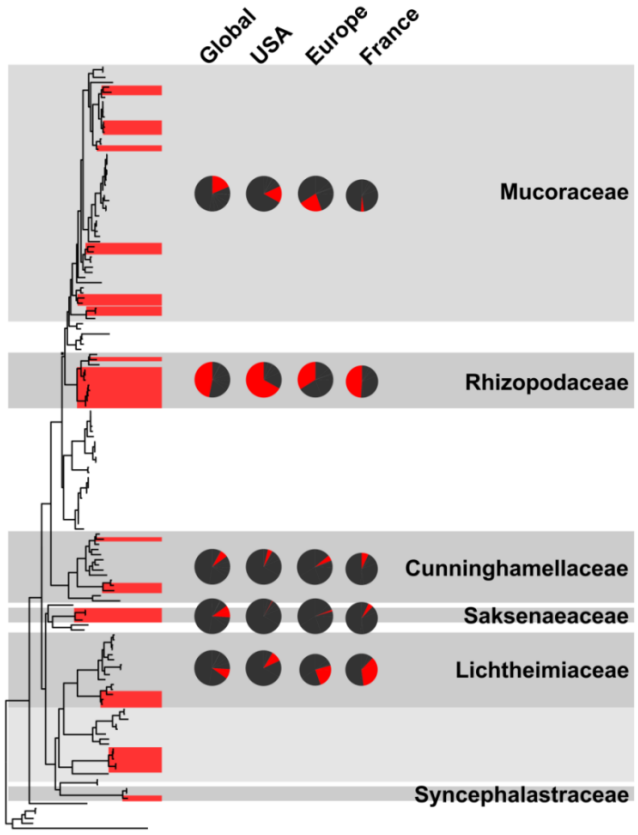

Figure 1.2: Phylogeny and frequency of mucoralean pathogens. The phylogeny and family concept are based on data published previously (Hoffmann et al, 2013). Clinically relevant species are indicated with red bars (de Hoog et al, 2010; Roden et al, 2005; Skiada et al, 2011; Vitale et al, 2012) and pie charts show the involvement of species of the indicated families in clinical infections based on comprehensive studies from different geographical regions (global: Roden et al, 2005; USA: Alvarez et al, 2009; Europe: Skiada et al, 2011; France: Lanternier et al, 2012a).

1.3 The genus *Lichtheimia*

1.3.1 Taxonomy and biology

1.3.1.1 Taxonomy

Lichtheimia species are part of the order Mucorales within the subphylum Mucoromycotina. To date, the genus comprises six species, namely, *L. corymbifera*, *L. ramosa*, *L. ornata*, *L. hyalospora*, *L. sphaerocystis* and *L. brasiliensis* (Alastruey-Izquierdo et al, 2010b; de A Santiago et al, 2014). *L. corymbifera* was described in 1884 as *Mucor corymbifer* and was the first species which was found to be involved in mammalian infections (Cohn, 1884; Platauf, 1885). In 1890 *L. ramosa* was described as *Rhizopus ramosus* (Vuillemin, 1903) but was later taken as a synonym for *L. corymbifera* (Nottebrock et al, 1974). *Lichtheimia* species underwent several taxonomic changes and were placed in the genus *Absidia* based on morphological similarities. However, phylogenetic analyses showed that thermotolerant species do not cluster with the core *Absidia* species but form a separate clade at the base of the Mucorales (Hoffmann et al, 2007). Based on these data the thermotolerant species were reclassified into *Mycocladus* spp., which was later corrected into *Lichtheimia* species in accordance with the International Code of Botanical Nomentclature (Hoffmann et al, 2009). *L. ramosa* was given back species rank in 2009 and a new species concept for the genus *Lichtheimia* was proposed in 2010 including five species (Garcia-Hermoso et al, 2009; Alastruey-Izquierdo et al, 2010b). Finally, a novel species was isolated from Brazilian soil and described as *L. brasiliensis* (de A Santiago et al, 2014).

1.3.1.2 Biology and distribution

Like most mucoralean fungi *Lichtheimia* species are saprotrophic fungi. To date, no comprehensive ecological data on the natural habitats of mucoralean species in the environment exist and due to the low number of isolates of *Lichtheimia* species, the distribution around the world is unclear. However, soil is believed to be the main habitat of these fungi but they are also found on different organic substrates like fruits, vegetables and dung.

Lichtheimia species are also present in many anthropogenic substrates and can be found regularly in farming environments including straw, grain and silage (Kotimaa et al, 1991; Reboux et al, 2006; Gbaguidi-Haore et al, 2009). In addition, they can be found on a diversity of fruits and food products. Recent studies identified *Lichtheimia* species as important contaminants of peanuts and cacao but also of processed food products like olive paste (Mphande et al, 2004; Copetti et al, 2011; Baffi et al, 2012). In contrast, Mucoralean fungi are also widely used in food production especially in Asian cuisine. Different species of *Lichtheimia* are associated with soy fermentation products but also starter cultures in rice wine production (Lee et al, 2010a; Yeun et al, 2011; Hong et al, 2012; Yang et al, 2011).

Like most other mucoralean fungi, *Lichtheimia* species propagate mainly by the formation of asexual, haploid and airborne spores, called sporangiospores. These spores are produced in high quantities during vegetative growth and form haploid mycelia, which then again produce asexual spores. Besides asexual reproduction also sexual reproduction has been observed in *Lichtheimia* species. To date it is unknown if zygospores play an important role in the reproduction of mucoralean fungi, since germination of zygospores was only rarely observed at least under laboratory conditions (Michailides and Spotts, 1988; Yi and Ko, 1997).

1.3.2 *Lichtheimia* infections and related diseases

Lichtheimia species are the second and third most common agent of mucormycosis in Europe and worldwide, respectively. Due to the low incidence of infections, species-specific data on the clinical presentations of different mucoralean pathogens is limited. Like most mucoralean pathogens the main route of infection appears to be the inhalation of asexual sporangiospores resulting in rhinoorbital and pulmonary infections (Lanternier and Lortholary, 2009; Petrikkos et al, 2012). Such infections are usually associated with unspecific symptoms like dyspnoea and pulmonary inflammation (Mattner et al, 2004; Zaki et al, 2014). Pulmonary *Lichtheimia* infections have been described in patients undergoing solid organ or bone marrow transplantation and patients who suffered from uncontrolled diabetes or leukaemia (Mattner et al, 2004; Barr et al, 2006; Däbritz et al, 2011; Kleinotiene et al, 2013; Zaki et al, 2013). Superficial infections including cutaneous and subcutaneous presentations are associated with similar risk groups as pulmonary infections but were also reported in persons without an underlying disease (Kobayashi et al, 1999; Skiada and Petrikkos, 2009). Typically, these infections develop following trauma and fractures due to accidents or following surgeries or burn injuries (Kobayashi et al, 2001; Thami et al, 2003; Pasticci et al, 2008; Almaslamani et al, 2009; Bibashi et al, 2012; Tyll et al, 2016). In addition, *Lichtheimia* infections occur in neonates and infants causing cutaneous, gastrointestinal but also pulmonary infections (Roilides et al, 2009; Irtan et al, 2013; Kleinotiene et al, 2013; Phulpin-Weibel et al, 2013). Disseminated infections are less common but have been reported in several cases independent from the original site of infection.

To date, only three of the six described species have been found in patient material, namely *L. corymbifera*, *L. ramosa* and *L. ornata* (Alastruey-Izquierdo et al, 2010b). *L. corymbifera* is most frequently reported to be involved in human infections representing more than 80 % of all cases in some studies (Skiada et al, 2011). However, since *L. corymbifera* and *L. ramosa* were used as synonyms for a long time, correct assessment of the frequency is problematic. Recent studies show a high incidence of incorrect species designation in *Lichtheimia* (Schwartze et al, 2012; Woo et al, 2012). Many cases which have been associated with *L. corymbifera* are actually caused by *L. ramosa* (Woo et al, 2012). While *L. corymbifera* and *L. ramosa* are relatively commonly found in

human infections, *L. ornata* was isolated from patient material only twice. Interestingly, all three clinical species showed comparable virulence in an alternative infection model using chicken embryos (Schwartze et al, 2012). In contrast, all strains of species which were never associated with human infection showed a lower virulence indicating differences in the pathogenic potential of *Lichtheimia* species (Schwartze et al, 2012; Schwartze et al, 2014a).

Besides their role in infectious diseases, *Lichtheimia* species seem also to be involved in Farmer's lung disease (FLD), a form of occupational hypersensitivity pneumonitis. The disease is caused by recurrent exposure to certain microorganisms and occurs mainly in farming personnel. *Lichtheimia* species are common contaminants of farming material and antibodies against *Lichtheimia* were found in sera of patients suffering from FLD (Reboux et al, 2001; Rognon et al, 2015). While this disease is not lethal, it causes severe health issues including influenza-like symptoms in its acute form; dyspnea and coughing in its chronic form (Selman et al, 2010).

1.4 Virulence-associated traits of mucoralean pathogens

The ability of an organism to cause disease in a susceptible host is called pathogenicity. Based on this concept, organisms can be classified as pathogenic or non-pathogenic. The severity of the infection and mortality caused by a pathogen is described as virulence (Pirofski and Casadevall, 2012). Microbial characteristics that contribute to the virulence of a pathogen are called virulence factors. Pathogenicity is multifactorial and fungal virulence factors include molecules involved in the adhesion, invasion and damage of host cells as well as factors which are involved in the immune evasion of the pathogen (Hogan et al, 1996; Abad et al, 2010; Behnsen et al, 2010; Mayer et al, 2013). In addition, contributory factors support the survival and growth of the fungus in the host such as metabolic flexibility and hydrolytic enzymes, the uptake of different nutrients, growth at elevated temperatures and resistance towards stresses (Hogan et al, 1996; Brock et al, 2009; Abad et al, 2010; Mayer et al, 2013; Brown et al, 2014). Due to the low attention which has been paid to mucoralean pathogens and the pathogenesis of these fungi in the last decades, little is known about the virulence factors involved in their infection process. Based on to the available genome

sequences and new technologies, recent studies on specific virulence factors in *Rhizopus* and *Mucor* species revealed the involvement of hydrolytic enzymes, iron uptake mechanisms, surface proteins but also morphological features in the pathogenicity. The features and their contribution during infection will be discussed in this section.

1.4.1 Hydrolytic enzymes

Fungi are osmotrophic organisms and degrade polymeric nutrients in the environment by enzymes in order to take them up. Such hydrolytic enzymes are also well-known virulence factors in a variety of fungal pathogens. They play a role in the nutrition of fungal pathogens inside the host but are also involved in the destruction of host tissue and immune evasion. Proteins are a major carbon and nitrogen source for the fungus during the infection and different proteases are well-known virulence factors of fungal pathogens. For example, secreted aspartyl proteases (SAPs) of *C. albicans* are involved in adhesion, invasion and damage in the host and are required for the full virulence of the fungus (Schaller et al, 2005). In addition, the secreted aspartyl protease Alp1 of *A. fumigatus* was shown to degrade factors of the complement system, which is an essential part of the innate immunity (Behnsen et al, 2010).

The genome of *R. arrhizus* was published in 2009 and analyses revealed a whole-genome duplication in this species which resulted in the expansion of different gene families including proteases (Ma et al, 2009). The number of SAP genes was twice as high as in *C. albicans* and all other fungi analysed. While there is no data available on the specific functions of these proteases, data from the closely-related *R. microspores* demonstrated a possible role for secreted protease in the pathogenesis of *Rhizopus* species. *In vivo* expression of two *R. microsporus* proteases was shown by the presence of specific antibodies in the serum of patients suffering from mucormycosis caused by *R. microsporus* (Schoen et al, 2002; Spreer et al, 2006). The purified enzyme of the aspartyl protease activated bovine blood coagulation factor X *in vitro* (Schoen et al, 2002). In addition, the enzyme shows also hydrolytic activity on human fibrinogen, which resulted in the formation of fibrinogen precipitates but not complete clots (Rüchel et al, 2004). However, complete

clotting could be observed in the presence of leukocyte elastase. The results suggest a role of proteases in the formation of thrombosis during infection.

1.4.2 Iron uptake mechanisms

Iron is essential for the development and growth of all organisms. Inside the host, free iron is limited and efficient iron uptake mechanisms are crucial for fungal pathogens in order to cause infections (Howard, 1999; Johnson, 2008; Sutak et al, 2008; Abad et al, 2010). Patients with elevated serum iron levels have been shown to be more susceptible to mucormycosis, which points out the importance of iron uptake in mucoralean pathogens (Sugar, 1992; Ibrahim, 2014).

Pathogenic fungi can take up iron by different mechanisms. The first one is the reductive pathway, where the ferric form of iron is reduced into its ferrous form and subsequently taken up by a permease (Howard, 1999; Ibrahim et al, 2008a). This system plays a crucial role in the virulence of *C. albicans* and consists of three elements: i) iron reductases ii) multicopper oxidases and iii) iron permeases (Ramanan and Wang, 2000; Almeida et al, 2009). Analyses of the reductive uptake system in *R. arrhizus* revealed the presence of a homologue of the high-affinity iron permease FTR1, which was highly expressed *in vitro* under low-iron conditions and in *in vivo* during infection in a mouse model (Fu et al, 2004; Ibrahim et al, 2010b). Inhibition of FTR1 expression resulted in reduced growth under low-iron conditions and reduced virulence in mouse infection experiments (Ibrahim et al, 2010b). Interestingly, the data suggest that the reductive pathway was involved in the utilisation of various iron sources including inorganic iron sulphate or iron chloride but also organic substances like haem (Ibrahim et al, 2010b).

The production and uptake of siderophores is the second common mechanism of iron acquisition in fungal pathogens (Howard, 1999). Siderophores play an important role in the virulence of a variety of fungi including *A. fumigatus*. While the reductive pathway is dispensable in this species, siderophore production is essential for virulence (Schrettl et al, 2004). Hydroxamate-class siderophores represent the majority of fungal siderophores (Haas, 2014). However, to date no

production of hydroxamate siderophore was found in mucoralean fungi. Instead carboxylate-type siderophores are produced by a variety of mucoralean species (Thieken and Winkelmann, 1992; Drechsler et al, 1995; Larcher et al, 2013). The role of these siderophores during infection is doubtful since they are not able to bind iron in the presence of human serum (Boelaert et al, 1993; de Locht et al, 1994). While the production of siderophores appears to be of little importance in the virulence of mucoralean species, the uptake of iron from siderophores produced by other species (xeno-siderophores) seems to play an important role. Patients undergoing therapy with the iron chelator deferoxamine (DFO) were found to be more susceptible to mucormycosis and infection experiments in mice showed that virulence of *R. arrhizus* was increased when animals were treated with deferoxamine (Ibrahim et al, 2007; Symeonidis, 2009; Ibrahim, 2014). It was shown that *Rhizopus* species could utilise iron-loaded deferoxamine (ferrioxamine) as an iron source even in the presence of human serum (de Locht et al, 1994; Boelaert et al, 1993). The process is energy dependent but did not rely on the uptake of the siderophore into the cell since albumin-bound ferrioxamine could also serve as iron source in these experiments (de Locht et al, 1994). In contrast, recent studies using a fluorescence-labelled derivate of deferoxamine showed uptake of the siderophore into the cells (Larcher et al, 2013). Interestingly, the reductive pathway seems to play a role in the utilisation of deferoxamine. Reduced expression of FTR1 in *R. arrhizus* resulted in a reduced ability to grow on ferrioxamine as sole iron source (Ibrahim et al, 2010b; Liu et al, 2015). In addition, a recent study could identify two genes which were highly expressed in the presence of ferrioxamine (Liu et al, 2015). The corresponding proteins, named Fob1 and Fob2, were located on the cell surface and were able to bind ferrioxamine *in vitro*. Reduced expression of FOB1 and FOB2 resulted in reduced virulence of *R. arrhizus* in deferoxamine-treated mice but not in a diabetic mouse model (Liu et al, 2015). A third mechanism for iron-uptake is the utilisation of organic iron-containing molecules like haem. While two haem oxygenases have been identified in the *R. arrhizus* genome, nothing is known about their involvement in the infection process (Ibrahim et al, 2012). However, the reductive pathway seems to be involved also in the utilisation of haem since reduced expression of FTR1 in *R. arrhizus* resulted in low growth on medium containing haem as sole iron source (Ibrahim et al, 2010b). To date, iron uptake mechanisms have only been analysed in *R. arrhizus* but no other mucoralean species.

1.4.3 Surface proteins

Rapid invasion of blood vessels is a major hallmark of mucormycoses (Sugar, 1992). *In vitro* experiments with human endothelial cells revealed that adhered spores or germlings of *R. arrhizus* enter endothelial cells by phagocytosis and subsequently cause cell damage (Ibrahim et al, 2005a). Viability of the fungus was not required for the observed interaction. Interestingly, no damage was observed when endothelial cells were confronted with clinical isolates of *Mucor* sp. indicating differences in the interaction of mucoralean fungi with endothelial cells (Ibrahim et al, 2005a). The endoplasmatic reticulum (ER) chaperone GRP78 was found to be one of the major surface proteins of endothelial cells involved in the invasion and damage caused by *R. arrhizus* (Liu et al, 2010). Increased expression of GRP78 in endothelial cell lines enhanced invasion and damage by *R. arrhizus*. Interestingly, GRP78 was found to be expressed at higher levels in the presence of high levels of iron or glucose, which can also be found in diabetic ketoacidosis patients (Liu et al, 2010). Binding of GRP78 to germlings was found in a variety of mucoralean pathogens. Recently, a family of proteins, containing a functional domain similar to the bacterial spore coat protein CotH, was identified as ligand for GRP78 (Gebremariam et al, 2014). Homologues of the corresponding genes were found in all mucoralean species investigated and two members were found to be interacting with GRP78 in *R. arrhizus* (Gebremariam et al, 2014). Both proteins are attached to the cell wall via a glycosylphosphatidylinositol (GPI) anchor. This post-translational modification is synthesized and transferred to the protein in the endoplasmatic reticulum and links the protein to the cell wall β-glucan (Orlean and Menon, 2007). Heterologous expression of CotH2 and CotH3 in *Saccharomyces cerevisiae* increased adhesion to and endocytosis by endothelial cells. Accordingly, reduced expression of CotH2 and CotH3 reduced the ability of *R. arrhizus* to invade and damage endothelial cells (Gebremariam et al, 2014). Blocking of the GRP78-CotH interaction by antibodies reduced the virulence of *R. arrhizus* in mouse infection experiments, underlining the importance of this mechanism in the pathogenicity of mucoralean pathogens (Liu et al, 2010; Gebremariam et al, 2014). While CotH was found in many mucoralean species, the role of the different copies has only been investigated in *R. arrhizus* so far.

1.4.4 Mating type dimorphism and spore size

Most mucoralean species are heterothallic and two different mating types, called (+) and (-), are required for sexual reproduction. The corresponding sex locus has been first identified in *Phycomyces blakesleeanus* (Idnurm et al, 2008). The core element in both mating types is a homeodomain transcription factor, which exists in two allelic versions called sexM and sexP in the (-) and (+) mating type, respectively. The transcription factor is flanked by a putative triose-phosphate transporter and a RNA helicase. Additional studies showed a similar architecture of the sex locus in other mucoralean fungi including *R. arrhizus* and *Mucor circinelloides* (Gryganskyi et al, 2010; Li et al, 2011). Interestingly, a link between mating types and virulence of strains was found in *M. circinelloides*. Strains of the (-) mating type produced bigger spores than (+) mating types and were more virulent in wax moth larvae (Li et al, 2011). Spores of the two mating types also differed in the interaction with macrophages. While the small (+) mating type spores stayed inside the macrophage, the large (-) type spores germinated and lysed the macrophages (Li et al, 2011). However, while the spore size was coupled to the mating type, the sex locus itself did not contribute to the differences in the spore size directly since deletion mutants of SexM showed no changes in morphology or virulence. In contrast, mutants defective in the calcineurin signalling pathway produced large spores, which also escaped from macrophages by the formation of hyphae. The results indicate that the differences in the cell signalling between the mating types may play a role in the morphological differences (Lee et al, 2013). The phenomenon has not been never been observed in other mucoralean species.

1.4.5 Dimorphism: Hyphal-to-yeast transition

The yeast-hyphal transition plays a role in the pathogenicity of several fungal pathogens such as *C. albicans*, *C. neoformans, Histoplasma capsulatum* and *Penicilliium marneffei* (Mayer et al, 2013; Boyce and Andrianopoulos, 2015). Some mucoralean fungi are known to undergo phenotypic switching under certain environmental conditions but only yeast stages of *Mucor* species have been

studied in more detail. *M. circinelloides* grows in the hyphal stage under high oxygen conditions but switches to the yeast growth in low oxygen and high carbon dioxide conditions (Lee et al, 2013). In addition, a variety of chemicals such as fungicides, metabolic inhibitors and antibiotics were shown to induce yeast-like growth in other *Mucor* species (Fisher, 1977). Analyses of the genetic background for the phenotypic switching revealed the involvement of the calcineurin signal transduction pathway in the process (Lee et al, 2013). Chemical or genetical inhibition of the calcineurin signalling resulted in a lock of the fungus in the yeast stage. The mutant strains were found to be less virulent, underlining the importance of hyphae formation in the infection process of *M. circinelloides* (Lee et al, 2013). While yeast stages of *M. circinelloides* were found in human samples (Cooper, 1987), it is unclear if they contribute directly to the infection process during mucormycosis.

1.5 Fungal genome projects and pathogenomics

The first completely sequenced eukaryotic genome was from the fungus *Saccharomyces cerevisiae* and was published in 1996 (Goffeau et al, 1996). The progress of fungal genome projects was initially slow and a total of only 23 genomes were sequenced in the following ten years. However, due to the decrease of costs for sequencing and the improvement of bioinformatics tools for assembly and gene predictions numerous new fungal genome projects were established.

1.5.1 Strategies and methods in fungal pathogenomics

Whole genome sequencing represents an important tool for all fields of biology. The availability of genome sequences and the encoded genes give first insights into genomic structure and help to identify genes which are involved in specific processes. Moreover, high-throughput screenings for gene expression, such as microarrays and RNA-sequencing, depend on the good quality of a reference genome. Also identification of proteins from proteomic screenings can be improved by

the availability of the genome of the corresponding species. Finally, the creation of mutants relies on data about the sequence of the gene of interest and its surrounding region, which can be provided by whole genome sequencing projects.

The term pathogenomics describes the application of genomics tools in order to study the pathogenicity mechanisms and the identification of virulence factors in plant, animal or human pathogenic species. Analyses and comparison of the genomes of pathogens with non- or less pathogenic isolates of the same species or closely-related species help to identify factors that are involved in the infection process (Demuth et al, 2008; Pallen and Wren, 2007). In addition, putative virulence factors can be identified by gene expression studies of the pathogen during the interaction with the host or under host-like conditions using transcriptomic and proteomic approaches.

Specific virulence factors of pathogenic species can be defined by the molecular Koch's postulates, which were introduced by Stanley Falkow (Falkow, 1988). Based on these postulates, virulence factors should fulfil several requirements. The genes should occur only in pathogenic strains of a species or pathogenic members of a genus. In addition, deletion of the genes should result in reduced virulence of the organism and re-introduction of the wildtype gene into the mutant should restore the wildtype virulence (Falkow, 1988). However, while these postulates represent good criteria for the identification of virulence factors, there are limitations since for a variety of pathogens no tools for genetic manipulation are available. Furthermore, putative virulence factors might be essential genes and mutants of these genes are not viable (Falkow, 2004). In such cases, the expression of the gene during the infection can be used as additional evidence for the role in infections (Falkow, 2004).

Pathogenomic studies are frequently applied for bacterial pathogens but become more and more important also for fungal species due to the decreasing costs and improved technologies for sequencing and analyses (Pallen and Wren, 2007; Demuth et al, 2008; Guttman et al 2014).

Due to their economic importance especially plant-pathogens have been studied extensively using pathogenomic approaches (Guttman et al, 2014; Schmidt and Panstruga, 2011). Important examples are species from the genera *Fusarium* (Chiara et al, 2015; Ma et al, 2013), *Verticillium*

(Klosterman et al, 2011; Tan et al, 2015) and *Magnaporthe* (Dean et al, 2005; Chen et al, 2013; Park et al, 2013).

The genomes of the most common human-pathogenic fungi are available and a lot of progress in the understanding of the diversity of infection strategies and the genes involved has been made during the last decade. Comparative genome analyses of species with different pathogenic potential have been performed for many important fungal pathogens such as species of *Aspergillus*, *Candida* and *Cryptococcus*, which increased the knowledge about genome evolution, adaptation to and the factors involved in pathogenicity (Galagan et al, 2005b; Moran et al, 2011). Interestingly, several common mechanisms were found to be involved in the emergence of pathogenicity factors. One of the main factors seems to be gene duplication, which increases the copy number of a certain gene in the genome. Duplicated genes can increase the expression levels of the gene, may undergo diversification resulting in lineage-specific neo-functionalization of the gene or may get lost during evolution.

Comparative analyses in fungal human pathogens revealed the presence of pathogen-specific genes, which most likely result from gene duplication and diversification. However, also increased copy numbers of certain genes and expansions of specific gene families with different biological functions were found in pathogenic species, that are involved in the infection process (Loftus et al, 2005; Fedorova et al, 2008; Butler et al, 2009; Gabaldón et al, 2013; Burmester et al, 2011; Muñoz et al, 2015; Farrer et al, 2015). Interestingly, these genes are located in close proximity in many cases and form so-called “genomic islands”, which were described for a variety of fungal pathogens and are often located in telomeric regions (Fedorova et al, 2008; Butler et al, 2009; Moran et al, 2011). In addition to the gain of genes in pathogenic species, loss of genes also plays a role in the adaptation to certain host niches but may also result in reduced virulence of certain strains or species. A recent study showed that *Malassezia* species, which are adapted to life on human skin, lost a large set of genes of the carbohydrate metabolism since they were dispensable for the growth on the carbohydrate-poor human skin (Wu et al, 2015). Such metabolic reductions have also been described in other human-adapted fungi like *Candida* species (Dujon et al, 2004; Galagan et al, 2005b; Moran et al, 2011). Specific loss of metabolic genes is mainly present in obligate host-associated pathogens and opportunistic commensals but not in saprophytic

opportunists. Comparative analyses of *Aspergillus* species revealed the presence of many pathogen-specific genes in *A. fumigatus*, which cluster in special genomic islands and may be involved in the infection process. The results of the studies indicate that the differences in the genetic makeup of the *Aspergillus* species are due to a lineage-specific combination of gene duplication, diversification and gene loss (Nierman et al, 2005; Fedorova et al, 2008). However, differences between pathogenic and non-pathogenic species can be more subtle due to positive selection of genes in one of the species resulting in a high dissimilarity of the amino acid sequences of the encoded genes (Moran et al, 2011). Studies on laboratory micro-evolution of fungi identified even single nucleotide polymorphisms (SNP) in genes which caused dramatic changes in the phenotype and virulence of strains (Janbon et al, 2014; Brunke et al, 2014; Wartenberg et al, 2014). The importance of subtle structural differences may be hard to determine especially when related species are compared since the number of SNPs between strains can be very high. In addition, differences can occur on the expression level of the genes, which cannot be detected by comparative genomic analyses alone. The use of transcriptomic or proteomic approaches allows the detection of differentially expressed genes between strains or species, which help to understand the molecular base of the differences in drug and stress resistance or virulence (Lelandais et al, 2008; Tsai et al, 2010; Moran et al, 2011; Chen et al, 2014; Linde et al, 2015). While pathogenomic approaches have been used to understand the evolution of pathogenicity and virulence factors in a variety of fungal pathogens, the use of pathogenomics in basal fungi is restricted by the small number of genomes sequences available.

1.5.2 Current state of genome sequencing projects of basal fungi

To date, more than 1,000 fungal genome sequences are publicly available (National Center for Biotechnology Information, NCBI) and almost 3,000 fungal projects are registered in the Genomes Online Database (GOLD) (Figure 1.1). The majority of available genomes and planed projects are dedicated to the derived fungi (Dikarya) representing around 95 % of all public and planned projects (Figure 1.1, Figure 1.3). In contrast, basal fungal genomes are still only poorly studied.

The genome of the most important mucoralean pathogen *R. arrhizus* was the first published genome of a member of the basal fungi (Ma et al, 2009). The authors found an unusual high amount of duplicated genes, which could be linked to a whole genome duplication event in this species. Several of the expanded gene families are putatively involved in the pathogenicity of this fungus including hydrolytic enzymes and enzymes involved in cell wall biosynthesis (Ma et al, 2009). Although additional genomes of different species of the Mucorales have been published during the last years (Figure 1.3), only few of them have been studied in more detail. Of the 22 mucoralean pathogens only *R. arrhizus* and *M. circinelloides* have been investigated and some virulence factors were described based on the genome sequences available (Ma et al, 2009; Ibrahim et al, 2010b; Gebremariam et al, 2014; Liu et al, 2015; Lee et al, 2013; Li et al, 2011; Lee et al, 2014). In contrast, there is a lack of information on pathogenicity mechanisms in more basal mucoralean pathogens.

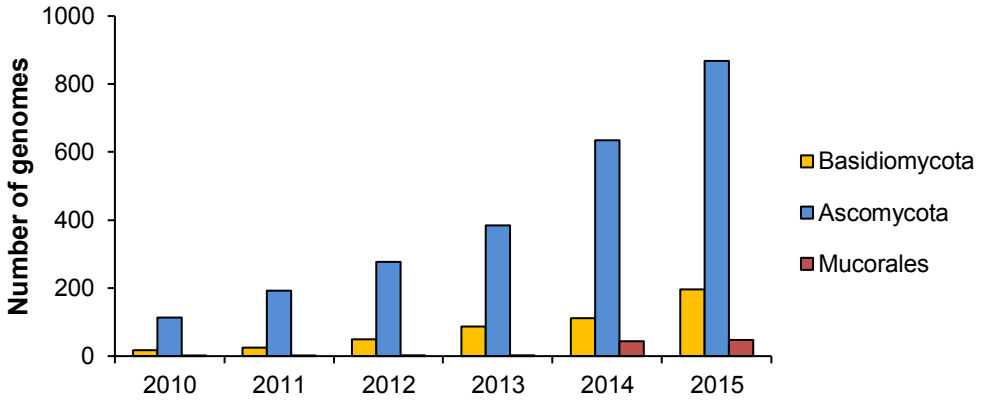

Figure 1.3: Number of fungal genomes published in NCBI. Bars show the accumulative number of available genomes of the respective taxon till the end of the indicated year based on the date of publishing in the Genome database of the National Center for Biotechnology Information (NCBI, as of October 2015).

Lichtheimia species are a good model as they represent the most common cause of infections with basal Mucorales. In addition, the phylogeny, physiology and virulence potential of various strains of the six described species have been studied (Alastruey-Izquierdo et al, 2010a; Alastruey-Izquierdo et al, 2010b; de A Santiago et al, 2014; Schwartze et al, 2012; Schwartze et al, 2014a).

2 Aims of this study

While life-threatening infections with members of the Mucorales are increasingly recognised in patients, little is known about the virulence mechanisms of these fungi. More importantly, the term mucormycosis is used as a general description for infections with mucoralean fungi, although these infections are caused by more than 20 different species which diverged from each other several hundred million years ago. Species-specific features of infections are only rarely discussed in clinical reviews and research is mainly limited to two pathogenic genera, namely *Mucor* and *Rhizopus* which are closely-related. In contrast, almost nothing is known about pathogenicity of more basal mucoralean pathogens.

The aim of this study was to investigate the pathogenicity and pathogenomics of *Lichtheimia* species which are among the most basal mucoralean pathogens and represent the second-most common cause of mucormycosis in Europe.

In order to get first insights into (i) the general genomic features and (ii) pathogenicity mechanisms of *Lichtheimia* species, the genome of *L. corymbifera* was sequenced and compared with other fully sequenced fungal genomes. Besides comparative genomics, transcriptomic analyses under infection-associated stress conditions were used to identify putative virulence factors which may play a role in the adaptation to growth inside the host.

Since the available genome sequences of mucoralean fungi are only from distantly related species, nothing is known about the differences in the genomic organisation within a single genus. No studies on the genomic differences of closely-related pathogenic and non-pathogenic species are published, which could help to understand the evolution of pathogenicity in mucoralean fungi and identify putative virulence factors. To get first insights into this topic, the genomes of two pathogenic and one non-pathogenic *Lichtheimia* species were sequenced and compared. Additional physiological and transcriptomic analyses were used to identify differences in stress adaptation of the species and to reveal new information on the stress response in basal fungi.

3 Summary of manuscripts

Manuscript A

Mucormycoses caused by *Lichtheimia* species

Schwartze VU, Jacobsen ID

Published in: Mycoses 57 (Suppl 3): 73-78, 2014

The review focuses on the clinical importance of *Lichtheimia* species. The epidemiology and clinical presentation of *Lichtheimia* infections in humans and animals are discussed. Since infection models are crucial for the understanding of the infection process, a brief overview over different infection models for *Lichtheimia* species and the main results of the experiments is given.

Volker U. Schwartze and Ilse D. Jacobsen wrote the manuscript.

Estimated proportion of work:

Volker U. Schwartze	50 %
Ilse D. Jacobsen	50 %

Manuscript B

Gene Expansion Shapes Genome Architecture in the Human Pathogen *Lichtheimia corymbifera*: An Evolutionary Genomics Analysis in the Ancient Terrestrial Mucorales (Mucoromycotina)

Schwartze VU, Winter S, Shelest E, Marcet-Houben M, Horn F, Wehner S, Linde J, Valiante V, Sammeth M, Riege K, Nowrousian M, Kaerger K, Jacobsen ID, Marz M, Brakhage AA, Gabaldón T, Böcker S, Voigt K

Published in: PLoS Genetics 10 (8): e1004496, 2014

Little is known about the genome structure and evolution of mucoralean fungi. The manuscript describes the genome of *L. corymbifera*, one of the most common causes of mucormycosis in humans. Comparative analyses showed the high dissimilarity of *L. corymbifera* from other sequenced mucoralean fungi and revealed several species-specific expansions, which could partially be linked to the presence of tandem duplications of genes. Based on comparative genomics and transcriptome analyses under infection-associated conditions putative virulence factors of *L. corymbifera* could be identified. This study gives first insights into genomics and virulence factors of basal mucoralean pathogens and represents an important resource for further research.

Volker U. Schwartze, Sascha Winter, Ekaterina Shelest, Marina Marcet-Houben, Fabian Horn, Michael Sammeth, Minou Nowrousian, Vito Valiante, Jörg Linde, Ilse D. Jacobsen, Manja Marz, Axel A. Brakhage, Toni Gabaldón, Sebastian Böcker and Kerstin Voigt conceived and designed the experiments.

Volker U. Schwartze, Sascha Winter, Ekaterina Shelest, Marina Marcet-Houben, Fabian Horn, Michael Sammeth, Minou Nowrousian, Vito Valiante, Jörg Linde, Ilse D. Jacobsen, Konstantin Riege, Kerstin Kaerger and Stefanie Wehner performed the experiments.

Volker U. Schwartze, Sascha Winter, Ekaterina Shelest, Marina Marcet-Houben, Fabian Horn, Michael Sammeth, Minou Nowrousian, Jörg Linde, Vito Valiante, Ilse D. Jacobsen, Konstantin

Riege, Stefanie Wehner, Manja Marz, Toni Gabaldón, Sebastian Böcker and Kerstin Voigt analysed the data.

Sebastian Böcker, Michael Sammeth, Sascha Winter, Konstantin Riege, Manja Marz, Fabian Horn, Jörg Linde, Marina Marcet-Houben and Toni Gabaldón designed the software used in the analyses.

Sascha Winter, Ekaterina Shelest, Fabian Horn, Michael Sammeth, Minou Nowrousian, Vito Valiante, Jörg Linde, Ilse D. Jacobsen, Konstantin Riege, Stefanie Wehner, Manja Marz, Axel A. Brakhage, Toni Gabaldón, Sebastian Böcker and Kerstin Voigt contributed reagents, materials or analysis tools.

Volker U. Schwartze, Sascha Winter, Ekaterina Shelest, Marina Marcet-Houben, Fabian Horn, Michael Sammeth, Minou Nowrousian, Vito Valiante, Manja Marz, Toni Gabaldón, Sebastian Böcker and Kerstin Voigt wrote the paper.

Estimated proportion of work:

Schwartze + Winter + Shelest + Marcet-Houben + Horn	55 %
Wehner + Linde + Riege + Sammeth + Valiante	25 %
Nowrousian + Kaerger + Jacobsen	10 %
Marz + Brakhage + Gabaldon + Böcker + Voigt	10 %

Manuscript C

To the limit and beyond: Comparative genomic and transcriptomic analyses reveal stress adaptation determinants of human pathogenic *Lichtheimia* species

Schwartze VU, Klassert TE, Riege K, Marcet-Houben M, Nowrousian M, Binder U, Grigoriev I, Grøtli M, Gryganskyi A, Fleischauer M, Lipzen A, Park H, Salamov A, Stajich J, Tamas M, Tritt A, Winter S, Böcker S, Gabaldón T, Marz M, Lass-Flörl C, Slevogt H, Voigt K

Intended for publication in: Genome Biology

Only three of the six described *Lichtheimia* species are known to cause infections in humans. Comparative genomics and transcriptomics of the two human pathogenic species *L. corymbifera* and *L. ramosa* with *L. hyalospora* reveal a high similarity in the genome structure between *Lichtheimia* species and show that major virulence determinants are conserved between clinically relevant species and clinically non-relevant species. However, differences in the thermotolerance of *Lichtheimia* species were observed, which could be linked to the accumulation of misfolded proteins. Stress resistance could be altered by the application of combinatory stresses. Transcriptome analyses under different stress conditions gave first insights into the stress response of mucoralean pathogens and helped to identify putative virulence factors in these fungi.

Volker U. Schwartze performed the biological experiments, partially with the help of Hea Reung Park.
Volker U. Schwartze performed the cultivation for the transcriptome analyses and extracted the mRNA. Tilman E. Klassert prepared all RNA libraries and performed the sequencing. Konstantin Riege and Manja Marz performed the mapping of reads and calling of the differentially expressed genes. Volker U. Schwartze analysed the differentially expressed genes.
Igor V. Grigoriev, Andrii Gryganskyi, Anna Lipzen, Asaf Salamov, Jason E. Stajich, and Andrew Tritt sequenced the genome and transcriptome of *L. hyalospora* and performed the functional annotation.

Konstantin Riege and Manja Marz performed the annotation of the non-coding RNAs.

Marina Marcet-Houben and Toni Gabaldón prepared the phylome and orthology prediction.

Marina Marcet-Houben, Toni Gabaldón and Volker U. Schwartze analysed the proteomes.

Marina Marcet-Houben and Toni Gabaldón performed the analyses of the amino acid frequencies of the protein-coding genes.

Minou Nowrousian performed the analyses of the repetitive elements.

Markus Fleischauer performed the ordering of the assemblies.

Sascha Winter analysed the syntenic clusters.

Ulrike Binder performed the infection experiments in *Galleria mellonella*.

Morten Grøtli and Markus Tamas provided the HOG1 inhibitor.

Volker U. Schwartze, Tilman E. Klassert, Konstantin Riege, Marina Marcet-Houben, Sebastian Böcker, Toni Gabaldón, Manja Marz, Cornelia Lass-Flörl, Hortense Slevogt and Kerstin Voigt planned the project.

Volker U. Schwartze, Marina Marcet-Houben, Toni Gabaldón, Kerstin Voigt and Jason E. Stajich wrote the manuscript.

Estimated proportion of work:

Schwartze	25 %
Klassert	15 %
Riege + Marcet-Houben	25 %
Nowrousian + Binder + Grøtli + Fleischauer + Park + Winter	10 %
Grigoriev + Gryganskyi + Lipzen + Salamov + Stajich + Tamas + Tritt	15 %
Böcker + Gabaldón + Marz + Lass-Flörl + Slevogt + Voigt	10 %

4 Manuscripts

Manuscript A

Schwartze VU, Jacobsen ID

Mucormycoses caused by *Lichtheimia* species

mycoses
Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Supplement article

Mucormycoses caused by *Lichtheimia* species

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Summary

Mucormycoses are life-threatening infections with fungi from the order Mucorales (Mucoromycotina). Although mucormycoses are uncommon compared to other fungal infections, e.g. aspergillosis and candidiasis, the number of cases is increasing especially in immunocompromised patients. *Lichtheimia* (formerly *Absidia*) species represent the second to third most common cause of mucormycoses in Europe. This mini review presents current knowledge about taxonomy and clinical relevance of *Lichtheimia* species. In addition, clinical presentation and risk factors will be discussed. Proper animal infection models are essential for the understanding of the pathogenesis and the identification of virulence factors of fungal pathogens. To date, several animal models have been used to study *Lichtheimia* infection. A brief overview of the different models and the main conclusions from the infection experiments is summarised in this review.

Key words: *Absidia*, *Lichtheimia*, mucormycosis, infection.

Introduction

Compared to the more frequent invasive fungal infections like cryptococcosis, candidiasis and aspergillosis, infections by mucormycetes (mucormycoses) are rather uncommon.[1] However, the number of mucormycosis cases is increasing, especially in patients with underlying immunosuppression.[2,3] Treatment of these infections is difficult and requires fast initiation of antifungal therapy, often in combination with extensive surgical debridement. Despite appropriate treatment, overall mortality still reaches approximately 50%.[4,5] More than 20 mucoralean species are known to cause infections in humans, with *R. oryzae* as the most frequently isolated species worldwide. In Europe, members of the genus *Lichtheimia* are the second to third most important cause of mucormycoses.[6,7] The following review will summarise the current taxonomy of the genus *Lichtheimia*, its role as human pathogen and cause of disease in other species, and will provide a brief overview of infection models used to study *Lichtheimia* infections.

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Systematics and biology of *Lichtheimia*

The genus *Lichtheimia* (ex *Absidia*, *Mycocladus*) belongs to the family *Lichtheimiaceae*, one of the most basal families in the fungal order Mucorales.[8,9] To date, six species have been described: *L. corymbifera*, *L. ramosa*, *L. ornata*, *L. hyalospora*, *L. sphaerocystis* and *L. brasiliensis*.[10] The taxonomy of the members of this genus has been changed repeatedly: *L. corymbifera* was originally described 1884 as *Mucor corymbifer* by Cohn[11] before being placed within the mesophilic genus *Absidia*. Based on their higher temperature optimum (>30 °C – 37 °C), morphology and molecular phylogeny, the thermophilic species within *Absidia*, including current members of *Lichtheimia*, were reclassified into the genus *Mycocladus*, resulting in the species designations *M. corymbifer*, *M. hyalosporus* and *M. blakesleeanus*.[8] However, the name had to be corrected to *Lichtheimia* to comply with the International Code of Botanical Nomenclature.[12] Finally, Alastruey-Izquierdo *et al.* described five species, *L. corymbifera*, *L.ramosa*, *L.*

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ornata, *L. hyalospora* and *L. sphaerocystis*, within the genus, based on physiological, morphological and phylogenetic data.[10] Recently, a new species, *L. brasiliensis*, has been described which represents the most basal species within *Lichtheima*.[13] All species of *Lichtheimia* grow well on artificial media and have a growth optimum between 30 °C and 37 °C.[10]

Mucoralean fungi are ubiquitous saprophytes and are globally distributed. Soil is believed to be the main habitat of most Mucorales, but some of these fungi can also be found in decaying vegetation and rotting fruits.[14] In addition, *Lichtheimia* species can be found in a variety of substrates including farming products like hay and straw as well as processed and unprocessed food products like flour and fermented soybeans.[15–21] Interestingly, *L. corymbifera* and *L. ramosa*, which represent the most important pathogenic *Lichtheimia* species, are also the most frequently isolated species in all of these food samples.

Human disease caused by *Lichtheimia*

Epidemiology

In contrast to *Rhizopus* species that are the main cause for mucormycoses worldwide, the frequency of *Lichtheimia* infections differs significantly between geographic regions (summarised in Table 1). In a global survey, and in a study from the USA, *Lichtheimia* species accounted for 5% of all mucormycoses.[5,22] In contrast, in recent studies from Europe *Lichtheimia* species were identified as the second most common cause of mucormycosis, causing 19–29% of the cases.[7,23] The majority of these cases appear to be caused by *L. corymbifera*, as 84% of all *Lichtheimia* isolates in a European study were identified as *L. corymbifera*.[7] Furthermore, *L. corymbifera* is the only *Lichtheimia* species isolated from patients in the USA.[22] However, since *L. ramosa* and *L. corymbifera* were used synonymously for a long time and *L. ornata* was only recently given species status, correct assessment of the frequency of the species is difficult. Indeed, a recent study revealed that a significant proportion of human infections originally assigned to *L. corymbifera* was, in fact, caused by *L. ramosa*.[24] While the pathogenic potential of both *L. corymbifera* and *L. ramosa* is well documented by human cases, only one clinical isolate of *L. ornata* has been described[10] and no infections with *L. hyalospora* or *L. sphaerocystis* have been reported to date. In addition, infection experiments in chicken embryos showed a lower virulence potential of *L. hyalospora* and *L. sphaerocystis*.[25]

Table 1 Involvement of *Lichtheimia* species in mucormycosis.

Geographical region	# *Lichtheimia* cases (% of total cases)	References
Europe	32 (19)	Skiada *et al.* [7]
Europe	29 (29)	Lanternier *et al.* [6]
USA	10 (5)	Alvarez *et al.* [22]
Egypt	4 (40)	Zaki *et al.* [31]
Worldwide	25 (5)	Roden *et al.* [5]
Worldwide	13 (8)	Rammaert *et al.* [83]

Clinical presentation of *Lichtheimia* infections

Inhalation of asexual spores (sporangiospores) is believed to be the main route of infection with mucormycetes and thus, infection commonly manifests in the respiratory tract.[2,3] Pulmonary infections with *L. corymbifera* have been reported in patients with different underlying diseases, including bone marrow and solid organ transplantation, uncontrolled diabetes and leukaemia.[26–32] The observed symptoms are generally unspecific, such as dyspnoea, pulmonary inflammation and occasionally pleuritis. Endobronchial bleeding is typical for pulmonary mucormycosis but not specific for *Lichtheimia* infections. Pulmonary *Lichtheimia* infections can disseminate to different internal organs, including the central nervous system, often associated with fatal outcome.[28,33–36] Pathological alterations resemble those observed in other cases of mucormycosis and are characterised by vascular invasion, thrombosis and tissue necrosis.

Another common clinical manifestation caused by *Lichtheimia* species is cutaneous and subcutaneous infections. These cases are generally associated with previous wounds or fractures due to traumatic accidents or surgery. Thus, contamination of wounds, either with plant material during accidents, or *via* non-sterile bandages or surgical dressings, is the most likely route of infection.[37–43] However, nosocomial infections due to person-to-person transmission also appear possible.[44] Interestingly, trauma-related infections have been described in patients without obvious underlying immunosuppression.[37,40,42] Superficial infections can occur in patients suffering from an immunosuppressive disorder, such as leukaemia or HIV, but also in premature infants and apparently healthy adult persons.[42,45–52] They are characterised by rapidly developing extensive tissue necrosis leading to purple to black discolouration of the skin.[45,53] In individual cases involvement of deeper tissue, leading to necrotising fasciitis and cellulitis, has also been reported.[40,46,54] In the most severe cases, cutaneous infections can progress to disseminated disease,

especially in immunocompromised patients and premature infants.[47,55] In premature infants, *Lichtheimia* infections furthermore commonly affect the gastrointestinal tract,[56] often resembling necrotising enterocolitis.[57]

Since most studies on mucormycosis do not examine the type of infection on a species-specific level, it is hard to assess the incidence of different types of infections for *Lichtheimia*. Only two studies include more detailed information about infections with *Lichtheimia* species. The study of Alvarez *et al.* included seven cases of *Lichtheimia* infections with pulmonary infection and infections of the sinuses as the most important presentations (6 of 7 cases).[22] Only one additional study focused on species-specific analysis of healthcare-associated mucormycosis. Cutaneous and pulmonary infections were the most common types of infection representing 70% and 20% respectively.[83] However, due to the limitations of the currently available studies, e.g. low numbers of cases or restriction to a special patient group, no clear conclusions can be drawn about the incidence of the different types of infections and underlying conditions for the development of *Lichtheimia* infections.

Farmer's lung disease

In addition to causing infections, *Lichtheimia* species have been implicated in the form of occupational hypersensitivity pneumonitis termed Farmer's lung disease (FLD). Farmer's lung disease is caused by recurrent exposure to certain microorganisms, especially in farming personnel. The acute form is characterised by influenza-like symptoms like sweating, chills, fever, nausea and headache. The (sub)chronic form is associated with coughing and dyspnoea for up to several weeks.[58] As mentioned above, *Lichtheimia* species represent a major contaminant of farming material like hay and straw. The occurrence of FLD has been associated with increased numbers of *L. corymbifera* in the farm environment and *L. corymbifera*-specific antibodies in affected patients.[59] Furthermore, *in vitro* experiments with lung epithelial cells revealed high expression of pro-inflammatory and allergic mediators (IL-8, IL-13) after exposure to extracts of *L. corymbifera*.[60] These results support the role of *Lichtheimia* in the development of hypersensitivity pneumonia.

Lichtheimia infections in animals

Lichtheimia species have been reported to cause infections in various warm-blooded animals (summarised in Table 2), especially bovines. In cattle, *L. corymbifera* can cause abortions and mastitis,[61] but also gastrointestinal mycoses. Jensen *et al.* identified *L. corymbifera* as the cause of bovine gastrointestinal mycoses in more than 60% of the cases.[62] As *Lichtheimia* species are present in high amounts in cattle feed, oral inoculation of fungal spores and hyphae seems to be the most likely rout of infection.[16] Furthermore, mucoralean species including *L. corymbifera* and *L. ramosa* represent the majority of filamentous fungi in rumen fluid of healthy cattle[63] and therefore endogenous infections might also occur. Limited spread from the intestinal tract is also the most likely explanation for the cases of mesenteric lymphadenitis caused by *L. corymbifera*. The affected animals appeared clinically healthy but displayed invasion of lymph nodes and subsequent necrosis and dystrophic calcification at slaughter.[64] Infections caused by *Lichtheimia* seem not to be restricted to bovines but might also affect other ruminants, as illustrated by a case of systemic infection in deer.[65]

Equine hosts can also be infected by *Lichtheimia* species. Two cases of *Lichtheimia* infections in ponies were reported by Guillot *et al.* [66]. While one of the animals suffered from localised cutaneous *Lichtheimia* infection and necrotic ulceration in the nostrils, the other died due to systemic mucormycosis. Postmortem examination revealed lesions in the lung, stomach, digestive tract and a large infarct in the brain.

Pulmonary, gastrointestinal and disseminated infections with *Lichtheimia* have also been described in birds.[67,68] In a recent study on stork chicks, *L. corymbifera* was identified as the second most common cause of fungal pneumonia, accountable for 18% of the cases.[69] In both mammalian and avian hosts, *Lichtheimia* can occur as coinfection with *A. fumigatus*.[62,69,70]

Table 2 *Lichtheimia* infections in animals.

Host species	Infection type	References
Cattle	Abortion	Piancastelli *et al.* [61]
	Gastrointestinal	Jensen *et al.* [62]
	Lymphadenitis	Ortega *et al.* [64]
Horse	Cutaneous	Guillot *et al.* [66]
	Systemic	Guillot *et al.* [66]
Bank vole (*Clethrionomys glariolus*)	Systemic	Corbel *et al.* [78]
Stork	Pulmonary	Olias *et al.* [69]

Infection models for *Lichtheimia*

Mouse models

Murine models are the most commonly used animal models for most fungal infections. Several different mouse models have been used to study *Lichtheimia* infections by various groups; however, a standardised and well-characterised model has not been established yet.

In immunocompetent mice infected either intravenously or intracerebrally, both *L. corymbifera* and *L. ramosa* were shown to cause lethal disease with lesions predominantly affecting the central nervous system and the kidneys.[71,72] In pregnant mice, the infection did also affect the placenta.[73] Immunosuppression by cortisone acetate increased the susceptibility to intravenous infection and led to more widespread organ pathology.[74] In contrast to systemic infection models, immunocompetent mice were resistant to oral and subcutaneous challenge with *Lichtheimia*.[74,75] Similarly, development of clinical disease after pulmonary challenge via the intranasal or intratracheal route depended on immunosuppression.[74–76] In general, clinical symptoms in mouse models are comparable to the clinical situation in human patients, suggesting suitability of this model to study human disease.

Other mammalian models

A variety of animal models have been used to investigate the virulence and pathogenicity of *Lichtheimia* species. Like in mice, intravenous infection leads to the development of disease and mortality in healthy rabbits and bank voles with kidney and brain being the main target organs.[77,78] Intranasal infection of bank voles did only rarely lead to mortality but fungi disseminated and could be isolated from lung, liver, brain and kidney at high infection doses.[78] In contrast, intratracheal infection of Asian water buffalo calves led to restricted, self-limiting lung infection without fatalities and dissemination.[79] These results demonstrate that *Lichtheimia* can infect a wide range of host species but that disease development depends on the route of infection and immunosuppression.

Chicken embryo model

Due to ethical and practical limitations of the use of mammals as infection models to analyse virulence in large numbers of strains, an alternative infection model using chicken embryos has been developed for different bacteria and fungi, including *Lichtheimia*.[25,80–82] To study virulence of *Lichtheimia* species, infection was performed via the chorioallantoic membrane.[25] The main features of infection in this model were penetration and destruction of blood vessels, comparable to human disease. Mortality and the extend of pathological alterations were higher in the clinical-relevant species *L. corymbifera*, *L. ramosa* and *L. ornata* compared to the non-clinical species *L. hyalospora* and *L. sphaerocystis*, suggesting that the different *Lichtheimia* species exhibit differences in their virulence potential.[25]

Conclusion

In summary, *Lichtheimia* species (especially *L. ramosa* and *L. corymbifera*) are important causes of mucormycoses. The clinical disease resembles infections with other mucoralean fungi; however, it remains unclear whether the same predisposing risk factors underlie mucormycoses caused by the different genera and species. Further epidemiological studies are needed to address these questions. Furthermore, the elucidation of pathogenesis mechanisms, assessment of risk factors and determination of the relative virulence of the different *Lichtheimia* species and strains would greatly benefit from the development of standardised mammalian infection models.

Conflict of interest

The authors declare that no conflict of interest exists.

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Gene Expansion Shapes Genome Architecture in the Human Pathogen *Lichtheimia corymbifera*: An Evolutionary Genomics Analysis in the Ancient Terrestrial Mucorales (Mucoromycotina)

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Abstract

Lichtheimia species are the second most important cause of mucormycosis in Europe. To provide broader insights into the molecular basis of the pathogenicity-associated traits of the basal Mucorales, we report the full genome sequence of *L. corymbifera* and compared it to the genome of *Rhizopus oryzae*, the most common cause of mucormycosis worldwide. The genome assembly encompasses 33.6 MB and 12,379 protein-coding genes. This study reveals four major differences of the *L. corymbifera* genome to *R. oryzae*: (i) the presence of an highly elevated number of gene duplications which are unlike *R. oryzae* not due to whole genome duplication (WGD), (ii) despite the relatively high incidence of introns, alternative splicing (AS) is not frequently observed for the generation of paralogs and in response to stress, (iii) the content of repetitive elements is strikingly low (<5%), (iv) *L. corymbifera* is typically haploid. Novel virulence factors were identified which may be involved in the regulation of the adaptation to iron-limitation, e.g. LCor01340.1 encoding a putative siderophore transporter and LCor00410.1 involved in the siderophore metabolism. Genes encoding the transcription factors LCor08192.1 and LCor01236.1, which are similar to GATA type regulators and to calcineurin regulated CRZ1, respectively, indicating an involvement of the calcineurin pathway in the adaption to iron limitation. Genes encoding MADS-box transcription factors are elevated up to 11 copies compared to the 1–4 copies usually found in other fungi. More findings are: (i) lower content of tRNAs, but unique codons in *L. corymbifera*, (ii) Over 25% of the proteins are apparently specific for *L. corymbifera*. (iii) *L. corymbifera* contains only 2/3 of the proteases (known to be essential virulence factors) in comparision to *R. oryzae*. On the other hand, the number of secreted proteases, however, is roughly twice as high as in *R. oryzae*.

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Introduction

The basal lineages of terrestrial fungi, formerly Zygomycota, were recently shown to be polyphyletic and were therefore separated into four separate subphyla [1]. Especially the order Mucorales of the Mucoromycotina encompasses several human pathogenic species. Although infections with mucoralean fungi (mucormycosis) are less common as compared to aspergilloses or candidioses, these fungi are increasingly recognized as the source of infection in immunocompromised patients [2]. Mucormycoses

Author Summary

Lichtheimia species are ubiquitous saprophytic fungi, which cause life-threating infections in humans. In contrast to the mucoralean pathogen *R. oryzae*, *Lichtheimia* species belong to the ancient mucoralean lineages. We determined the genome of *L. corymbifera* (formerly *Mycocladus corymbifer* ex *Absidia corymbifera*) and found high dissimilarities between *L. corymbifera* and other sequenced mucoralean fungi in terms of gene families and syntenies. A highly elevated number of gene duplications and expansions was observed, which comprises virulence-associated genes like proteases, transporters and iron uptake genes but also transcription factors and genes involved in signal transduction. In contrast to *R. oryzae*, we did not find evidence for a recent whole genome duplication in *Lichtheimia*. However, gene duplications create functionally diverse paralogs in *L. corymbifera*, which are differentially expressed in virulence-related compared to standard conditions. In addition, new potential virulence factors could be identified which may play a role in the regulation of the adaptation to iron-limitation. The *L. corymbifera* genome and the phylome will advance further research and better understanding of virulence mechanisms of these medically important pathogens at the level of genome architecture and evolution.

are associated with rapid blood vessel invasion and massive destruction of tissue (necrosis) [3,4]. Mortality rates are high (~50%) and treatment mainly includes a combination of antifungals and extensive surgery [2,5–7]. In addition, mucoralean pathogens are resistant to a variety of antifungals including voriconazole which makes treatment even more complicated [8].

The order Mucorales comprises 240 described species, of which at least 20 have been found to be involved in mucormycosis. Genome sequences have been published for only two important pathogenic species within the Mucorales, namely *Rhizopus oryzae* (=*R. arrhizus*) and *Mucor circinelloides*. These species are closely related and represent derived lineages within the group. However, a large proportion of pathogenic Mucorales (10 species) belong to more basal groups including the genera *Lichtheimia*, *Rhizomucor*, *Apophysomyces*, *Saksenaea* and *Syncephalastrum*. Recently, the first report of the involvement of *Thamnostylum lucknowense*, an ancient mucoralean fungus, in human infections has been published [9]. To date, almost nothing is known about the genomic structure and pathogenicity mechanisms of these basal groups.

Lichtheimia species are ubiquitous saprophytic molds and represent the second and third most common cause of mucormycosis in Europe and worldwide, respectively [2,7,10,11]. The genus *Lichtheimia* was formerly included in the genus *Absidia* based on morphological similarities [12]. However, based on the higher growth optimum as well as morphological and molecular data *Lichtheimia* species were separated from the mesophilic *Absidia* species [13]. Today the genus encompasses five thermotolerant species, of which three are known to be clinically relevant, namely *L. corymbifera*, *L. ramosa* and *L. ornata* [14]. In addition to the distinct phylogenetic position at the base of mucoralean fungi, *Lichtheimia* species exhibit differences in physiology compared to the sequenced pathogens *M. circinelloides* and *R. oryzae*, including a higher maximum growth temperature (48–52°C vs 37°C and <45°C) and differences in susceptibility to certain antifungals [8,15]. Moreover, filamentously growing *Mucor* and *Rhizopus* species have been shown to be able to form yeast cells which were also found in patient material and thus might be of relevance during infection [16–18]. In contrast, no yeast-like growth forms of *Lichtheimia* species have been observed to date. In addition, pulmonary *Lichtheimia* infections following solid organ transplantation seem to be associated with a higher risk to develop disseminated disease [19]. Besides its role in human infections, *L. corymbifera* is also believed to be associated with Farmer's lung disease (FLD), a hypersensitivity disorder resulting from frequent contact of mouldy material in agriculture [20]. Nothing comparable has been described for other mucoralean species. In addition to their pathogenicity towards humans, several *Lichtheimia* species are known as contaminants of several food products (e.g. cocoa, peanuts, olive products) [21–23]. However, despite the known role of *Lichtheimia* species in infection and diseases, several *Lichtheimia* species play an important role in the fermentation of soy products in Asian cuisine [24].The large evolutionary distance and notable differences in infection strategies between *Lichtheimia* and the two sequenced mucoralean pathogens indicate that they independently evolved their ability to infect humans by developing specific pathogenesis mechanisms. To gain insight into the genomic differences between these groups of pathogens, here we report the genome sequence of the type-strain of *L. corymbifera* (FSU 9682, CBS 429.75, ATCC 46771) which has been shown to be a typical strain in terms of virulence and physiology for this species [25] and compare it to published genomes of mucoralean fungi and other fungal phyla.

Results/Discussion

Genome assembly and structure

The genome of the type-strain of *L. corymbifera* (FSU 9682, CBS 429.75, ATCC 46771) was sequenced by a combination of 454 sequencing of a shotgun and 8 kb paired-end library in combination with Illumina sequencing of a paired-end read library (Materials and methods, Table S1). The final assembly comprises 209 scaffolds with a N50 scaffold size of 367,562 nt and a total length of 33.6 Mb (Table 1), which is comparable to the genome size of other zygomycetous fungi [26]. Mucoralean genomes are generally believed to contain large amounts of repetitive elements representing around 35% of the genome [27]. However, analysis of the *L. corymbifera* genome shows a much smaller content of repetitive elements, with only 4.7% of the assembly representing repetitive elements including DNA transposons, LTR and non-LTR retrotransposons (Table S2). This finding is consistent with the results of the k-mer analyses on the Illumina reads where only low amounts of potential repetitive regions were found. Of note, all previous estimates of repetitive elements in mucoraleans correspond to species with large genomes such as *R. oryzae* (46 Mb; 20% repetitive elements), *Absidia glauca* (52 Mb; 35%) and *P. blakesleeanus* (54 Mb; 35% repetitive elements) [27,28]. Interestingly, a *Lichtheimia*-specific gene expansion in the heterokaryon incompatibility genes was discovered (see section gene expansion) which are involved in the recognition of non-self DNA and may contribute to the low amount of repetitive elements. Another mechanism of protection against transposons and viruses is RNA interference resulting in sequence specific RNA degradation [29]. Several predicted proteins with functional domains associated with this mechanism were found including a dicer-like protein, one argonaute-2 protein and a translation initiation factor 2C homolog. However, the exact effects of these mechanisms on the amount of repetitive elements remain to be determined.

Heterozygosity was shown for several fungi including the basal lineage fungus *Batrachochytrium dendrobatidis* [30]. In order to

Table 1. Statistics of the *L. corymbifera* genome.

Assembly statistics	
Total scaffold length (Mb)	33.6
Scaffolds	209
N50 contig length (nt)	66,718
N50 scaffold length (nt)	367,562
G+C content	43.4%
Predicted protein-coding genes	
Predicted genes	12,379
Average coding sequence size (nt)	1,287
Average G+C content in coding sequence	46.2%
Total introns	48,663
Introns per gene (median)	4.8
Average intron length (nt)	258
Predicted non-coding RNA genes	
Predicted genes	213
Average G+C content in non-coding RNAs	49.2%
Total introns	3

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test for potential heterozygous regions and estimate the genome size of *L. corymbifera*, k-mer analyses based on the Illumina reads were performed using an algorithm described previously [31] (Material and Methods). Analysis resulted in a relatively clear single peak with a slightly trailing left flank for all k-values (Figure S1). The distribution could be dissected into three components, each showing a normal distribution with similar variance, but different means and different proportions. The main component represents the potential homozygous part of the genome (94%), whereas two small components represent the potential heterozygous part of the genome (4%), and most likely some repeat regions that occur at relatively low frequency (2%). It has to be noted that the potential heterozygous part is rather small and could as well be explained e.g. by regions that are difficult to sequence and therefore have lower k-mer coverage. The lack of heterozygocity is in accordance with the general assumption that mucoralean fungi are haploid during vegetative growth. Based on the k-mer analysis for different k-mer lengths (41, 59, 69, and 79 nt) a total genome size of around 35 Mb was predicted which is close to our total scaffold length of 33.6 Mb (96% of k-mer predicted size).

Non-coding RNA prediction and annotation

We annotated 174 tRNAs in *L. corymbifera*. Although *R. oryzae* (239) comprises many more tRNAs, we found unique anticodons among the basal fungi in *Lichtheimia*: CCC (Gly), AAA (Phe) and GAT (Ile). In contrast, only *L. corymbifera* misses the anticodons CAC (Val), CCT (Arg) and TAT (Ile). Three GTA (Tyr) tRNAs were predicted with introns in *L. corymbifera*, while the number was higher in other mucoralean fungi (up to 10). No selenocysteine and possible suppressor tRNAs were predicted. We found the downstream half of 28S rRNA only, but no 18S rRNA in the current assembly. We expect at least two operons (18S – 5.8S – 28S rRNA) as found in *R. oryzae*. In addition to 5S rRNAs located close to the operons, we were able to identify several independent 5S rRNA copies (Table S3). Another housekeeping ncRNA, present in all kingdoms of life, is the ribozyme RNase P, which processes tRNAs by cleaving off nucleotides on the 3′ end of tRNAs [32]. We detected this gene as expected in a single copy per genome, but two identical copies are apparently present in the genome of *R. oryzae*, which may result from whole genome duplication in *R. oryzae* [28]. The pseudoknot in the centre of the molecule is accredited with the catalytic function and highly conserved in evolution [33]. However, the *L. corymbifera* candidate varies exceptionally in sequence, while the secondary structure is maintained. Whether the function of the molecule is affected has to be analyzed. The evolutionary related RNase MRP was invented at the origin of eukaryots with dual function: (a) initiation of mitochondrial replication and (b) separation of 18S rRNA from 5.8S rRNA [34]. One copy per basal fungal genome was detected. The signal recognition particle containing a ncRNA part (SRP RNA) guides proteins to the endoplasmatic reticulum [35]. One copy was detected in *Lichtheimia*, whereas two copies were identified in the genome of *R. oryzae*. Surprisingly, the covariance model of mucoralean fungi, in agreement with *Rhizopus*, *Batrachochytrium* and *Monosiga* (RF00017) is much closer related to metazoans than to other known fungi SRP RNAs. We detected the RNA components of the major spliceosome and collected indications for a functional minor spliceosome. Except for U4 snRNA all five RNAs involved in U2-splicing were detected in *Lichtheimia*. U4 snRNA was not part of the assembly; however an U4-candidate was identified in the originally sequenced read data. Additionally, four of five RNAs involved in AT-AC-splicing were found. However, several special secondary structures were discovered, which may alter the functionality of the minor spliceosome: (i) The third stem of U12 snRNA is atrophied and the last stem is shorter than expected for all basal fungi. (ii) U4atac is not detected in *Lichtheimia*. The other basal fungi show one inconspicuous copy, which is assumed to to be an assembly mistake. However, no similar homologous gene was detected in reads either. (iii) The second half of U6atac is highly divergent (Figure S2 and supplemental material: http://www.rna.uni-jena.de/supplements/lichtheimia/index.html). Eleven CD-box snoRNAs and 3 H/ACA snoRNAs were identified, which are mainly conserved in sequence and structure among basal fungi. For further details we refer to the supplemental material (www.rna.uni-jena.de/supplements/lichtheimia/index.html). Additionally, several ncRNA candidates could be proposed, which have to be functionally characterized in future experiments. A riboswitch, binding to thiamine pyrophosphate (TPP) was found in all basal fungi. For *Lichtheimia* a potential telomerase RNA is suggested, which is surprisingly closely related to the shortest known telomerase RNAs in ciliates (150 nt *Tetrahymena paravorax*). This is unexpected, since the longest telomerase is known from the fungus *Saccharomyces cerevisiae* (1,220 nt). Although the alignment of the usually extremely divergent telomerase RNA is very convincing in sequence and secondary structure (see supplemental material), no homologs in another basal fungus and no interacting ciliate protein homolog were found in our current assembly. U7 snRNA is known to interact with the downstream region of histone mRNA for inhibition of degradation. Four similar candidates for this short ncRNA were identified. In eukaryotes, polymerase III transcripts (e.g. U6 snRNA, RNase P, RNase MRP, SRP RNA, U6atac snRNA) usually display a typical promoter region: −10 nt TATA box, PSE element, Oct region [36]. Therefore, a search for conserved motifs was conducted in *Lichtheimia* promoter regions. However, we were not able to identify even one of these motifs. This highlights a possible modified polymerase III activity for basal fungi and has to be investigated in detail in further work. A phylogeny among basal fungi and *Schizosaccharomyces pombe* as outgroup based on ncRNAs (except 18S and 28S rRNA) was reconstructed, see Figure S2 (B and C). In accordance with protein and traditional

rRNA phylogeny *Lichtheimia* groups basal to *P. blakesleeanus* and the other two investigated fungi.

Protein-coding gene prediction and annotation

To aid prediction of protein-coding genes, RNA-seq analyses were performed for three different growth conditions in three biological replicates (see Material and Methods). The use of different conditions should ensure a higher number of expressed genes, thereby allowing evidence-based gene predictions for many gene models. On average, each replicate has a 70-fold genome coverage, which sums up to a 630-fold genome coverage (Table S4). Prediction of protein-coding genes was performed using AUGUSTUS [37], resulting in 12,379 predicted genes. Genes were functionally annotated by comparing to GenBank sequences using BLASTp (E-value$\leq 10^{-25}$), and by scanning for the presence of conserved domains using the InterProScan function of BLAST2GO [38]. BLAST hits were obtained for 7,917 genes, InterProScan results were found in 10,066 genes and at least one Gene Ontology (GO) term was assigned to 7,435 genes based on the union of BLAST and InterProScan results. The raw reads of the DNA- and RNA-seq experiments, the final genome assembly, the structural and functional gene prediction are available at http://www.ebi.ac.uk/ena/data/view/PRJEB3978). The genome data are also accessible *via* HKI Genome Resource (http://www.genome-resource.de/).

Comparison of protein-coding genes between *L. corymbifera* and other completely sequenced genomes

An exhaustive comparison of *L. corymbifera* genome with other 24 completely sequenced genomes including the major fungal groups (Chytridiomycota, Mucoromycotina, Asco- and Basidiomycota) was performed. This comparison included the reconstruction of *L. corymbifera* phylome, which encompasses the complete set of evolutionary histories of *L. corymbifera* genes (Material and Methods). It was carried out using the previously described PhylomeDB pipeline [39]. In brief, for each *L. corymbifera* protein-coding gene we searched for homologs, and multiple sequence alignments were built, and Maximum Likelihood analyses were performed to reconstruct a phylogenetic tree. The phylome is available through phylomeDB (http://phylomedb.org), with the phylome ID 245. The phylome was used to establish phylogeny-based orthology and paralogy relationships among genes in the species considered [40], and to detect gene expansions (see below). In addition, we used two complementary approaches, gene concatenation and super-tree [41], to reconstruct the species tree that represents the evolution of the 25 species considered. In the first approach, 58 genes were selected that were present in 21 out of 25 in single copy. Their corresponding alignments were then concatenated and a maximum-likelihood species tree was reconstructed (Material and Methods). In the second approach, 9,478 trees present in the phylome were used to build a super-tree using a gene tree parsimony approach, a method which finds the topology that minimizes the total number of duplications in the phylome [42]. Both resulting trees presented a similar topology, which placed *L. corymbifera* at the base of the other Mucorales species (Figure 1). The only difference found between the trees by the complementary approaches was the position of *Schizosaccharomyces pombe*, which appeared at the base of Ascomycota in the super-tree tree while in the concatenated tree it grouped with *S. cerevisiae*. To assess the level of overlap in genetic content between the different species an all-against-all comparison of the 25 genomes was performed. The results indicate between 50% and 75% of the proteins encoded in the other three Mucorales species had homologs in *L. corymbifera* (Figure 1). Surprisingly, this percentage of shared gene content with *L. corymbifera* was similar to that of *Schizosaccharomyces pombe* (60.6%), which is higher than that found in the more closely related chytrid *B dendrobatidis* (an average of 41.1%). Figure 1 also shows how these homologs are distributed in differently defined groups. Most interestingly, the fraction of species-specific proteins (grey bars in the figure) is particularly high in large genomes (e.g., over half of the largest genomes *Laccaria bicolor* and *Puccinia graminis*). Over 25% of the proteins apparently are specific for *L. corymbifera*.

Conserved gene regions in *L. corymbifera* and other mucoralean genomes

Since the *Lichtheimia* lineage separated early in mucoralean evolution we can expect that severe genomic re-arrangements have taken place during evolution, causing substantial differences between the genome structures of *L. corymbifera* and other Mucorales. Only 57.7% of the gene families present in *Lichtheimia* are also present in at least one of the other mucoralean genomes while only 36.7% were found in all four genomes representing 70.4% and 53.7% of the total *L. corymbifera* genes, respectively (Figure 2 A). Conserved regions, in terms of gene order, between mucoralean genomes were examined and evaluated with respect to the amount of conserved genes of these regions. A total of 230 regions with a minimum of 3 conserved genes of *L. corymbifera* were found that were present in at least one of the other genomes. These regions were interspersed over 41.1% of the scaffolds but covered only 7.6% of the *L. corymbifera* genome reflecting the high dissimilarity between the mucoralean genomes (Figure 2 B). Only 6 regions were shared with all species. The total number of shared clusters was found to be consistent with the phylogenetic distance between the species (Figure 2 B+C). Genes in the conserved regions are members of different gene families and contain a variety of functional domains.

Gene duplications in *L. corymbifera*

In accordance with former results [28] a higher number of gene families with two members were detected for *R. oryzae* but also for *L. corymbifera* as compared to other fungi (6.99%±0.58%) (Figure 3 A). Whole genome duplication has been previously described for *R. oryzae* based on the presence of gene duplications and duplication of large genomic regions (segmental duplications) [28]. To investigate whether segmental duplications and thus a potential WGD also occur in *L. corymbifera*, the genomes were scanned for the presence of duplicated regions using GECKO2 [43,44]. Consistent with the former findings, our analysis showed a high number of segmental duplications in *R. oryzae* covering more than 10% of the genome [28] while fewer duplicated regions were found in *L. corymbifera* covering less than 4% of its genome (Figure 3 B). Thus, the gene duplications seem not to result from recent WGD as in *R. oryzae* but may result from an ancient genome duplication in mucoralean fungi as suggested by Marcet-Houben et al. [45] which is no longer detectable in the duplicated gene clusters. The possibility of ancient WGD in mucoralean genomes is currently investigated in more detail (Corrochano et al., pers. comm.). The genome of *L. corymbifera* also shows increased numbers of gene families with a higher number of genes indicating that gene duplication and the preservation of the gene copies seem to be a common process in mucoralean genomes and may be independent from WGD. This will be further addressed in the next section.

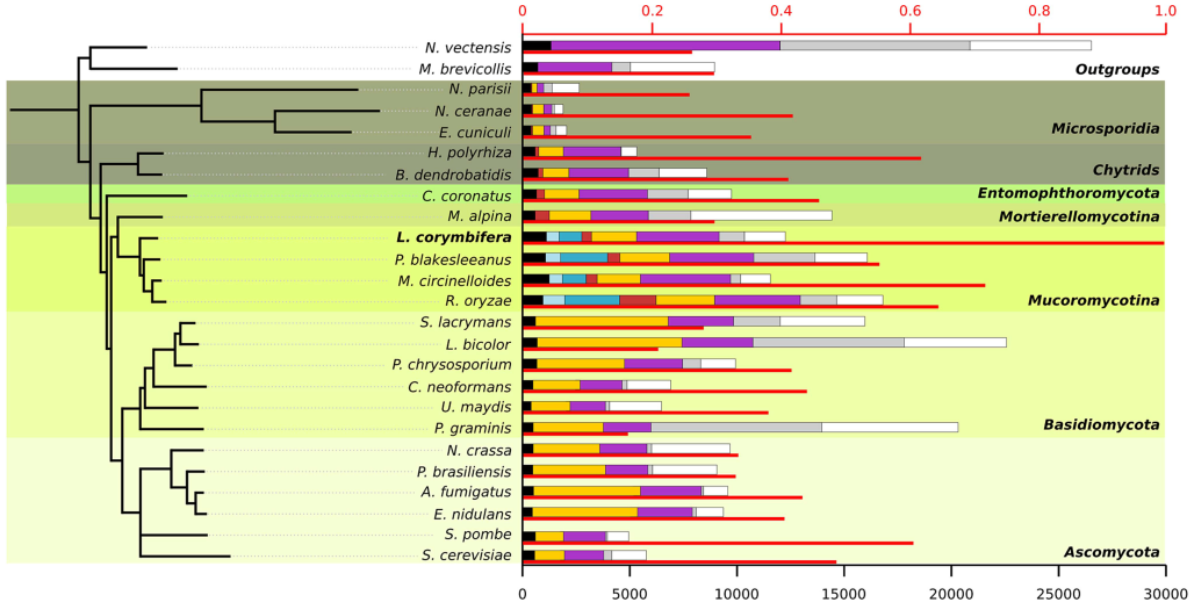

Figure 1. Species tree including the 25 species used during phylome reconstruction. For each species the thin red bar represents the proportion of proteins that have a homolog in *L. corymbifera* (upper axis). The coloured bars represent the number of proteins found in different ranges of species (lower axis): black: wide-spread proteins found in at least 23 of the 25 species, light blue: proteins found exclusively in all four Mucorales species, darker blue: proteins found only in Mucorales species, red: proteins found in early diverging fungi, yellow: proteins found in fungi, purple: proteins found in fungi and at least one of the outgroups, grey: species-specific proteins without orthologs in other species but with paralogs within the genome, white: proteins with no homologs. All nodes in the tree have a bootstrap support of 100.
doi:10.1371/journal.pgen.1004496.g001

Gene expansions in the *L. corymbifera* genome

In addition to gene duplications shared by all mucoralean fungi a high amount of species-specific duplications was detected. Therefore, the phylome was scanned in search of expansions of protein families that occurred specifically in *L. corymbifera*. For each tree, ETE [46] was used to find nodes that contained at least five *L. corymbifera* sequences and no other fungal sequence (Figure 4 A and B). Overlapping expansions were fused when they shared more than half of their members. We found 75 expansions that fulfilled those requirements. Five expansions contained more than 30 members, with the largest containing 331 paralogous genes. In contrast, the large genome of *R. oryzae* contains approximately twice the number of expansions, with the largest encompassing 1,888 members. As some of those expansions are likely the result of the presence of transposons, we scanned them for the presence of transposon-linked domains using the Pfam

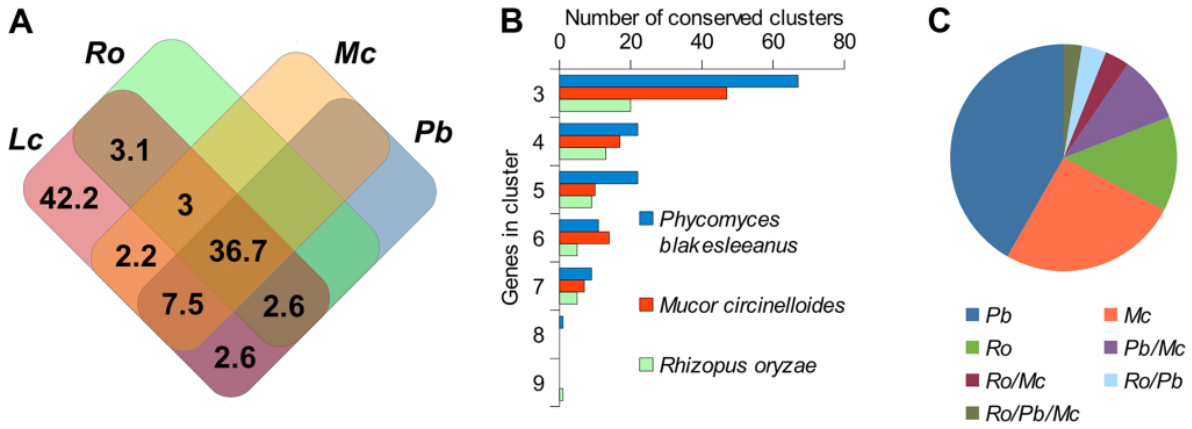

Figure 2. Conserved regions of the *L. corymbifera* genome with other mucoralean genomes. (A) Venn diagram of shared gene families between *L. corymbifera* and other mucoralean fungi based on GhostFam gene families. Numbers indicate percentage of *L. corymbifera* gene families. (B) Number and size of conserved clusters of *L. corymbifera* with other mucoralean genomes. (C) Proportions of conserved clusters of *L. corymbifera* shared with different mucoralean genomes. Occurrences in more than one of the genomes are indicated by a slash between the species.
doi:10.1371/journal.pgen.1004496.g002

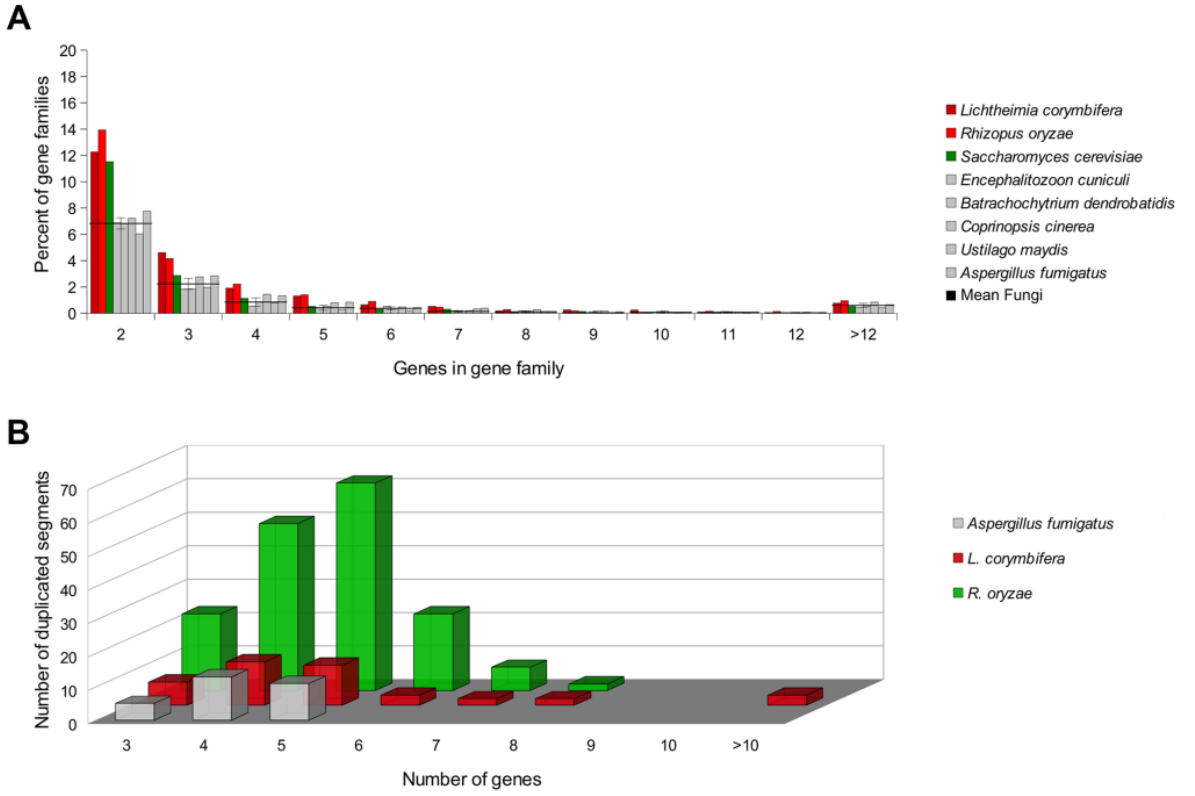

Figure 3. Gene duplication and duplication of genomic regions within mucoralean genomes in comparison to the genome of *A. fumigatus*, which (i) inhabits the same natural habitats and (ii) causes similar symptomatology in human like *L. corymbifera* and (iii) serves as model organism for causative agents of invasive mycoses [56,75]. The genome of *A. fumigatus* serves as measure for low incidences of singular and segmental gene duplications [115,116]. (A) Comparison of gene families between *L. corymbifera*, *R. oryzae* and non-mucoralean fungi. Gene families are based on GhostFam homology families. Values for *L. corymbifera* and *R. oryzae* are excluded from the "mean fungi" value. (B) Regions with a minimum of 3 genes were tested for multiple occurrences within the genomes by GECKO2.
doi:10.1371/journal.pgen.1004496.g003

database and HMMER3 [47,48]. If the expansions that contain transposons were excluded, 66 groups of paralogous proteins will be left in *L. corymbifera* comprising a total of 820 genes (Figure 4 B).

The most abundant expanded groups (with 331 and 242 members) are rather heterogeneous in terms of functional domains, thus there is no particular function that could be assigned to them. The largest group with a dominating domain contains in total 56 members, of which 52 possess a heterokaryon incompatibility protein (HET) domain (PF06985) (Figure 4 C). Interestingly, this domain was so far attributed nearly exclusively to ascomycetes (with only one exception for the basidiomycete *Moniliophthora perniciosa*), where the HET proteins control somatic allorecognition (non-self-recognition) during the formation of heterokaryons [49]. However, Mucorales, opposite to ascomycetes, do not form heterokaryons by fusion of somatic cells but only during sexual reproduction and zygospore-formation. Since HET domain proteins are absent in all other sequenced zygomycetous genomes it is unlikely that they play a general role in the sexual reproduction but seem to be specific for *Lichtheimia*. Interestingly, these HET genes were differentially regulated under stress conditions. Several copies of the HET domain proteins were down-regulated under iron-depletion and hypoxia. Since these genes are absent in all other mucoralean fungi it is unclear which functions they serve in *L. corymbifera*. In addition it is unclear where these genes originate since they do not occur in other basal fungi and show only very weak similarity with the HET proteins of dikaryan fungi.

Several expansions contain transporters: major facilitator superfamily (MFS, PF07690, PF12832, PF05977, PF13347), ABC transporters (PF00005, PF00501, PF01061, PF00664, PF06422), sugar (and other) transporters (PF00083). In addition, some interesting expansions are connected to the transcription regulation function, which is discussed in more detail in a separate section and signal transduction pathways (see supplemental Material, Table S5 and S6). Four expanded groups are characterized by the cytochrome P450 (PF00067) domain (Figure 4 C). Interestingly, mucoralean pathogens like *L. corymbifera* have been shown to be resistant to several antifungals including voriconazole [8,50] which could be explained by high copy numbers and isoforms of the target genes. Thus, gene duplication and expansion might be important for the success of *L. corymbifera* in human infections.

Strikingly, these domains (MFS transporters, HET and cytochrome P450) were also the dominant domains in genes which were localized in tandem duplications (Figure S3). Tandem duplicated genes were found to be present in 42 of the 66 gene expansion groups covering 38% of all genes in the expanded

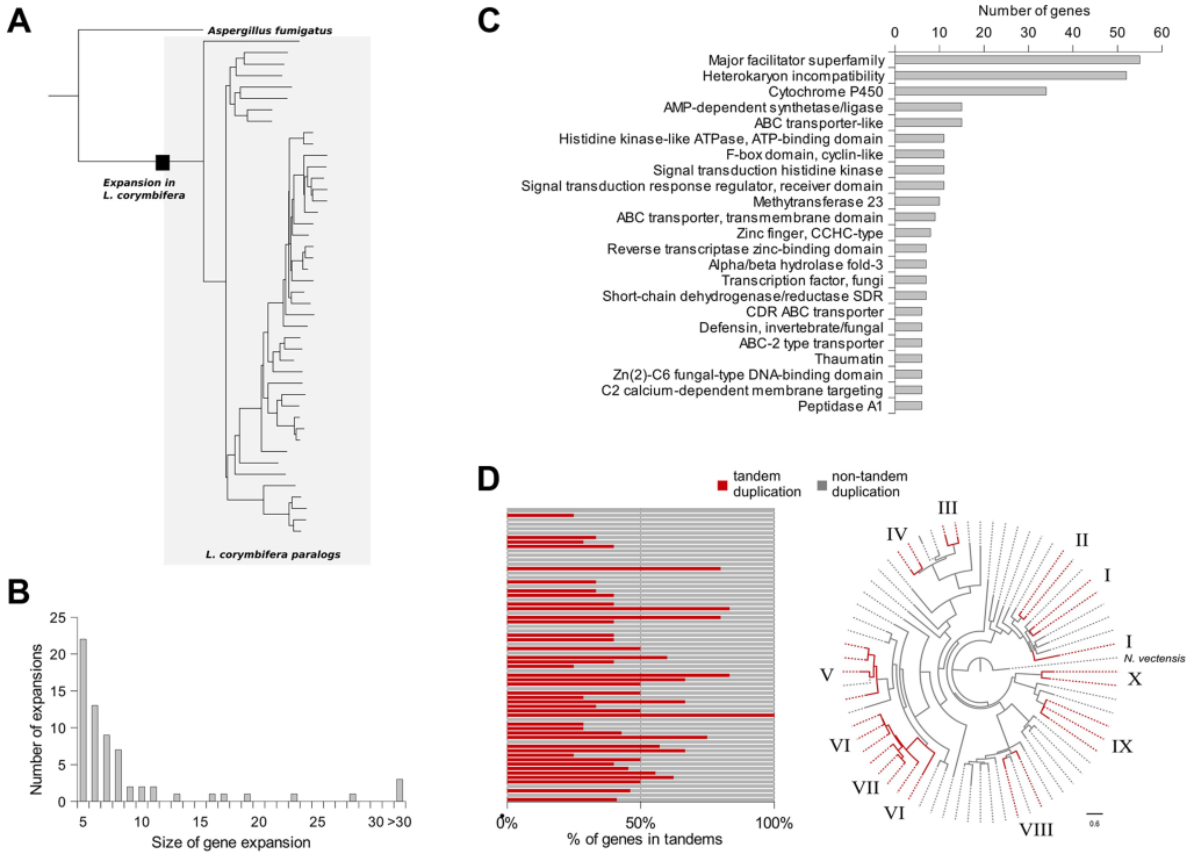

Figure 4. Gene expansions and tandem duplications found in *L. corymbifera*. (A) Tree representing an expansion of HET proteins in *L. corymbifera*. Branches enclosed in the grey shaded area represent paralogs of *L. corymbifera*. The black square represents the point where the expansion started. (B) Number and size of gene expansions in the *L. corymbifera* genome. (C) Main functional domains of gene expansions based on PFAM annotation. The numbers of genes with the different functional domains were combined if a domain was present in more than one expansion. (D) Proportion of genes within gene expansions which are arranged in tandem duplications. Each bar represents an expansion with the red part as the percentage of tandem duplicated genes (left). Clustering of tandem duplicated genes of cytochrome P450 genes in *L. corymbifera* (based on reconstruction with RaxML [130]). Red branches represent tandem duplicated genes. Numbers at the branch tips indicate different tandems.
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groups (Figure 4 D). However, additional smaller tandems were found which did not fit the criteria of gene expansions. A total of 701 genes are organized in such tandem repeats (Figure S3). In addition, duplicated genes were frequently found to be located on the same scaffold which may result from older tandem duplications.

Thus, tandem duplications and a high amount of gene retentions may give an additional explanation for the high amounts of duplicated genes in *L. corymbifera* comparable to the observations in plant genomes where segmental duplications (resulting from WGD) and tandem duplications play different roles in the enrichment of genes of several gene families [51,52]. Tandem duplication and the retention of duplicated genes would be an explanation for the severe differences in the size of gene families between mucoralean fungi with only 53% of gene families with the same size in *L. corymbifera* and *R. oryzae* (see Figure S3 C).

To investigate if the different gene copies may have different functions and thus may contribute to rapid adaptations to different environmental conditions we analysed the expression of tandem duplicated genes under infection-associated stress conditions (iron depletion and hypoxia; see Table S7). Differential expression of at least one gene of the tandem clusters under at least one of the conditions was found for 71 tandems. Strikingly, only 7 tandems were co-regulated while in 64 cases expression of the copies was different including six cases were copies were antithetically regulated (Figure S4). These results are consistent with the hypothesis that the high prevalence and maintenance of duplicated genes leads to diversification of gene functions.

Alternative splicing

Duplicated genes can lead to the diversification of gene functions of the two copies which has been discussed in the section above. In addition, alternative splicing (AS) can increase the functional diversity. Gene prediction resulted in 841 alternative splicing events in a total of 683 genes (5.5% of total genes) comparable to the situation in *S. cerevisiae* [53]. Based on the analysis of the RNAseq data alternative splicing could be verified for 273 genes (2.2% of total genes) (Figure S5 A and Table S8). Alternative donor and acceptor are the dominant groups of alternative splicing events (>75% of the total events) which is similar to the situation in several higher eukaryotes [54] (Figure S5

A). Comparison of alternatively spliced genes with genes in tandem duplications and gene expansions showed that only 12 (4.4% of genes with AS) in these groups are also alternatively spliced. If AS occurs in tandem duplicated genes, it occurs in only one of the copies except in one case. This is in accordance with recent results in *S. cerevisiae* which show that duplicated genes can replace one alternatively spliced gene and that alternative splicing is often lost after gene duplication [55]. To test if AS plays a role in the stress adaptation of *L. corymbifera* we analysed the potential alteration in alternative splicing pattern during stress adaptation. Significant changes were only detected for 16 and 23 genes under iron depletion and hypoxia (<0.2% of the total genes), respectively (Figure S5 B). Based on the high incidence of gene duplication and the differential expression of the copies as well as the comparably low number of alternatively spliced genes, maintenance of duplicated genes seems to play a more important role for the generation of functionally distinct paralogs than alternative splicing.

Identification and expression of potential virulence factors under infection-related conditions

a) Iron uptake genes. Iron is an essential trace element for all organisms and plays a crucial role in fungal pathogenicity [56–58]. Elevated host iron levels are an important prerequisite for mucormycosis and the iron permease FTR1 has been shown to be crucial for virulence in *R. oryzae* [59–62]. The genome of *L. corymbifera* contains four copies of FTR1, of which three are located next to a multicopper oxidase and may share the same promoter. Both FTR1 and multicopper oxidase are important players of the reductive pathway and have been shown to form functional complexes in *C. albicans* [63]. Thus, co-expression of both genes and maintenance of their proximity may contribute to effective iron uptake and, hence, to the virulence of *L. corymbifera*. The higher copy number of FTR1 in *L. corymbifera* compared to *R. oryzae* (one copy) suggests more efficient employment of this pathway and, probably, optimisation of the different copies to different environmental conditions in *L. corymbifera*. To investigate the expression of iron-uptake genes under iron limited conditions we added the iron chelator bathophenanthrolinedisulfonic acid (BPS) to overnight cultures of *L. corymbifera* (200 µM final concentration) and analysed gene expression via RNA sequencing (see Material and Methods for details). Consistent with this hypothesis only one of the copies of FTR1 (LCor01036.1) was up-regulated under iron limitation in the gene expression experiments while another copy (LCor06326.1) was moderately down-regulated (Figure 5 A). In addition, all multicopper oxidases which are co-localized with FTR1 were regulated in the same manner as the corresponding FTR1 gene and expressed at comparable levels (Figure 5 A). The two remaining copies (LCor00518.1, LCor04103.1) were expressed constitutively at low levels and may be either specific for other conditions or generally silenced. In addition to FTR1 and multicopper oxidases, ferric reductases are key elements in the reductive pathway of iron acquisition. Two of the three ferric reductases were up-regulated under iron limitation (LCor07115.1, LCor11373.1), whereas the third one was constitutively expressed (Figure 5 B).

Besides the role of the reductive pathway little is known about the iron uptake systems in mucoralean pathogens. Our analysis uncovered the presence of additional genes involved in iron uptake including zinc/iron permeases, heme oxygenases and siderophore transporters (Table 2). Heme utilization may contribute to growth within the host, since mucoralean pathogens rapidly invade blood vessels [3] and may use hemoglobin as iron source [60]. Accordingly, one of the heme oxygenases (LCor09772.1) was strongly up-regulated under iron limitation (Figure 5 B). All available mucoralean genomes, including *L. corymbifera*, lack non-ribosomal peptide synthetases (NRPSs) and are therefore unable to produce hydroxamate siderophores. Instead they produce polycarboxylate siderophores (rhizoferrin), which have a much weaker binding activity compared to hydroxamate siderophores and are produced by direct fermentation [64,65]. In addition, mucoralean fungi are also able to utilize deferoxamine, a bacterial siderophore which is used as an iron-chelator in human therapy [66,67]. Interestingly, zygomycetous species have been shown to live in close relationship with bacteria, including cases containing bacterial endosymbionts indicating that xenosiderophores might play a role in the development of siderophore uptake systems [68,69]. However, *L. corymbifera* has also been shown to be a potent producer of siderophores itself [70]. Under iron-limitation the putative siderophore transporter of *L. corymbifera* (LCor01340.1) was up-regulated (Figure 5 B) supporting the role of siderophores in the iron acquisition of mucoralean fungi. Interestingly, based on the expression data we found an additional gene (LCor00410.1) that may be involved in the siderophore metabolism of *L. corymbifera*, containing functional domains which are typical for genes involved in regulation and synthesis of siderophores in bacteria (lucA/lucC PF04183 and FhuF PF06276). Thus, the gene may encode a novel candidate for a regulator of siderophore biosynthesis in mucoralean fungi.

Although fungi generally lack ferritin as intracellular iron storage, ferritin has been found in several mucoralean species [62,71]. Based on orthology searches, ferritin genes could be identified in all mucoralean genomes. In addition, domain search in *Spizellomyces punctatus* and *Allomyces macrogynus* (Origins of Multicellularity, BROAD) revealed also the presence of potential ferritin genes. Since these two groups represent the most basal fungal lineages, apart from the highly derived microsporidians [72], ferritin seems to have been lost in some microsporidians and the higher fungi. Interestingly, in higher fungi, the loss of ferritin coincides with the appearance of sidA, an important gene in hydroxamate siderophore production (Figure S6). Siderophores are known to serve as intracellular iron storages in several Asco- and Basdiomycota species [73,74]. Thus, a plausible hypothesis is that maintenance of ferritin was not necessary in derived fungi due to the gain of importance of siderophores. The expression of the two ferritin genes (LCor08103.1, LCor11038.1) was slightly decreased under iron-limitation (~1.6×, Fig. 5B). Nothing is known about the dynamics and functions of fungal ferritins. The slight decrease may be sufficient to stabilize free iron concentrations in the cytoplasm.

Only three transcription factors were up-regulated under iron depleted conditions (Figure S7). Interestingly, one of these genes (LCor08192.1) shows similarities to GATA type regulators which are known to be involved in the adaptation to iron limitation in higher fungal pathogens (e.g. *A. fumigatus and Histoplasma capsulatum*) [75,76]. Thus, this transcription factor may represent a key regulator in iron acquisition, and therefore an important virulence factor of *L. corymbifera*. A second transcription factor (LCor01236.1) resembles CRZ1, which is a calcineurin regulated TF and indicates a possible involvement of the calcineurin pathway in the adaption to iron limitation.

b) Secreted proteases. Besides the iron uptake systems, hydrolytic enzymes like proteases are known as important virulence factors in fungal pathogens, e. g., *R. microsporus* and *C. albicans* [77–79]. In addition, gene expansion of secreted proteases was observed in the *R. oryzae* genome [28]. The genome of *L. corymbifera* contains a total of 413 predicted proteases representing 3.3% of all genes comparable to the situation in *R.*

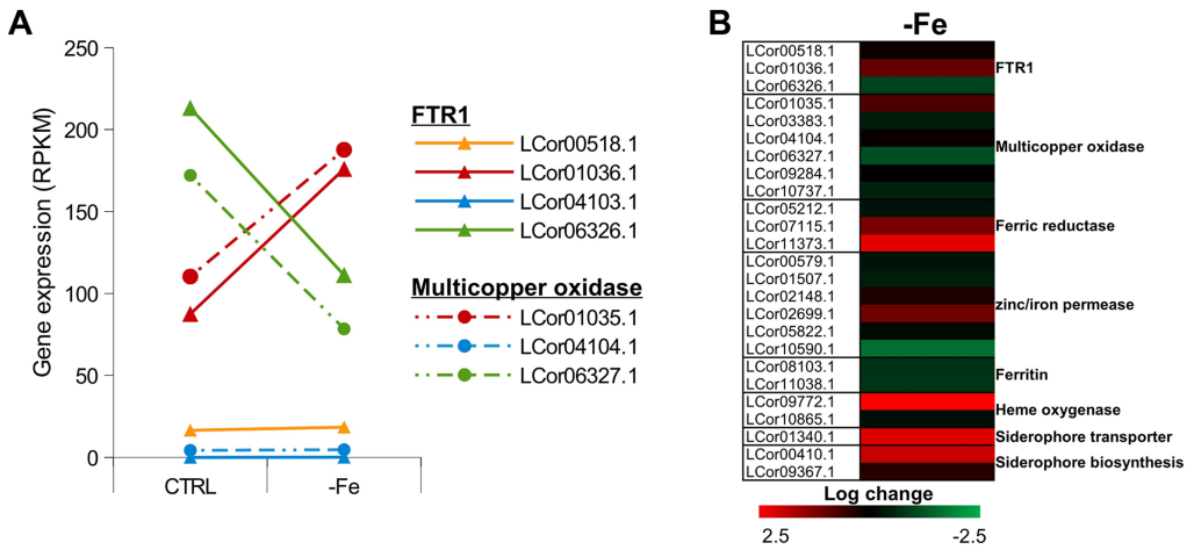

Figure 5. Expression of iron uptake genes under iron limited conditions. (A) Expression levels of FTR1 domain genes and their corresponding multicopper oxidases under standard conditions and iron limitation. (B) Heat map showing the regulation of iron uptake genes under iron-limited conditions.
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oryzae which contains 630 proteases (3.6% of all genes). However, the number of secreted proteases differs between the two species, moreover, in *L. corymbifera* the relative amount of secreted proteases is nearly twice as high as in *R. oryzae*: in *L. corymbifera* 13% (53) of the proteases are predicted to be secreted while in *R. oryzae* this number reaches only 7% (44). The most important classes of secreted proteases are serine and aspartate proteases representing 55% and 36% of total secreted proteases, respectively (Table 3). Comparison of secreted aspartic proteases (SAP) of *R. oryzae* revealed an enrichment of SAPs compared to other fungal genomes [28]. However, the number of SAPs is comparable in *L. corymbifera* (24) and *R. oryzae* (28) indicating that the presence of a higher number of these enzymes is a general feature of mucoralean pathogens. Several secreted proteases were activated under infection-related stress conditions (iron depletion, hypoxia). While iron depletion affected mainly the expression of aspartic and serine proteases, hypoxic conditions induced the expression of serine-, metallo- and some aspartic proteases.

c) Transcription factors. The *Lichtheimia* genome encodes 768 putative transcription factors (TFs) representing 6.2% of total genes. This amount is comparable to the situation in *R. oryzae* (6.4% of total genes) but higher than the average content of TFs in other fungi (4.5%) [80]. Basic BLASTp analyses showed that 37 of the TFs (4.8% of total TFs) are specific for *L. corymbifera*.

The TFs were assigned to 53 families of DNA-binding domains (based on the InterProScan predictions). The great majority of these families have been described previously in fungal species [81]. However, 4 families have not been found in true fungal species before (putative representatives of 2 families, PF01167 (Tubby) and PF02319 (TDP), were predicted in microsporidia; the other 2 were described in plants and bacteria). Of these "new" TFs one (PF03106, DNA-binding WRKY domain), represented in 7 *Lichtheimia* proteins, has been previously described only in plants. Interestingly, in plants these TFs are numerous and have diverse functions, including pathogen defense [82]. In *L. corymbifera* we found three of the seven members differentially regulated under hypoxic conditions (down: LCor09690.1; up: LCor02851.1, LCor08197.1) indicating a function in the stress response of this species (Figure S7).

Of 6 fungal-specific TF families [81], 2 families are not predicted in *L. corymbifera* genome, namely PF04769 (mating-type protein MAT a1) and PF02292 (APSES domain). The lack of

Table 2. Iron uptake genes in the *L. corymbifera* genome.

Pathway	Iron uptake gene	Number of genes
Reductive pathway	FTR1	4
	multicopper oxidase	8
	ferric reductase	3
Low affinity iron uptake	zinc/iron permease	6
Siderophore uptake	siderophore transporter	1
Heme utilization	heme oxygenase	2
Iron storage	ferritin	2

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Table 3. Protease families in the *L. corymbifera* genome.

	Proteases (% of total proteases)	Secreted (% of secreted proteases)
Aspartate	60 (14.5)	19 (35.8)
Cysteine	68 (16.5)	2 (3.8)
Metallo	146 (35.4)	3 (5.6)
Serine	124 (30)	29 (54.7)
Threonine	14 (3.4)	0
Unknown	1 (0.2)	0
Total	413	53

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MAT gene is expected because mating in Mucorales is regulated via sex plus and sex minus HMG transcription factors [83]. The absence of APSES domain may be compensated by the APSES-type DNA binding domain PF04383.

It is also noteworthy that the traditional proportion of the Zn fingers Zn(2)Cys(6) (Zn cluster) and Cys(2)His(2) is inverted in *Lichtheimia*. In all fungi observed so far, the Zn clusters are more abundant than C2H2 TFs, in fact they are normally the most numerous in the fungal genomes. In *Lichtheimia*, on the contrary, the number of C2H2 Zn fingers is nearly 1.5 times larger than the number of Zn clusters.

Comparative phylome-based analysis reveals several expanded TF families in *L. corymbifera* including MADS box TFs with 11 representatives instead of the usual 1–4 members. MADS box genes are known to play a role in a variety of functions (e.g, cell cycle, stress response, development [84]). Presumably, the expansion of the MADS box genes in *Lichtheimia* was accompanied by the delegation of some functions from other TFs or even neofunctionalization. The functional basis for such expansion as well as the potential roles of these TFs cannot be elucidated from their primary structure, because MADS box genes are not conserved except for the MADS domains. But at the epxressional level, we could find significant up-regulation of two of the 11 MADS box TFs (LCor03918.1, LCor08105.1). Thus, the copies do not seem to have completely overlapping functions.

Analysis of the phylomes allowed us to detect another exciting expansion, which is evidently characteristic for all Mucorales: the duplication of TBP, TATA binding protein (PF00352). As it has been recently shown for higher eukaryotes, core promoter recognition factors can be involved in modulating gene- and cell-type-specific programs of transcription, such as tissue differentiation, development, etc. [85]. These new functions are associated with a gene duplication of the TBP, resulting in TRF2 (and other) factors, which are highly similar to TBP but do not bind the TATA box. In fungi, the event of TBP duplication is exceptionally rare. A survey of all so far sequenced genomes revealed only 4 examples of such duplication: 3 in Ascomycetes/Sordariomycetes (*Chaetomium globosum*, *Grosmannia clavigera* and *Podospora anserina*) and 1 in the basidiomycete *Laccaria bicolor*. In contrast, in Mucorales all 4 considered species possess 2 copies of the TBP gene. It can be supposed that the duplicated TBP-like factors may play an additional role in condition-specific responses and thus may be of interest as potential virulence factors.

Temperature tolerance is an essential prerequisite for the infection of warm-blooded animals and was shown to be connected to the virulence of *Lichtheimia* species [25,86]. The genome was surveyed for the presence of heat shock transcription factors (HSF). The total number of these TFs in *L. corymbifera* genome is 24, which is the highest number among all so far investigated fungi. This is in accordance with the known tolerance of *L. corymbifera* to high temperatures [13,14]. However, it seems that this family expansion is not a specific trait of *Lichtheimia* but is characteristic for all Mucorales. Interestingly, HSF genes were also up-regulated under hypoxic conditions indicating additional functions of the different members of the HSF family in the response to different stresses and growth conditions.

It is curious that additionally to the abundant heat shock factors also a cold shock TF (PF00313) was found, which was not previously described in fungi. This can explain why *Lichtheimia*, although it does not grow at low temperatures, can tolerate cold as it was shown to survive periods of more than 5,000 years in ice [87].

The importance of the *L. corymbifera* genome for studying the infection biology of mucoralean pathogens: Concluding remarks

The genome of *L. corymbifera* represents the first insight into the genome structure of basal mucoralean pathogens. Despite the growing recognition of Mucorales as life-threatening clinically important human pathogens, little is known about the virulence traits of these fungi. The high dissimilarity between *L. corymbifera* and the other sequenced mucoralean pathogens *R. oryzae* and *M. circinelloides* in both evolutionary and functional sense underlines the importance of additional genome projects.

This study revealed a high proportion of duplicated and expanded genes in the *L. corymbifera* genome comparable to the situation in *R. oryzae*. However, clear evidence for a WGD can be detected only for *R. oryzae*, but not for *L. corymbifera* indicating that additional mechanisms contribute to the higher incidence of duplicated genes in mucoralean fungi. Tandem repeats seem to be an important source for gene duplication in *L. corymbifera* and may explain the rapid development of lineage-specific gene duplication and expansion in mucoralean fungi. Several species-specific gene duplications point at potential virulence traits including iron uptake genes, hydrolytic enzymes and genes which may contribute to resistance against antifungal agents like azoles (cytochrome P450 gene expansion). In contrast, alternative splicing does not seem to play an important role in the generation of orthologs and the adaption to stress conditions.

Based on these results, we postulate a relationship between genome fluidity by the generation and retention of additional gene copies and dynamics of adaptation to new environments. Higher genome flexibility results in a higher likelihood for a saprobic zygomycete to become a pathogen.

In addition we were able to shed light on the genes involved in iron uptake, which is a crucial step for virulence and thus for the

development of an infection. We could identify additional genes which might be involved in iron-uptake besides the known virulence factor FTR1 *L. corymbifera* including transcription factors, siderophore transporters and a potential regulator involved in siderophore biosynthesis that has not been described in mucoralean fungi.

Our data represent a valuable resource for future research and the understanding of infection-associated mechanisms of mucoralean pathogens.

Materials and Methods

Genome sequencing and assembly

A combination of Illumina and 454 sequencing was used for the *L. corymbifera* genome. A shotgun library and an 8 kb paired-end library were created and sequenced on a half plate on a Roche GS FLX Titanium each resulting in 1,168,226 shotgun reads (505,023,982 nt) and 519,989 paired-end reads (76,603,029 nt). In addition, a standard paired-end read library was prepared and sequenced in one channel Illumina HiSeq2000 (100 bp paired-end reads) resulting in 264,907,616 raw reads (26,490,761,600 nt) and 12,614,650 filtered and downsampled reads (1,261,465,000 nt). The 454 reads were separately assembled using Newbler (454 Life Sciences) and Mira [88] and both assemblies were unified using minimus2 [89]. The Illumina reads were used to solve homopolymeric regions using Nesoni (http://bioinformatics.net.au/software). This approach resulted in a total of 1,214 contigs ($\geq$ 500 nt) with a total of 41,405,106 nt and a N50 of 66.718 nt. Finally, the contigs were mapped on Newbler predicted scaffolds using MUMmer [90] resulting in 209 scaffolds with a total length of 33.6 Mb (for statistics refer to Table 1). The raw DNA-seq reads and the resulting genome assembly is available at EMBL under the study accession number PRJEB3978 (http://www.ebi.ac.uk/ena/data/view/PRJEB3978).

Detection of transposable elements

Scaffolds of *L. corymbifera* were searched for repeats by Repbase and the server version of Censor [91,92] (http://www.girinst.org/censor/index.php) using the eukaryotic repeat database.

K-mer analysis

The analysis was performed on the Ilumina reads with an algorithm described in the potato genome paper [31]. The algorithm was used to write a custom perl program. Based on the fastq data of the Illumina reads k-mers of 41, 59, 69, and 79 nt were detected and analyzed. Component estimation was done manually in R.

Non-coding RNA prediction, synteny and phylogeny

A local version of tRNAscan-SE v.1.23 [93] with parameters –omlfrF was used for the detection of tRNAs. Ouput files are supported in the supplemental material (http://www.rna.uni-jena.de/supplements/lichtheimia/index.html). With RNammer -S euk -m lsu,ssu,tsu -gff (v.2.1) [94] rRNAs were detected. The 1973 ncRNA classes currently available at RFAM (v.10.1) [95] were downloaded for homologous search. These classes were predicted with (I) BLAST (v.2.2.25) [96] with an E-value$<10^{-4}$ (II) with infernal [97] using covariance models from RFAM and (III) by hand as indicated in main text. Genes discovered in the reads only were found with rnabob [98] in combination with various programs of the RNAViennaPackage v.2.0.2 (http://www.tbi.univie.ac.at/~ivo/RNA/). All ncRNA genes are available at the supplemental material in gff and fasta format (http://www.rna.uni-jena.de/supplements/lichtheimia/index.html). Additionally, sequence-structure-alignments for each RFAM-ncRNA class in stockholm format are provided. Motif search in promoter regions of polymerase III transcripts was performed with MEME (v.4.8.1) [99], rnabob and by hand. Synteny analysis: for all of our identified ncRNA positions in *L. corymbifera* and *R. oryzae*, five direct upstream and downstream located genes and their function were extracted, according to protein-annotation files. Pairwise alignments of syntenic proteins with a) -p blastn and b) -p tblastn and a minimum E-value of E$<10^{-4}$ were performed. For ncRNA-phylogeny reconstruction the best scored ncRNA per ncRNA family was joined, which was identified in all species, except 18S and 28S rRNA, and *S. pombe* used as outgroup. A multiple alignment was created by Mafft with the L-INS-i method, 1000 iterations as module in the EPoS framework for phylogenetic analysis [100]. Out of this alignment we constructed a Neighbour Joining Tree (Kimura correction model, 1000 bootstrap replicates) and Mr. Bayes (v.3.1.2; two runs with each four chains and 5,000,000 generations).

Prediction of protein-coding genes and functional annotation

Evidence-driven gene prediction was performed using AUGUSTUS v2.7 [37] using the gene models from *Rhizopus oryzae* prediction was supported by the incorporate pooled Illumina RNA-seq data from three biological replicates of three different physiological conditions (control, hypoxia, iron depletion) sequenced on Illumina HiSeq 2000. After the raw RNA-Seq data were quality trimmed- using btrim [101], the data were pooled and mapped using the splice-junction mapper tophat2 [102]. From this mapping data the AUGUSTUS protocol (http://bioinf.uni-greifswald.de/bioinf/wiki/pmwiki.php?n = IncorporatingRNAseq. Tophat) was followed to create hints for gene structures in an iterative manner. Finally, the hints were incorporated during the AUGUSTUS predcition based on the *Lichtheimia* genome using the metaparameters of *R. oryzae*. For functional annotation predicted protein-coding genes were analyzed by BLASTp in BLAST2GO [38] with a minimum E-value of E$\leq 10^{-25}$ and a HSP length cut-off of 33 amino acids. Conserved domains were identified using the InterProScan function of BLAST2GO and GO mapping was performed based on the BLAST and InterProScan results. Genome annotations are available at the ENA under the study accession number PRJEB3978 (http://www.ebi.ac.uk/ena/data/view/PRJEB3978).

Detection of differentially expressed genes under infection-related conditions

Paired-end RNA-seq data for three biological replicates of two infection-associated conditions (i.e., iron-depletion and hypoxia) and a control treatment was obtained.

L. corymbifera was grown on SUP agar [103] plates for 7 days at 37°C. Spores were washed off with sterile PBS, washed with PBS and counted using a Thoma chamber. Erlenmeyer flasks (500 ml) containing 100 ml of chemical defined medium (1.7 g/l YNB w/o amino acids and ammonium sulphate, 20 g/l Glucose, 5 g/l ammonium sulphate, 50 mg/l arginine, 80 mg/l aspartic acid, 20 mg/l histidine, 50 mg/l isoleucine, 100 mg/l leucine, 50 mg/l lysine, 20 mg/l methionine, 50 mg/l phenylalanine, 100 mg/l threonine, 50 mg/l tryptophane, 50 mg/l tyrosine, 20 mg/l valine) [60] were inoculated with 10^7 spores and grown for 16 h at 37°C under shaking. Afterwards (i) cultures were grown for additional 2 h under these conditions, (ii) the iron chelator bathophenanthroline-disulfonic acid (BPS, Sigma) was added to a final concentration of

200 µm and cultures were incubated for additional 2 h under previous conditions or (iii) cultures were subjected to hypoxic conditions (1% oxygen, 5% CO_2) and incubated for 2 h at 37°C under shaking. The mycelium was separated from the medium using a miracloth filter (Millipore) and immediately frozen in liquid nitrogen. For RNA isolation the mycelium was grounded using mortar and pestle under liquid nitrogen and total RNA was isolated using the RNAeasy Plant kit (Quiagen) according to the manufacturer's instructions.

Sequencing was performed using Illumina HiSeq 2000. Raw reads were quality-filtered using btrim [101] and mapped to the genome using tophat2 [102] (parameters: –no-discordant –no-mixed –b2-very-sensitive –max-intron-length 5000). Differentially expressed genes were identified with EdgeR [104] which also adjusted obtained p-Values for multiple testing. Transcripts with an absolute fold-change≥2 and an adjusted p-Value≤0.01 were considered differentially expressed. Results are available in Table S7.

Phylome reconstruction

The phylome, meaning the complete collection of phylogenetic trees for each gene in a genome, was reconstructed for the genome of *L. corymbifera*. 24 other fungal species were included in the reconstruction. A rough draft of the proteome of *Mortierella alpina* ([26]; PUBMED ID:22174787) was predicted using AUGUSTUS [32] due to the lack of a publicly available proteome. The phylome was reconstructed using an automated pipeline previously described in [39]. Briefly, for each protein in the *L. corymbifera* genome a Smith-Waterman search was performed against the fungal proteome database. Results were filtered using an e-value cut-off $E<1e^{-5}$ and a continuous overlapping region of 0.5. At most 150 homologous sequences for each protein were accepted. Homologous sequences were then aligned using three different programs: MUSCLE v3.8 [105], MAFFT v6.712b [106], and kalign (http://www.biomedcentral.com/1471-2105/6/298/)]. Alignments were performed in forward and reverse direction (i.e. using the Head or Tail approach [107]), and the 6 resulting alignments were combined with M-COFFEE [108]. This combined alignment was trimmed with trimAl v1.3 [109] (consistency-score cut-off 0.1667, gap-score cut-off 0.9). Trees were reconstructed using the best-fitting evolutionary model. The selection of the model best fitting each alignment was performed as follows: a Neighbour Joining (NJ) tree was reconstructed as implemented in BioNJ [110]; the likelihood of this topology was computed, allowing branch-length optimization, using 7 different models (JTT, LG, WAG, Blosum62, MtREV, VT and Dayhoff), as implemented in PhyML v3.0 [111]; the model best fitting the data, as determined by the AIC criterion [112], was used to derive ML trees. Four rate categories were used and invariant positions were inferred from the data. Branch support was computed using an aLRT (approximate likelihood ratio test) based on a chi-square distribution. Resulting trees and alignments are stored in phylomeDB [39] (http://phylomedb.org), with the phylomeID 245. Trees were scanned using ETE v2 [46].

Orthology prediction

Orthologs between *L. corymbifera* and the other species included in the phylome were based on phylogenies obtained during phylome reconstruction. A species-overlap algorithm, as implemented in ETE v2 [46], was used to infer orthology and paralogy relationships. Briefly the algorithm decides whether a node in a tree is a speciation of a duplication node depending on the overlap of the species branching from the node. Overlap between those species will indicate a duplication node. Otherwise a speciation node will be considered.

Species tree reconstruction

The species tree was build using a concatenation method. 58 single-copy proteins that appeared in at least 21 of the 25 genomes were selected. After concatenation, the alignment was trimmed using trimAl [109]. Columns with more than 50% of gaps were removed. A conservation score of 50% of the alignment was used. The final alignment contained 46,793 positions. The tree was reconstructed using phyML [111]. LG model [113] was selected and a 4-categories GAMMA distribution was used. Bootstrap was obtained by creating 100 random sequences using SeqBoot from the phylip package. A tree was then reconstructed for each sequence and the consensus tree was inferred using phylip. All the nodes in the species tree had a bootstrap of 100. Additionally a species tree based on the super-tree reconstruction program DupTree [42] was reconstructed. The input contained the 9,478 trees obtained during phylome reconstruction. Both species trees showed a similar topology. The only difference pertained to the position of *S. pombe*. In the concatenated tree it appeared grouped with *S. cerevisiae* while in the super-tree it appeared in its correct position at the base of Ascomycota. This difference was collapsed into a multifurcation for the tree in figure 1.

Detection of conserved regions

For the detection of conserved regions, all genomes were modeled as strings of integers. BLAST analyses [96] were performed for all proteins in the four mucoralean genomes all-against-all, with an E-value threshold of 0.1. Homology families IDs were assigned to the protein-coding genes using GhostFam [114] with default parameters. Genomes were transformed into strings of gene IDs, which were then used as input for the reference gene cluster implementation in Gecko2 [43,44]. The two parameters for the algorithm were the minimum size of the reference cluster/hypothetical conserved region "s" and the maximal distance "δ" (insertion or deletion of a gene). For every hypothetical gene cluster larger than s on the reference genome, all other genomes were tested for approximate occurrences of this reference gene cluster. The *L. corymbifera* genome was used as a reference genome and searched for gene clusters with parameters $s=3$ (minimum size of the reference gene cluster) and $\delta=0$ (number of insertions and deletions), $s=4/\delta=1$, $s=5/\delta=2$, $s=6/\delta=3$ and $s=7/\delta=4$. Results of the different filter settings were combined and overlapping clusters were eliminated. Local rearrangements and duplications within the cluster occurrences were not punished. All regions that had approximate occurrences in at least one other genome were reported. If multiple occurrences did intersect, only the best scoring one was reported.

Detection of duplicated regions (segmental duplications)

To detect duplicated regions in the mucoralean species, each genome was analysed individually by using the single contigs as reference. As for the detection of conserved regions, the same homology assignment and parameters of $s=5$ and $\delta=2$ were used. All regions with approximate occurrences in at least one other contig or the reference contig were reported, unless they intersected.

Detection of tandem duplications

Tandem duplications were defined by at least two genes assigned to the same GhostFam gene family and a maximum of three genes between the copies.

Prediction of alternative splicing

Predicted transcripts of the genomes were separated in alternative splicing events by Astalavista [24]. Events of predicted transcripts that contain splice-junctions have been confirmed by the number of split-mappings that confirm each of the exon-exon junctions (Table S8). For a read to support a splice-junction, the left part of the read was required to be included in one exon, and the right part had to be included in the other exon of a splice junction, with the first/last position before/after the split matching exactly the position of the predicted intron.

Genome resources

Genome data of *Aspergillus fumigatus* [115], *Aspergillus nidulans* [116], *Batrachochydrium dendrobatidis*, *Cryptococcus neoformans*, *Encephalitozoon cuniculi* [117], *Rhizopus oryzae* [28], *Paracoccidioides brasiliensis*, *Schizosaccharomyces pombe* [118], *Nosema ceranae* [119], *Nematocida parisii* [120], *Puccinia graminis* [121], *Ustilago maydis* and *Coprinus cinerea* [122] are genome sequencing projects of the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) (see Table S9 for detailed citations). *Phycomyces blakesleeanus*, *Phanerochaete chrysosporium* [123], *Laccaria bicolor* [124], *Mucor circinelloides*, *Nematostella vectensis* [125], *Monosiga brevicollis* [126] and *Serpula lacrymans* [127] genomic data were obtained from Joint Genome Institute (JGI). These sequence data were produced by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/ in collaboration with the user community. The genomes of *Homoloaphlyctis polyrhiza* [128] and *Mortierella alpina* [26] were obtained from Genbank (*Hp*: PRJNA68115; *Ma*: PRJNA41211). The *Neurospora crassa* genome [129] was obtained from UniProt reference genomes. The *Saccharomyces cerevisiae* genome was obtained from *Saccharomyces* Genome database (SGD) (see Table S9) [53].

Supporting Information

Figure S1 K-mer frequency distribution for *Lichtheimia corymbifera*. The k-mer frequency distribution (black line) was calculated for all k-mers of length of 59, i.e. for all possible 59-mers derived from the original Illumina/Solexa reads. The number of k-mers (y-axis) is plotted against the frequency at which they occur (x-axis). The distribution shows a main peak (shaded in light gray) and a steep rise to the left (shaded in dark gray). This left-most rise of k-mers at lower frequencies represents mostly k-mers with randomly occuring sequencing errors. The main peak represents k-mers derived from (putatively) correct sequencing reads. This main peak can be dissected into three normal distributions (red, blue and orange lines) the sum of which (green line) matches the observed distribution (black line). The three component distributions represent the 'homozygous' part of the genome (blue line, major component), the 'heterozygous' part of the genome (red line), and most likely some repeat regions that make up a minor proportion of the observed k-mers (orange line). Component estimation was done manually in R. The component distributions have the same variance (21), but different means (blue 116, red 65, orange 165) and proportions (blue 94%, red 4%, orange 2%).
(TIFF)

Figure S2 Structure of spliceosomal RNAs and ncRNA based phylogeny. (A) The last stem (IV) of *Lichtheimia* U11 snRNA is extended in comparison to other U11 snRNAs and to U1 snRNA. U2 snRNA folds into an expected secondary structure. In contrast, U12 snRNA shows an extended stem II, and misses the third stem (III). Stem IV/V is much shorter as in other known U12 snRNAs. U5 snRNA is used by both spliceosomes, with the general eukaryotic secondary structure. 2D structures were computed using RNAfold (RNA Vienna Package). Boxes indicate sm binding sites. Phylogeny of *L. corymbifera*, *M. circinelloides*, *P. blakesleeanus*, *R. oryzae* and *S. pombe* (outgroup) based on ncRNAs (except 18S and 28S rRNA). Alignment computed via Mafft L-INS-i with 1000 generations; Tree construction via (B) Neighbour Joining: Kimura: 1000 bootstrap replicates and (C) Mr. Bayes: two runs with each four chains and 5,000,000 generations.
(TIFF)

Figure S3 Tandem duplications in *L. corymbifera*. (A) Number and size of tandem duplication in the *L. corymbifera* genome. (B) Functional classes of genes in tandem duplications based on PFAM annotation. Asterisk indicates classes which are enriched in tandem duplications (Fisher test, $P<0.05$). (C) Gene family size comparison of *L. corymbifera* and *R. oryzae*. Gene families are indicated as larger in *Lichtheimia* (L>R), smaller in *Lichtheimia* (L<R) or as large as in *Rhizopus* (L = R).
(TIFF)

Figure S4 Expression of tandem duplicated genes. Tandem duplicated genes were analysed based on the RNA-seq data. Tandems were regarded as (i) not regulated if no copy in the cluster was up/down-regulated under the tested conditions, (ii) co-regulated if all copies in the clusters were up/down-regulated under at least one of the conditions, (iii) not co-regulated if one of the copies was differently regulated than the other(s), (iv) antithetically regulated if one copy was up- and the other down-regulated. Genes were regarded as differentially regulated if there was a two-fold change of expression and $P<0.01$ (edgeR).
(TIFF)

Figure S5 Alternative splicing in *L. corymbifera*. (A) Number and proportion of different classes of alternative splicing events based in evidence driven gene prediction (outer ring) and confirmed events (inner ring). (B) Proportion of AS genes where AS patterns were changed under stress conditions compared to control.
(TIFF)

Figure S6 Distribution of genes involved in iron uptake within the fungal kingdom. Orthologs of iron uptake genes were identified using the phylome of *L. corymbifera* (indicated in blue). If no ortholog was found BLASTp analysis was performed using the *L. corymbifera* protein sequence and an E-value $E \leq 10^{-10}$ (indicated in pink). Intracellular iron storages besides ferritin are indicated as 's' (siderophores) or 'v' (vacuolar) according to previous results (1 Silva et al., 2 Haas et al.). The presence of a sidA ortholog is indicated as "+", the absence as "−" according to previous results (1 Silva et al., 2 Haas et al.).
(TIFF)

Figure S7 Differential expression of transcription factors under iron depletion and hypoxia. Bar charts on top represent TFs grouped according to their functional domains (domain combinations). Up- and down-regulated genes are indicated in red and green respectively. The bar chart on the bottom shows the total amount of TFs regulated under the conditions.
(TIFF)

Table S1 Sequencing statistic of the *L. corymbifera* genome and transcriptome.
(PDF)

Table S2 Transposable and repetitive elements in the *L. corymbifera* genome.
(PDF)

Table S3 Overview of ncRNAs found in basal fungi. op – Close/Part to Operon; pg – Pseudogene; Ror – *R. oryzae*; Lco – *L. corymbifera*; ? – candidate.
(PDF)

Table S4 Sequencing and mapping statistics of RNA sequencing.
(XLS)

Table S5 Signalling pathway components included in the study and orthologues identified in *L. corymbifera*.
(PDF)

Table S6 Classification and gene IDs of putative protein phosphatases in the *L. corymbifera* genome.
(PDF)

Table S7 RNA-Seq mapping and differentially expressed genes.
(XLS)

Table S8 Potential alternatively spliced genes in *L. corymbifera* and confirmation of alternative transcripts by RNA-Seq data.
(XLSX)

Table S9 Genomes used in this study.
(PDF)

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Author Contributions

Conceived and designed the experiments: VUS SWi ES MMH FH MS MN VV JL IDJ MM AAB TG SB KV. Performed the experiments: VUS SWi ES MMH FH MS MN VV JL IDJ KK KR SWe. Analyzed the data: VUS SWi ES MMH FH MS MN VV JL IDJ KR SWe MM TG SB KV. Contributed reagents/materials/analysis tools: SWi ES FH MS MN VV JL IDJ KR SWe MM AAB TG SB KV. Wrote the paper: VUS SWi ES MMH FH MS MN VV MM TG SB KV. Designed the software used in analysis: SB MS SWi KR MM FH JL MMH TG.

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Figure S1

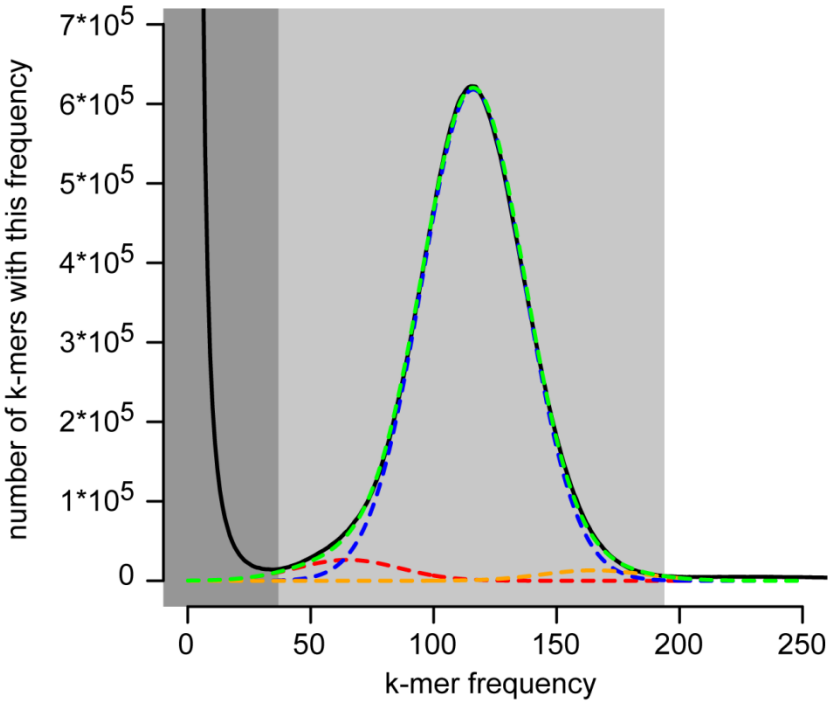
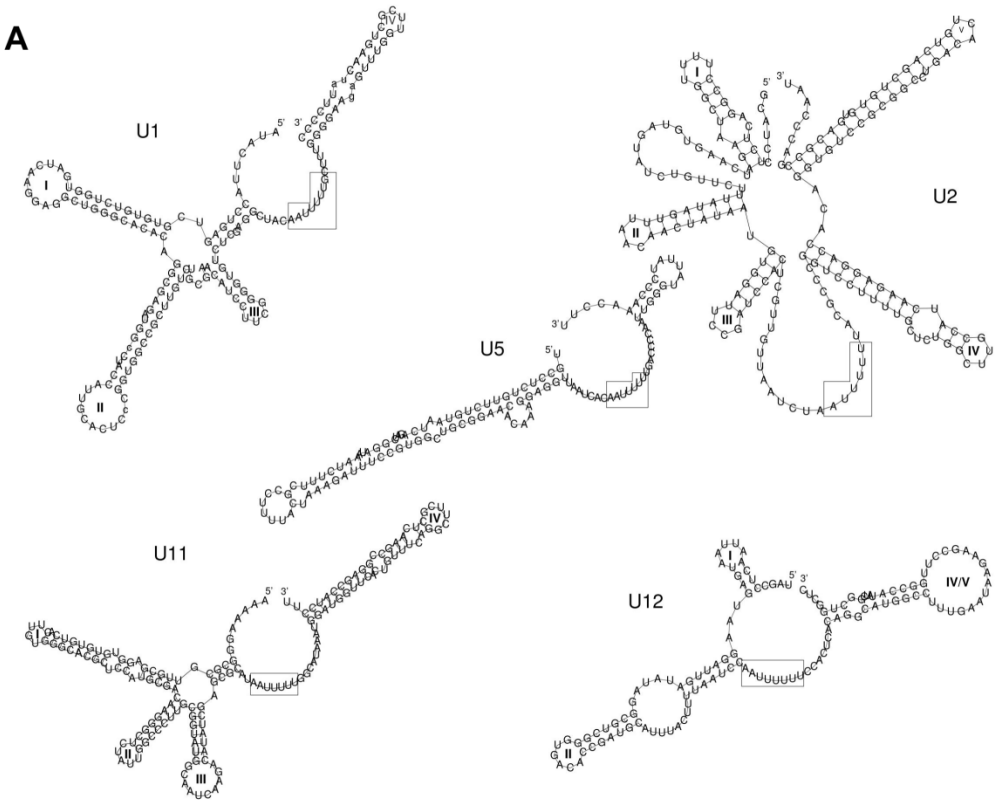
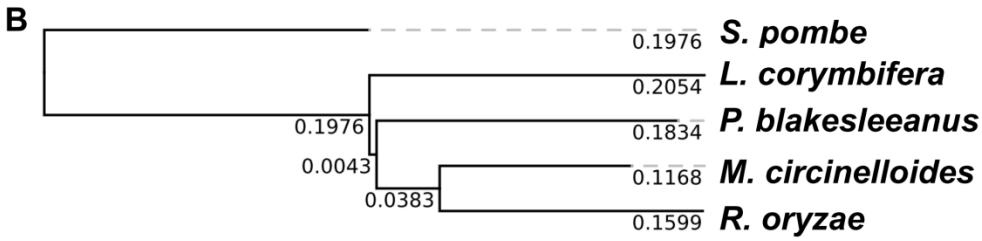

number of k-mers with this frequency
7*10^5
6*10^5
5*10^5
4*10^5
3*10^5
2*10^5
1*10^5
0
0
50
100
150
200
250
k-mer frequency

Figure S2


A
U1
U2
U5
U11
U12


B
0.1976
S. pombe
0.2054
L. corymbifera
0.1976
0.1834
P. blakesleeanus
0.0043
0.1168
M. circinelloides
0.0383
0.1599
R. oryzae

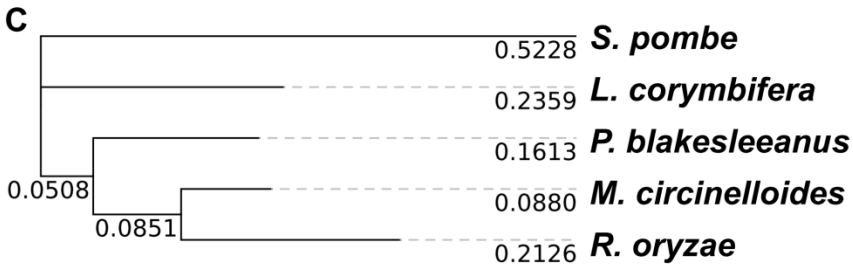

C
0.5228
S. pombe
0.2359
L. corymbifera
0.1613
P. blakesleeanus
0.0508
0.0880
M. circinelloides
0.0851
0.2126
R. oryzae

Figure S3

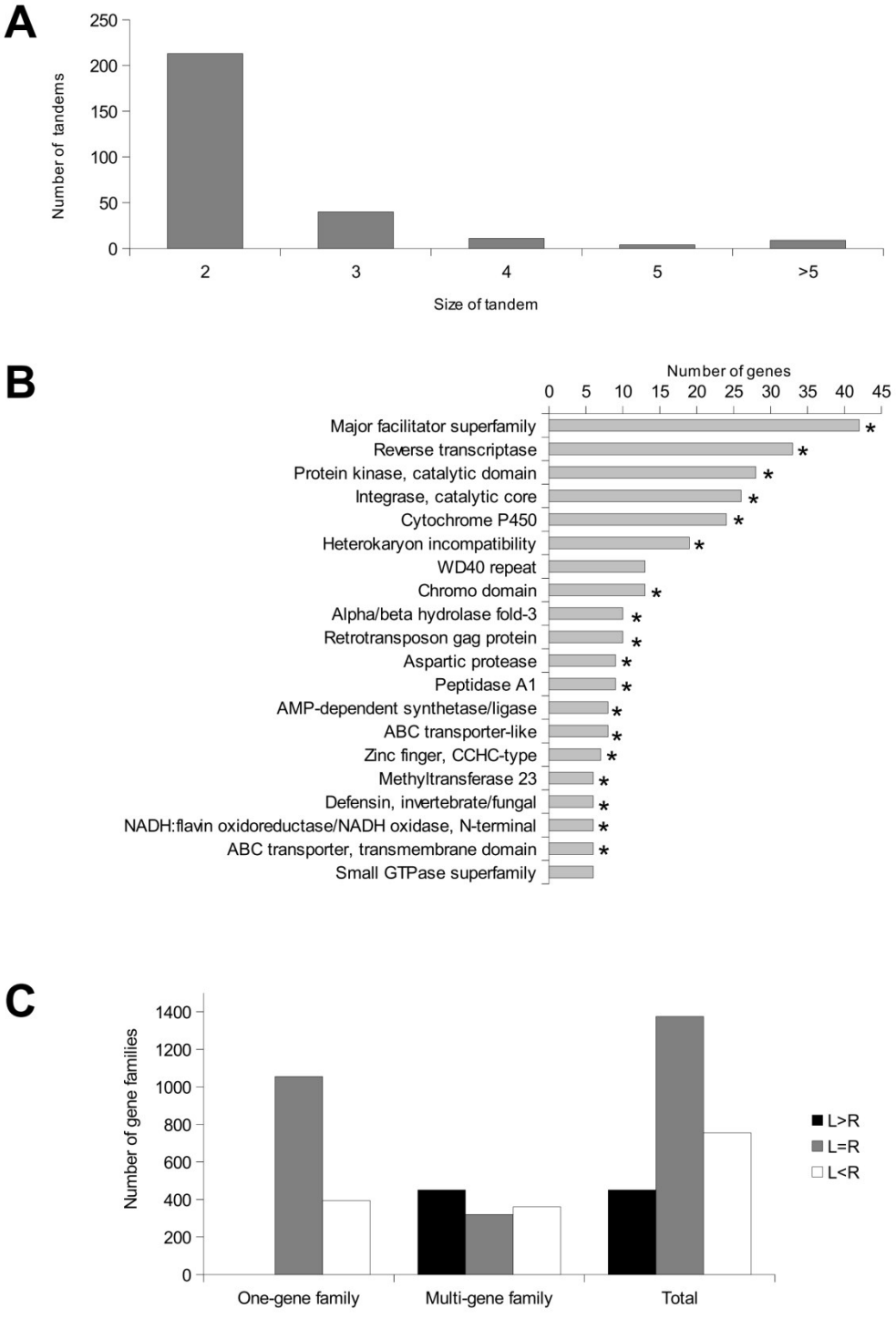

A
Number of tandems
250
200
150
100
50
0
2
3
4
5
>5
Size of tandem
B
Number of genes
0 5 10 15 20 25 30 35 40 45
Major facilitator superfamily
Reverse transcriptase
Protein kinase, catalytic domain
Integrase, catalytic core
Cytochrome P450
Heterokaryon incompatibility
WD40 repeat
Chromo domain
Alpha/beta hydrolase fold-3
Retrotransposon gag protein
Aspartic protease
Peptidase A1
AMP-dependent synthetase/ligase
ABC transporter-like
Zinc finger, CCHC-type
Methyltransferase 23
Defensin, invertebrate/fungal
NADH:flavin oxidoreductase/NADH oxidase, N-terminal
ABC transporter, transmembrane domain
Small GTPase superfamily
C
Number of gene families
1400
1200
1000
800
600
400
200
0
One-gene family
Multi-gene family
Total
L>R
L=R
L<R

Figure S4

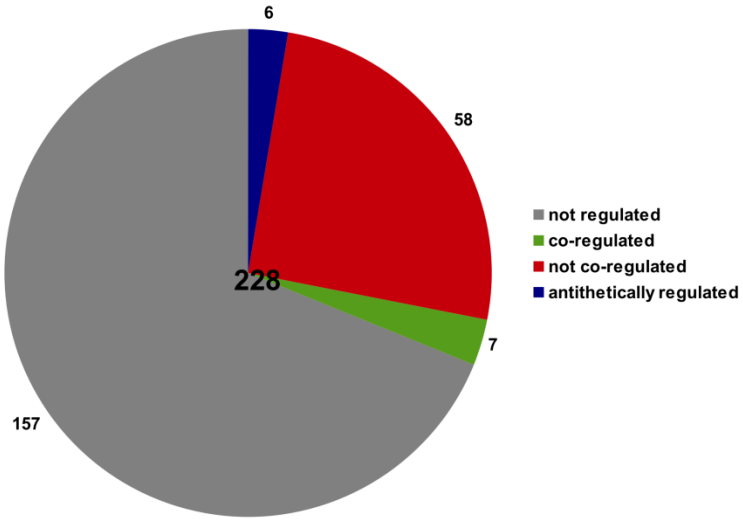


Figure S5

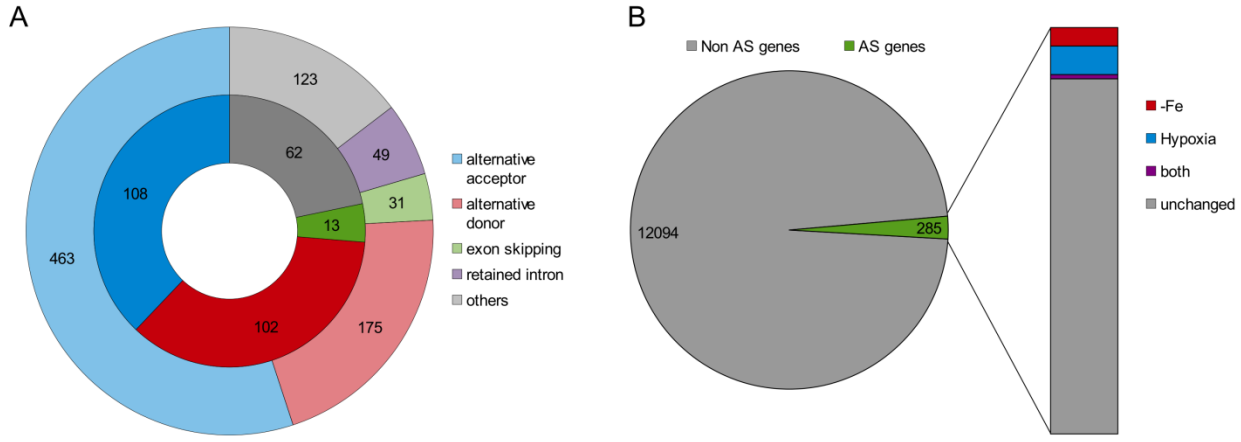


Figure S6

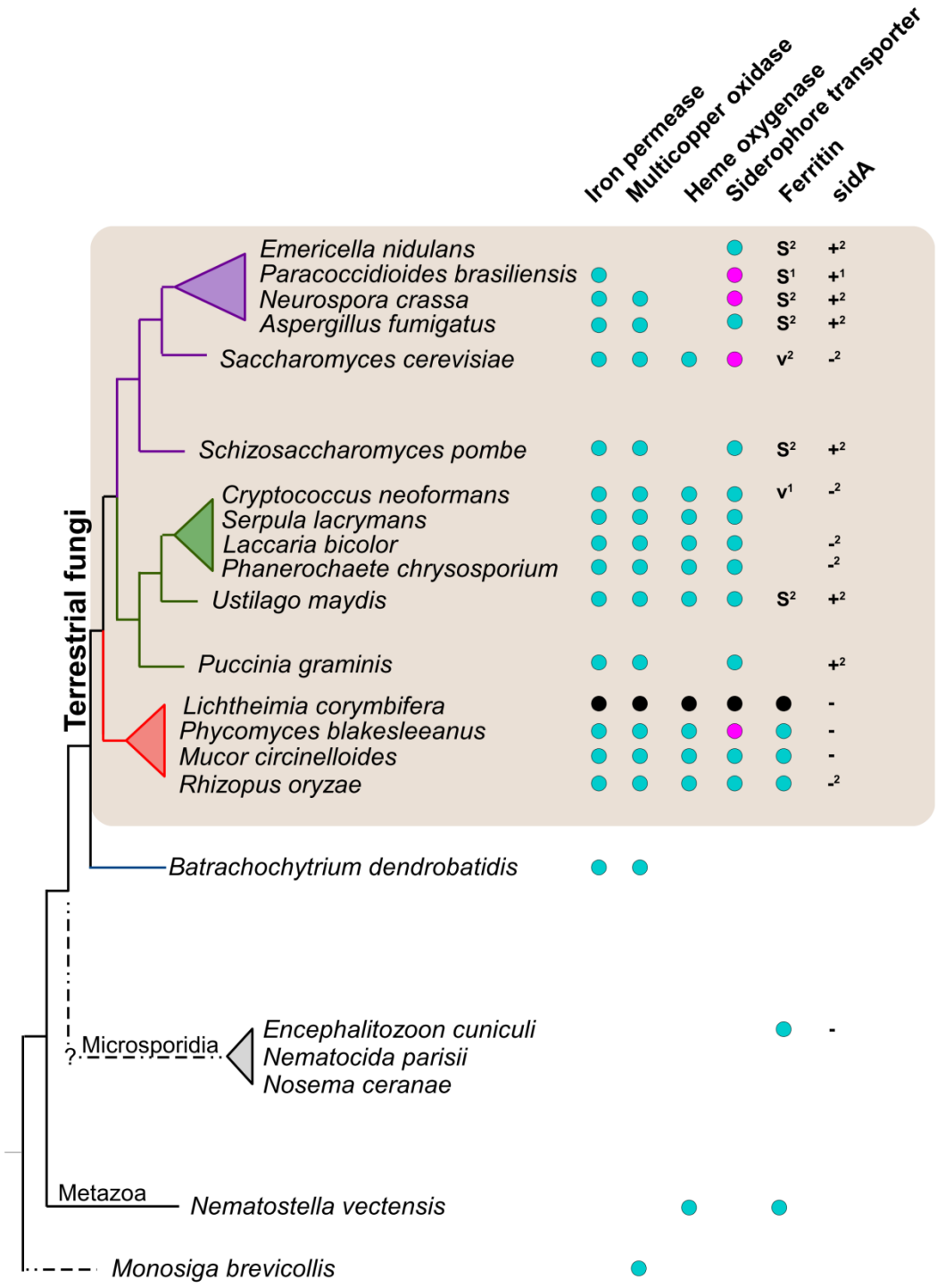

Iron permease
Multicopper oxidase
Heme oxygenase
Siderophore transporter
Ferritin
sidA
Terrestrial fungi
Emericella nidulans
Paracoccidioides brasiliensis
Neurospora crassa
Aspergillus fumigatus
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Cryptococcus neoformans
Serpula lacrymans
Laccaria bicolor
Phanerochaete chrysosporium
Ustilago maydis
Puccinia graminis
Lichtheimia corymbifera
Phycomyces blakesleeanus
Mucor circinelloides
Rhizopus oryzae
Batrachochytrium dendrobatidis
? Microsporidia
Encephalitozoon cuniculi
Nematocida parisii
Nosema ceranae
Metazoa
Nematostella vectensis
Monosiga brevicollis

Figure S7


YABBY protein
Transcription factor CBF/NF-Y/archaeal histone
Zinc finger, CCCH-type
Zinc finger, CCHC-type
Zn(2)-C6 fungal-type DNA-binding domain/Transcription factor, fungi
Zn(2)-C6 fungal-type DNA-binding domain
Homeodomain-like / Glucocorticoid receptor-like
Zinc finger, GATA-type / Glucocorticoid receptor-like
Homeodomain-like / Beta-beta-alpha zinc fingers
Winged helix / Nucleic acid-binding, OB-fold
NDT80 DNA-binding domain / p53-like transcription factor, DNA-binding
KilA, N-terminal / APSES-type HTH, DNA-binding
Transcription factor E2F / dimerisation partner (TDP) / Winged helix
DNA-binding RFX-type winged-helix domain / Winged helix
Zinc finger, A20-type / Glucocorticoid receptor-like
Heat shock factor (HSF)-type, DNA-binding / Winged helix
Cold-shock protein, DNA-binding / Nucleic acid-binding, OB-fold
Transcription factor, fork head / Winged helix
SANT/Myb domain / Homeodomain-like
Zinc finger, C2H2 / Beta-beta-alpha zinc fingers
Homeodomain
Myc-type, basic helix-loop-helix (bHLH) domain
Glucocorticoid receptor-like
Zn(2)-C6 fungal-type DNA-binding domain
Beta-beta-alpha zinc fingers
ACT-like
Transcription regulator HTH, APSES-type DNA-binding domain
ssDNA-binding transcriptional regulator
Nucleic acid-binding, OB-fold
p53-like transcription factor, DNA-binding
Myc-type, basic helix-loop-helix (bHLH) domain
DNA binding domain, putative
Winged helix
Homeodomain-like
Basic-leucine zipper domain
SGT1
Zinc finger, GRF-type
GCN5-like 1
YL1 nuclear
CP2 transcription factor
Transcription factor, fungi
CCR4-Not complex component, Not1, C-terminal
SART-1 protein
DNA-binding WRKY
Zinc finger, C5HC2-type
Zinc finger, BED-type predicted
Zinc finger, MIZ-type
DDT domain
CCAAT-binding transcription factor, subunit B
Zinc finger, NF-X1-type
Helix-turn-helix
TEA/ATTS
Tubby, C-terminal
Copper fist DNA-binding
Zinc finger, GATA-type
Transcription factor, MADS-box
Zn(2)-C6 fungal-type DNA-binding domain
Basic-leucine zipper domain
Zinc finger, C2H2
0 20 40 60 80
Number of genes
0 20 40 60 80
Number of genes
up-regulated
not regulated
down-regulated
100%
75%
50%
25%
0%
% of TFs
SID
HYP

Table S1

Roche 454	
Number of Shotgun Reads	1,168,226
Number of Bases in Shotgun Reads	505,023,982
Number of Paired-End Reads	519,989
Number of Bases in Paired-End Reads	76,603,029
Illumina	
Number of Raw Paired-End Reads	264,907,616
Number of Bases in Raw Paired-End Reads	26,490,761,600
Number of Filtered Paired-End Reads	240,264,256
Number of Bases in Filtered Paired-End Reads	24,026,425,600
Number of downsampled Paired-End Reads	12,614,650
Number of Bases in downsampled Paired-End Reads	1,261,465,000
Assembly	
Newbler	
Number of large Contigs (≥500 bp)	1,936
Number of Bases in Large Contigs	35,683,536
N50 Size of Large Contigs	57,255
Largest Contig Size	226,585
Mira	
Number of large Contigs (≥500 bp)	2,117
Number of Bases in Large Contigs	37,540,862
N50 Size of Large Contigs	46,267
Largest Contig Size	158,689
Minimus2	
Number of large Contigs (≥500 bp)	1,214
Number of Bases in Large Contigs	41,405,106
N50 Size of Large Contigs	66,718
Largest Contig Size	308,811

Table S2

Transposable element	Total bases	Percent of assembly	Major superfamilies (% of class)
DNA transposons	512,554	1.52	
			EnSpan (23)
			Harbinger (7)
			hAT (14.7)
			Helitron (4.3)
			Mariner/Tc1 (5.3)
			MuDR (10.6)
			Polinton (9.8)
			Sola (4.8)
LTR retrotransposons	612,555	1.82	
			Gypsie (59.7)
			Copia (25.8)
			DIRS (6.4)
Non- LTR retrotransposons	359,496	1.06	
			Jockey (17.8)
			L1 (35.9)
			CR1 (14.6)
			R1 (7.4)
Viruses*	65,316	0.19	
Satellite and microsatellite	10,469	0.03	
Simple repeats	18,155	0.05	
Other repeats	17,634	0.05	
Total	1,596,179	4.74	

*DNA, integrated, endogeneous retrovirus

Table S3

ncRNA	Function	*Ror*	*Lco*	Remarks
5S rRNA	component of the large ribosomal subunit	2op	0op+1	
18S rRNA	small subunit of ribosomal RNA	2op	0	Within the assembly of *L. corymbifera*, several 100 nt fragments were found.
5.8S rRNA	component of the large ribosomal subunit	2op+1	1-4	In *L. corymbifera* found in read data only. Last hairpin divergent.
28S rRNA	component of the large ribosomal subunit	2op	0.5	In *L. corymbifera*, only 3' half was detected.
tRNA	transfer RNA	237+1pg	164+10pg	
Rnase P	tRNA processing	2	1	Central Pseudoknot of *L. corymbifera* diverse.
RNase MRP	rRNA processing	1	1	
SRP	directs ribosome to endoplasmatic reticulum	2	1	closer to metazoan than fungal SRP RNA
U1	major spliceosomal RNA	3	2	
U2	major spliceosomal RNA	4	2	
U4	major spliceosomal RNA	1-3	0-1	*L. corymbifera* gene was found in read data only.
U5	major spliceosomal RNA	3	2	
U6	major spliceosomal RNA	4	4	
U11	major spliceosomal RNA	1	1	*P. blakesleeanus* contains a 138 nt intron. Last stem longer.
U12	major spliceosomal RNA	1	1	Third stem atrophied. Last stem much shorter.
U4atac	minor spliceosomal RNA	1	0	
U6atac	minor spliceosomal RNA	1	1	Second half divergent.
U3	C/D box snoRNA, cleaves rRNA	4	3	
TPP	riboswitch, binds to thiamine pyrophosphate	4?	1?	
Telomerase	telomerase RNA component	0	1?	closer to ciliate than fungal telomerase RNA; No known proteins found
U7	histone processing RNA	-	4?	

Tables S4 and S5 are too large for print but can be found on the CD attached to this thesis.

Table S6

	S. cerevisiae	*R. oryzae*	*L. corymbifera*
CDC25	SCRG_01933	none	none
DSPs	SCRG_05285 SCRG_05575 SCRG_05152 SCRG_02694 SCRG_03190 SCRG_03261	RO3G_01445 RO3G_00853 RO3G_00502 RO3G_07214 RO3G_03637 RO3G_12794 RO3G_15103 RO3G_16335 RO3G_04063 RO3G_08234	LCor00777.1.t1 LCor02142.1.t1 LCor04949.1.t1 LCor05281.1.t4 LCor05433.1.t1 LCor05489.1.t1 LCor06286.1.t1 LCor08356.1.t1 LCor08468.1.t1 LCor09285.1.t1 LCor09981.1.t1 LCor10898.1.t1 LCor10947.1.t1 LCor11198.1.t1
FCP1	SCRG_02173	RO3G_04929 RO3G_16681	LCor02318.1.t1
LmwPTP	SCRG_02557	RO3G_16977	LCor09244.1.t1
Myotubularin	SCRG_03760	RO3G_06890	LCor05331.1.t1
PTPs	SCRG_00717 SCRG_04551 SCRG_01596 SCRG_03193 SCRG_03158 SCRG_00448 SCRG_03235	RO3G_00326 RO3G_04458 RO3G_06503 RO3G_07589 RO3G_08421 RO3G_11155 RO3G_02273 RO3G_14132 RO3G_13499 RO3G_13912	LCor05981.1.t1 LCor09442.1.t1 LCor06192.1.t1 LCor08202.1.t1 LCor09938.1.t1
STPPs	SCRG_04788 SCRG_00547 SCRG_04609 SCRG_01879 SCRG_02318 SCRG_01842 SCRG_04371 SCRG_03111 SCRG_00440 SCRG_00679 SCRG_00630	RO3G_01038 RO3G_02253 RO3G_07220 RO3G_13180 RO3G_14156 RO3G_15330 RO3G_16219 RO3G_02173 RO3G_04542 RO3G_04836 RO3G_00528	LCor00604.1.t1 LCor03810.1.t2 LCor04784.1.t2 LCor00797.1.t1 LCor01164.1.t1 LCor01800.1.t1 LCor02322.1.t1 LCor02879.1.t2 LCor03163.1.t1 LCor03480.1.t1 LCor03495.1.t1

Table S6 continued

	SCRG_00895	RO3G_12129	LCor03616.1.t2
	SCRG_00101	RO3G_02565	LCor06144.1.t1
	SCRG_03343	RO3G_08858	LCor06724.1.t1
		RO3G_10916	LCor07635.1.t1
		RO3G_14499	LCor08272.1.t1
		RO3G_13122	LCor09474.1.t1
		RO3G_13385	LCor09841.1.t1
		RO3G_12832	LCor10112.1.t1
		RO3G_14483	LCor10351.1.t1
		RO3G_08936	
		RO3G_00628	
		RO3G_04139	
		RO3G_12341	
		RO3G_13249	
		RO3G_13891	
		RO3G_14955	
		RO3G_16278	
		RO3G_10591	
		RO3G_03043	
PP2Cs			
	SCRG_04566	RO3G_01402	LCor00327.1.t1
	SCRG_02843	RO3G_06856	LCor00363.1.t1
	SCRG_00513	RO3G_06853	LCor01216.1.t1
	SCRG_01488	RO3G_10282	LCor01254.1.t1
	SCRG_03018	RO3G_02940	LCor04110.1.t1
	SCRG_05468	RO3G_04097	LCor07086.1.t1
		RO3G_01541	LCor07733.1.t1
		RO3G_06955	LCor09007.1.t1
		RO3G_11555	LCor09282.1.t1
		RO3G_14392	LCor07825.1.t1
		RO3G_16439	
Total	**37**	**65**	**52**

Tables S7 to S9 are too large for print but can be found on the CD attached to this thesis.

Manuscript C

Schwartze VU, Klassert TE, Riege K, Marcet-Houben M, Nowrousian M, Binder U, Grigoriev IV, Grøtli M, Gryganskyi A, Fleischauer M, Lipzen A, Park H, Salamov A, Stajich JE, Tamas M, Tritt A, Winter S, Böcker S, Gabaldón T, Marz M, Lass-Flörl C, Slevogt H, Voigt K

To the limit and beyond: Comparative genomic and transcriptomic analyses reveal stress adaptation determinants of human pathogenic *Lichtheimia* species

Title:

To the limit and beyond: Comparative genomic and transcriptomic analyses reveal stress adaptation determinants of human pathogenic *Lichtheimia* species

Running title:

Comparative genomics of *Lichtheimia* species

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Abstract

Background

Lichtheimia species are ubiquitous saprophytic fungi which can cause life-threatening infections in both animals and humans. To date, only three of the six described species in the genus have been associated with human infections. Species that are clinically relevant or not relevant differ in virulence and stress tolerance. In order to get broader insight into the molecular basis of pathogenicity, we compared the genomes of the clinical species *L. corymbifera* and *L. ramosa* with *L. hyalospora* and analysed the transcriptomes of the species under different stress conditions.

Results

The genomes of the species were similar in structure and gene content, but highly dissimilar from all other fungal genomes which were compared in this study. Our analyses showed that virulence-associated traits are also well conserved in the clinically not relevant species. However,

L. hyalospora showed major defects in growth at elevated temperatures which are caused by the accumulation of misfolded proteins. Comparative transcriptomic analyses under heat and endoplasmatic reticulum stress revealed similarities in the general stress response but also species-specific responses. Interestingly, the sensitivity of *L. hyalospora* to heat stress could be reduced by the simultaneous application of elevated osmotic pressure. Transcriptome analyses under combinatorial stress conditions revealed only minor differences in the gene expression compared to heat stress alone.

Conclusions

The genus-wide comparative genomics and transcriptomics of *Lichtheimia* give insights into its phylogeny, physiology and evolution of pathogenicity factors. Our results show that all *Lichtheimia* species have the genetic make-up enabling them to cause infections but clinically relevant species differ in stress response to elevated temperatures. However, the stress response can be altered by the presence of additional stresses leading to growth recovery of *L. hyalospora*. This is the first study providing insights into the genomic and transcriptomic transition to stress resistance and pathogenicity of mucoralean pathogens. The ability of clinically relevant *Lichtheimia* species to cope with the accumulation of misfolded proteins contributes to fast growth at elevated temperatures and their ability to cause human infections.

Keywords

Mucormycosis, comparative genomics, phylome, synteny, iron uptake, misfolded proteins, chaperones, thermotolerance, combinatorial stress

Background

The fungal order Mucorales (formerly Zygomycota) encompasses a variety of human pathogenic fungi. Life-threatening infections caused by mucoralean fungi (mucormycosis) have been

increasingly recognized in immunocompromised hosts [1]. Mucormycoses are uncommon compared to aspergillosis and candidiasis but cause severe clinical problems due to the rapid progress of infections, difficult diagnosis and the fact that treatment often includes extensive surgery in addition to antifungals [1–4]. A hallmark of mucormycosis is the rapid invasion of blood vessels by the fungus resulting in massive thrombosis and tissue necrosis [5, 6].

Lichtheimia species represent the second and third most common cause of mucormycosis in humans in Europe and worldwide, respectively [1, 4, 7, 8]. In addition, cases of *Lichtheimia* infections have been described in a variety of warm-blooded animals, e.g. horses, cattle and birds [9–11]. Recent studies also suggest that *L. corymbifera* is involved in Farmer's lung disease (FLD), a hypersensitivity disorder [12, 13]. The genus encompasses six described species but, to date, only three have been associated with human infections, namely *L. corymbifera*, *L. ramosa* and *L. ornata* [9, 10, 14]. Interestingly, these species have also shown higher virulence potential in infection experiments using the embryonated chicken egg [11, 15]. Up until now, there have only been limited data available regarding the ecology of *Lichtheimia* species and studies have often not distinguished between species. However, several *Lichtheimia* species are associated with food production in the Asian cuisine and have been isolated from fermented soy products [10, 16]. In addition, they are also contaminants of a variety of food products, such as peanuts and cocoa [10, 17, 18].

Little is known about the pathogenicity and virulence factors of mucoralean fungi. Only a few genomes of distantly related species have been sequenced and analysed so far. A previous study showed that the genome structure and the gene content of *L. corymbifera* are highly dissimilar compared to other mucoralean genomes including other pathogenic species, such as *Mucor circinelloides* and *Rhizopus arrhizus* [19]. In addition, the genome of *L. corymbifera* showed high rates of duplicated genes involving several putative virulence factors which could be identified [19]. Since virulence evolved likely several times independently in mucoralean fungi no conclusions about the evolution of virulence factors in certain species can be drawn from these comparisons. To date, nothing is known about the intra- and interspecific genomic flexibility of mucoralean fungi. Virulence factors described so far are mainly ubiquitously distributed genes in mucoralean fungi

which occur in pathogenic as well as non-pathogenic species. Prominent examples of this are the high-affinity iron permease Ftr1 and the surface protein CotH [20, 21]. While these genes help to understand the molecular factors involved in infections by pathogenic species, they are not sufficient to explain virulence since non-pathogenic species carrying these genes are unable to cause infection.

Recent studies have addressed this issue based on the physiological comparison of closely-related, pathogenic and non-pathogenic species. A study by Kaerger et al. focusing on several *Rhizopus* species showed that evolutionary emergence of virulence coincided with the adaptation to high temperatures [22]. However, non-thermophilic species were also less virulent in wax moth larvae kept at permissive temperatures for these species indicating that additional changes in metabolism may contribute to the high virulence of thermotolerant species [22]. Studies on the virulence and physiology of *Lichtheimia* species revealed differences in the adaptation compared to Rhizopus species. In experimental virulence tests, the three clinically relevant species of *Lichtheimia* showed higher virulence compared to the other *Lichtheimia* species [11, 15]. However, while physiological assays, growth experiments and stress tests revealed major differences in growth at elevated temperatures, there were no significant differences in metabolic flexibility and stress resistances between *Lichtheimia* species [11, 14]. Adaptation to elevated temperatures is a major prerequisite for fungal pathogens in order to cause invasive infections in warm-blooded animals and reduced ability to cope with heat stress correlates with reduced virulence [23–27]. Molecular adaptations that support the ability to grow at elevated temperatures include the sensing of elevated temperatures but also the presence of chaperones which stabilize other proteins and help to prevent or reverse misfolding of proteins [24, 27–30]. In addition, functionality of the endoplasmatic reticulum (ER) is crucial for thermoadaptation since immature and misfolded proteins are (re-)folded and modified in this compartment. Moreover, misfolded proteins in the ER can be transported to the cytoplasm and are degraded by the proteasomes. Mutants with defects in ER function are generally reduced in growth at elevated temperatures and reduced in virulence [25, 26, 31–35].

In this study we compared the genome sequences of the clinically relevant species *L. corymbifera* and *L. ramosa* with the genome of *L. hyalospora* which is not involved in human infections. We analysed a combination of physiological traits as well as genomic and transcriptomic data to get insights into putative virulence factors and stress response of *Lichtheimia* species to detect differences which could explain the virulence differences of the species. Finally, the results of the study show that stress-resistance in *Lichtheimia* species can be modified and is defined by gene expression rather than genome structure or gene content.

Results and discussion

General features and comparative analyses of *Lichtheimia* genomes

The assemblies of all three sequenced species of *Lichtheimia* are similar in size ranging from 30.7 Mb for *L. ramosa* to 33.3 Mb and 33.6 Mb for *L. hyalospora* and *L. corymbifera*, respectively (Table 1) [19, 36]. This is comparable to the size of genomes of other published mucoralean genomes such as M. circinelloides (36.4 Mb) and *Rhizomucor miehei* (27.6 Mb) but smaller than the genome of *R. arrhizus* (45.3 Mb) [37–39]. Heterozygosity of the genomes was estimated by k-mer analyses of the Illumina reads as described previously [19, 40]. Similar to our previous results analysing the *L. corymbifera* genome, reads from the three genomes showed a single main peak and evidence for only a small proportion of potentially heterozygous regions, supporting the general assumption that *Lichtheimia* genomes are haploid during vegetative growth (Figure S1) [19]. In previous works it has been shown that, in contrast to other mucoralean fungi, *L. corymbifera* seems to possess only a low number of repetitive elements [19]. The three *Lichtheimia* genomes were screened for repetitive elements using RepeatMasker using libraries from RepBase and *de novo* repeat libraries (see material and methods) [41, 42]. Using only curated repeats from RepBase libraries repeat content was comparable in all three species ranging from 1.5 % to 2.1 % of the genomes. However, when *de novo* repeats were included in the analyses the repeat content ranged from 4.5 % in *L. ramosa* to 6.2 % and 10.6 % in *L. hyalospora* and *L. corymbifera*, respectively (Table S1). This content is comparable to the available genome of the most closely-related species, *R. miehei* (3.17

%) but much lower than in *R. arrhizus* (~35 %) [38, 39]. Low complexity regions, simple repeats and non-classified repeats account for the majority of repetitive elements in *Lichtheimia* (Table S1).

We annotated 225, 213 and 168 ncRNAs for *L. corymbifera*, *L. ramosa* and *L. hyalospora*, respectively (Table 2). The number of spliceosomal RNAs, snoRNAs, SRP RNA, RNase MRP, riboswitch TPP and Hammerhead 1 differ only marginally between the three fungi. None of the three assemblies contain all four types of rRNAs. Contrary, the number of tRNAs varies significantly between the pathogenic fungi (*L. corymbifera* 175, *L. ramosa* 171) and *L. hyalospora* (130 tRNAs). The full annotation and supporting material (fasta sequences, stk alignments, gff annotations) are provided in the supplemental material (File S1).

Since the assembly quality varied between the genomes, fragmented whole genome alignment by Gegenees was used to compare the assemblies [43]. The amount of alignable fragments was similar between *Lichtheimia* species, while only few fragments could be aligned to *R. miehei* (Figure 1A). Fragments of *Lichtheimia* that could be aligned showed comparable average similarities of 56.2 to 58 %. In order to further analyse synteny between the *Lichtheimia* genomes the scaffolds of the three assemblies were ordered according to alignments produced by MAUVE and r2Cat software [44, 45]. Based on these results, 31 clusters were generated containing 49, 128 and 368 scaffolds of *L. ramosa*, *L. corymbifera* and *L. hyalospora*, representing 99 %, 95.3 % and 89.9 % of the assemblies, respectively (Table S2). While there is a lack of synteny between *Lichtheimia* spp. and the most closely related fungus *R. miehei*, the genomes of the different *Lichtheimia* species show strong synteny, thereby underlining the high similarity between the genomes (Figure 1 B + C).

Comparison of protein-coding genes of *Lichtheimia* genomes

Prediction of protein-coding genes resulted in 11,510, 12,062 and 12,379 genes in *L. ramosa*, *L. hyalospora* and *L. corymbifera*, respectively (Table 1) [19, 36].

To investigate the conservation of the proteomes of the *Lichtheimia* species, BLASTP was used to identify homologous proteins among 14 fully sequenced genomes from different fungal phyla (see material and methods and table S15). The majority of proteins found in the *Lichtheimia* species were conserved in at least some other fungal species (Figure 2A). However, more than 14 % of proteins were only shared with mucoralean fungi and approximately 5 % were *Lichtheimia*-specific (Figure 2A).

In addition, phylomes for all three *Lichtheimia* species and *R. miehei* were reconstructed using the PhylomeDB pipeline as described previously [19, 46]. The phylomes are available through phylomeDB with phylome IDs 257, 258, 259 and 260 (http://www.phylomedb.org). A species tree was reconstructed from the phylome based on 21 proteins found in single copy in at least 27 of the 28 species (Figure 2B). The low number of single-copy genes reflects the past history of rampant duplications in the genome [19]. The resulting phylogeny supports that *Lichtheimia* species are an ancient group of mucoralean fungi, closely related to *R. miehei*, as shown previously [19, 47]. However, it was not possible to resolve the topology of the clade formed by the three *Lichtheimia* species (Figure S2). The phylome support [48] (i.e. fraction of trees supporting a topology) was calculated for the three possible arrangements of the three *Lichtheimia* species. All alternative topologies showed similar supports (Figure S2A). A super-tree derived from the combination of all trees in the phylome using a gene tree parsimony approach, as implemented in duptree [49], resulted in a phylogeny supporting a closer relatedness of *L. hyalospora* and *L. corymbifera* and the exclusion of *L. ramosa*. To investigate the arrangement of the three *Lichtheimia* species in more detail, we performed a phylogenetic analysis on the concatenated alignment of 5,332 single-copy orthologues (3,326,163 homologous positions) present in single-copy in all three *Lichtheimia* species and the closest outgroup *R. miehei*. The resulting topology provided full support for the association of *L. hyalospora* and *L. corymbifera* (Figure S2B). This phylogenetic arrangement would indicate that the ability to infect humans has appeared at least twice independently in this clade or got lost during evolution in some species. Similarly, pathogenicity evolved independently several times among pathogens of the *Nakaseomyces* clade [50]. The close resemblance of the genomes indicates that *Lichtheimia* species diverged recently and divergence times were calculated based on the species tree using R8S-PL [51]. The calibration point was set for the split

between Asco- and Basidiomycota at 798 MyA. Based on the calculation, *Lichtheimia* species separated from *R. miehei* 320 MyA (Figure S2). However, the split between the *Lichtheimia* species occurred approximately 30 MyA for the first and 20 MyA for the second split (clades depending on the topology used). Thus, currently known *Lichtheimia* species evolved recently and show high similarity, while the genus itself seems to be highly dissimilar from other sequenced fungal species. To obtain further insight into the similarity of *Lichtheimia* genomes, the proteomes of the three species were compared.

Basic BLAST comparison showed that more than 80 % of all genes have homologues in all three *Lichtheimia* genomes (Figure 2C). Species-specific genes account for 0.9 % in *L. ramosa*, 4.2 % in *L. hyalospora* and 12.6 % in *L. corymbifera* but putative function could only be assigned to a minority of these genes (4.2 % to 16 %, see table S3). In order to also include a measure for the similarity between the proteins, BLAST score ratio analysis was performed [52]. Comparison of the *L. corymbifera* proteome resulted in a similar conservation of *L. corymbifera* proteins in *L. hyalospora* and *L. ramosa* (Figure 2D). Gene gain and loss was also reconstructed from the phylome data of the *Lichtheimia* species and support this distribution (Figure 2E; Table S3). Additional analyses of the synteny of the genes showed that the majority of genes in the *Lichtheimia* genomes were located in clusters which were conserved in at least one of the other *Lichtheimia* genomes encompassing 82.6 %, 70.1 % and 78.2 % of the total genes in *L. ramosa*, *L. corymbifera* and *L. hyalospora*, respectively (Table S4). In accordance with other mucoralean genomes, all *Lichtheimia* species showed a higher abundance of multi-gene families compared to other fungal phyla (Figure S3). The previously described tandem duplications of *L. corymbifera* which were linked to gene expansion in this species are also highly conserved in the other two species [19].

Since only *L. ramosa* and *L. corymbifera* have been associated with human infections, proteins exclusively present or which retained additional copies in these two species were analysed in more detail. Less than half of these genes contain known functional domains (Table S3). Most genes with functional domains are specific for basal fungi (41 %), represent transposons (19 %) or have homologues in other fungi which are not further characterized (21 %). To prevent false positive

results caused by differences in the assembly and gene prediction, genes were searched in the assemblies and the presence of the surrounding regions in *L. hyalospora* was confirmed. Only 31 non-transposon genes fulfilled these requirements. The best characterised genes are involved in the excretion of excess amino acids (LRAMOSA08662, LRAMOSA04899), translation (LRAMOSA04087) or protein folding and modification (LRAMOSA04444, LRAMOSA06320) as well as transport (LRAMOSA08686, LRAMOSA10993). Further genes contain functional domains indicating functions in membrane transport and stress response. Comparison of conserved virulence factors of mucoralean fungi including hydrolytic enzymes [23, 53, 54] and iron-uptake genes [20, 55, 56] but also additional putative virulence factors based on similarity to other pathogens (PHI-base, [57, 58]) revealed no obvious differences between the species (Table S6 + S7, Figure S4). The results indicate that *Lichtheimia* species may differ in the physiology and stress response, while classical virulence factors are conserved between the species.

Stress adaptation in *Lichtheimia* species

In accordance with previous results only minor differences were detectable on infection-associated stresses, including hypoxia, oxidative, osmotic, cell wall and ER-stress induced by dithiothreitol (DTT) (Figure S5 + S6) [11]. All *Lichtheimia* species were able to assimilate a wide range of iron sources including host-iron proteins which indicates that the found iron uptake genes are functional in all species (Figure S5 + Table S7). Furthermore, the three species showed comparable virulence in an invertebrate infection model supporting the hypothesis that important virulence-associated traits are conserved between the species (Figure S5). However, *L. hyalospora* showed major defects in growth at temperatures above 37°C (Figure 3A). At 45°C no growth was observed. When plates were further incubated at permissive temperatures after 16h at 45°C no additional colonies were found, indicating that this temperature is lethal to *L. hyalospora*. In contrast, growth of *L. corymbifera* and *L. ramosa* is significantly enhanced at temperatures between 37°C and 42°C compared to 30°C (Figure 3B). Short-term incubation at 56°C efficiently kills *L. hyalospora* after 15 minutes, while *L. corymbifera* and *L. ramosa* are not affected (Figure 3C). These data support results from previous studies indicating that clinically relevant *L. corymbifera* and *L. ramosa* show

better growth at high temperatures than *L. hyalospora* [14]. However, our results also show that growth is not only inhibited in *L. hyalospora* but that high temperatures are lethal for that species. It has been shown that thermotolerance contributes at least partially to the virulence of fungal pathogens [11, 23, 24, 27]. Thus, the cellular basis for the differences in the thermotolerance of the three *Lichtheimia* species was surveyed. High temperatures cause a variety of problems in filamentous fungi including disturbed cell wall integrity, altered membrane fluidity and the accumulation of misfolded proteins [23, 24, 26]. Several stressors were tested in combination with increased temperatures to see whether synergistic effects occurred. While at elevated temperatures all species were more sensitive to several stressors, only ER-stress induced by DTT resulted in an almost complete inhibition of growth which was limited to *L. hyalospora* (Figure 3D, Figure S6). This indicates that *L. hyalospora* has defects in the stabilization or degradation of misfolded proteins at elevated temperatures. One of the major factors involved in protein stabilisation and folding is the chaperone HSP90 [24, 28, 29]. To investigate whether blocking of HSP90 function is sufficient to inhibit growth of *Lichtheimia* species, the strains were grown in the presence of the HSP90 inhibitor geldanamycin. Growth of *L. hyalospora* was inhibited by all concentrations of geldanamycin at 42°C, whereas there was no inhibition for the other two species (Figure 3D). Based on these results, it was unclear whether the more thermotolerant species accumulated less misfolded proteins or if they could degrade misfolded proteins more efficiently. Misfolded proteins in fungi are normally transported to the cytosol and degraded in the proteasomes [31]. In order to investigate if degradation of misfolded proteins is required for the growth at elevated temperatures, *Lichtheimia* species were grown in the presence of the proteasome inhibitor MG132. There was no inhibition of growth for *L. corymbifera* or *L. ramosa*, while growth of *L. hyalospora* was reduced at 42°C (Figure 3D). Thus, accumulation of misfolded proteins seems to be lower in the two thermotolerant species compared to *L. hyalospora*. Similar phenotypes have been described for mutant strains which lack major components in the ER-stress and unfolded proteins response (UPR) [25, 26, 59]. A typical feature of such mutants is a reduced ability to secrete hydrolytic enzymes and to grow on complex nutrients [26, 32]. Additionally, cultivation on polymers such as maltose has been shown to induce gene expression in a manner resembling ER-stress response in *A. niger* [60]. Growth experiments on different complex nutrients at 37°C and 42°C were carried out to investigate the effect of heat on the ability to degrade these

nutrients. While growth of *L. hyalospora* on olive oil and gelatine was significantly reduced, no major changes in growth were observed on complex carbohydrates such as maltose and chitin (Figure S7). Interestingly, growth on amino acids was also reduced at higher temperatures for *L. hyalospora*. However, the results do not clearly indicate disturbed ER function since several hydrolytic enzymes appear to be properly secreted.

The increased accumulation of misfolded proteins can also be caused by a lower thermostability of the proteins. Thermoadaptation in eukaryotes is often associated with a higher frequency of isoleucine, valine, tryptophan, tyrosine, arginine, glutamic acid and leucine [61]. In the case of *Lichtheimia* few significant differences in the amino acid frequencies (alanine, asparagine and isoleucine) were observed between the clinically relevant species and *L. hyalospora*. In addition, more differences between the two thermotolerant species were found than between any of them and *L. hyalospora*. Based on these results it seems unlikely that specific adaptations in the amino acid composition of proteins play a role in *Lichtheimia* species.

Comparative transcriptomics under stress conditions

The physiological tests clearly showed that *L. hyalospora* suffers from a major growth defect when grown at elevated temperatures which could be linked to misfolding of proteins and ER-stress. To obtain further insights into the stress response of the different *Lichtheimia* species, we sequenced mRNA under multiple stress conditions. Strains were pre-grown at 37°C and shifted to one of the following conditions: (I) 6h at 42°C, (II) 2h with 5.5µM geldanamycin or (III) 2h with 5mM DTT (see Material and Methods).

The long (6 h) incubation time at 42°C was used to characterize the transcriptome during the establishment of growth at elevated temperatures, instead of initial heat shock response. The three *Lichtheimia* species differed in the number of genes with significant expression changes in response to the stresses. *L. hyalospora* showed a higher transcriptional reactivity (9 % of total genes) compared to the two other species (1.4 – 2.9 % of total genes) which reflects the results of

the physiological tests showing that growth at this temperature is a stronger stress condition for this species (Figures 4A and S8, Tables S8 – S10).

To get further insights into the regulation of orthologues in each of the species under stress conditions the gene expression profile of 8,127 3-way shared orthologous genes was analysed. Interestingly, only a minor overlap between the heat stress responses of the three *Lichtheimia* species was found. The two thermotolerant species did not show a common distinctive gene expression pattern and also species-specific changes in the expression did not help to explain their higher growth optimum (Figure 4B). Rather, it seems that incubation at 42°C did not require extensive changes in gene expression in these species.

Chaperones play a crucial role in the stabilization and folding of newly synthesized proteins and are known to be involved in the adaptation to increased temperatures [24, 27–30]. All three *Lichtheimia* species contain a similar set of heat shock proteins and heat shock factor (HSF) transcription factors in their genomes with multiple copies for most of the genes (Table 4). In order to identify which of the genes play a role during heat stress and to identify if there are differences between the three species in the expression of these genes, transcriptome data were searched for chaperones and HSF transcription factors. Transcriptional changes under heat stress indicate that only few play a role during heat stress (Figure 4C). Only one orthologues (LCor02456.1, L*RAMOSA*04008, jgi.p|Lichy1|190525) of HSF was up-regulated in all three species under heat stress conditions. Regulation of heat shock proteins was similar in the three species and no specific regulation of heat shock proteins in the two thermotolerant species could be observed (Figure 4C). Major gene expression changes observed in all species included the up-regulation of hsp20, hsp90 and an orthologue of hsp60 (Figure 4C). While Hsp90 and Hsp60 are known to play essential roles in heat adaptation, the role of small heat shock proteins (sHsps) is less clear. In *S. cerevisiae* and *C. albicans*, expression of sHsps is increased under heat stress. However, deletion of the gene only affected growth of *C. albicans* but not *S. cerevisiae* under heat stress [27]. Small heat shock proteins could be a good target for antifungal therapy since the sequence of sHsps is not well conserved between fungi and mammals compared to other Hsps, like Hsp90. In contrast to the regulation in hsp90 and hsp20, where several copies are regulated under heat-stress, only one

copy of hsp60 was altered in expression. According to our phylome data, this gene (LCor09740.1, LRAMOSA05508, jgi|Lichy1|171566) is the orthologue of hsp60 in other fungi and seems to be conserved in its function.

To further explore which responses are conserved between the species, gene ontology (GO) term enrichment of genes regulated in *L. hyalospora* and at least one of the other species was performed as implemented in FungiFun (Table S11) [62]. Up-regulation of typical heat shock associated genes involved in, for example, protein folding, unfolded protein binding and ER function were found to be conserved in *L. hyalospora*. KEGG pathway reconstruction showed that these genes are involved in the endoplasmic-reticulum-associated protein degradation (ERAD) pathway but not the unfolded protein response (UPR) indicating that all species are able to suffer from accumulation of misfolded proteins but respond to the stress in a similar way. Besides chaperones, genes involved in trehalose synthesis are also up-regulated in *L. hyalospora*. In conclusion, typical heat shock response seems to be conserved in all three *Lichtheimia* species and the lack of typical heat shock response seems not to be the reason for the lower growth of *L. hyalospora*. Direct comparison of expression levels between the three species revealed significant differences between *L. hyalospora* and the two thermotolerant species but only few genes were associated with thermotolerance and proteins folding (Table S12). However, several chaperone genes were found to be higher expressed including homologues of Mdj1 and Pim1 which are involved in the degradation of misfolded proteins in the mitochondria. In addition, genes with lower expression levels in *L. hyalospora* included four F-box-like proteins (PF12937; PF00646.28) and ubiquitin (PF00240) involved in ubiquitin-mediated protein degradation as well as a FMO-like protein which could be involved in protein folding in the ER (jgi|Lichy1|79920).

We further investigated which genes were specifically up-regulated in *L. hyalospora* and found several significantly enriched GO terms (Table S12). KEGG analyses showed that many genes involved in amino acid biosynthesis were up-regulated when *L. hyalospora* grew at 42°C. In addition, eight amino acid transporters were found to be down-regulated supporting the hypothesis that heat induces changes of the amino acid metabolism in *L. hyalospora*. However, other membrane transporters for the uptake of nitrogen sources were also down-regulated including four

of the seven oligopeptide transporters (PF03169) and two of the three ammonium transporters (PF00909) encoded in the genome. In addition, several genes involved in lipid metabolism (PF04734, PF03982, PF03694, PF01222, PF00487) were down-regulated under heat stress which may explain the lack of growth on olive oil at elevated temperatures (Figure S7).

Inhibition of HSP90 by geldanamycin had only small effects on *L. corymbifera* and *L. ramosa* but induced significant changes in the gene expression of *L. hyalospora* (Figure 4A and D, Tables S8 – S10). This is consistent with the hypothesis that *L. hyalospora* suffers from a certain level of heat stress at 37°C and thus relies on Hsp90 function. As expected, inhibition of Hsp90 function induces expression of hsp90. Interestingly, many genes that were differentially regulated under heat stress or ER-stress were also regulated when Hsp90 function was inhibited (Figure 4C, Figure S8). These data indicate that Hsp90 function is necessary even at 37°C in *L. hyalospora* and that inhibition of Hsp90 induces transcriptional changes similar to heat stress and ER-stress response.

ER-stress induced by DTT induced the strongest changes in gene expression affecting around one third of the genes in all three species (Figure 4A, Tables S8 – S10). In contrast to the other two stresses ER-stress induced a similar response in all three species (Figure 4A + B). More than two thirds of the differentially regulated genes with orthologues in all three species are similarly regulated between the species. ER stress has been extensively studied in *S. cerevisiae* [30] and orthologues for most of the corresponding genes could be found in *Lichtheimia* species (Table S13). All genes were found to be up-regulated under ER-stress in all three *Lichtheimia* species indicating a conserved function in basal fungi. Although ER-stress causes strong induction of chaperones, several genes are not required for ER stress, despite being activated under heat stress (Figure 4C). While hsp60 and hsp20 were strongly induced under heat stress, the expression was not changed under ER-stress conditions. Interestingly, other hsp60 domain genes were up-regulated under ER-stress but not under heat stress (Figure 4C) showing the functional divergence of this gene family in *Lichtheimia* species.

In addition, only one copy of hsp90 (LCor07667.1, L*RAMOSA*10189, jgi|Lichy1|204946) was up-regulated under ER-stress in all three *Lichtheimia* species. The gene has orthologues in Metazoa, Microsporidia, basal fungi and Basidiomycota but is missing in Ascomycota. The corresponding

orthologues have not been further characterized in any species yet, but may play a role in ER-stress adaptation.

A significant enrichment of several GO terms was found in the regulated genes shared between all species including terms associated with ER and Golgi function as well as protein folding and posttranslational modification (Figure S9A, Table S12). No specific GO enrichments were found in genes which were exclusively regulated in only one of the species. The results show that ER-stress response of *Lichtheimia* species is conserved between the species and do not indicate that *L. hyalospora* has a major defect in ER-stress response itself but has a specific defect under heat stress conditions.

Rescue of *L. hyalospora* heat stress induced phenotype

Physiological and transcriptional data suggested that accumulation of misfolded proteins contributes to the lower thermotolerance of *L. hyalospora*. Several chemical chaperones are able to stabilize proteins and alleviate ER-stress [63–67]. Trimethylamine-N-oxide and proline supported growth in general, but could not prevent the strong growth reduction at elevated temperatures. Interestingly, addition of sodium chloride to the culture medium increased growth at elevated temperatures and shifted the growth optimum to higher temperatures comparable to the situation in the two thermotolerant species (Figure 5 and Figure 3B). Sodium chloride not only increased growth at 42°C but also enabled *L. hyalospora* to grow at otherwise lethal temperatures ≥45°C and in the combination of heat and hypoxic conditions (Figure 5A + B). Similar but reduced effects can be seen for other *Lichtheimia* species at temperatures >47°C. Since increased temperatures strongly inhibited or prevented growth on certain nutrients (Figure S7), it was tested if addition of NaCl to the medium was able to increase growth. Addition of NaCl to medium with olive oil as carbon source reduced growth at 37°C. However, at 42°C at which temperature *L. hyalospora* was unable to grow properly on olive oil, NaCl restored growth on this medium (Figure 5C). Results from transcriptomics and physiological tests showed that amino acid metabolism was strongly influenced by elevated temperatures. As for growth on olive oil, addition of NaCl improved growth significantly,

indicating that osmotic stress normalizes the metabolism of *L. hyalospora* at elevated temperatures (Figure 5C).

Short term heat treatment of spores showed also increased survival of *L. hyalospora* the presence of 0.5 M NaCl (Figure 5D). Pre-treatment with certain stresses have been shown to cause cross-protection to other stressors in fungi such as *C. albicans* [29]. To investigate if cross-protection plays a role in the higher survival of *L. hyalospora*, spores were pre-treated with NaCl prior to heat stress or NaCl was added immediately before heat incubation. Only the presence of NaCl during heat stress resulted in increased survival of spores, whereas pre-treatment had no effect (Figure 5D). In order to investigate whether osmotic stabilisation rather than osmotic stress may have caused the increased survival of *L. hyalospora* spores, short term incubation at was carried out in hypotonic buffer. Survival of spores was not reduced in the hypotonic environment indicating that the effect is not based on osmotic stabilisation (Figure 5D + E). It is known that osmotic stress causes the intracellular accumulation of protective osmolytes like glycerol or trehalose which play essential roles in heat stress response and protein stabilization [23, 27, 68, 69]. Trehalose had no beneficial effect on growth but growth was tendentially increased in the presence of glycerol (Figure S10). The high-osmolarity glycerol (HOG) pathway was originally described as being activated by osmotic stress resulting in the increased production of glycerol [69]. The HOG pathway is also involved in the adaptation to a variety of stresses including heat, reactive oxygen species and ER-stress in fungi [68–71]. However, species-specific differences in the function of Hog1 and its orthologues in stress adaptation between fungal species have been described [29, 72, 73]. Inhibition of Hog1 function by the chemical inhibitor BPTIP [74] reduced growth of *Lichtheimia* strains in the presence of osmotic stress but had no effect on the growth under heat stress conditions (Figure S10). Activation of the HOG pathway does not seem to play a role in the increased growth on NaCl containing media since the supportive effect was visible even when Hog1 inhibitor was present in the medium (Figure S10). The inhibitor had minor effect on the growth under cell wall stress and oxidative stress but not under hypoxia. Based on these results, only minor cross-talk between the pathways seems to occur and Hog1 is mainly restricted to the response to osmotic stress and plays no role in the rescue of *L. hyalospora* growth under heat stress.

To further investigate the effects of osmotic stress on the thermotolerance of *L. hyalospora*, transcriptome analyses under heat stress alone and in combination with osmotic stress were performed. Only 406 genes were differentially regulated in samples which were incubated with 0.5 M NaCl at 42°C compared to samples incubated at 42°C only (Figure 6A). As expected, genes related to osmotic stress response were strongly up-regulated in the presence of NaCl including ER-located and vacuolar P-type ATPases, putative glycerol transporters and genes involved in cell wall synthesis namely chitosan synthases (Table S14). Despite the increased growth only few up-regulated genes were found to be directly involved in protein folding including an FMO-like protein (jgi|Lichy1|79920) and hsp20 (jgi|Lichy1|181425) (Table S14).

While no specific enrichment of GO terms among the differentially regulated gens was found, genes encoding membrane, lysosomal, secreted and ER-located proteins are over-represented based on TargetP and Predotar predictions (Figure 6B) [75, 76]. Membrane proteins represented mainly transporters with different substrates including amino acids, oligopeptides and sugars (Table S14). Secreted proteins often did not contain functional domains and were only conserved in mucoralean species or even specific for *Lichtheimia* species. Predicted ER-located genes included no major genes involved in protein folding but genes with a variety of functions including transport, cell wall synthesis and polyamine synthesis. Polyamines are known to be involved in stress response to a variety of stresses in fungi and accumulation of polyamines was shown to protect yeast cells from elevated temperatures [77]. In addition, three key enzymes of proline biosynthesis were found to be up-regulated (Table S14). Proline is known to act as a chemical chaperone and to stabilise proteins [65, 66]. Thus, higher production of proline due to osmotic stress may help to increase the stability of the proteins under heat stress conditions. Additional genes were involved in the synthesis of glycerol and the biosynthesis of lipids which are also known to play a role in the function of the ER and the stabilisation of proteins [71, 78].

No major changes in the expression of genes involved in amino acid metabolism which were up-regulated specifically in *L. hyalospora* under heat stress. These results indicate that amino acids were still needed when growth was improved by the addition of NaCl. In accordance with these results combination of amino acids and NaCl in the medium resulted in fast growth of *L. hyalospora*

even at 42°C (Figure 5). Poor growth of *L. hyalospora* on medium containing amino acids without the presence of NaCl was therefore likely caused by a lack of ability to take up or utilise amino acids properly at 42°C (Figure S7).

Addition of NaCl could directly or indirectly change the physical or chemical characteristic of the cellular environment and thus prevent or reduce the effects of increased temperatures. In order to investigate this possibility, the differentially regulated genes were compared to genes which were up- or down-regulated under heat stress alone compared to control conditions (37°C). If the hypothesis would be true the strong stress response should be reduced by the addition of NaCl and the expression pattern should get more similar to 37°C incubation. A total of 177 genes overlapped in heat compared to control and heat + NaCl compared to heat only (Figure 6). The majority (>75 %) of these overlapping genes showed antithetical regulation between the two conditions. It is noteworthy that among the down-regulated genes three chaperones were found, namely hsp20 (jgi|Lichy1|171634), hsp60 (jgi|Lichy1|171566) and an orthologue of the cytoplasmatic chaperone Sse1 of *S. cerevisiae* (jgi|Lichy1|228816) indicating that NaCl indeed alleviates the effects of heat stress at least partially. Therefore, treatment with NaCl rather reversed effects caused by increased growth temperature rather than simply amplifying the response. However, these effects could be secondary effects resulting from the regulation of other genes protecting the cells from heat induced stress, for example by the production of osmolytes.

Since there were several genes expressed on higher or lower levels in *L. hyalospora* compared to the two more thermotolerant species, which could potentially cause differences in the thermotolerance of the species, changes in the expression of these genes upon addition of NaCl were analysed. Less than 10 % of the differentially expressed genes among species were also affected by the addition of NaCl to the experimental setting (Figure 6D). The majority of these genes (40) had lower expression levels in *L. hyalospora* than in the other two species and was up-regulated by addition of NaCl. However, only one of the genes was directly associated to ER function and protein folding a FMO-like gene (jgi|Lichy1|79920). In summary, these results show that *L. hyalospora* is able to grow at otherwise inhibitory or even lethal temperatures in the presence of osmotic stress. A similar phenomenon was observed in a hacA mutant of *A. fumigatus*

[26]. Addition of sorbitol and KCl supported growth of the mutant at 45°C. However, the authors found that the effect is due to osmotic stabilisation, while *L. hyalospora* showed also good survival in hypotonic environments indicating that the effect is not based on osmotic stabilisation. In addition, a hsp21 mutant of *C. albicans* showed no growth at 42°C unless NaCl or sorbitol were added to the medium [27]. The effect was based on the increased production of osmolytes which also seems to be a likely explanation in the case of *L. hyalospora*.

Conclusions

In summary, this study provides first insights into genomics and differences in stress response of mucoralean fungi on a species level. The comparison of clinically relevant *Lichtheimia* species and the clinically not relevant *L. hyalospora* showed high similarity between the genomes and revealed that virulence-related traits evolved in a common ancestor of all three species. While the *L. hyalospora* was more sensitive to heat stress, the core heat-shock response was conserved. Importantly, growth at high temperatures could be restored by combining heat with osmotic stress. Only minor transcriptional changes seem to be necessary to restore growth at high temperatures which do not include major heat shock proteins. The results from this study represent a valuable resource for future studies and may help to understand the evolution of heat adaptation and pathogenicity of mucoralean fungi. In addition, transcriptomic analyses revealed several candidate genes (e.g. genes encoding sHsps, Hsp60, HSF transcription factors) which are important for the adaptation to infection-related stresses in all *Lichtheimia* species and represent potential targets for the development of novel antimycotic drugs.

Methods

Fungal strains and cultivation

L. corymbifera (JMRC:FSU:9682, CBS 429.75), *L. ramosa* (JMRC:FSU:6197) and *L. hyalospora* (JMRC:FSU:10163, CBS 173.67) were used for this study. Strains were cultivated on modified SUP medium [79] (55 mM glucose, 30 mM potassium dihydrogen phosphate, 20 mM ammonium chloride, 5 mM di-potassium hydrogen phosphate, 1 mM magnesium sulphate and 0.5 % yeast extract) at 37°C. Spores for additional experiments were washed of fully grown plates (5-7 days) with sterile PBS and washed at least two times with PBS. Spores were counted microscopically with a Thoma counting chamber and diluted to desired concentrations in PBS.

Iron-source utilization

Growth on different iron sources was investigated on minimal medium (YNB, 0.5 % NH_4SO_2, 1 % glucose, 50 mM HEPES, 7.6 mg/l Bromocresol green, pH 7 [80], bacteriological agar) in 6-well plates. For iron-depletion 450 µM bathophenanthrolinedisulfonic acid (BPS) was added to the medium. Spores were prepared as described above, but PBS containing 50 µM BPS was used for all washing and dilution steps to deplete spores of iron. A total of 100 spores were plated in each well and cultures were incubated for 24 h at 37°C. Iron-containing proteins and control proteins were prepared as described previously [80]. Briefly, haemoglobin, ferritin and apoferritin (all Sigma) were diluted to 2 mg/ml in 5 mM HEPES + 0.1 M NaCl (pH7) and washed two times using Roti-Spin Mini columns (10 kDa). The flow-through from the last washing step was used as a control in the growth experiments to see if unbound iron was present in the samples. Solutions of ferric chloride and ferric ammonium sulphate were prepared in the same buffer (final concentration 10 mM). Iron-loaded deferoxamine (Sigma) was prepared by mixing deferoxamine with ferric chloride in a molar ratio of 2:1 (final concentration 10 mM $FeCl_2$ + 20 mM DFO). All solutions were filter sterilized. Afterwards 50 µl of the iron-sources was added to the wells and buffer was added as a negative control. Plates were further incubated for 48 h at 37°C. Experiments were performed three times.

Growth experiments

For all growth experiments spores were prepared as described above. Radial growth at different temperatures was screened on SUP or SUP medium containing sodium chloride (NaCl), trehalose or glycerol at indicated concentrations. Five microliter of spore suspensions (10^5 spores per ml) were placed in the centre of a plate and incubated at indicated temperatures for up to 48 h. Colony diameters were measured twice a day. All experiments were performed three times in technical triplicates.

For drop dilution tests ten-fold dilution series were prepared ($2x10^6$ – $2x10^3$ spores per ml) in PBS. Five microliter of spore suspensions were placed on squared petri dishes containing SUP medium with or without addition of NaCl. Plates were incubated at indicated temperatures for 24 h to 48 h. All growth experiments were performed under normoxic conditions if no hypoxic conditions are stated (1 % O_2/5 % CO_2 or 0.2 % O_2/5 % CO_2). All experiments were performed at least three times.

Stress assays

Broad stress resistance screenings were performed in a 96-well format. Liquid SUP medium containing different stressors including calcofluor-white (CFW), dithiothreitol (DTT), hydrogen peroxide (H_2O_2), sodium dodecyl sulphate (SDS) and sodium chloride (NaCl) at indicated concentrations (total volume 200 µl) was inoculated with 500 spores per well. Plates were incubated for 48 h at 37°C or 42°C and growth was evaluated by optical inspection.

Resistance towards DTT, geldanamycin and MG132 (Sigma) was tested in 24 well plates. Stocks of DTT (1 M) were prepared in water, geldanamycin (10 mg/ml) and MG132 (50 mM) stocks were prepared in DMSO. Liquid SUP medium containing indicated concentrations of the stressors was prepared and medium containing equal amounts of the solvents as the highest concentrations were used as controls (max 0.1 % DMSO). All wells (total 1 ml medium) were inoculated with $5x10^3$ spores and incubated at 37°C or 42°C for 48 h.

For short-term heat stress treatments, spores were prepared in PBS buffer as described above and diluted to $5x10^2$/ml. Spore suspensions were treated at 56°C for indicated times. Non-treated spores were used a control (=100 % survival). For quantification of survival 100 µl of the suspensions was plated onto SUP agar and incubated overnight at 37°C. Growing colonies were counted and compared to colony formation of control suspensions. Experiments with variations in the osmolarity were performed in a similar fashion. For parallel stressing in hypo- or hyperosmotic buffer spore suspensions of $5x10^2$ spores per ml were prepared in PBS + 0.5 M NaCl or 100-fold diluted PBS. For pre-treated spores, initial suspensions of 10^6 spores per ml were prepared in PBS + 0.5 M NaCl and incubated for 30 min at room temperature. Control samples were prepared in the same way but without the addition of NaCl. Spores were collected via centrifugation, resolved in PBS, diluted 1:2000 in PBS and subjected to the same treatment as described above. For the combined treatment, spore suspensions ($5x10^2$/ml) were prepared in PBS + 0.5 M NaCl, pre-incubated for 30 min at room temperature and then subjected to heat stress in the same buffer. All experiments were performed three times in technical triplicates.

Utilization of complex nutrients

Utilization of complex nutrients was assayed on minimal medium (YNB, 2 % carbon source, 0.5 % nitrogen source, bacteriological agar). Casamino acids, chitin, glucose, maltose and olive oil were autoclaved. Gelatine was solved in warm water and filter-sterilized. YNB was prepared according to the manufacturer's instructions and added to the medium after autoclaving. Spore suspensions were prepared as described previously, diluted to $2x10^5$ spores per ml and plates were inoculated with 5 µl of the suspensions. For indicated experiments 1 M or 1.25 M NaCl was added to the medium. Cultures were incubated at 37°C or 42°C for up to 7 days.

HOG1 inhibitor studies

Stock solutions of the HOG1 inhibitor BPTIP [74] were prepared in DMSO (final concentration 75 mM). Different stressors were added to autoclaved SUP agar from stock solutions, except for NaCl which was directly autoclaved in the medium. Final concentrations in the agar were 1.25 M NaCl, 50 µg/ml SDS, 100 µg/ml Congo red or 0.25 mM Menadione. BPTIP was added to a final concentration of 75 µM. For control media, DMSO was added to an equal volume (= 1 µl/ml). Cultures were grown at 37°C under normoxic conditions except for thermic stress which was induced by incubation at 42°C and hypoxia growth was performed at 37°C under a 1 % O_2/5 % CO_2 atmosphere.

Virulence tests in the invertebrate infection model *Galleria mellonella*

Virulence assays in *G. mellonella* were carried out according to Fallon et al. [81]. *G. mellonella* larvae (K. Pechmann, Biologische Wurmzucht, Langenzersdorf, Austria) were kept in the dark at 18°C before use. 1 x 10^6 spores in 20 µl insect physiological saline (IPS) were injected into the hemocoel via one of the hind pro-legs of larvae weighing between 0.3 and 0.4 g. Untreated larvae and larvae injected with 20 µl of IPS served as controls. Larvae were incubated at 30°C or 37°C, respectively, in the dark and monitored daily up to 6 days. Significance of survival data was evaluated by using Kaplan-Meier survival curves, analysed with the log-rank (Mantel Cox) test utilizing GraphPad Prism software. Results represent the average of three independent experiments.

Genome sequencing, assembly and annotation

Both *Lichtheimia hyalospora* genome and transcriptome were sequenced using the Illumina platform. For genome sequencing, 500 ng of DNA was sheared to 270 bp using the covaris E210 (Covaris) and size selected using SPRI beads (Beckman Coulter). The fragments were treated with

end-repair, A- tailing, and ligation of Illumina adapters using the TruSeq Sample Prep Kit (Illumina). For transcriptome, stranded cDNA libraries were generated using the Illumina Truseq Stranded RNA LT kit. mRNA was purified from 1 µg of total RNA using magnetic beads containing poly-T oligos, fragmented and reversed transcribed using random hexamers and SSII (Invitrogen), followed by second strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, adapter ligation, and 8 cycles of PCR. Both libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were multiplexed and the pools were then prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v3, and Illumina's cBot instrument to generate a clustered flowcell for sequencing. Sequencing of the flowcell was performed on the Illumina HiSeq2000 sequencer using a TruSeq SBS sequencing kit 200 cycles, v3, following a 2x150 indexed run recipe.

Genomic reads were QC filtered for artifact/process contamination and subsequently assembled with Velvet [82]. The resulting assembly was used to create a simulated 3 Kbp insert long mate-pair library, which was then assembled together with the original Illumina library with AllPathsLG release version R42328 [83]. Stranded RNA-seq data were *de novo* assembled into consensus sequences using Rnnotator (v. 2.5.6 or later) [84].

The genome was annotated using the JGI Annotation Pipeline and made available via the JGI fungal portal MycoCosm [85]. Both genome assemblies and annotation were also deposited at DDBJ/EMBL/GenBank under accession XXXXXX (TO BE PROVIDED UPON PUBLICATION).

K-mer analyses and prediction of repetitive elements

The analysis was performed on the Illumina reads with an algorithm described previously [40]. The algorithm was used to write a custom Perl program. Based on the fastq data of the Illumina reads, k-mers of 59 nt were generated and analysed. Data for *L. corymbifera* were taken from a previous study [19].

Repetitive elements were identified using Repeatmasker 4.0.5 and libraries from Repbase (repeatmaskerlibraries-20140131) [41, 42]. In addition, *de novo* repeats were identified using RepeatModeler 1.0.7, RepeatScout 1.0.5 and RECON 1.07 [86–89] and resulting libraries were used in RepeatMasker. A consensus repeat file based on repeats identified *de novo* or using RepBase libraries was created for each species.

Whole genome alignments and contig ordering

Fragmented whole genome alignments were performed using Gegenees (v2.2.1) [43]. Genomes were fragmented in the accurate setting (fragment size: 200 bp; step size: 100 bp) resulting in 336,081, 307,057, 331727 and 270,584 fragments for *L. corymbifera*, *L. ramosa*, *L. hyalospora* and *R. miehei*, respectively.

Contig ordering was performed using MAUVE 2.3.1 and r2Cat [44, 45]. In both cases *L. ramosa* was used as reference in the first ordering cycle since the assembly showed the best quality. The contigs of the *L. corymbifera* genome was first ordered according to the *L. ramosa* genome. Then, three additional contig orderings between *L. corymbifera* and *L. ramosa* were performed with each genome as reference sequence on a rotating basis. In a last step the *L. hyalospora* genome was ordered using *L. ramosa* as a reference. Contig ordering with r2Cat was performed in the same way. Ordered genomes were aligned with progressive Mauve and contigs were manually clustered based on overlapping regions.

Prediction of non-coding genes

The annotation of non-coding RNAs was performed by homology search of Rfam database (v 12) [90] using the genome-wide RNA annotation pipeline (GORAP), including a combination of BLAST (v 2.2.27+) [91], Infernal (v 1.1) [92], Bcheck (v 0.6) [93], RNAmmer (v 1.2) [94], and tRNAscan-SE (v 1.3.1) [95]. We used family specific parameters and Rfam-provided noise cutoffs or taxonomic

specific thresholds, as well as subsequent applied filter steps regarding secondary structure and primary sequence conservation. The prediction of snoRNAs was performed using the snoStrip pipeline [96]. All resulting Stockholm alignments were hand-curated using the Emacs RALEE mode [97].

Proteome comparison and phylome reconstruction

Homologues of *Lichtheimia* proteins in other fungal species (see table S15) were identified by global BLAST analyses (E-value cut-off E≤10^{-5}, similarity ≥30 %) [91]. To prevent false negative results between *Lichtheimia* species due to differences in gene predictions, an additional tBLASTn search was performed (E-value cut-off E≤10^{-5}, similarity ≥30 %). BLAST score ratios were calculated from BLASTp results as described previously [52]. A phylome for each of the four species (*L. corymbifera*, *L. ramosa*, *L. hyalospora* and *R. miehei*) was reconstructed as described previously [19, 98]. A total of 24 genomes additional genomes was used for each phylome similar to the existing *L. corymbifera* phylome (phylome ID: 245) [19]. Briefly, for each protein in the genomes, a Smith-Waterman search was performed against the fungal proteome database. Results were filtered using an E-value cut-off E<10^{-5} and a continuous overlapping region covering 0.50 % of the query sequence. At most 150 homologous sequences for each protein were accepted. Homologous sequences were then aligned using three different programs: MUSCLE v3.8 [99], MAFFT v6.712b [100], and kalign [101]. Alignments were performed in forward and reverse direction (i.e. using the Head or Tail approach [102]), and the six resulting alignments were combined with M-COFFEE [103]. This combined alignment was trimmed with trimAl v1.3 [104] (consistency-score cut-off 0.1667, gap-score cut-off 0.9). Trees were reconstructed using the best-fitting evolutionary model. The selection of the model best fitting each alignment was performed as follows: a Neighbour Joining (NJ) tree was reconstructed as implemented in BioNJ [105]; the likelihood of this topology was computed, allowing branch-length optimization, using seven different models (JTT, LG, WAG, Blosum62, MtREV, VT and Dayhoff), as implemented in PhyML v3.0 [106]; the model best fitting the data, as determined by the AIC criterion [107], was used to derive ML trees. Four rate categories were used and invariant positions were inferred from the data. Branch

support was computed using an aLRT (approximate likelihood ratio test) based on a chi-square distribution. Results are stored in the phylomeDB (phylome IDs: 257, 258, 259 and 260) [98]. Trees were scanned using ETE v2 [108].

Orthology prediction

Orthologues between the *Lichtheimia* genomes and the other species included in the phylome were based on phylogenies obtained during phylome reconstruction. A species-overlap algorithm, as implemented in ETE v2 [108], was used to infer orthology and paralogy relationships. Briefly the algorithm decides whether a node in a tree is a speciation of a duplication node depending on the overlap of the species branching from the node. Overlap between those species would indicate a duplication node. Otherwise a speciation node would be considered. Predictions obtained from the four phylomes were fused into a single orthology prediction using the metaPhOrs pipeline [109]. Only predictions with an orthology consistency score above 0.50 were considered.

Comparison of amino acid frequencies

Orthologous proteins that had a one-to-one relationship between the three *Lichtheimia* species were used to calculate the frequency of use of each of the 20 amino acids. For each protein the number of times each amino acid appeared was divided by the total length of the protein. Distributions were compared using a t-test to assess for significant differences. Amino acids with frequencies with a p-value below 0.01 were considered as significantly different. Comparisons were performed between each pair of species and for comparing *L. hyalospora* with the average frequencies for the two thermotolerant *Lichtheimia* species.

Species tree reconstruction

The species tree was build using a concatenation method. 21 single-copy proteins that appeared in at least 27 of the 28 genomes were selected. After concatenation, the alignment was trimmed using

trimAl [104]. Columns with more than 50 % of gaps were removed. A conservation score of 50 % of the alignment was used. The tree was reconstructed using phyML [106]. LG model [110] was selected and a 4-categories GAMMA distribution was used. Bootstrap was obtained by creating 100 random sequences using SeqBoot from the phylip package. A tree was then reconstructed for each sequence and the consensus tree was inferred using phylip. Additionally, a super-tree based on 11030 phylogenetic trees reconstructed for the *L. ramosa* phylome was reconstructed using the program DupTree [49]. Both species trees showed a similar topology. Bootstrap support was low in the branches of the genus *Lichtheimia*. Therefore, phylome support was calculated for this region [48]. The phylome support is a measure that calculates the number of trees reconstructed in a phylome that support one topology in a given node and its alternatives. For the node at the base of *Lichtheimia*, the number of trees that supported the three topologies was calculated. Calculation of divergence times were carried out using R8S-PL [51], with the split between Asco- and Basidiomycota 798 MyA as calibration point (Timetree of life) [111, 112]. Cross-validation was performed to obtain the optimal smoothing parameter.

Detection of conserved clusters

For the detection of conserved regions, all genomes were modelled as strings of integers. BLAST analyses [96] were performed for all proteins in the three *Lichtheimia* genomes and additional three mucoralean genomes (*M. circinelloides*, *P. blakesleeanus* and *R. arrhizus*) all-against-all, with an E-value threshold of 0.1. Homology families of the genes were assigned using Transclust [113]. All genomes were transformed into strings of gene IDs and used for the reference gene cluster implementation in Gecko2 [114, 115]. All three *Lichtheimia* genomes were used as references and clusters were calculated as described previously [19]. Results of the filter settings were combined and overlapping clusters were eliminated. Local rearrangements and duplications within the cluster occurrences were not punished. All regions that had approximate occurrences in at least one other genome were reported. If multiple occurrences did intersect, only the best scoring one was reported.

Detection of tandem duplications

Tandem duplications were defined by at least two genes assigned to the same gene family based on Pfam annotation and a maximum of three genes between the copies.

Detection of differentially expressed genes / RNA-Sequencing

Spores of the three *Lichtheimia* species for RNA-Seq experiments were prepared as described above. Erlenmeyer flasks (500 ml) containing 100 ml of SUP medium were inoculated with $5x10^5$ spores and incubated for 16 h (heat stress) or 20 h (GdA and DTT treatment) at 37°C under shaking. After that time (i) heat samples were transferred to 42°C under shaking for 6 h, (ii) geldanamycin was added to a final concentration of 5.5 µM from a 10 mg/ml stock in DMSO and cultures were further incubated for 2 h at 37°C under shaking, (iii) DTT was added to a final concentration of 5 mM from a freshly prepared 1 M stock solution and cultures were further incubated at 37°C for 2 h under shaking. For *L. hyalospora*, additional cultures were prepared and pre-grown for 16 h at 37°C under shaking. Afterwards NaCl or an equal volume of distilled water was added to the cultures (final concentration: 0.5 M NaCl, 5 M stock) and cultures were further incubated at 42°C under shaking for 6 h.

After incubations mycelia were separated from the medium using a miracloth filter (Millipore) and immediately frozen in liquid nitrogen. RNA isolation was performed by grinding the mycelium using mortar and pestle under liquid nitrogen and subsequent use of the RNAeasy Plant kit (Quiagen) according to the manufacturer's instructions. Total RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Quality of the RNA samples was measured using a Tape Station 2200 (Agilent Technologies, USA). All samples showed RNA Integrity Number (RIN) values ≥ 8.2. Poly (A) RNA was purified from 5 µg of total RNA using the Dynabeads mRNA DIRECT Micro Purification Kit (Ambion, USA), according to the manufacturer's instructions. Quality control for the depletion of rRNA was carried out using High Sensitivity RNA Screen Tapes (Agilent Technologies, USA).

Strand-specific whole transcriptome libraries were prepared using the Ion Total RNA-Seq Kit v2.0 (Life Technologies, USA). RNAse III was employed to fragment the purified RNA. Ion adapters were ligated to the resulting fragments, and reverse transcription was performed using the SuperScript III Enzyme Mix (Life Technologies, USA). Barcoded primers were used to amplify the libraries with the Platinum PCR High Fidelity polymerase (Life Technologies, USA). Size distribution analysis and quantification of the final barcoded libraries was performed on D1000 Screen Tapes on the Tape Station 2200 (Agilent Technologies, USA). Library templates were clonally amplified on Ion Sphere particles using the Ion PI IC 200 Seq Kit (Life Technologies, USA), loaded into Ion PI Chips and sequenced on an Ion Proton Sequencer (Life Technologies, USA). Raw reads were quality controlled with FastQC (v 0.1.0.1) and trimmed using QTrim (v 1.1) [24479419] with a quality threshold of 20, a sliding window of length 10 and minimum read length of 20. Mapping was performed by TopHat –max-segment-intron=5000 (v 2.0.11) [23618408] and segemehl -S (v 2.0) [19750212]. The latter one was mainly used for DGE, due to a flexible handling of mismatches resulting in a high mapping rate (up to 95 % for segemehl against 75 % for TopHat). *De novo* transcripts were detected with Cufflinks (v2.1.1) and Cuffmerge (v 2.1.1) [20436464] constrained by gene annotations. Resulting transcripts were assigned with a function by homology search against the NCBI NR nucleotide database via BLAST v2.2.27+ [2231712] with an E-value threshold of E<10-10 in combination with the Panther database tool [23193289, 16912992]. We used non-rRNA unique-mapped reads for DESeq (v 1) [20979621] with a fold change of at least 2 and an adjusted p-value < 0.05. Proportional VENN diagrams were calculated and drawn using the VENN diagram plotter (http://omics.pnl.gov/software/venn-diagram-plotter). Hypergeometric tests for the enrichments of subcellular localisations were performed using geneprof [116, 117] based predictions by predotar [75] and targetp (cut-off: 0.8) [76] as implemented in the secretool pipeline [118].

Genome resources

Genome data of *Aspergillus fumigatus* [119], *Aspergillus nidulans* [120], *Batrachochydrium dendrobatidis*, *Cryptococcus neoformans*, *Encephalitozoon cuniculi* [121], *Rhizopus oryzae* [39],

Paracoccidioides brasiliensis, *Schizosaccharomyces pombe* [122], *Nosema ceranae* [123], *Nematocida parisii* [124], *Puccinia graminis* [125], *Ustilago maydis* and *Coprinus cinerea* [126] are genome sequencing projects of the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) (see Table S15 for detailed citations). *Phycomyces blakesleeanus*, *Phanerochaete chrysosporium* [127], *Laccaria bicolor* [128], *Mucor circinelloides*, *Nematostella vectensis* [129], *Monosiga brevicollis* [130] and *Serpula lacrymans* [131] genomic data were obtained from Joint Genome Institute (JGI). These sequence data were produced by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/ in collaboration with the user community. The genomes of *Homoloaphlyctis polyrhiza* [132], *Mortierella alpina* [133] and *Rhizmomucor miehei* [38] were obtained from Genbank (Hp: PRJNA68115; Ma: PRJNA41211). The *Neurospora crassa* genome [134] was obtained from UniProt reference genomes. The *Saccharomyces cerevisiae* genome was obtained from Saccharomyces Genome database (SGD) (see Table S15) [135].

Competing interests

The authors declare that there are no conflicts of interest.

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Figure 1. Comparative genomic analyses of *Lichtheimia* species.

(A) Fragmented whole genome alignment of *L. corymbifera*, *L. ramosa* and *L. hyalospora* with *R. miehei* as outgroup by Gegenees. The percent of fragments which could be aligned and average similarities are indicated for each comparison using a 10 % threshold. Taxa are ordered according to a phylogenetic tree based on the LSU region of the species (GenBank accession numbers indicated). (B) Progressive MAUVE alignment of the largest contig of *L. ramosa* and the corresponding contigs of *L. corymbifera* and *L. hyalospora* ordered by MAUVE2 (left). Colours indicate aligned regions. Inverted (lower) and co-linear (upper) regions are indicated (C) Dot plots of genome-wide alignments of ordered contigs using r2cat (*L. ramosa* genome as reference).

Figure 2. Proteome comparison and phylogenomic analyses of *Lichtheimia* species.

(A) Distribution of homologues of *Lichtheimia* proteins in other fully sequenced fungal genomes. Bars show the number of proteins found also in other fungal phyla (grey), only in Mucorales (blue), only in Lichtheimiaceae (yellow), only in *Lichtheimia* spp. (green) and only in the corresponding species (red). (B) Species tree derived from the concatenation of 21 proteins found in single copy in at least 27 out of the 28 species included in the tree. The tree was reconstructed using phyML and bootstrap support was calculated. (C) VENN diagram of shared proteins between *Lichtheimia*

species based on tBLASTn analyses (E≤ 10^{-5}; ≥30 % similarity). (D) Scatter blot of BLAST score ratio analysis of *Lichtheimia* species. The red number in the lower left corner indicates species-specific proteins. Black numbers in the centre indicate the proportion of proteins shared with both species (green), only *L. ramosa* (red), only *L. hyalospora* (blue) and only weakly conserved in both other species (grey). (E) Gene gain and loss in *Lichtheimia* species based on phylome data. Numbers in green indicate gene gain and numbers in red gene loss at the internal nodes or at the tips.

Figure 3. Thermotolerance of *Lichtheimia* species.

(A) Drop dilution tests of *Lichtheimia* species at different temperatures. Spores were diluted and spotted in decreasing amounts ($2x10^4$, $2x10^3$, $2x10^2$, $2x10^1$) and incubated for 24 h. (B) Radial growth of *Lichtheimia* species at different temperatures. Bars represent colony diameters after 48 h and error bars show the standard deviation (three biological replicates with three technical replicates). Statistically significant results according to a two-sided T-test (P ≤ 0.05) are indicated with an asterisk. (C) Survival of spores of *Lichtheimia* species after heat treatment. Spores were incubated at 56°C for up to 45 min and plated on SUP agar. Values represent the average proportion of grown colonies compared to an untreated control (three biological replicated, three technical replicates). Error bars show the standard deviation and statistical significant results according to two-sided T-test (P ≤ 0.05) are indicated with an asterisk. (D) Growth assays of *Lichtheimia* species on ER-stress inducing DTT, HSP90 inhibitor geldanamycin and proteasome inhibitor MG132. Wells were inoculated with 5 x 10^3 spores and photographs were taken after 48 h growth at indicated temperatures. The pictures show representative outcomes of three independent experiments.

Figure 4. Comparative transcriptomics of *Lichtheimia* species under stress conditions.

(A) Number of up- and down-regulated genes in three *Lichtheimia* species under different stress conditions. (B) VENN diagrams of differentially regulation of orthologues in *Lichtheimia* species.

Orthology prediction was based on phylome data. (C) Heat map of expression changes of chaperone and HSF transcription factor genes in response to different stress conditions. Species are indicated as LC (*L. corymbifera*), LR (*L. ramosa*) and LH (*L. hyalospora*).

Figure 5. Rescue of the *L. hyalospora* heat phenotype by osmotic stress.

(A) Drop dilution tests of *L. hyalospora* at different temperatures with or without addition of 1.25 M NaCl to the medium. Spores were diluted and spotted in decreasing amounts ($2x10^4$, $2x10^3$, $2x10^2$, $2x10^1$) and incubated for 24 h. Additionally, plates were incubated at 42°C under hypoxia (1 % O_2/5 % CO_2). (B) Radial growth of *L. hyalospora* at different temperatures with or without addition of indicated concentrations of NaCl to the medium. Bars represent colony diameters after 48 h and error bars show the standard deviation (three biological replicates with three technical replicates). Statistical significant results according to two-sided T-test ($P \leq 0.05$) are indicated with an asterisk. (C) Growth of *L. hyalospora* on minimal medium with different C- and N-sources at different temperatures with or without addition of NaCl to the medium (1 M for olive oil/KNO_3 and 1.25 M for glucose/ casamino acids). Photos were taken after 5 days for glucose/CAA and after 7 days for olive oil/KNO_3. (D) Survival of *L. hyalospora* spores after heat treatment for 30 min at 56°C in the presence or absence of 0.5 M NaCl. Spores were pre-treated with PBS + 0.5 M NaCl for 30 min at room temperature and afterwards heat treated in standard PBS buffer (Pre), heat treated in PBS + 0.5 M NaCl (parallel) or a combination of both (combined). Spores were plated on SUP agar. Values represent the average proportion of grown colonies compared to an untreated control (three biological replicated, three technical replicates). Error bars show the standard deviation and statistical significant results according to two-sided T-test ($P \leq 0.05$) are indicated with an asterisk. (E) Survival of *L. hyalospora* spores after heat treatment for 30 min at 56°C in a hypotonic buffer. Spores were plated on SUP agar. Values represent the average proportion of grown colonies compared to an untreated control (three biological replicated, three technical replicates). Error bars show the standard deviation and statistically significant results according to a two-sided T-test ($P \leq 0.05$) are indicated with an asterisk.

Figure 6. Transcriptome of *L. hyalospora* under combinatorial stress.

(A) Scatter Plot of average expression levels of *L. hyalospora* cultures grown under heat stress (42°C) and osmotic stress (0.5 M NaCl) or heat stress alone. Differentially expressed genes (at least 2-fold expression change and P≤0.05) are shown in red (up) and green (down). Numbers indicate the amount of differentially expressed genes. (B) Subcellular localization of proteins encoded by differentially expressed genes. Significantly over- or underrepresented groups as identified by hypergeometric test are indicated with an asterisk. Subcellular localizations were predicted by TargetP (upper 9) or Predotar (lower 2). (C) VENN diagram of genes which were differentially expressed in heat stress compared to control conditions (42°C vs. 37°C) and in combinatorial stress compared to heat stress only (42°C + NaCl vs. 42°C). The direction of expression changes of shared genes in both datasets is shown in the pie chart at the bottom. (D) VENN diagram of genes which were lower or higher expressed in *L. hyalospora* compared to the two more thermotolerant *Lichtheimia* species and which were differentially expressed by combinatorial stress. The differences in gene expression levels and regulation under combinatorial stress for the shared genes are indicated in the chart at the bottom.

Figure S1. K-mer analyses.

The k-mer frequency distributions for the three *Lichtheimia* species were calculated for all k-mers of length of 59, i.e. for all possible 59-mers derived from the original Illumina reads. The number of k-mers (y-axis) is plotted against the frequency at which they occur (x-axis). The distribution shows a main peak and a steep rise to the left. This left-most rise of k-mers at lower frequencies represents mostly k-mers with randomly occurring sequencing errors. The main peak represents k-mers derived from (putatively) correct sequencing reads.

Figure S2. Topologies and dating of *Lichtheimia* species based on multi-gene trees.

Phylome support for the three different possible topologies of *Lichtheimia* species are indicated in percentage for each possible variant. For the first two versions divergence times were calculated with R8S-PL using the split between Asco- and Basidiomycota (798MyA) as calibration point.

Figure S3. Gene and tandem duplications in *Lichtheimia* species.

(A) Gene family sizes show the typical distribution of multi-gene families in mucoralean fungi for all three *Lichtheimia* species. Gene family designations were based on Transclust results. (B) Functional annotation of tandem duplicated genes in *Lichtheimia* species. Each bar represents a Pfam domain and shows the number of genes which were found in tandem duplicated groups. (C) Ring diagram of the 20 most abundant Pfam domains found in tandem duplicated genes. (D) Example cluster shows synteny and copy-number variations of tandem duplicated genes in the three *Lichtheimia* species. Genes with the same colour represent orthologues/paralogues in the other genomes. Pfam domains of the corresponding genes are indicated at the bottom.

Figure S4. Distribution of putative virulence-associated genes in *Lichtheimia* species.

(A) VENN diagram of putative virulence factors based on comparison to the pathogen-host interaction database (PHI-database) and their homologues in *Lichtheimia* species. (B) Distribution of the most abundant BLAST hits of different genes from the PHI-database in *Lichtheimia* species. (C) Distribution of the most abundant Pfam domains associated with putative virulence factors in *Lichtheimia* species based on comparison to the PHI-database.

Figure S5. Virulence-associated traits in *Lichtheimia* species.

(A) Growth of *Lichtheimia* species on different iron sources in the presence of the iron chelator BPS. (B) Growth of *Lichtheimia* species under hypoxic conditions (1 % O_2/5 % CO_2 and 0.2 % O_2/5

% CO_2) on SUP agar at 37°C. Spores were diluted and spotted in decreasing amounts ($2x10^4$, $2x10^3$, $2x10^2$, $2x10^1$). Pictures were taken after 24 h and are representative results from three independent experiments. (C) Virulence of *Lichtheimia* species in Galleria mellonella larvae. Twenty sixth-instar larvae per group were infected each via injection in the hemocoel with 10^6 spores. Spore-free IPS was used as negative control. Larvae were incubated at 30°C. Experiments were performed 3 times; curves represent average survival rates over a period of 6 days post infection.

Figure S6. Stress tolerance of *Lichtheimia* species at different growth temperatures.

SUP medium with increasing concentrations of different stressors was inoculated with spores of *Lichtheimia* species and incubated at 37°C or 42°C for 48 h. Growth was evaluated via optical inspection and rated according to the amount of mycelium.

Figure S7. Effect of heat on the growth of *Lichtheimia* species on complex nutrients.

Growth of *Lichtheimia* species on YNB medium containing different combinations of carbon- and nitrogen-sources (2 % C-source/0.5 % N-source). Bars represent average percent of growth at 42°C compared to 37°C and error bars show the standard deviation (three biological replicates with three technical replicates). Statistical significant results according to two-sided T-test ($P \leq 0.05$) are indicated with an asterisk (glucose/KNO_3 as reference).

Figure S8. Transcriptome of *Lichtheimia* species under different stress conditions.

VENN diagrams show shared and exclusive genes which are up- and down-regulated under heat, ER-stress (DTT) and Hsp90 inhibition (GdA) in three different *Lichtheimia* species.

Figure S9. Conserved response of *Lichtheimia* species towards ER-stress induced by DTT.

Enriched gene ontology (GO) terms of genes involved in ER-stress response in all three *Lichtheimia* species based on the GO annotation of *L. ramosa* (P≤0.05 in Fisher's exact test).

Figure S10. Rescue of growth of *L. hyalospora* under heat stress and involvement of Hog1.

(A) Effect of osmolytes and NaCl on growth of *Lichtheimia* species under heat stress. Bars represent average percent of growth at 42°C compared to 37°C and error bars show the standard deviation (three biological replicates with three technical replicates). Statistical significant results according to two-sided T-test (P ≤ 0.05) are indicated with an asterisk (SUP as reference). (B) Effect of a HOG1 inhibitor on growth of *Lichtheimia* species under stress conditions. Spores of the *Lichtheimia* species were placed on SUP agar with or without different stressors and HOG1 inhibitor BPTIP ('+', 75 µM) or an equal volume of the solvent ('-', DMSO). Plates were incubated for 24 h at 37°C except for heat stress samples which were grown at 42°C. Stresses included NaCl (1.25 M), heat (42°C), hypoxia (1 % oxygen), SDS (50 µg/ml), Congo red (100 µg/ml) and menadione (0.25 mM). (C) Effect of BPTIP on the growth supporting effect of NaCl at elevated temperatures. Spores of *L. hyalospora* were placed on SUP medium with or without NaCl and with 75 µM BPTIP or an equal volume of DMSO. Plates were incubated for 24 h at 42°C. Pictures show representative results from two independent experiments.

Table S1. Repetitive elements

Table S2. Contig ordering and clustering.

Table S3. Conserved and species-specific genes and gene duplications.

Table S4: GECKO Clusters. Number of syntenic clusters and average number of genes per cluster.

Table S5. Protease families of *Lichtheimia* species.

Table S6. Iron uptake genes in *Lichtheimia* species.

Table S7. Orthologous genes between *Lichtheimia* species.

Table S8. RNA-Seq mapping and differentially expressed genes in *L. corymbifera*.

Table S9. RNA-Seq mapping and differentially expressed genes in *L. ramosa*.

Table S10. RNA-Seq mapping and differentially expressed genes in *L. hyalospora*.

Table S11. GO enrichment of differentially expressed genes under heat stress.

Table S12. RNA-Seq mapping and differentially expressed orthologues between *L. hyalospora* and the two clinically relevant *Lichtheimia* species.

Table S13. Conserved genes of the ER-stress response in *S. cerevisiae* and expressional changes of their orthologues in *Lichtheimia* species after DTT treatment.

Table S14. Functional annotation of significantly up-regulated genes in *L. hyalospora*.

Table S15. Genomes used in this study.

File S1. Annotations and alignments of ncRNAs.

Table 1: Genome statistics

	L. ramosa	*L. hyalospora*	*L. corymbifera*
Assembly statistics			
Total scaffold length (Mb)	30.7	33.3	33.6
Scaffolds	74	2222	209
G+C content	41.2 %	43.4 %	43.3 %
Repetitive elements (Mb)	1.38	3.59	2.06
Predicted protein-coding genes			
Predicted genes	11,510	12,062	12,379
Average CDS size (nt)	1,390	1,251	1,287
Average G+C content	43.7 %	43.8 %	46.2 %

Table 2: Non-coding RNAs of *Lichtheimia* species.

ncRNA class	*L. corymbifera*	*L. ramosa*	*L. hyalospora*
5S rRNA	1	0	0
5.8S rRNA	0	1	0
18S rRNA	0	1	0
28S rRNA	1	1	0
tRNA	174	171	130
U1	2	2	2
U2	2	3	3
U4	0	1	1
U5	2	3	3

U6	3	3	2
U11	1	1	1
U4atac	1	1	1
U6atac	1	1	1
CD snoRNA	31	35	36
HACA snoRNA	4	5	4
SRP	1	1	1
RNase MRP	1	1	1
Hammerhead 1	0	1	1
TPP	1	1	1

Table 3: Distribution and copy numbers of classical heat shock related proteins.

Pfam ID	Domain	*L. corymbifera*	*L. hyalospora*	*L. ramosa*
PF00011	HSP20	3	2	2
PF00012	HSP70	17	21	20
PF00183	HSP90	4	5	5
PF02861	Clp_N	1	1	1
PF10431	ClpB_D2-small	5	7	6
PF00166	Cpn10	0	1	1
PF00118	Cpn60_TCP1	12	13	13
PF00226	DNAJ	35	34	35
PF02996/PF01920	Prefoldin	8	10	8
PF06825	HSBP1	0	1	1
PF00447	HSF_DNA-bind	24	24	24
PF02358	Trehalose_PPase	6	7	7
PF00982	Glyco_transf_20	6	7	7

Figure 1

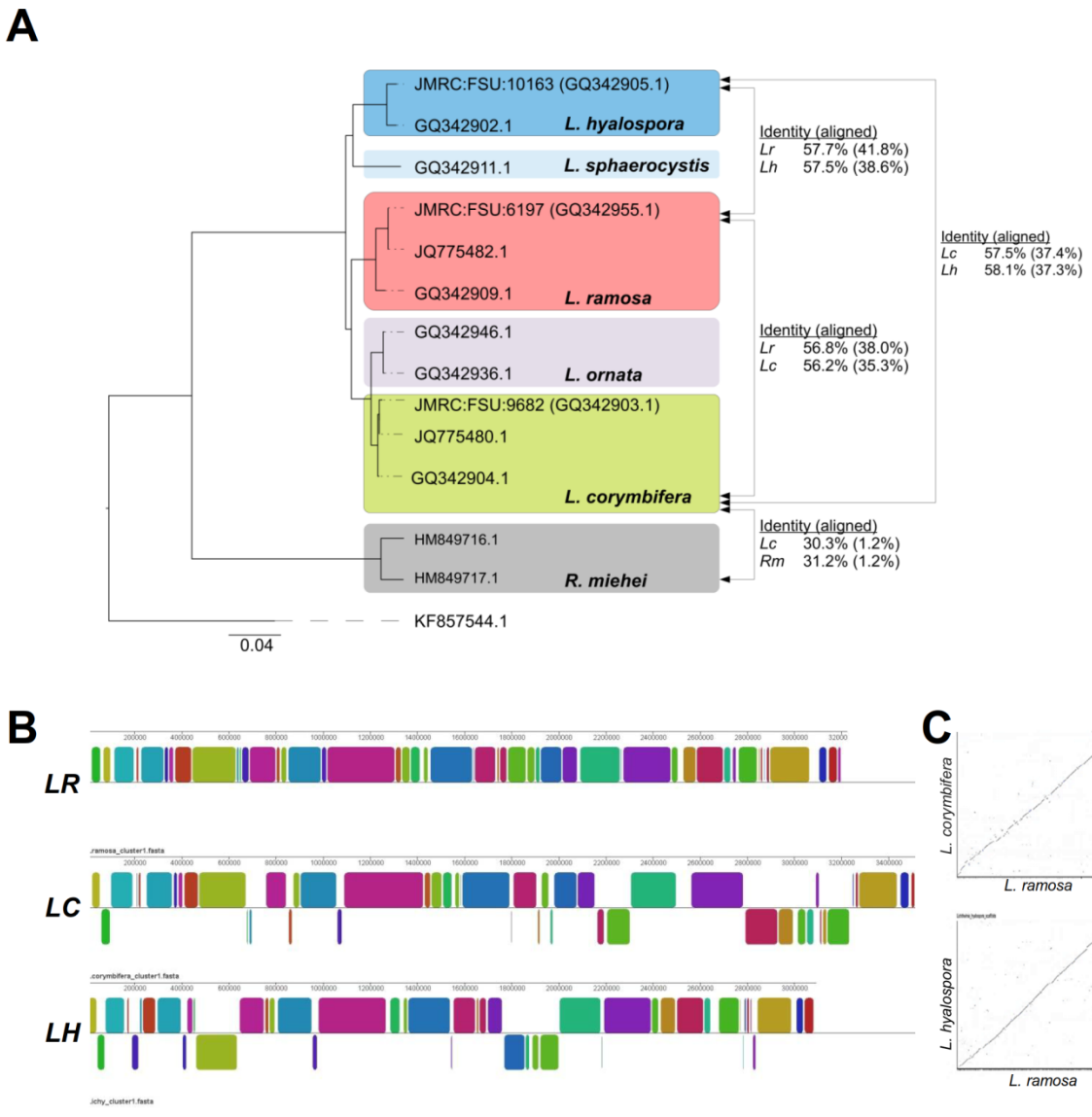

A
JMRC:FSU:10163 (GQ342905.1)
GQ342902.1
L. hyalospora
GQ342911.1
L. sphaerocystis
JMRC:FSU:6197 (GQ342955.1)
JQ775482.1
GQ342909.1
L. ramosa
GQ342946.1
GQ342936.1
L. ornata
JMRC:FSU:9682 (GQ342903.1)
JQ775480.1
GQ342904.1
L. corymbifera
HM849716.1
HM849717.1
R. miehei
KF857544.1
0.04
Identity (aligned)
Lr 57.7% (41.8%)
Lh 57.5% (38.6%)
Identity (aligned)
Lc 57.5% (37.4%)
Lh 58.1% (37.3%)
Identity (aligned)
Lr 56.8% (38.0%)
Lc 56.2% (35.3%)
Identity (aligned)
Lc 30.3% (1.2%)
Rm 31.2% (1.2%)
B
LR
LC
LH
C
L. corymbifera
L. ramosa
L. hyalospora
L. ramosa

Figure 2

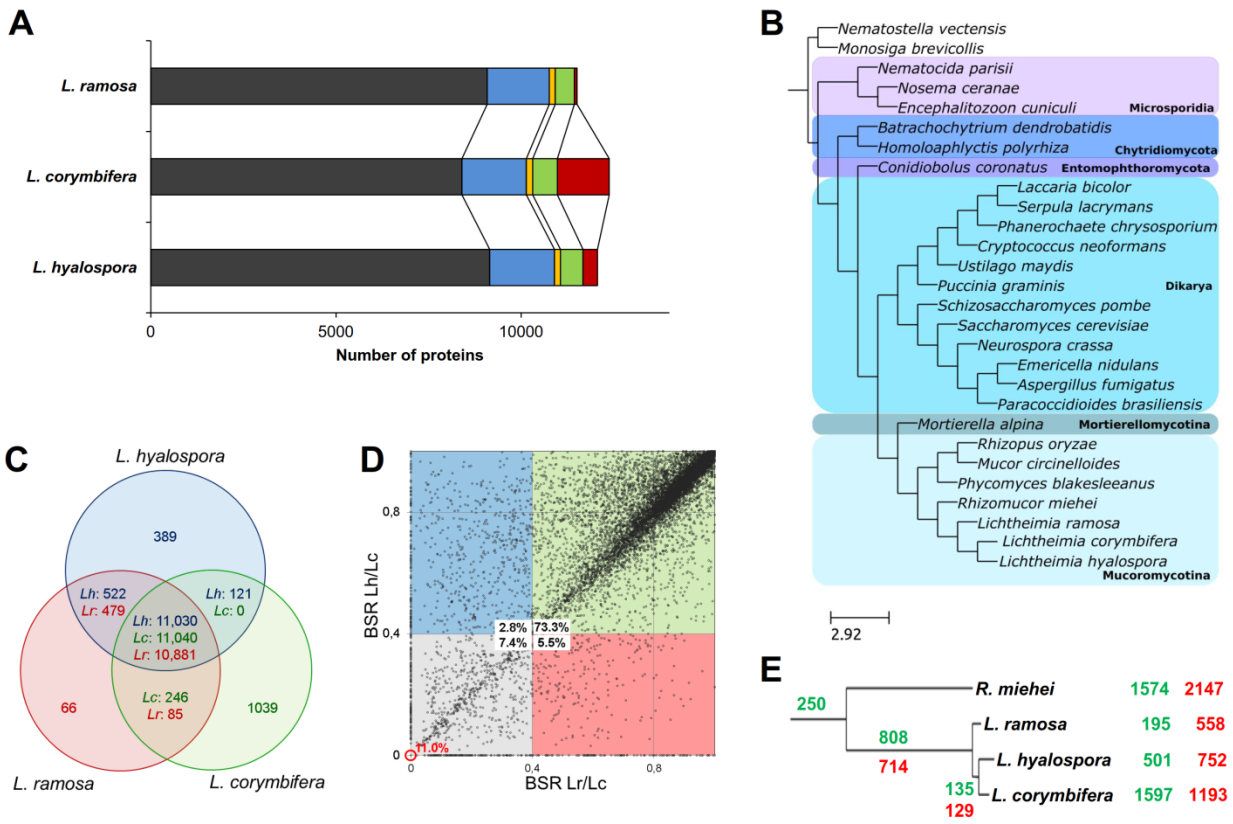

A
L. ramosa
L. corymbifera
L. hyalospora
0
5000
10000
Number of proteins
B
Nematostella vectensis
Monosiga brevicollis
Nematocida parisii
Nosema ceranae
Encephalitozoon cuniculi
Microsporidia
Batrachochytrium dendrobatidis
Homoloaphlyctis polyrhiza
Chytridiomycota
Conidiobolus coronatus
Entomophthoromycota
Laccaria bicolor
Serpula lacrymans
Phanerochaete chrysosporium
Cryptococcus neoformans
Ustilago maydis
Puccinia graminis
Dikarya
Schizosaccharomyces pombe
Saccharomyces cerevisiae
Neurospora crassa
Emericella nidulans
Aspergillus fumigatus
Paracoccidioides brasiliensis
Mortierella alpina
Mortierellomycotina
Rhizopus oryzae
Mucor circinelloides
Phycomyces blakesleeanus
Rhizomucor miehei
Lichtheimia ramosa
Lichtheimia corymbifera
Lichtheimia hyalospora
Mucoromycotina
2.92
C
L. hyalospora
389
Lh: 522
Lr: 479
Lh: 121
Lc: 0
Lh: 11,030
Lc: 11,040
Lr: 10,881
66
Lc: 246
Lr: 85
1039
L. ramosa
L. corymbifera
D
BSR Lh/Lc
BSR Lr/Lc
0,8
0,4
0
0,4
0,8
2.8%
73.3%
7.4%
5.5%
11.0%
E
250
808
714
135
129
R. miehei
1574
2147
L. ramosa
195
558
L. hyalospora
501
752
L. corymbifera
1597
1193

Figure 3

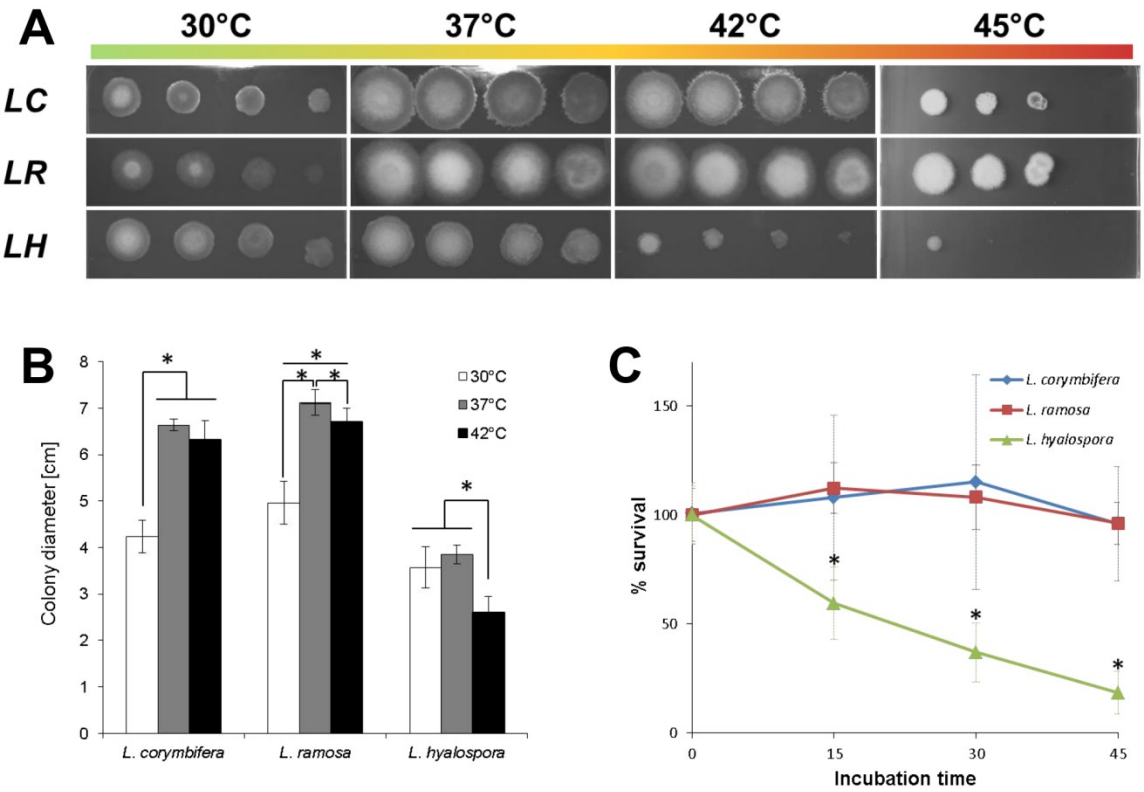

A
30°C
37°C
42°C
45°C
LC
LR
LH
B
Colony diameter [cm]
30°C
37°C
42°C
L. corymbifera
L. ramosa
L. hyalospora
C
% survival
L. corymbifera
L. ramosa
L. hyalospora
Incubation time
D
DTT [mM]
Geldanamycin [µg/ml]
MG132 [µM]
37°C
42°C
LC
LR
LH

Figure 4

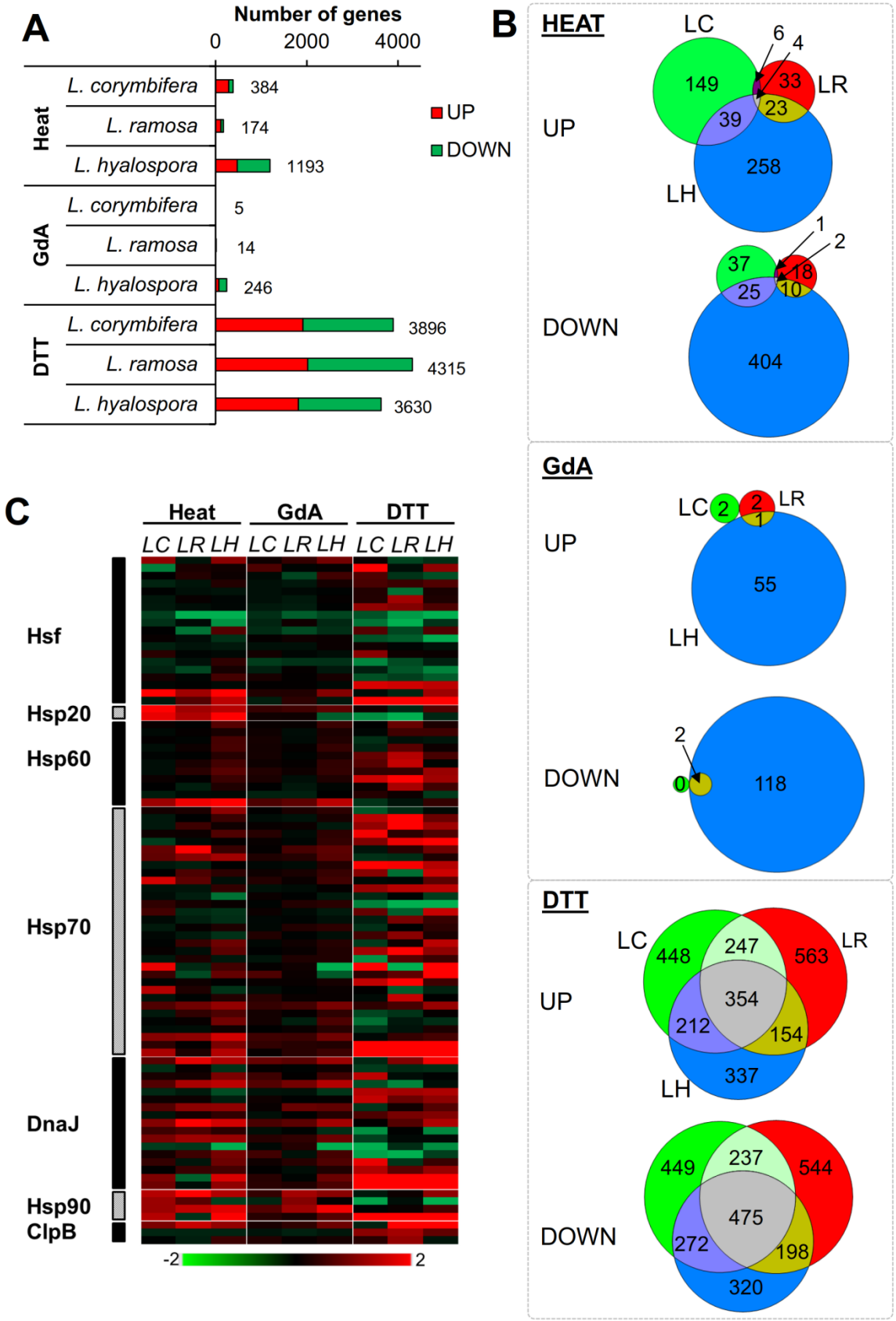

A
Number of genes
0
2000
4000
Heat
GdA
DTT
L. corymbifera
L. ramosa
L. hyalospora
384
174
1193
5
14
246
3896
4315
3630
UP
DOWN
B
HEAT
UP
DOWN
LC
LR
LH
149
33
23
39
258
6
4
37
18
25
10
404
1
2
GdA
55
118
2
0
DTT
448
247
563
354
212
154
337
449
237
544
475
272
198
320
C
Heat
GdA
DTT
LC LR LH LC LR LH LC LR LH
Hsf
Hsp20
Hsp60
Hsp70
DnaJ
Hsp90
ClpB
-2
2

Figure 5

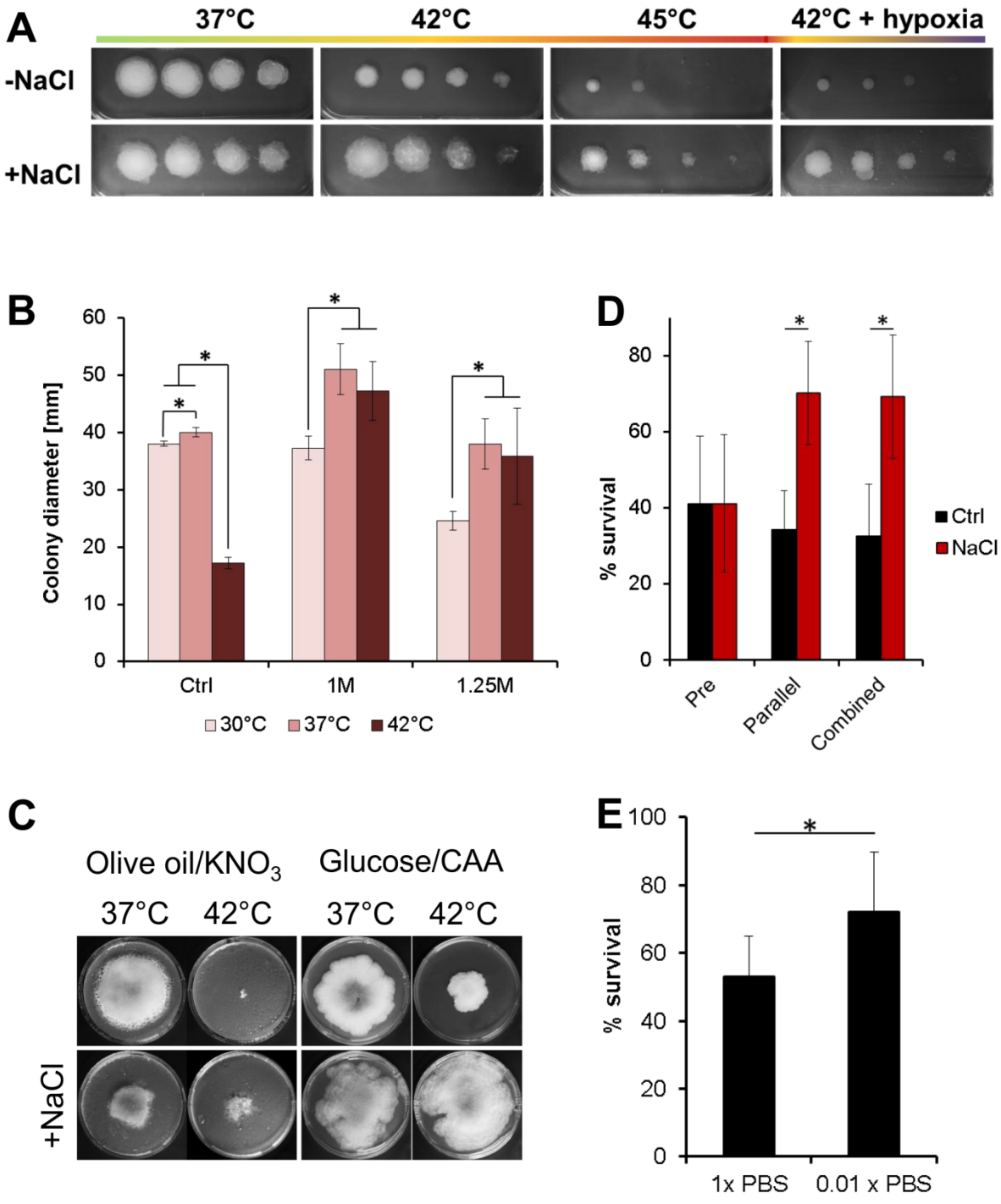

A
37°C
42°C
45°C
42°C + hypoxia
-NaCl
+NaCl
B
Colony diameter [mm]
Ctrl
1M
1.25M
30°C
37°C
42°C
D
% survival
Pre
Parallel
Combined
Ctrl
NaCl
C
Olive oil/KNO3
Glucose/CAA
37°C
42°C
37°C
42°C
+NaCl
E
% survival
1x PBS
0.01 x PBS

Figure 6

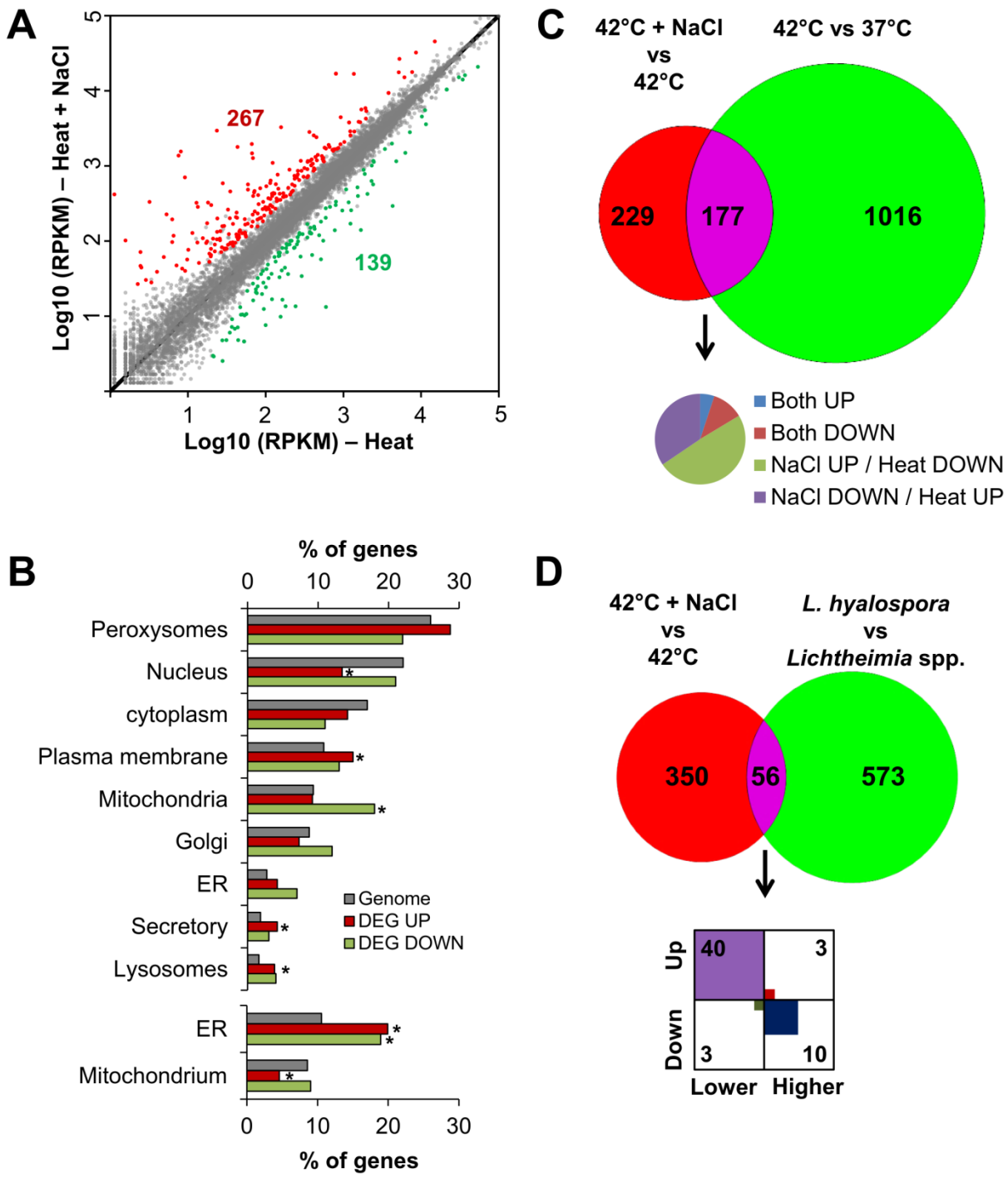

A
Log10 (RPKM) – Heat + NaCl
Log10 (RPKM) – Heat
267
139
C
42°C + NaCl vs 42°C
42°C vs 37°C
229
177
1016
Both UP
Both DOWN
NaCl UP / Heat DOWN
NaCl DOWN / Heat UP
B
% of genes
Peroxysomes
Nucleus
cytoplasm
Plasma membrane
Mitochondria
Golgi
ER
Secretory
Lysosomes
ER
Mitochondrium
Genome
DEG UP
DEG DOWN
D
42°C + NaCl vs 42°C
L. hyalospora vs Lichtheimia spp.
350
56
573
Up
Down
40
3
3
10
Lower
Higher

Figure S1

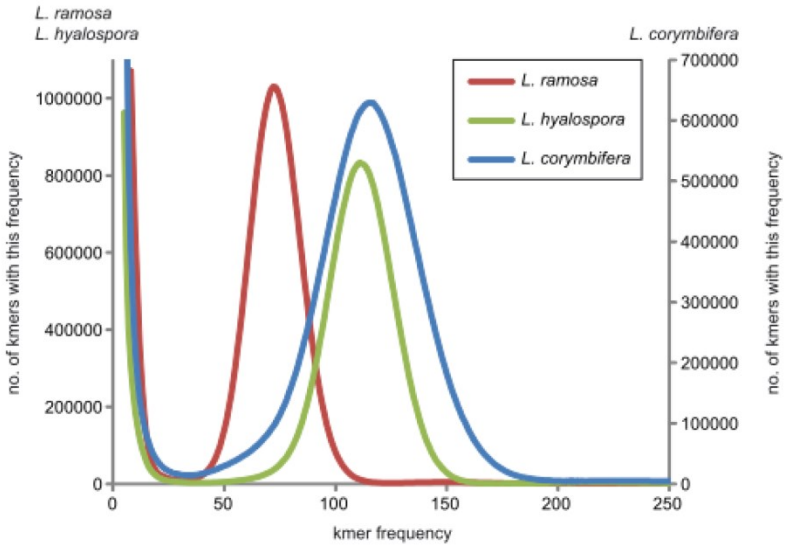
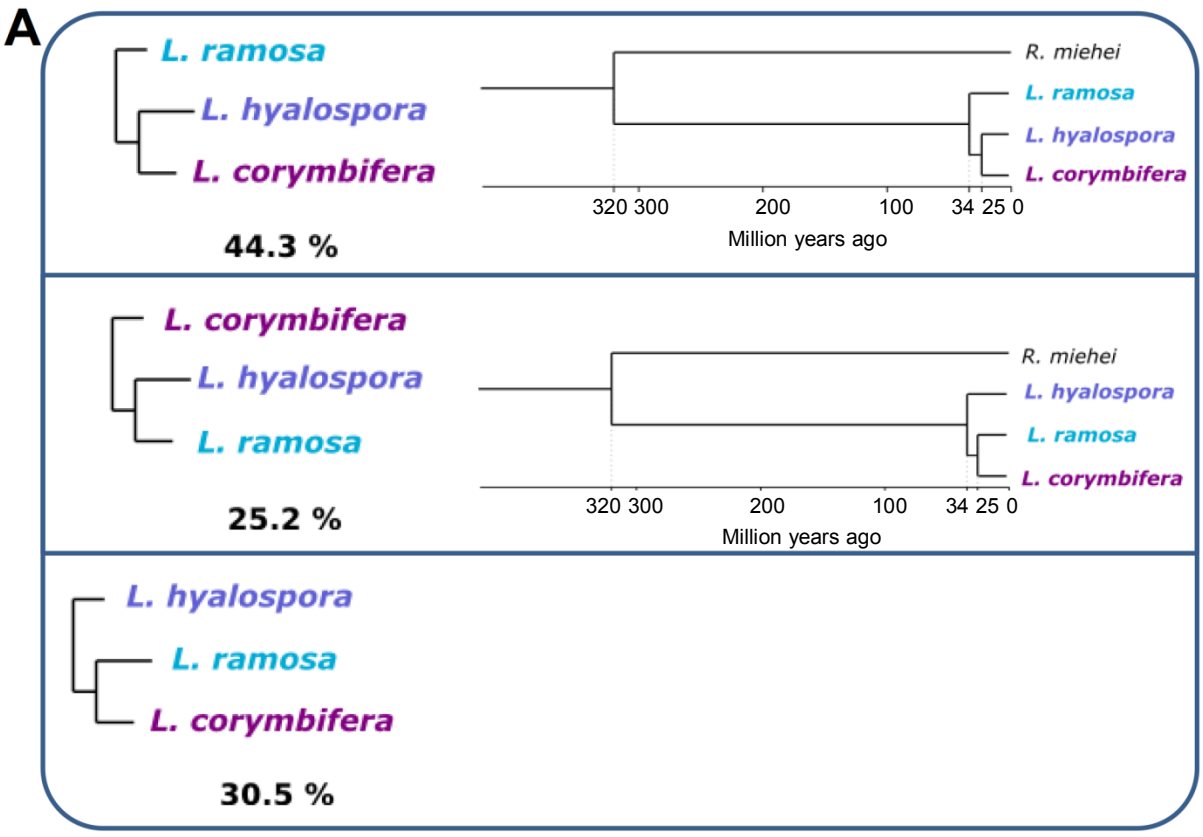

L. ramosa
L. hyalospora
L. corymbifera
L. ramosa
L. hyalospora
L. corymbifera
no. of kmers with this frequency
no. of kmers with this frequency
kmer frequency
0
200000
400000
600000
800000
1000000
100000
200000
300000
400000
500000
600000
700000
0
50
100
150
200
250

Figure S2


A
L. ramosa
L. hyalospora
L. corymbifera
44.3 %
R. miehei
L. ramosa
L. hyalospora
L. corymbifera
320 300 200 100 34 25 0
Million years ago
L. corymbifera
L. hyalospora
L. ramosa
25.2 %
R. miehei
L. hyalospora
L. ramosa
L. corymbifera
320 300 200 100 34 25 0
Million years ago
L. hyalospora
L. ramosa
L. corymbifera
30.5 %

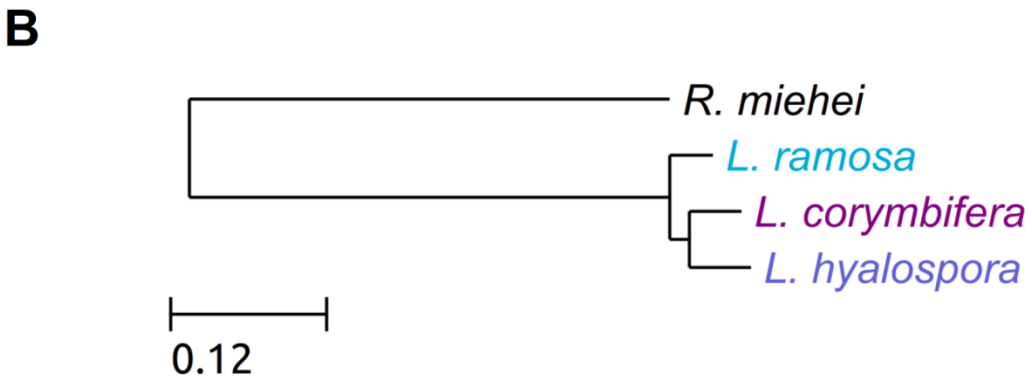

B
R. miehei
L. ramosa
L. corymbifera
L. hyalospora
0.12

Figure S3

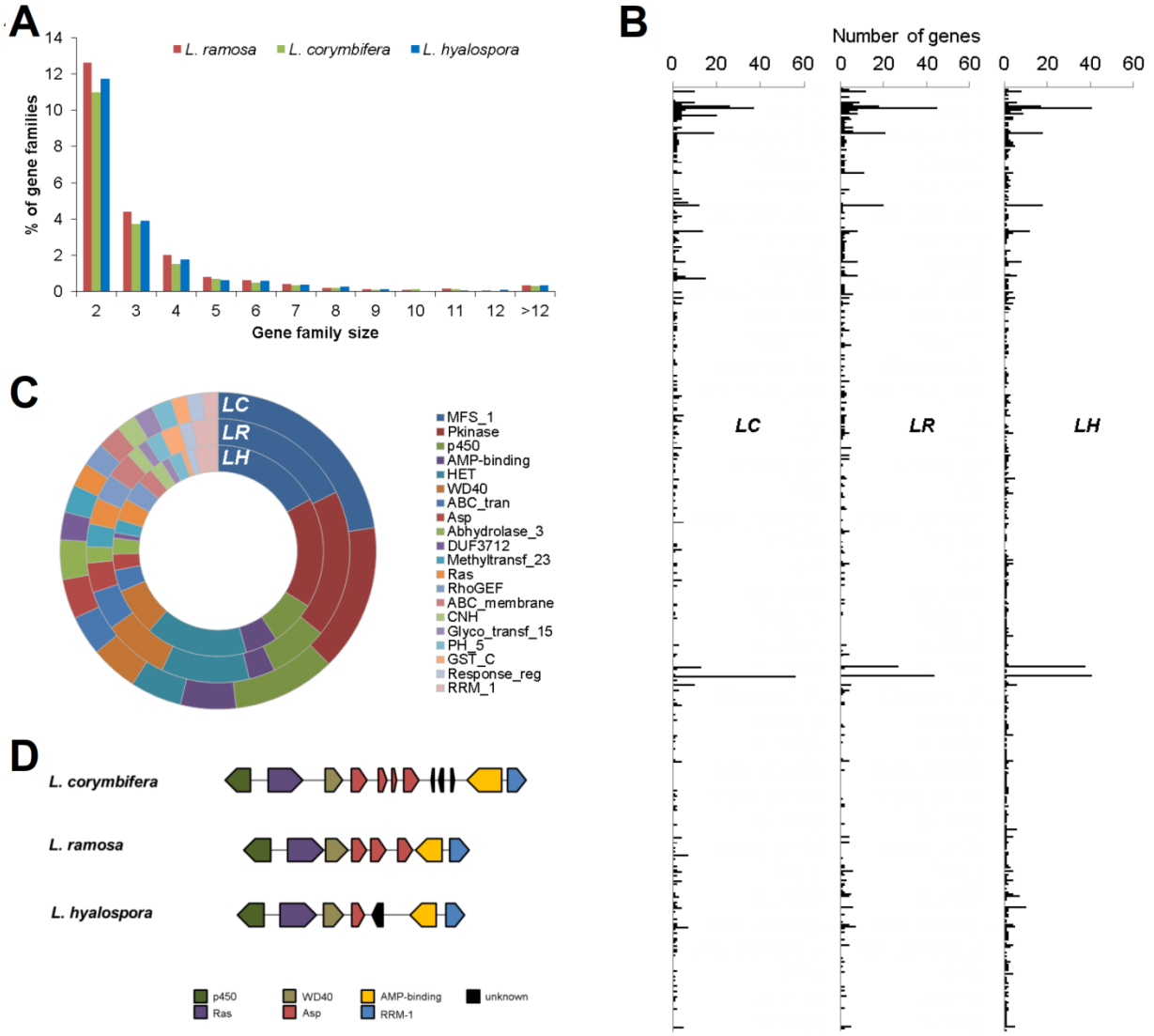

A
L. ramosa
L. corymbifera
L. hyalospora
% of gene families
Gene family size
B
Number of genes
LC
LR
LH
C
LC
LR
LH
MFS_1
Pkinase
p450
AMP-binding
HET
WD40
ABC_tran
Asp
Abhydrolase_3
DUF3712
Methyltransf_23
Ras
RhoGEF
ABC_membrane
CNH
Glyco_transf_15
PH_5
GST_C
Response_reg
RRM_1
D
L. corymbifera
L. ramosa
L. hyalospora
p450
Ras
WD40
Asp
AMP-binding
RRM-1
unknown

Figure S4

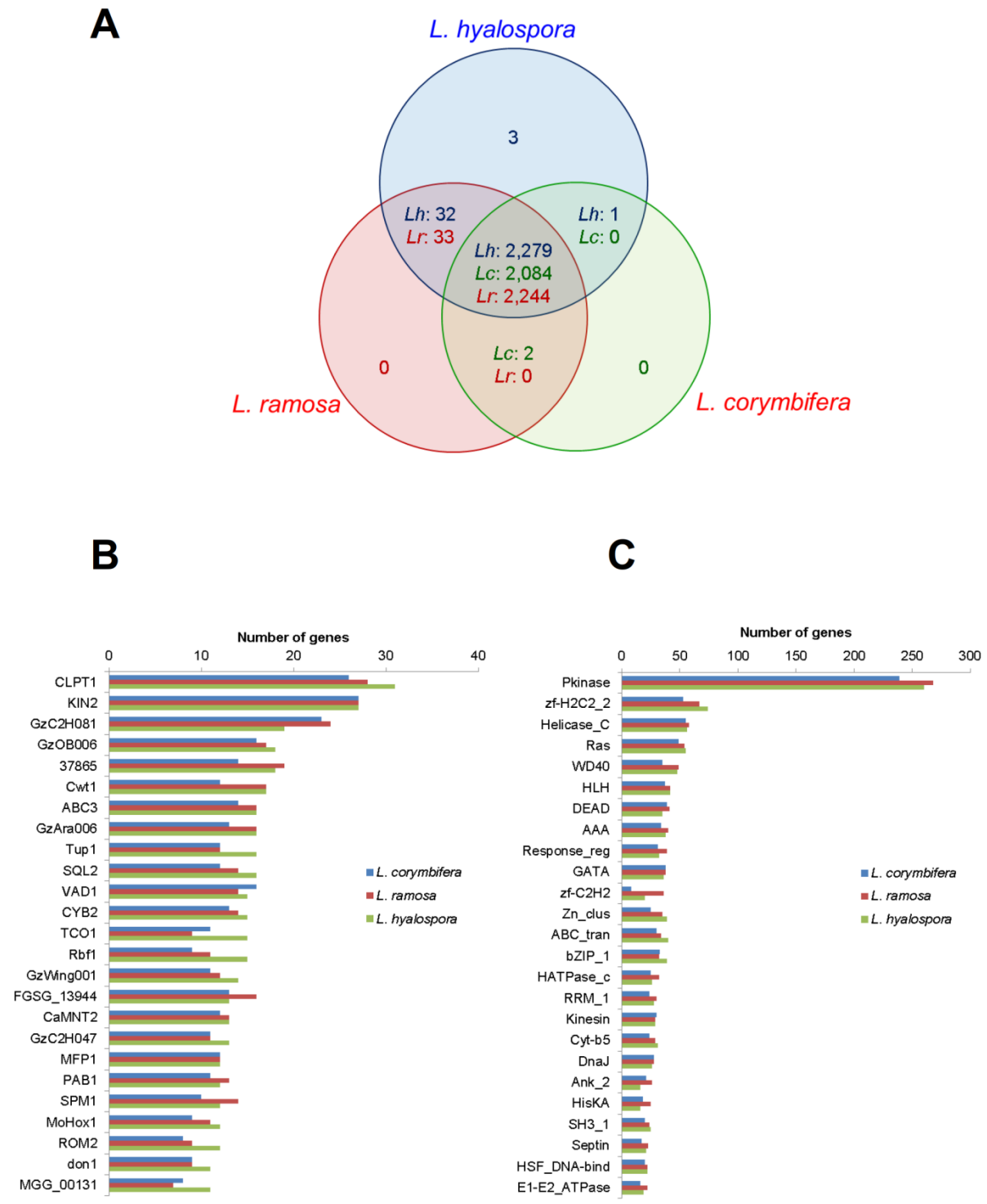

A
L. hyalospora
3
Lh: 32
Lr: 33
Lh: 1
Lc: 0
Lh: 2,279
Lc: 2,084
Lr: 2,244
Lc: 2
Lr: 0
0
0
L. ramosa
L. corymbifera
B
Number of genes
0
10
20
30
40
CLPT1
KIN2
GzC2H081
GzOB006
37865
Cwt1
ABC3
GzAra006
Tup1
SQL2
VAD1
CYB2
TCO1
Rbf1
GzWing001
FGSG_13944
CaMNT2
GzC2H047
MFP1
PAB1
SPM1
MoHox1
ROM2
don1
MGG_00131
L. corymbifera
L. ramosa
L. hyalospora
C
Number of genes
0
50
100
150
200
250
300
Pkinase
zf-H2C2_2
Helicase_C
Ras
WD40
HLH
DEAD
AAA
Response_reg
GATA
zf-C2H2
Zn_clus
ABC_tran
bZIP_1
HATPase_c
RRM_1
Kinesin
Cyt-b5
DnaJ
Ank_2
HisKA
SH3_1
Septin
HSF_DNA-bind
E1-E2_ATPase
L. corymbifera
L. ramosa
L. hyalospora

Figure S5

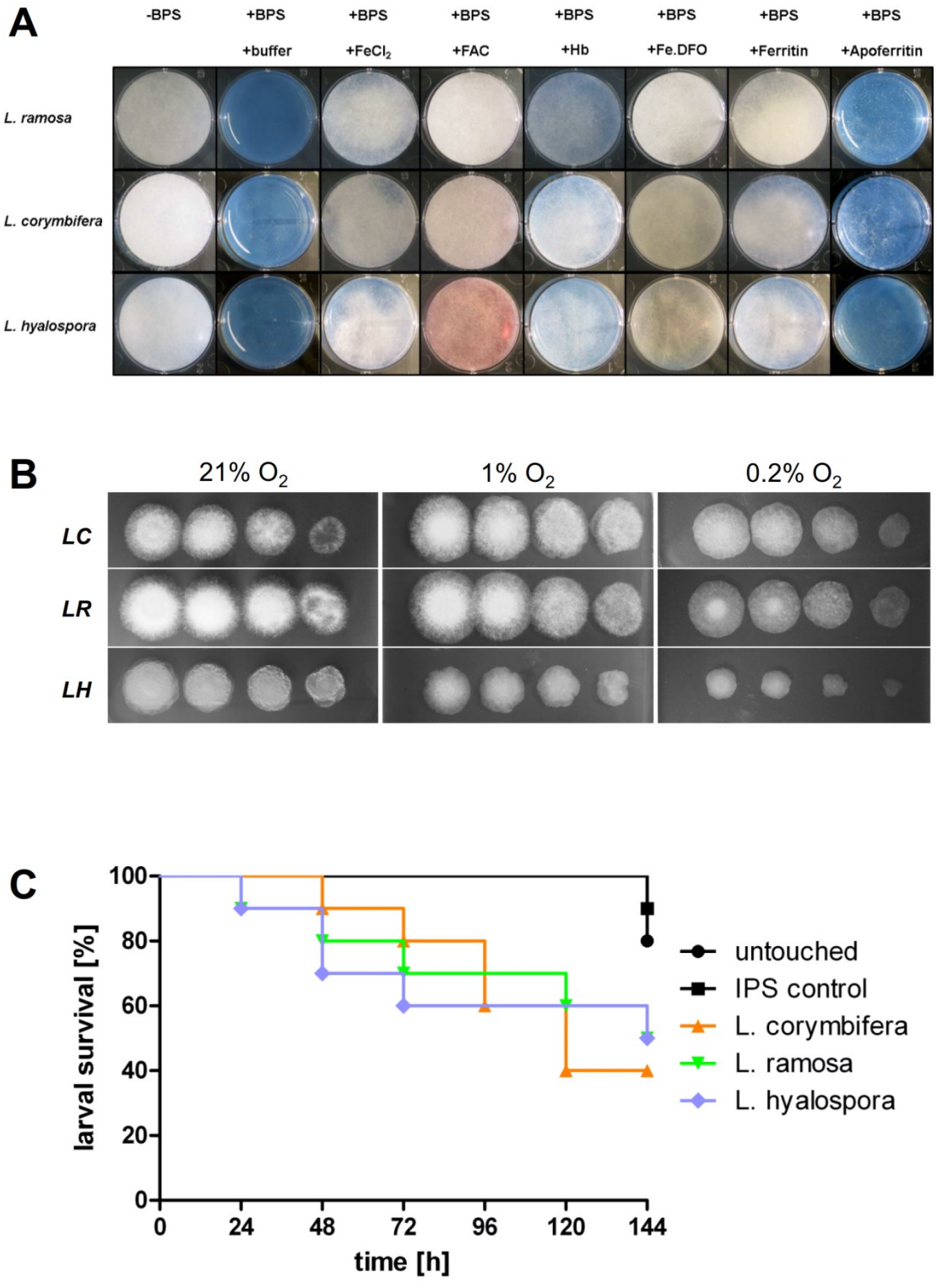

A
-BPS
+BPS
+buffer
+BPS
+FeCl2
+BPS
+FAC
+BPS
+Hb
+BPS
+Fe.DFO
+BPS
+Ferritin
+BPS
+Apoferritin
L. ramosa
L. corymbifera
L. hyalospora
B
21% O2
1% O2
0.2% O2
LC
LR
LH
C
larval survival [%]
100
80
60
40
20
0
0
24
48
72
96
120
144
time [h]
untouched
IPS control
L. corymbifera
L. ramosa
L. hyalospora

Figure S6

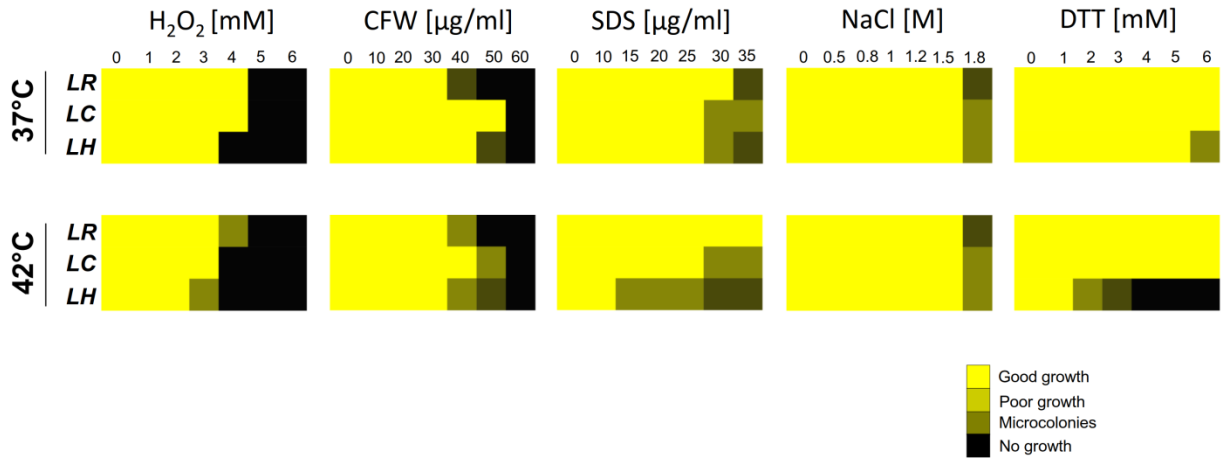


Figure S7

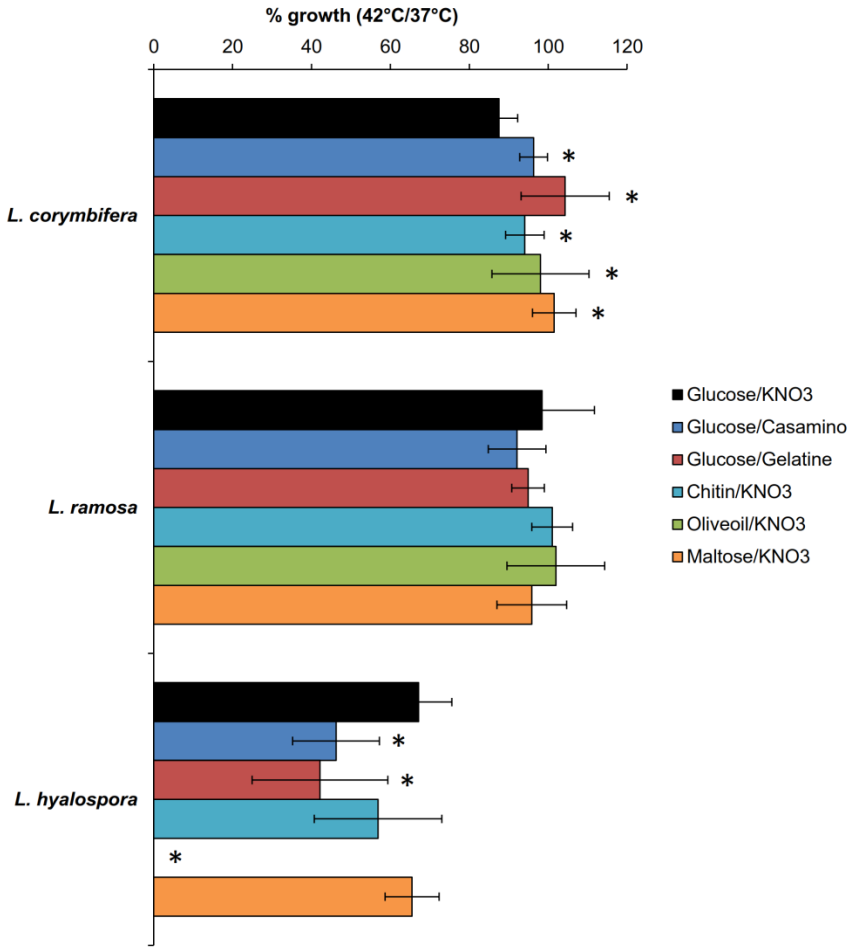


Figure S8

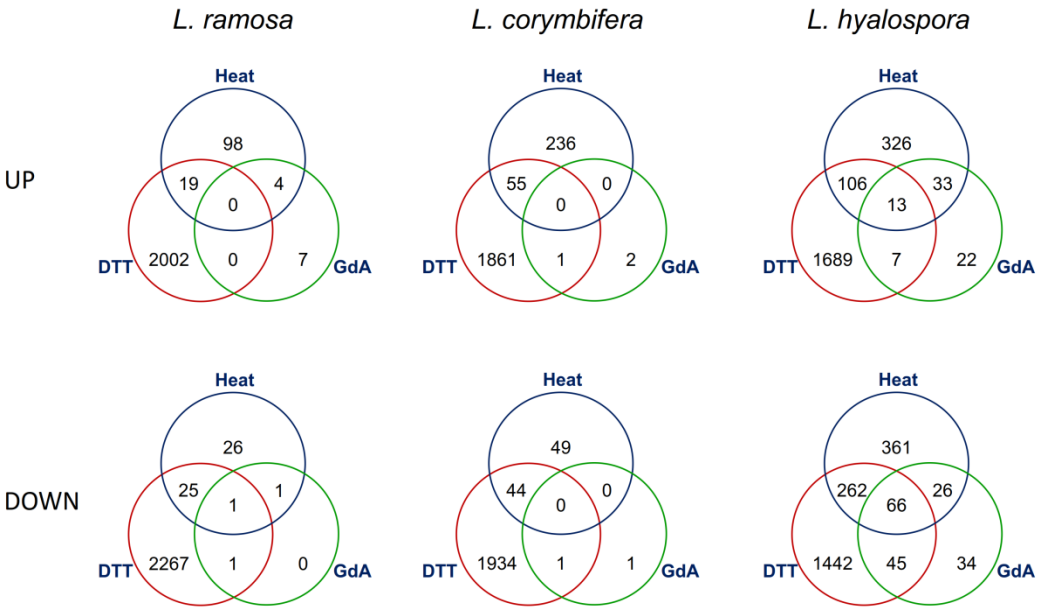


Figure S9

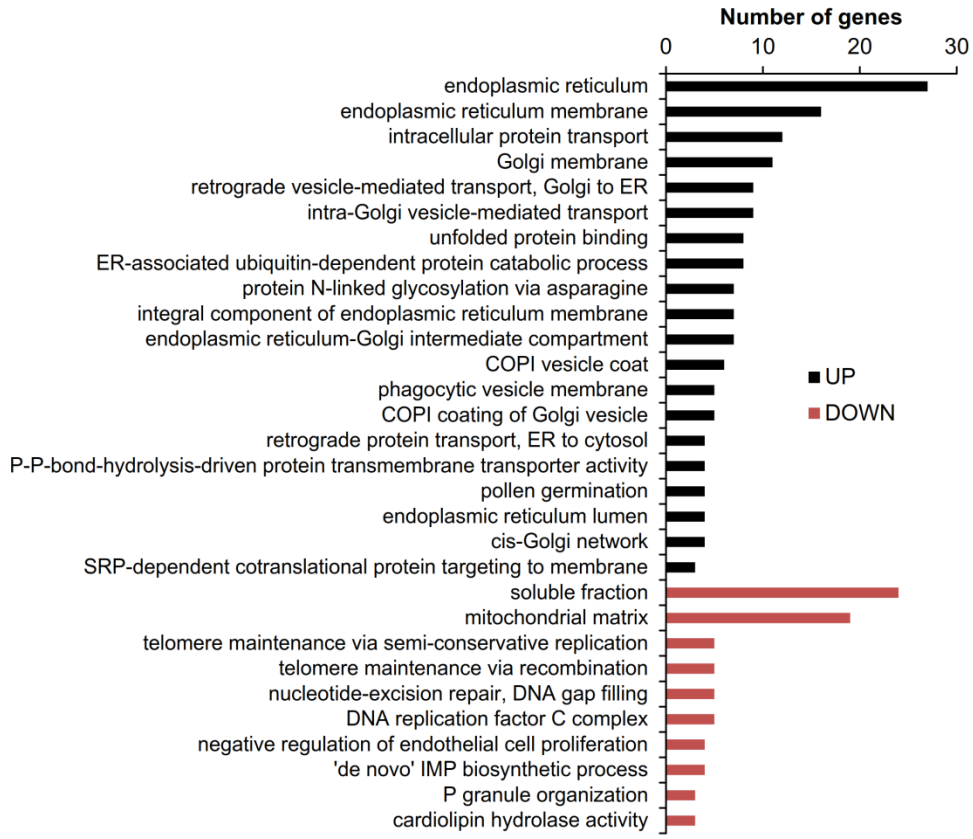


Figure S10

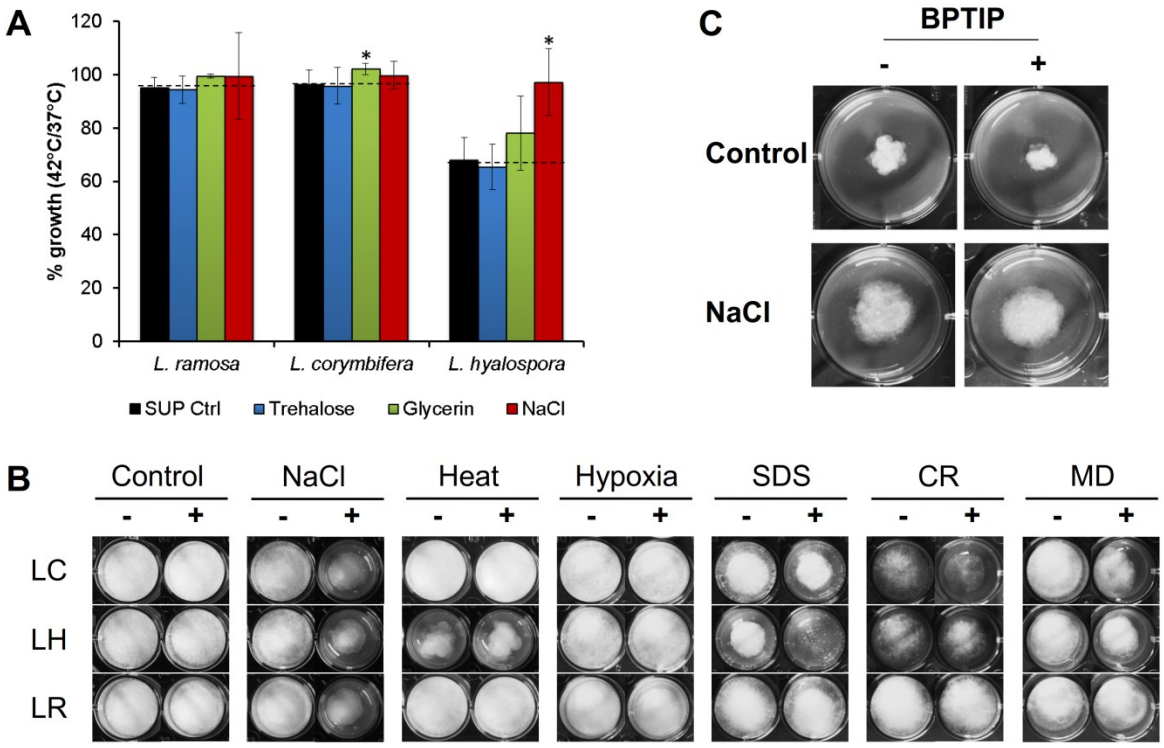

A
120
100
80
60
40
20
0
% growth (42°C/37°C)
*
*
L. ramosa
L. corymbifera
L. hyalospora
SUP Ctrl
Trehalose
Glycerin
NaCl
C
BPTIP
-
+
Control
NaCl
B
Control
NaCl
Heat
Hypoxia
SDS
CR
MD
- + - + - + - + - + - + - +
LC
LH
LR

Table S1

		L. ramosa	*L. corymbifera*	*L. hyalospora*
DNA transposons	Number	132	44	1
	Total length	151,845	69,906	203
	% of assembly	0.4944	0.2079	0.0006
LTR retrotransposons	Number	24	245	18
	Total length	18,283	250,916	17,472
	% of assembly	0.0595	0.7463	0.0525
non-LTR retrotransposons	Number	3	2	20
	Total length	1,063	821	21,252
	% of assembly	0.0035	0.0024	0.0639
Simple repeats	Number	373	180	18
	Total length	297,696	100,682	4,791
	% of assembly	0.9694	0.2995	0.0144
Low complexity	Number	2	0	2
	Total length	417	0	504
	% of assembly	0.0014	0.0000	0.0015
unknown	Number	833	3,052	2,077
	Total length	401,301	2,402,194	1,289,393
	% of assembly	1.3068	7.1454	3.8741
Total	Number	1,365	3,523	2,136
	Total length	870,188	2,824,519	1,333,615
	% of assembly	2.8337	8.4016	4.0070

Table S2 and S3 are too large for print but can be found on the CD attached to this thesis.

Table S4

	L. corymbifera	*L. ramosa*	*L. hyalospora*
L. corymbifera	-	510 (15.7)	570 (13.6)
L. ramosa	649 (13.2)	-	678 (13.9)
L. hyalospora	625 (12.8)	575 (15.8)	-
Mucorales	167 (4.35)	198 (4.27)	187 (4.36)

Table S5

Protease family	*L. corymbifera*	*L. ramosa*	*L. hyalospora*
Aspartate	60	38	29
Cysteine	70	72	76
Metallo	148	156	131
Serine	124	122	127
Threonine	14	18	18
Unknown	1	1	1
Total	417	407	382

Table S6

Pathway	**Iron uptake gene**	***L. corymbifera***	***L. ramosa***	***L. hyalospora***
Reductive pathway	FTR1	4	3	4
	multicopper oxidase	8	7	6
	ferric reductase	3	3	3
Siderophores	siderophore transporter	1	1	1
	DFO receptor (Fob2)	1	1	1
Heme utilization	heme oxygenase	2	2	2
Iron storage	ferritin	2	2	2

Tables S7 to S12 are too large for print but can be found on the CD attached to this thesis.

Table S13

Gene ID				log2 fold expression change		
S. cerevisiae	**LC**	**LR**	**LH**	**LC**	**LR**	**LH**
Pdi1 / Eug1	LCor09401	LRAMOSA03317	jgi.p|Lichy1|142528	5.2	3.6	4.0
Eps1	-	-	-	-	-	-
Mpd1	LCor06134	LRAMOSA03126	jgi.p|Lichy1|163781	3.7	3.2	2.1
Kar2	LCor11316	LRAMOSA01351	jgi.p|Lichy1|144160	5.4	4.4	4.3
Lhs1	LCor02606	LRAMOSA05196	jgi.p|Lichy1|157586	5.1	4.2	4.4
Scj1	LCor11099	LRAMOSA10793	jgi.p|Lichy1|230640	3.6	3.6	2.2
Cne1	LCor02382	LRAMOSA03926	jgi.p|Lichy1|156437	4.2	4.2	1.1
Sil1	-	-	-	-	-	-
Jem1	-	-	-	-	-	-
Sec63	LCor10730	LRAMOSA01203	jgi.p|Lichy1|178115	1.3	2.0	1.1
Tom70	LCor01231	LRAMOSA06859	jgi.p|Lichy1|237386	5.1	4.4	3.9
Ire1	LCor06590	LRAMOSA01609	jgi.p|Lichy1|164429	1.0	1.0	1.1

Tables S14 and S15 are too large for print but can be found on the CD attached to this thesis.

5 Unpublished data

5.1 The sex locus of *Lichtheimia* species

The sex locus of mucoralean fungi was first identified first *Phycomyces blakesleeanus* in 2008 (Idnurm et al, 2008). As described in the introduction, the allelic version of a homeodomain transcription factor, called SexM or SexP, defines the (-) and (+) mating type, respectively. The transcription factor is flanked by a putative triose-phosphate transporter and a RNA helicase. This structure of the sex locus was also found in other mucoralean fungi (Gryganskyi et al, 2010; Li et al, 2011). It is known that the morphology and virulence of *M. circinelloides* differs between the mating types (Li et al, 2011). However, it has never been tested if this is also true for other mucoralean species.

In order to determine the mating types of clinical *Lichtheimia* strains, two strains with known mating types of each species were used as tester strains. Mating conditions were optimized using the *L. corymbifera* strains CBS 429.75 (FSU 9682) and CBS 100.31, which represent the (-) and (+) mating type, respectively (Alastruey-Izquierdo et al, 2010b). The strains were cultured on modified SUP agar (55 mM glucose, 30 mM potassium dihydrogen phosphate, 20 mM ammonium chloride, 5 mM di-potassium hydrogen phosphate, 1 mM magnesium sulphate and 0.5 % yeast extract; Wöstemeyer, 1985) at 37°C for 7 days. Small pieces of mycelium of both strains were placed on one corner of a plate containing SUP medium, 10 % malt extract agar or sabouraud agar (110 mM glucose, 1 % peptone) at 37°C or 42°C and were incubated for three days in the dark. Zygospores could only be observed on SUP medium at 37°C. Based on these conditions a total of 21 strains from different origins were tested (Table 5.1). Mating types could be identified for all strains except one based on zygospore formation with one of the tester strains (Table 5.1). Both mating types were equally represented in the 12 clinical isolates tested (Table 5.1, Figure 5.1 A). In contrast, the 8 tested veterinarian isolates were mainly (+) mating types (Figure 5.2 A).

Virulence of almost 50 strains of *Lichtheimia* species was determined in a previous study in an embryonated chicken egg model (Schwartze et al, 2012). In contrast to the results in *M. circinelloides* (Li et al, 2011), comparison of the virulence of the strains based on their mating type

revealed no obvious differences between the mating types (Figure 5.1 B). Mating of *L. ramosa* could not be observed under any condition tested.

Table 5.1: Strains used for mating experiments. Mating types are indicated as (+) and (-) for successful matings or (?) if no zygospores could be observed. Mating types of tester strains ([1]) were taken from Alastruey-Izquierdo et al, 2010b. Strains are deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS); the strain collection of the Institute for Bacteriology and Mycology, Faculty of Veterinary Medicine at the University of Leipzig (IBML), the Mold Collection of the Spanish National Center for Microbiology, Instituto de Salud Carlos III, Spain (CNM-CM) and the Jena Microbial Resource Collection (FSU).

Strain	Equivalent strain designation	Substrate/origin	Mating type
FSU 9682	CBS 429.75	Soil	- [1]
FSU 10164	CBS 519.71	environmental, kurone	+
FSU 10178	IBML 4 - Probe M 10012	cattle, gut	+
FSU 10179	IBML 5 - Probe M 10005	horse, gut	+
FSU 10180	IBML 6 - Probe D 10005	horse, gut	+
FSU 10563	623	stork	+
FSU 10564	829	stork	-
FSU 10565	612B	stork	+
FSU 10567	909B	stork	+
FSU 938	CBS 100.31	bovine foetus	+[1]
FSU 10058	CBS 101040	human, keratomycosis	+
FSU 10061	CBS 109940	human, finger tissue	+
FSU 10073	CBS 120580	human, lung	+
FSU 10074	CBS 120581	human, bronchia	+
FSU 10239	CNM-CM 3346	human, sputum	-
FSU 10240	CNM-CM 3415	human, ear swab	+
FSU 10244	CNM-CM 4738	Human, bronchoalveolar lavage	?
FSU 10247	CNM-CM 5039	human, peritoneal drainage	-
FSU 10252	CNM-CM 5538	human, sputum	-
FSU 10253	CNM-CM 5637	human, skin	-
FSU 10255	CNM-CM 5738	human, abscess	+
FSU 10257	CNM-CM 5861	human, cutaneous wound	-
FSU 6250	-	human, scale	-

Since the genomes of *Lichtheimia* species are available, they were searched for the presence of the transcription factors SexM or SexP. In the *L. corymbifera* genome (CBS 429.75) a homologue of SexM was found based on bidirectional BLAST and the phylome of this strain. Interestingly, the gene was not flanked by a helicase or a putative triose-phosphate transporter (TPP) as described in other mucoralean genomes (Figure 5.1. C). In order to exclude that the structure represents an artefact resulting from genome assembly or gene prediction, the sex locus was searched in an unpublished genome assembly of the (+) mating type strain *L. corymbifera* CBS 519.71. Preliminary *de novo* gene prediction of the strain was performed using the web interface of AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/submission.php) with the *Rhizopus arrhizus* settings (Stanke et al, 2008). The transcription factors SexM and SexP from *Phycomyces blakesleeanus* (ABX27909.1; ABX27912.1) were searched against the assembly and the predicted genes of *L. corymbifera* CBS 519.71. A homologue of SexP was found on contig 74 of the assembly via tBLASTn and for a predicted gene on the same contig in a BLASTp search. The protein sequence of the gene showed a similarity of 27.9 % and an e-value of 6e-31. BLASTp search against the Genbank sequences revealed SexP as the best hit, indicating that the gene really represents the orthologue of the sex locus gene. Analyses of the surrounding region in this strain also showed the lack of the helicase and the triose-phosphate transporter but a high similarity to the (-) mating type strain of *L. corymbifera* CBS 429.75 (FSU 9682) (Figure 5.1 C). However, the mating locus seems to be functional as both strains produce zygospores when they are cultivated together (Figure 5.1 D).

To investigate whether this structure is conserved also in other *Lichtheimia* species, the sex locus of *L. corymbifera* including the flanking genes as well as SexM and SexP of *P. blakesleeanus* were searched against the proteomes of *L. ramosa* FSU 6197 (Linde et al, 2014) and *L. hyalospora* CBS 173.67 (FSU 10163) (JGI; Schwartze et al, 2016). While all parts of the *L. corymbifera* sex locus could be identified in *L. hyalospora* (Figure 5.2), the transcription factor was missing in the proteome of *L. ramosa*. The region between the flanking genes (LRAMOSA8467 and LRAMOSA8468) was re-annotated using the web interface of AUGUSTUS with the *R. arrhizus* settings (Stanke et al, 2008). Based on this annotation the SexM transcription factor could also be identified in *L. ramosa* (Figure 5.2).

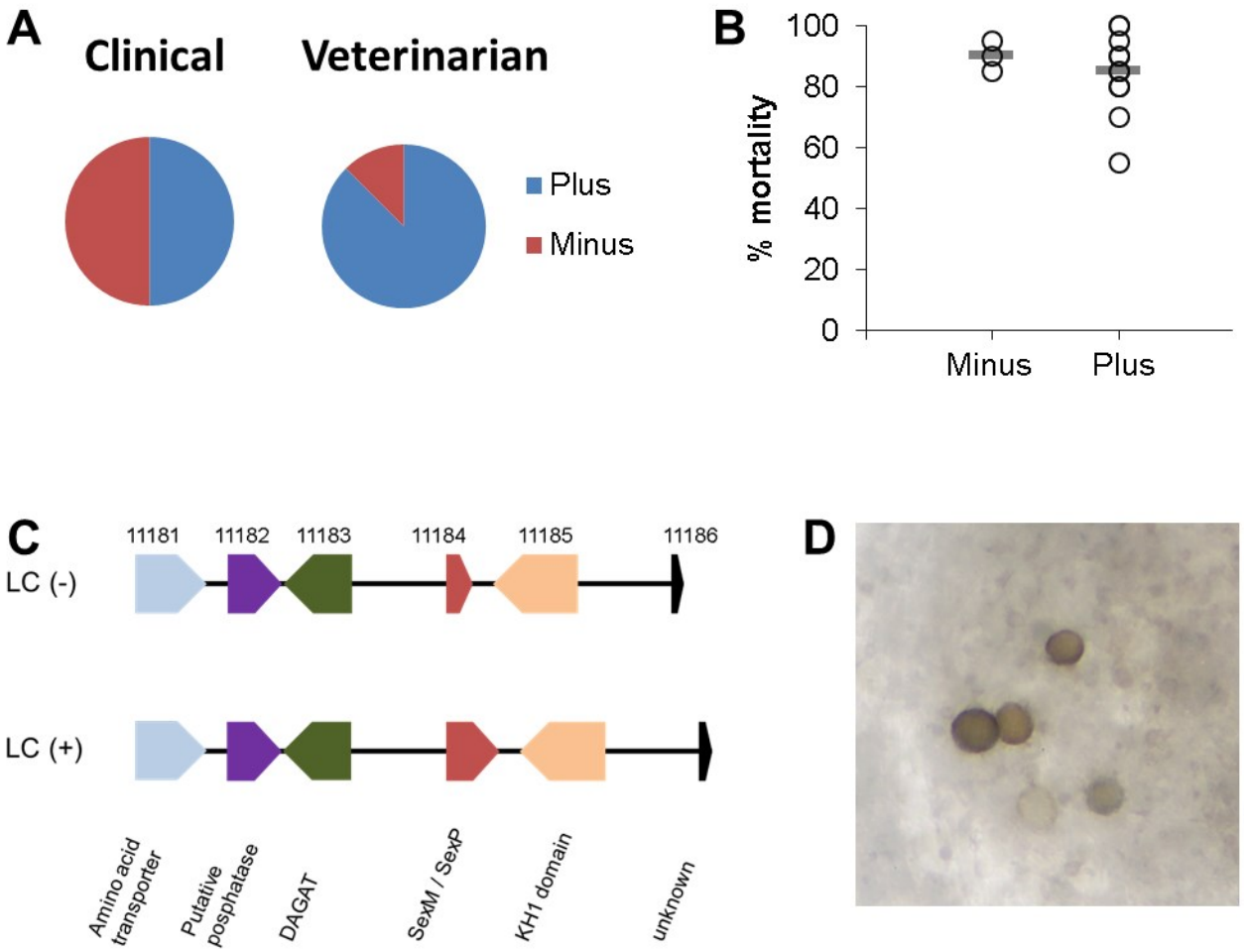


Figure 5.1: Distribution and organisation of the sex locus in *L. corymbifera*. (A) Distribution of (+) and (-) mating types in clinical and veterinarian isolates. (B) Mortality rates in the embryonated chicken egg model after 6 days of infection with different (+) and (-) mating type strains. Virulence data were taken from Schwartze et al (2012). (C) Organisation of the locus around SexM and SexP in the *L. corymbifera* strains CBS 429.75 (FSU 9682) (-) and CBS 519.71 (+). Numbers on top represent the protein IDs and descriptions of the genes based the Pfam domains are indicated at the bottom. (D) Zygospores formed by mating of *L. corymbifera* CBS 429.75 (FSU 9682) and CBS 519.71 after 3 days on SUP agar at 37°C.

In addition to the SexM and SexP genes, also the two other genes of the sex locus of other mucoralean fungi were searched in the *Lichtheimia* genomes based on the phylome data of the three species (see Schwartze et al, 2016). Orthologues of the helicase and the triose-phosphate transporter (TPP) could be identified in all three *Lichtheimia* species but were not located in proximity of the SexM / SexP gene (Figure 5.2).

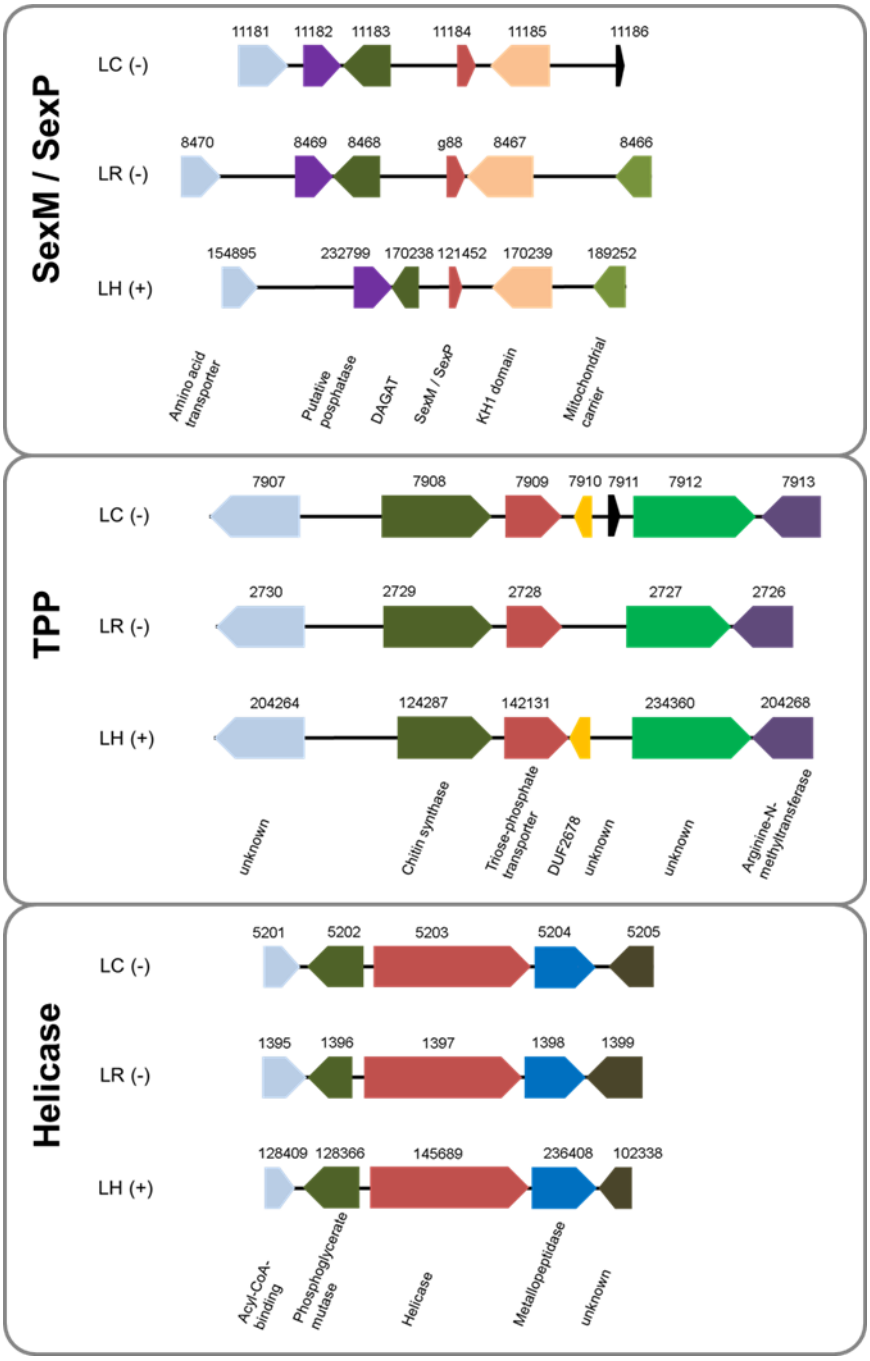


Figure 5.2: Organisation of sex locus genes in *Lichtheimia* species. Orthologues of the three genes located in the sex locus of other mucoralean fungi were identified based on the phylomes of the three species. Numbers indicate protein IDs and descriptions for all genes are indicated at the bottom based on Pfam domains or BLAST.

5.2 Extracellular matrix binding and identification of putative adhesins

Adhesion to host cells is the first step of the infection process and is required for subsequent tissue invasion and damage by pathogens (Tronchin et al, 2008; de Groot et al, 2013; Mayer et al, 2013). Pathogenic fungi are able to adhere directly to host cells but also to extracellular matrix (ECM) proteins like laminin and collagens (Singh et al, 2012; de Groot et al. 2013).

Spores of *R. arrhizus* were shown to be able to bind to laminin and collagen but not other ECM proteins like fibronectin (Bouchara et al, 1996). Adhesion to ECM proteins was strongly reduced after germination (Ibrahim et al, 2005a). In contrast, spores and germlings were able to adhere to endothelial cells. Little is known about the adhesion of other mucoralean pathogens and the molecular mechanisms involved in the process.

To investigate whether *Lichtheimia* species show similar adhesion patterns, binding to ECM proteins was tested *in vitro*. The tests were performed in 96 well plates using a modified protocol of the method of Bouchara et al (1996). ECM and control proteins (BSA, Carl Roth; Collagen C9791, Fibronectin F1141 and Laminin L2020; Sigma Aldrich) solutions with a final concentration of 50 µg/ml were prepared in PBS buffer, filter sterilised (0.2 µM pore size) and 100 µl of the solutions were added to the wells. Control wells were filled with PBS without proteins. The plates were incubated for 1 h at 37°C and afterwards overnight at 4°C. The protein solutions were removed and the wells were washed three times with 200 µl PBS. Afterwards the wells were blocked with 100 µl of 1 % (w/v) BSA in PBS for 1h at 37°C and the wells were washed three times with 200 µl PBS.

Spores of *L. corymbifera* (FSU 9682) were washed of fully grown plates (5-7 days at 37°C on SUP agar; Wöstemeyer, 1985) with PBS. The spore suspensions were washed three times with PBS, counted in a Thoma chamber and diluted to 10^7 spores per millilitre with PBS. For each protein three wells were filled with 100 µl of the spore suspension and the plates were incubated for 1 h at 37°C. To wash off unbound spores, the wells were washed six times with PBS + 0.05 % Tween 20. The samples were fixed with 4 % formaldehyde in PBS overnight at 4°C. At the next day all wells were washed twice with 200 µl PBS. Pictures of five view fields of each well were taken using the Zeiss Axiovert 40 inverse microscope (200 x magnification) and the Moticam 5MP. Pictures were

analysed using ImageJ (Schneider et al, 2012). The results of the experiment revealed a higher binding of *L. corymbifera* spores to wells which were coated with ECM proteins compared to the control wells (Figure 5.3). In contrast to the results using *R. arrhizus* spores, *L. corymbifera* was also able to bind to fibronectin. First experiments with *L. corymbifera* germlings indicate a strong reduction in the binding to ECM proteins after germination, similar to the situation in *R. arrhizus*.

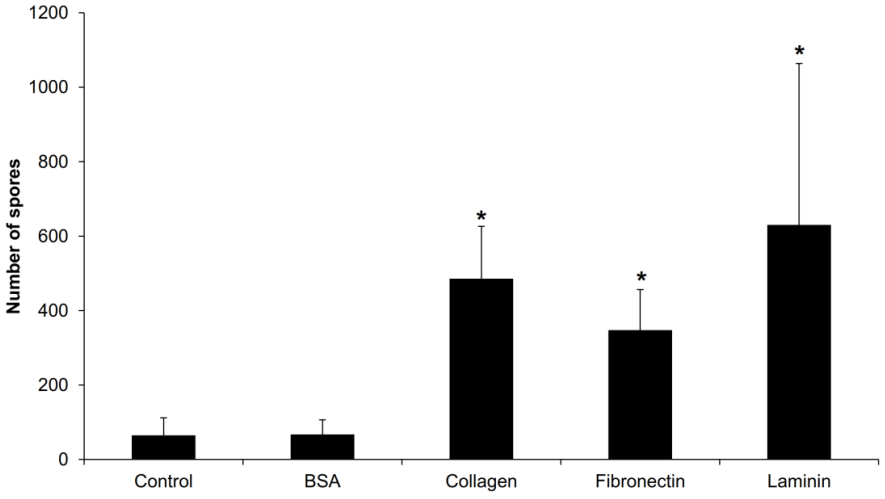


Figure 5.3: Binding of *L. corymbifera* spores to ECM proteins. The total number of spores in the five view fields is shown. Bars represent average values of three independent experiments with three technical replicates each. Error bars show the standard deviation between the replicates. Significant differences to the control, according to two-sided T-test ($P < 0.05$), are indicated with an asterisk.

Since *L. corymbifera* shows the ability to adhere to host proteins, the genome was used to identify proteins, which may play a role in the adhesion process. Glycosylphosphatidylinositol (GPI)-anchored proteins are cell wall proteins, which are mainly bound to β-glucan and include many fungal adhesins known to date (Tronchin et al, 2008; de Groot et al, 2013). The GPI anchors are synthesised and attached to the C-terminus of the protein in the ER.

To identify proteins which may play a role in the adhesion of *L. corymbifera* spores, the proteome was scanned for the presence of (GPI)-anchored proteins *in silico* using PredGPI (http://gpcr2.biocomp.unibo.it/gpipe/index.htm) (Pierleoni et al, 2008). To confirm the location of the putatively GPI-anchored proteins they were scanned for the presence of signal peptides by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/; default settings), Phobius (http://phobius.sbc.su.se/)

and TargetP 1.1 via the Secretool pipeline (cut-off: 0.8) (Petersen et al, 2011; Käll et al, 2007; Cortázar et al, 2014). A total of 120 putative GPI-anchored proteins were predicted to be secreted by at least one of the tools. Only 62 of the proteins contain any functional domains (Figure 5.4 A). The majority of these proteins are most likely involved in cell wall synthesis of the fungus such as chitin deacetylases (Table 5.2).

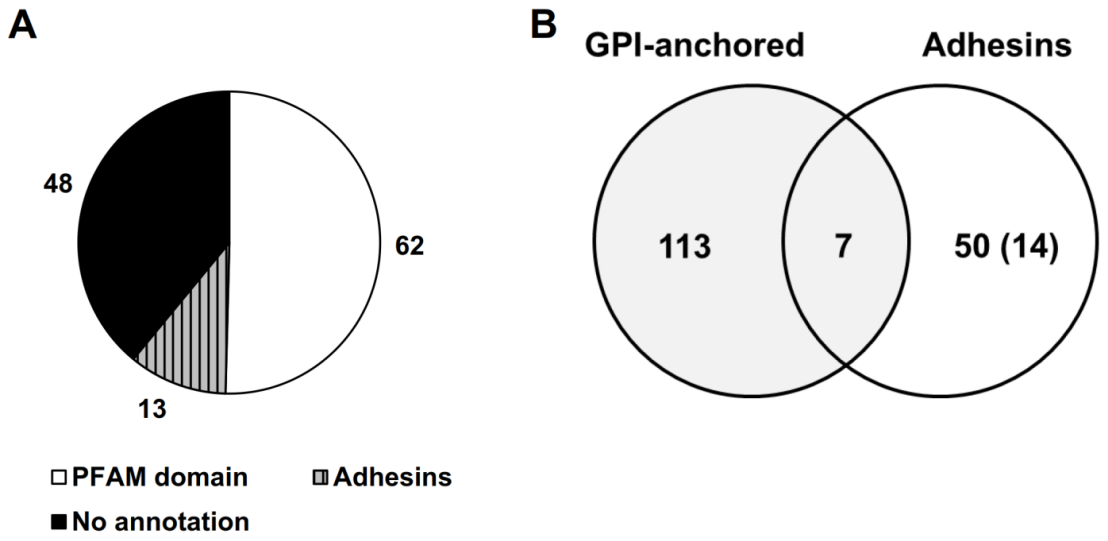


Figure 5.4: Putative GPI-anchored proteins and adhesins of *L. corymbifera*. (A) Level of functional annotation of putative GPI-anchored proteins. (B) VENN diagram of GPI-anchored proteins and all adhesins predicted in the *L. corymbifera* genome via FungalRV (Chaudhuri et al, 2011). The number of predicted adhesins with signal peptides is indicated in brackets.

In addition, two genes with similarities to the fasciclin family (PF02469) were found, which is known to play a role in the adhesion of many organisms. A study on fasciclin-like proteins in the rice blast fungus, *Magnaporthe oryzae*, revealed that the deletion of the corresponding genes resulted in reduced conidia formation, adhesion and pathogenicity of the fungus (Liu et al, 2009).

Six proteins belong to the CotH family (PF08757) of mucoralean fungi, which has been shown to play a role in the invasion of *R. arrhizus* into epithelial cells (Gebremariam et al, 2014). Due to the lack of functional annotation for many of the proteins or the poor characterisation of the domains found, putative adhesins were also identified based on *in silico* predictions using FAApred

(http://bioinfo.icgeb.res.in/faap/) and FungalRV (http://fungalrv.igib.res.in/query.php) using the recommended thresholds of 0.5 and 0.511, respectively (Ramana and Gupta, 2010; Chaudhuri et al, 2011). Based on the predictions, three genes with an assigned functional domain were predicted to be adhesins (Table 5.2) including one protease and two proteins containing a serine/threonine rich domain of GPI-anchored protein (PF10342), which has been described in other fungal adhesins like *C. albicans* ALS family proteins (Trochin et al, 2008; de Groot et al, 2013). In addition, eight GPI-anchored proteins without any functional annotation were predicted to be adhesins of *L. corymbifera* (Table 5.2).

Since not all cell wall located proteins are linked via a GPI anchor, the whole proteome of *L. corymbifera* was searched for the presence of putative adhesins via FungalRV. A total of 57 proteins were predicted to be adhesins and 14 of them contained signal peptides supporting an extracellular location (Figure 5.4 B). Only 8 of the proteins contained any functional domains based on PFAM predictions and only the serine/threonine rich domain of GPI-anchored protein (PF10342) was found in the secreted proteins. The complete identifier lists and annotations can be found in a complete table on the CD attached to this thesis.

Table 5.2: Functional annotation of GPI-anchored proteins of *L. corymbifera*. GPI-anchors were predicted by PredGPI (>99 %). Putative adhesins were predicted by FAApred and FungalRV using a threshold of 0.5 and 0.511, respectively. Results of adhesins predictions are indicated with "Y" (positive) and "N" (negative). For all proteins secretion was predicted by at least one of the prediction tools (SignalP, Phobius, TargetP). Only sequences with functional annotation or predicted adhesion function are shown.

Gene ID	FAApred	FungalRV	PFAM ID	PFAM description
LCor02157	N	N	PF00026	Asp
LCor02158	N	N	PF00026	Asp
LCor02509	Y	N	PF00026	Asp
LCor08268	N	N	PF00026	Asp
LCor05604	N	N	PF00082	Peptidase_S8
LCor07832	N	N	PF00089	Trypsin
LCor06503	N	N	PF00498	FHA
LCor03231	N	N	PF00704	Glyco_hydro_18
LCor00937	N	N	PF00722	Glyco_hydro_16
LCor09252	N	N	PF00722	Glyco_hydro_16
LCor09342	N	N	PF00722	Glyco_hydro_16
LCor10839	N	N	PF00722	Glyco_hydro_16
LCor00433	N	N	PF00759	Glyco_hydro_9
LCor03198	N	N	PF01161	PBP
LCor10663	N	N	PF01266	DAO
LCor00417	N	N	PF01522	Polysacc_deac_1
LCor00550	N	N	PF01522	Polysacc_deac_1
LCor02064	N	N	PF01522	Polysacc_deac_1
LCor03136	N	N	PF01522	Polysacc_deac_1
LCor03341	N	N	PF01522	Polysacc_deac_1
LCor03404	N	N	PF01522	Polysacc_deac_1
LCor03932	N	N	PF01522	Polysacc_deac_1
LCor04079	N	N	PF01522	Polysacc_deac_1
LCor06208	N	N	PF01522	Polysacc_deac_1
LCor06264	N	N	PF01522	Polysacc_deac_1
LCor06312	N	N	PF01522	Polysacc_deac_1
LCor07600	N	N	PF01522	Polysacc_deac_1
LCor09590	N	N	PF01522	Polysacc_deac_1
LCor09984	N	N	PF01522	Polysacc_deac_1
LCor10833	N	N	PF01522	Polysacc_deac_1
LCor11766	N	N	PF01522	Polysacc_deac_1
LCor03979	N	N	PF02469	Fasciclin
LCor04548	N	N	PF02469	Fasciclin
LCor10590	N	N	PF02535	Zip
LCor05248	N	N	PF03198	Glyco_hydro_72
LCor07549	N	N	PF03330	DPBB_1
LCor10090	N	N	PF03601	Cons_hypoth698
LCor02146	N	N	PF05426	Alginate_lyase

LCor02952	N	N	PF07987	DUF1775
LCor02436	N	N	PF08757	CotH
LCor04095	N	N	PF08757	CotH
LCor08001	N	N	PF08757	CotH
LCor12343	N	N	PF08757	CotH
LCor12344	N	N	PF08757	CotH
LCor05507	N	N	PF08757	CotH
LCor00529	N	N	PF10342	GPI-anchored
LCor00772	N	N	PF10342	GPI-anchored
LCor01214	N	N	PF10342	GPI-anchored
LCor02247	N	N	PF10342	GPI-anchored
LCor02549	N	N	PF10342	GPI-anchored
LCor02893	N	N	PF10342	GPI-anchored
LCor04000	N	N	PF10342	GPI-anchored
LCor06467	N	N	PF10342	GPI-anchored
LCor07306	N	N	PF10342	GPI-anchored
LCor07760	N	Y	PF10342	GPI-anchored
LCor09240	N	Y	PF10342	GPI-anchored
LCor10856	N	N	PF13347	MFS_2
LCor00171	N	N	PF13499	EF-hand_7
LCor10642	N	N	PF13855	LRR_8
LCor10197	N	N	PF13886	DUF4203
LCor06520	N	N		homologue of SPS2/22
LCor12329	N	N		homologue of SPS2/22
LCor00494	N	Y		
LCor00497	Y	N		
LCor06540	N	Y		
LCor06815	Y	N		
LCor08404	Y	Y		
LCor08937	N	Y		
LCor09400	Y	N		
LCor09666	N	Y		

6 Discussion

The fungal order Mucorales represents one of the most basal fungal groups and encompasses 270 described species with a variety of life-styles (Hoffmann et al, 2013). While most species are ubiquitous saprotrophs, several species are able to infect other fungi, plants, animals and also humans. Only 22 of the 270 mucoralean species are described as human pathogens (de Hoog et al, 2010). However, human pathogenic species belong to different families of the Mucorales and diverged from each other several hundred million years ago.

Despite the broad range of species causing infections, the term mucormycosis is used for all infections with mucoralean fungi independent of the species involved. In addition, research is mainly focused on two human pathogenic species, namely *Mucor circinelloides* and *Rhizopus arrhizus* (Ibrahim et al, 2010b; Li et al, 2011; Gebremariam et al, 2014; Lee et al, 2014; Liu et al, 2015), which are closely related and belong to the derived groups of mucoralean fungi (Hoffmann et al, 2013). In contrast, *Lichtheimia* species belong to the most basal groups of mucoralean pathogens and are the second-most common cause of mucormycosis in Europe.

The aim of this work was to establish *Lichtheimia* as a new model for basal mucoralean pathogens, in order to get further insights into genome evolution and pathogenicity factors of mucoralean fungi. Since *Lichtheimia* species differ in their virulence potential, the structure of the genomes and the gene contents were analysed by comparative and functional genomics in order to identify virulence-associated traits, which may help to understand the molecular base of these differences.

Genome analyses of *Lichtheimia* species reveal profound differences but also conserved characteristics of mucoralean genomes.

Rhizopus, *Mucor* and *Lichtheimia* species cause up to 80 % of all mucormycosis cases (Gomes et al, 2011) but only the first two genera have been studied in more detail during the last years. Besides the large phylogenetic distance of these species, also remarkable differences in the physiology of the three genera were found, including temperature optimum and susceptibility towards certain antifungals (de Hoog et al, 2000; Vitale et al, 2012). In addition, yeast-like growth stages have been described in *Mucor* species and may play a role in their pathogenicity (Haidle

and Storck, 1966; Hesseltine and Featherston, 1985; Cooper, 1987; Lee et al, 2013). In contrast, such yeast stages have never been described in *Lichtheimia* species and only few ambiguous observations are reported for *R. arrhizus*.

The results of this study showed that also the genomes of the three most important mucoralean pathogens differ greatly from each other. Less than two third of the gene families found in the *L. corymbifera* genome were also present in at least one other mucoralean species and almost 30 % of the total genes were found to be absent in the genomes of *M. circinelloides* and *R. arrhizus* (Schwartze et al, 2014b). Similar results were obtained in an independent study on the genome structure of *Rhizomucor miehei,* which is closely related to *Lichtheimia* species (Zhou et al, 2014). Moreover, only few and short syntenic regions with similar gene order were detected between these mucoralean genomes. In the case of *L. corymbifera* less than 8 % of the genes were found to be organised in syntenic regions, which were conserved in at least one other mucoralean genome (Schwartze et al, 2014b). *R. miehei* represents one of the most closely related species to *Lichtheimia* and is sometimes also included in the family of *Lichtheimiaceae* (Hoffmann et al, 2013). The divergence of the genera *Lichtheimia* and *Rhizomucor* was estimated approximately 300 million years ago based on protein-coding genes and only a weak synteny between the genomes was found (Schwartze et al, 2016).

It can be concluded that the large evolutionary distance between the genome sequenced mucoralean species leads to a high diversity in the genome structure and the gene content. Therefore, results from genome studies in one species are only poorly transferable to other species, which underlines the need for additional genome projects for mucoralean pathogens. Despite the profound differences in the gene content and the genomic structure, some genomic features were shared between mucoralean genomes. The genome of *R. arrhizus* was published in 2009 and represented the first analysed genome of a mucoralean fungus (Ma et al, 2009). The authors of the study found a high proportion of duplicated genes, which was similar to the situation in *S. cerevisiae*, and could be explained by a recent whole genome duplication (WGD) event in this species. Genome analyses of *L. corymbifera* showed that, although the genome was smaller than the genome of *R. arrhizus* (33.6 Mb vs. 45.3 Mb) and contained a smaller number of genes, the amount of multi-gene families was similar in both genomes (Schwartze et al, 2014b). Similar results

were also obtained in the genome projects of *Mortierella alpina* (Wang et al, 2011) and *Conidiobolus coronatus* (Shelest and Voigt, 2014) indicating that the high proportion of duplicated genes is a general feature of basal fungi. Interestingly, no signs for a recent whole genome duplication event were found in the *L. corymbifera* genome, suggesting that other mechanisms besides WGD contribute to the increased number of duplicated genes (Schwartze et al, 2014b). Reconstruction of the *L. corymbifera* phylome revealed that many gene duplications occurred early in the evolution of mucoralean fungi and may result from an ancient whole genome duplication event followed by selective gene loss in the different lineages (Schwartze et al, 2014b). This hypothesis is supported by the results of a study on the evolution of genes involved in oxidative phosphorylation in fungi (Marcet-Houben et al, 2009). However, the sizes of gene families differ between the mucoralean species and only half of the gene families found in *L. corymbifera* and *R. arrhizus* contained the same amount of genes (Schwartze et al, 2014b). While the high amount of duplicated genes seems to result from at least one ancient whole genome duplication event, additional lineage-specific events of gene loss and duplication shape the genomes of mucoralean fungi.

Gene duplications lead to the expansion of certain gene families in *L. corymbifera*, which belong to different functional groups such as transporters and genes involved in signal transduction (Schwartze et al, 2014b). Many genes of the expanded families are organised in tandems indicating that tandem duplication is a mechanism for the creation of lineage-specific gene copies in the genome of *Lichtheimia*. The patterns of tandem duplication are similar between different *Lichtheimia* species suggesting that they occurred at the base of this genus and were mostly retained in the different species (Schwartze et al, 2016). The retention of the duplications points to the fact, that the additional copies of the genes serve important functions in the cell, which are not known in detail, yet. Tandem duplications are not specific for *Lichtheimia* and occur also in other mucoralean and non-mucoralean genomes with a similar frequency (Shelest and Voigt, 2014).

However, tandem duplications alone could not explain the high amount of duplicated genes and other mechanisms contribute to the accumulation of additional gene copies. Studies in plant genomes have shown similar trends and revealed that whole genome duplication and additional gene duplication mechanisms including tandem duplications shape the genomes of plants and play

different roles in the evolution of various gene families (Cannon et al, 2004; Hanada et al, 2008; Conant et al, 2014). Especially tandem duplications were shown to be involved in the generation of lineage-specific duplications, similar to the situation in *Lichtheimia* species (Hanada et al, 2008; Schwartze et al, 2014b). In the fungal pathogen *C. albicans*, tandem duplications are known to play a role in the evolution of important virulence factors like secreted aspartyl proteases (SAP) and a family of surface proteins (ALS) (Jackson et al, 2009). It is not known if and how gene duplications contribute to the pathogenicity of mucoralean fungi but several gene duplications in the *L. corymbifera* genome involve gene families which are known to play a role during infections including transporters, hydrolytic enzymes and iron uptake genes (Schwartze et al, 2014b). In addition, expansions in certain other gene families may contribute to the stress-resistance of mucoralean pathogens. Resistance toward azole antifungals has been described frequently in mucoralean pathogens and gene expansions in the target gene family cytochrome P450 were found in the *L. corymbifera* genome (Alastruey-Izquierdo et al, 2010a; Vitale et al, 2012; Schwartze et al, 2014b). Additionally, duplications and expansions were found in a variety of transcription factors, which seems to be a common feature of basal lineage fungi (Schwartze et al, 2014b; Shelest and Voigt, 2014). However, further research is needed to investigate the impact of gene duplications on the virulence of mucoralean species.

Besides gene duplication, alternative splicing plays an important role in the diversification of the proteome in higher eukaryotes (Matlin et al, 2005; Sammeth et al, 2008; Roy et al, 2013). Alternative splicing has been described in several fungal species but little is known about the exact role of this mechanism in fungi. A recent study found that there is a tendency towards higher rates of alternative splicing in pathogenic species and several genes involved in the virulence of human and plant pathogenic fungi are alternatively spliced (Grützmann et al, 2014). The results of the study also showed that the rate of alternative splicing differs between but also within fungal phyla. Many genes of mucoralean fungi contain introns and could therefore undergo alternative splicing (Ma et al, 2009; Schwartze et al, 2014b). However, analyses in *L. corymbifera* identified alternative splicing for only 2 % of total genes (Schwartze et al, 2014b). A similar low proportion of alternatively spliced genes was predicted in *R. arrhizus*, the only other mucoralean fungus which has been analysed to date (Grützmann et al, 2014). While the role of alternative splicing in fungi is still

unclear, it has been associated with stress adaptation in several plants (Filichkin et al, 2015). In *L. corymbifera*, only small changes of alternative splicing under stress conditions were detected involving less than 0.2 % of the genes (Schwartze et al, 2014b). Thus, alternative splicing does not seem to play a major role during stress adaptation. In contrast, several transcriptional changes of multi-copy genes were detected under stress conditions and different copies were often regulated independently (Schwartze et al, 2014b). Based on these results, gene duplication but not alternative splicing seems to be a major factor in the generation of functionally distinct proteins in mucoralean fungi. Gene duplications may play a role in the rapid adaptation to new environments and contribute to the virulence of mucoralean pathogens.

Sex and virulence are not generally connected in mucoralean pathogens.

Studies on virulence factors are usually restricted to single species of mucoralean pathogens and it is not known whether these results can be transferred to other species. One remarkable example is the mating-type dependent virulence difference of *M. circinelloides* described by Li et al (2011).

While the association of sex and virulence was also described in other fungal pathogens, it seems to be species-specific. In addition, it is not always clear if the effect really depends on the mating type itself or if other effects might play a role. Clinical cases of *Cryptococcus neoformans* are highly associated with the α-mating type (Mora et al, 2010), which was also found to be more virulent than the a-mating type in *C. neoformans* serotype D (Kwon-Chung et al, 1992). However, the effect was not observed in strains of the serotype A (Nielsen et al, 2003). Data about the association of virulence and mating types in other fungal pathogens is also often conflicting. While some studies could link the Mat1-1 mating type to a higher virulence of *Aspergillus fumigatus* strains, other studies could not find a similar effect (Alvarez-Perez et al, 2010; Cheema and Christians, 2011; Losada et al, 2015). No clear correlation of mating types and virulence was found in *Candida albicans* (Ibrahim et al, 2005b). However, mating of two homozygous diploid strains of *C. albicans* leads to the formation of tetraploid strains, which show reduced virulence compared to the diploid parental strains (Ibrahim et al, 2005b).

To date, the relation of mating type and virulence in mucoralean pathogens has exclusively been studied in *M. circinelloides*. Like *M. circinelloides*, *Lichtheimia* species are heterothallic organisms and require two mating types for sexual reproduction, namely (+) and (-). Mating types were known for only a few strains of *Lichtheimia* species and mating is difficult to induce in this species (Alastruey-Izquierdo et al, 2010b). Using various conditions, successful mating could be observed in 21 of 22 tested strains of *L. corymbifera* in this study (unpublished data, 5.1). However, no mating could be observed for *L. ramosa* under the same conditions indicating species-specific demands for the induction of sexual interaction. The distribution of (+) and (-) mating types among clinical samples was equal and the results of infection experiments showed no obvious differences in virulence between the mating types (unpublished data, 5.1; Schwartze et al, 2012). Thus, the observed differences in virulence between the mating types of *M. circinelloides* do not seem to represent a general association of mating type and virulence in mucoralean fungi. In addition, virulence of the mating types was associated with differences in spore size and the interaction with macrophages, regardless of a functional sex locus (Li et al, 2011). This indicates that other genomic differences are associated with the mating types in *M. circinelloides*, which are unknown, missing or divergent in *L. corymbifera*.

Most interestingly, the structure of the sex locus of *Lichtheimia* species was found to be different from *M. circinelloides* and other mucoralean fungi (unpublished data, 5.1). The classical sex locus consists of a homeodomain transcription factor, called SexM or SexP, which is flanked by a putative triose-phosphate transporter and a helicase (Idnurm et al, 2008; Gryganskyi et al, 2010; Li et al, 2011; Idnurm, 2011; Wetzel et al, 2012; Lee and Heitman, 2014). While all three components were found in *Lichtheimia* genomes, they are not co-localized in a similar cluster (unpublished data, 5.1). However, the sex locus seems to be functional in *Lichtheimia* species since the formation of zygospores was observed in different species (Alastruey-Izquierdo et al, 2010b; unpublished data, 5.1). The function of the different genes in the sex locus is still unclear and only one study could show that at least the transcription factor is activated during sexual interaction (Wetzel et al, 2012).

All mucoralean sex loci were analysed in species, which are distantly-related to *Lichtheimia* species and it is unclear whether the structure in *Lichtheimia* species is specific for this lineage or represents an ancient structure of the locus.

The combination of comparative and functional genomics approaches gives first insights into putative virulence factors of *Lichtheimia* species.

Only a few virulence factors of mucoralean fungi have been described to date. These studies are almost exclusively restricted to *Rhizopus* and *Mucor* species. The use of comparative genomics helps to identify orthologues of virulence factors in new species. In addition, it enables the detection of copy number variations of known genes but also the identification of new, specific virulence factors, based on similarity.

Many virulence factors of pathogenic organisms have been described and several of them are conserved among different pathogens. Databases containing information about genes involved in the pathogenesis of different species have been created and are a valuable resource for the identification of putative virulence factors in newly sequenced genomes. The search for homologues of known virulence factors in *Lichtheimia* species revealed more than 2,000 putative target genes (Schwartze et al, 2016). However, many genes represented conserved gene families involved in basal cell function or were only weakly similar to the reference genes. While the method helps to identify putative targets, it lacks specificity in basal fungi due to large phylogenetic distance to other fungi. Despite the low number of known virulence factors of mucoralean fungi comparative analyses of more closely-related species results in more specific target genes. One of the most common features of mucoralean pathogens is the rapid invasion of blood vessels where they cause massive thrombosis (Sugar, 1992; Schoen et al, 2002; Spreer et al, 2006; Mantadakis and Samonis, 2009). The mechanism behind the thrombosis formation is still unclear. However, a study on *R. microsporus* identified a secreted aspartyl protease, which was expressed during infection and was able to activate components of the blood coagulation cascade indicating that proteases of mucoralean pathogens play a role in thrombus formation (Schoen et al, 2002, Rüchel et al, 2004). Proteases are also well-known virulence factors in other fungal pathogens and are involved in the acquisition of nutrients but also in adhesion, invasion, cell damage and the protection from the immune system (Schaller et al, 2005; Behnsen et al, 2010). The genome of *R. arrhizus* contained different gene expansions involving secreted proteases compared to other fungal genomes (Ma et al, 2009). Interestingly, the proportion of proteases was similar in *L. corymbifera* (Schwartze et al, 2014b; Schwartze et al, 2016) and *M. circinelloides* (unpublished data based on PRJNA172437).

Thus, a high content of proteases seems to be a common feature of mucoralean genomes indicating that proteases are not specifically acquired in pathogenic species but belong to the general genetic make-up of these fungi and probably play a role in the acquisition of nutrients in the environment. Due to the high copy numbers of proteases and the lack of information on the function of specific copies in closely-related species, it is complicated to identify genes which are directly involved in the virulence process. While a homologue of the fibrinogen-cleaving protease of *R. microsporus* is also present in *L. corymbifera*, further research is necessary to understand the function of the enzyme in *L. corymbifera* since both fungi are divergent and a high number of paralogues was found.

Fungal cell wall proteins play an essential role during adherence and invasion of host cells by pathogens (Tronchin et al, 2008; de Groot et al, 2013). To date, only little is known about the surface proteins of mucoralean fungi and their involvement in the pathogenicity. The only identified molecular factors are two invasins of *R. arrhizus*, which contain a CotH domain and play a role in the invasion of endothelial cells (Liu et al, 2010; Gebremariam et al, 2014). Similar to the situation in proteases, the cell wall protein CotH was present in up to 10 copies in the genomes of *Lichtheimia* species (Schwartze et al, 2014b; Schwartze et al, 2016). Orthologues of the described CotH-coding genes of *R. arrhizus* were found but also *Lichtheimia*-specific paralogues. The different copies of CotH in *R. arrhizus* were shown to differ in their function (Gebremariam et al, 2014) and structural data are insufficient to understand the specific functions of these genes in *Lichtheimia* species. The combination of functional genomics with proteomics approaches can help to get further insights into the function of the different gene copies in order to identify single genes, which may play a role during infection. One possible approach is based on the characterisation of strains with defects in virulence, stress tolerance or the interaction with immune cells, which can be used to study the underlying mechanisms (Park et al, manuscript in preparation; Schwartze et al, 2012). In an ongoing project, two *L. corymbifera* strains were selected, which are differently phagocytosed by macrophage cell lines. Since surface proteins may play a role during this interaction, the surface proteomes of spores were analysed (Park et al, manuscript in preparation). The genome of *L. corymbifera* was used as a resource for the identification of the proteins and gave insight into their putative function. Several differences in the spore cell wall proteins between

the strains could be identified including two copies of CotH, which were paralogues of the ones found in *R. arrhizus* (Park et al, manuscript in preparation; Gebremariam et al, 2014). Only little is known about the exact function of the different CotH copies in *R. arrhizus* and only two of them are known to be involved in the invasion of endothelial cells (Gebremariam et al, 2014). However, other copies might play a role during the interaction with other host cells such as macrophages. Proteomic approaches are useful for primary screenings to identify virulence factors but they are limited by the availability of strains with defects in the pathway of interest and by the limitations in the identification of subtle changes in protein structure or abundance.

Besides the identification of known virulence factors, the genomic data were used to predict additional cell wall proteins, which may be involved in the infection process. Many cell wall proteins are linked to the cell wall via GPI anchors and more than 100 putative GPI-anchored proteins could be identified in *L. corymbifera* (unpublished data 5.2). The best characterised proteins are proteases and enzymes involved in cell wall synthesis. Besides the described proteins of the CotH family, two fascilin-like proteins were predicted to be cell wall located. In *Magnaporthe oryzae*, fascilin-like proteins were shown to be involved in the adhesion and pathogenicity of the rice blast fungus (Liu et al, 2009). Despite the lack of functional domains, additional proteins were predicted to be adhesins and are interesting targets for further research (unpublished data 5.2). While the identified target proteins represent a valuable resource for future studies, stable genetic transformation of mucoralean fungi is still a difficult task and no transformation system has been described for *Lichtheimia* species (Skory, 2002; Ibrahim et al, 2010b; Li et al, 2011; Gebremariam et al, 2014). However, the function of surface proteins can also be studied by heterologous expression of the target proteins on the cell surface of *S. cerevisiae* (Pepper et al, 2008; Nobbs et al, 2010; de Groot et al, 2013; Kutty et al, 2013) and the approach was successfully used to determine the role of CotH in the invasion of endothelial cells by *R. arrhizus* (Gebremariam et al, 2014).

Besides the analysis of the presence and structure of the genes, gene expression studies under virulence-associated conditions can give insights into genes, which are involved in the adaptation to the host. Such approaches have been used to identify novel factors contributing to the pathogenicity and stress-adaptation in several fungal pathogens (Albrecht et al, 2010; Burmester et

al, 2011; Park et al, 2013; Barker et al, 2012; Krishnan et al, 2014; Kaloriti et al, 2014; Hillmann et al, 2015). In this study, differentially regulated genes were identified by RNA sequencing approaches under infection-associated conditions including heat stress, endoplasmatic reticulum stress, hypoxia and iron starvation (Schwartze et al, 2014b, Schwartze et al, 2016). Several conserved but also novel genes, involved in the adaptation to stresses, could be identified in *Lichtheimia* species and the results will be discussed in the following sections.

Lichtheimia species possess a variety of iron-uptake genes, which enable them to grow on a wide spectrum of iron sources.

Iron is an essential element for all living cells. However, iron is limited in the host and effective iron uptake systems are essential for fungal pathogens (Howard, 1999; Johnson, 2008; Sutak et al, 2008; Abad et al, 2010). Elevated serum iron levels have been associated with the development of mucormycosis, underlining the importance of iron and iron uptake in mucoralean fungi (Sugar, 1992; Ibrahim, 2014). Physiological experiments revealed that *Lichtheimia* species are able to grow on a variety of iron-sources, including the host-iron proteins haemoglobin and ferritin (Schwartze et al, 2016). Despite the important role of iron uptake in mucoralean pathogens, only little is known about the mechanisms involved in the process.

Fungi can take up iron by different pathways including the reductive pathway, the production and uptake of siderophores and the uptake of iron from organic iron-containing molecules (Howard, 1999; Ibrahim et al, 2008a). The high-affinity iron permease Ftr1 is involved in reductive iron uptake and was described as a main virulence factor in *R. arrhizus* and other fungal pathogens (Ramanan and Wang, 2000; Lian et al, 2005; Ibrahim et al, 2010b). Interestingly, gene duplications for this important gene were detected in the genomes of *Lichtheimia* species. While FTR1 seems to be present as a single copy in *R. arrhizus*, four FTR1 genes were found in the genome of *L. corymbifera* (Schwartze et al, 2014b). Three of the four genes were found next to a gene encoding a multicopper oxidase, which has been described to form a functional complex with Ftr1 in *C. albicans* (Ziegler et al, 2011). Transcriptome analyses revealed that only two of the copies were expressed on high levels, while the other two were almost silent (Schwartze et al, 2014b). In

all cases the corresponding multicopper oxidase was expressed on a similar level to FTR1, supporting the hypothesis that both also form a functional complex in *Lichtheimia* species. In addition, the two highly expressed copies were regulated in a different manner. While one copy was up-regulated under iron-limited conditions, the other was down-regulated indicating that both copies serve different functions in *L. corymbifera* (Schwartze et al, 2014b). Similar results were obtained for two copies of iron permeases in *C. albicans.* Ftr1 was strongly induced under iron-limited conditions and was required for growth under iron deficiency (Ramanan and Wang, 2000). In contrast, Ftr2 showed decreased expression in the absence of iron and played no role in the growth under iron-limitation. The two copies of *Lichtheimia* species may have a similar functional divergence based on the observed expressional changes. In contrast, it is unclear whether the other two FTR1 paralogues still serve a function. Gene duplications were also found in iron reductases, which are a third component of the reductive iron uptake pathway. Three copies were found in the genome of *L. corymbifera* with similar expression levels but only one of them was strongly induced under iron-starvation (Schwartze et al, 2014b). The results show that gene duplication occurs in important virulence factors leading to neo- or subfunctionalisation but also silencing of the new gene copies. Besides the role in the uptake of molecular iron, the reductive pathway has been shown to be involved in the uptake of iron from a variety of sources including haem and the bacterial siderophore deferoxamine (Ibrahim et al, 2010b). However, the exact role of the reductive pathway in the utilisation of the different iron sources in *L. corymbifera* is still unclear.

Recently, additional genes were found to be involved in the utilisation of deferoxamine in *R. arrhizus*, namely FOB1 and FOB2 (Liu et al, 2015). The gene products of both genes serve as a receptor for deferoxamine and iron is subsequently taken up by the reductive pathway (Liu et al, 2015). Only FOB2 was found in *Lichtheimia* species but all tested strains were able to grown on iron-loaded deferoxamine as sole iron source (Liu et al, 2015; Schwartze et al, 2016). Thus, a single functional copy seems to be sufficient for the utilisation of deferoxamine by *Lichtheimia* species. A second mechanism for deferoxamine utilisation has been proposed, which depends on the uptake of deferoxamine into the cell by siderophore transporters (Larcher et al, 2013; Liu et al, 2015). One transporter of *L. corymbifera* showed high similarity to the siderophore transporter mirB of *Aspergillus* species (Schwartze et al, 2014b). The gene showed increased expression levels

under iron starvation conditions, indicating a conserved function of siderophore transporters in the iron metabolism in *L. corymbifera* (Schwartze et al, 2014b). Since mucoralean fungi are unable to produce hydroxamate siderophores, the transporter is most likely used to take up siderophores produced by other fungi or bacteria. Conserved siderophore transporters were also found in *Candida* and *Saccharomyces* species, despite their inability to produce siderophores. In *C. albicans*, the gene was required during the invasion of epithelial cells and during the interaction with macrophages (Heymann et al, 2002; Nevitt et al, 2011). According to the phylome data of *Lichtheimia* species, the transporter and its orthologues in other species follow the species tree topology and it is unlikely that the transporter was acquired by horizontal gene transfer or evolved independently in the different species (Schwartze et al, 2014b). A more likely hypothesis is that the siderophore transporter is a basal feature of terrestrial fungi, independent of their inability to produce high-affinity siderophores. Thus, the siderophore uptake system may have evolved before the ability to produce siderophores in fungi and the utilisation of xeno-siderophores produced by bacteria may be the evolutionary origin of this uptake system. Mucoralean fungi are known to live in close relationship with bacteria and *Rhizopus microsporus* was found to carry *Burkholderia* species as bacterial endosymbionts (Partida-Martinez et al, 2007; Ibrahim et al, 2008b; Torres-Cortéz et al, 2015). Since many bacteria are potent siderophore producers, siderophore uptake may be an efficient way for mucoralean fungi to access iron in the environment. Further investigation is needed to understand the exact role of the siderophore transporters in mucoralean fungi and the evolution of the uptake systems in fungi.

The transcriptome analyses performed during this study revealed additional genes, which may play a role in the iron metabolism of *Lichtheimia* species (Schwartze et al, 2014b). Since invasion of blood vessels is an important feature of mucormycosis, haem and haemoglobin are likely iron sources of mucoralean fungi during infection. *In vitro* assays showed that *Lichtheimia* species are able to grow on haemoglobin as sole iron source (Schwartze et al, 2016). The utilisation of haem in *R. arrhizus* seems to be at least partially dependent on the function of the reductive pathway (Ibrahim et al, 2010b) but also two haem oxygenases could be identified in *Lichtheimia* genomes (Schwartze et al, 2014b, Schwartze et al, 2016). Growth under iron-limited conditions resulted in a change in the expression level of one of the haem oxygenases while the other copy was

constitutively expressed on high levels. This resembles the sub- or neo-functionalisation of the FTR1 genes in *L. corymbifera*. While the exact role of haem oxygenases in mucoralean pathogens is still unclear and requires further investigation, the haem oxygenase of the fungal pathogen *C. albicans* has been extensively studied and was shown to be required for haem utilisation and full virulence (Pendrak et al, 2004; Navarathna and Roberts, 2010). The *in vitro* data of this study show that *Lichtheimia* species can grow also on other host proteins like ferritin but it is unknown, which genes are involved in the utilisation of this iron source (Schwartze et al, 2016). The identification of the mechanisms involved is complicated since fungal pathogens developed different techniques to access iron from host ferritin. *C. albicans* possesses a specific surface receptor and uses the reductive pathway to take up the iron, which is released by ferritin after the acidification of the environment (Almeido et al, 2008). In contrast, *A. fumigatus* has no specific mechanism for the utilisation of ferritin and seems to access ferritin-bound iron by siderophores (Schrettl et al, 2004).

Contrary to iron uptake genes, which are usually well-conserved in their structure, nothing is known about the transcriptional regulators involved in the response towards iron starvation in basal fungi. Only few transcription factors were differentially regulated under iron starvation in *L. corymbifera*. One of them belonged to the GATA type transcription factors, which are known to be involved in iron metabolism in several fungal pathogens and may have similar functions in mucoralean fungi (Haas et al, 1999; Pelletier et al, 2007, Chen and Noble, 2012; Chung et al, 2012; Hwang et al, 2012; Schwartze et al, 2014b).

The comparative analyses in this study did not only help to identify putative virulence factors of mucoralean fungi but also gave additional insights into the evolution of the iron metabolism of fungi. Ferritins are iron-storage proteins, which are conserved in bacteria, plants and animals but are lacking in most fungi (Howard, 1999; Hintze and Theil, 2006). However, ferritins have been described in some mucoralean fungi (Carrano et al, 1996; Ibrahim et al, 2012). The phylome analyses performed in this study revealed that ferritins are present in mucoralean fungi but also in the basal Chytridiomycota and the fungi-related microsporidia (Schwartze et al, 2014b). Derived fungi store iron in the vacuole or use siderophores as intracellular iron storage (Howard, 1999; Haas et al, 2008; Haas, 2014; Silva et al, 2011) and the loss of ferritin coincides with the occurrence of siderophore biosynthesis genes (Schwartze et al, 2014b). This suggests that the

presence of ferritin is a basal feature of fungi, which got lost during evolution when alternative mechanisms took over the iron storage function. The results show that mucoralean fungi differ profoundly from derived fungal pathogens in iron metabolism and further studies are needed to get insights into the genes involved in iron uptake and storage in basal fungi. Understanding the iron metabolism would help to find new therapeutic targets since iron starvation of the fungus increases the survival of the host during mucormycosis (Ibrahim et al, 2007; Ibrahim et al, 2010a).

Analyses of the stress response of *Lichtheimia* species reveal differences compared to derived fungi.

The signal transduction and stress response of fungi has been analysed in detail in several model organisms, which belong mainly to the derived Asco- or Basidiomycota. However, little is known about signal transduction and stress adaptation in basal fungi. Analyses on signal transduction pathways showed a high conservation of these pathways in mucoralean genomes but revealed also specific duplications and losses of genes (Schwartze et al, 2014b). Gene duplications could be identified particularly in transcription factors as well as protein kinases and phosphatases in *L. corymbifera*.

The growth at elevated temperatures is a prerequisite for pathogens to cause infections in warm-blooded animals. High temperatures have severe effects on the membrane fluidity and the cell wall stability of fungi (Verghese et al, 2012; Leach and Cohen, 2013). In addition, heat induces the misfolding of proteins, which leads to a loss of function and aggregation of proteins inside the cell (Richie et al, 2009; Verghese et al, 2012; Leach and Cowen, 2013). Central components of the heat adaptation in pathogenic fungi are the heat shock transcription factor (HSF) and a variety of heat shock proteins (HSPs), which are mainly chaperones and help to stabilise, re-fold or degrade client proteins (Verghese et al, 2012; Leach and Cowen, 2013; Brown et al, 2014). The genomes of *Lichtheimia* species contain 24 copies of the HSF transcription factor, which is the highest number among fungi investigated so far (Schwartze et al, 2014b). However, this seems to be a general feature of basal fungi as other mucoralean fungi showed also a high copy number of this transcription factor. It is not clear if all copies are still functional and which role they play in the

stress adaptation of mucoralean fungi. Transcriptome analyses indicate that only a small number of HSF genes contribute to the adaptation to heat stress conditions, while other copies may play a role under different stress conditions (Schwartze et al, 2016). A similar situation was found for Hsp90, a central chaperone of the stress response in fungi. Like other mucoralean fungi, *Lichtheimia* species contain up to five copies of the HSP90 gene, while only a single copy can be found in fungal pathogens like *C. albicans* or *A. fumigatus*. Since stress adaptation in basal fungi was never analysed before, structural data were insufficient to identify genes and specific gene copies involved in stress signalling. Transcriptome analyses showed that there is a functional heterogeneity within the gene families and different members are activated under different stress conditions (Schwartze et al, 2016). Major changes in the gene expression under heat stress in *Lichtheimia* species were found in different chaperones including genes encoding Hsp20, Hsp60 and Hsp90 (Schwartze et al, 2016). Of the five HSP90 copies found in *Lichtheimia* species, only two closely related paralogues seem to play a major role in the heat adaptation. Both were up-regulated under heat stress and in the presence of an Hsp90 inhibitor in at least two of the species. In contrast, these genes did not play a role in the response to misfolded proteins, where a third copy of HSP90 was highly up-regulated. This copy has orthologues in Metazoa, Microsporidia, basal fungi and Basidiomycota but is missing in Ascomycota (Schwartze et al, 2016). The corresponding orthologues have not been further characterised in any species but may play a conserved role in ER stress adaptation. The results suggest that the different paralogues of heat shock proteins differ in their function (Schwartze et al, 2016).

While the function of Hsp60 and Hsp90 in the heat stress response is well conserved among fungi, the role of small heat shock proteins such as Hsp20 is less clear. Deletion of the HSP21 gene in *C. albicans* reduced the heat resistance of this species, while no such effect could be observed in deletion mutants of *S. cerevisiae* (Mayer et al, 2012). The transcriptional up-regulation of the HSP20 genes suggests a function in heat shock response but verification of the exact function by the deletion of the genes is necessary. Such small heat shock proteins would represent a good drug target since they are only poorly conserved between fungal species and humans, which increases the specificity of the drug and helps to reduce side effects.

The main reaction of all *Lichtheimia* species under heat stress was the activation of pathways involved in protein folding and stabilisation (Schwartze et al, 2016), which is similar to the situation in other fungal pathogens (Albrecht et al, 2010; Brown et al, 2014). An additional mechanism involved in the heat stress response of fungal pathogens is the production of different osmolytes like glycerol and trehalose (Singer and Lindquist, 1998; Perlmutter, 2002; Abad et al, 2010; Rajan et al, 2011; Mayer et al, 2012). The production of glycerol under stress conditions in fungi is mainly activated by the high osmolarity glycerol (HOG) pathway. The central part of this pathway is the stress activated protein kinase Hog1 (Saito and Posas, 2012; Brewster and Gustin, 2014). The HOG pathway was first described to be involved in the resistance towards osmotic stress but was later found to be involved in the response to oxidative and heat stress in fungi (Winkler et al, 2002; Bahn et al, 2005; Brown et al, 2014; Nimmanee et al, 2015). Inhibition of the Hog1 function in *Lichtheimia* species resulted in reduced growth under osmotic stress but did not alter the growth under other stress conditions like oxidative and cell wall stress (Schwartze et al, 2016). In addition, no effect on the growth at elevated temperatures was found. Similarly, Hog1 function played a minor role in the thermotolerance of some fungal species such as *A. nidulans* and *C. neoformans* serotype D (Miskei et al, 2009; Bahn et al, 2005). However, even in these organisms Hog1 was involved in different stress responses, while it seems to be restricted to osmotic stress in *Lichtheimia* species (Schwartze et al, 2016). Further experiments with deletion mutants are necessary to finally elucidate the function of Hog1 in basal fungi but the results indicate only a minor cross-talk of stress response pathways via Hog1 compared to other fungi. Besides the characterised heat shock proteins, a variety of other genes putatively involved in heat adaptation of *Lichtheimia* species could be identified but functional annotation is missing in many cases and the exact function of these genes needs to be determined in future studies.

If prolonged exposure to heat results in the accumulation of misfolded proteins and ER stress, cells activate the unfolded protein response (UPR). The UPR induces processes to reduce the load with misfolded proteins by the reduction of translation and the increased expression of chaperones to stabilise proteins (Gardner et al, 2013). In addition, misfolded proteins can be degraded by the ubiquitin-proteasome system and the UPR has been shown to be important for the growth at elevated temperatures and the virulence of fungal pathogens (Wimalasena et al, 2008; Richie et al,

2009; Askew, 2014; Glazier and Panepinto, 2014; Krishnan and Askew, 2014; Miyazaki and Kohno, 2014). Endoplasmatic reticulum stress in *Lichtheimia* species induced genes which are typical markers for ER stress in other fungi such as the ER chaperones KAR2 and LHS1 (Verghese et al, 2012; Cheong et al, 2014; Schwartze et al, 2016). These chaperones are also involved in the regulation of the transmembrane kinase Ire1, which performs the unconventional splicing of the bZIP transcription factor HAC1, a major regulator of the UPR (Chen et al, 2005; Cheong et al, 2011; Feng et al, 2011; Cheon et al, 2014). Ire1 was present in all *Lichtheimia* species and was significantly up-regulated under ER stress conditions. However, no homologue of Hac1 was found in the *Lichtheimia* genomes, which is similar to the situation in *R. arrhizus* (Hooks and Griffiths-Jones, 2011; Schwartze et al, 2016). *C. neoformans* also lacks a Hac1 homologue but uses the unique transcriptional regulator Hxl1 for UPR signalling (Cheon et al, 2011). The lack of Hac1 and Hxl1 homologues suggests that other transcription factors and additional Ire1 targets are involved in the UPR signalling of basal fungi. The high copy numbers of bZIP transcription factors and the lack of information on the function of the different copies hamper the identification of the corresponding gene in *Lichtheimia* species (Schwartze et al, 2014b; Schwartze et al, 2016). It has been described that the unconventional splicing sites of HAC1, HXL1 and other Ire1 spliced genes are well conserved and can be predicted by *in silico* modelling approaches (Oikawa et al, 2010; Nagashima et al, 2011) but no conserved splicing sites could be found in the bZIP transcription factors of *L. corymbifera*. However, splicing sites can be as small as 20 nucleotides and might possess specific features in basal fungi (Hooks and Griffiths- Jones, 2011). Further studies are needed to investigate the signalling involved in the ER stress response of basal fungi and the data obtained during this study are an important resource for future analyses.

Lichtheimia species diverged recently and are similar in gene content and genome organisation.

Due to the large phylogenetic distance of available genome sequenced mucoralean fungi, comparison of pathogenic and non-pathogenic species could not be used to understand the evolution of pathogenicity (Schwartze et al, 2014b; Zhou et al, 2014). Since *Lichtheimia* species

differ in their virulence potential, the genomes of closely-related clinically relevant *Lichtheimia* species and their sibling species were compared in order to identify factors that contribute to the virulence differences of the species (Schwartze et al, 2016). The three analysed genomes are similar in genome size, the number of protein-coding genes and are highly co-linear. As described previously, mucoralean genomes encompass an unusual high amount of multi-gene families and tandem duplications seem to play a role in the lineage-specific expansion of gene families in mucoralean fungi (Schwartze et al, 2014b). Comparative genomics of *Lichtheimia* species showed that the tandem duplication patterns are highly conserved between the species indicating that the corresponding duplication events took place in a common ancestor of all *Lichtheimia* species and additional copies were mostly retained (Schwartze et al, 2016). The involved gene families encompass transporters, protein kinases and cytochrome p450. In addition, genes which carry a heterokaryon incompatibility protein (HET) domain were highly duplicated in all genomes with 45 to over 70 copies (Schwartze et al, 2014b; Schwartze et al, 2016). This domain is mainly known in ascomycetes, where they control non-self-recognition during heterokaryon formation (Paoletti et al, 2007). In contrast, mucoralean fungi do not form a heterokaryon except for the formation of sexual spores. The HET genes of *Lichtheimia* species are only weakly similar to the ascomycete genes and the function remains to be determined. The genes are specific for *Lichtheimia* species and do not have homologues in the genomes of other basal fungi indicating that they do not play a fundamental role in the sexual interaction of these fungi (Schwartze et al, 2014b; Schwartze et al, 2016).

The high similarity between the species of *Lichtheimia* raises the possibility that they represent evolutionary young species. Based on protein-coding genes, the divergence of the three investigated species occurred only approximately 20 to 30 million years ago (Schwartze et al, 2016). Thus, the split between the *Lichtheimia* species occurred around the same time as the divergence of the variances of *C. neoformans* (Ngamskulrungroj et al, 2009) and later than the divergence of pathogenic and non-pathogenic species of *Aspergillus* or *Candida* (Hedges et al, 2006; Hedges et al, 2015). Proteome comparison revealed a high overlap in the presence of previously described virulence factors of mucoralean pathogens such as iron-uptake genes and hydrolytic enzymes (Schwartze et al, 2014b; Schwartze et al, 2016). Small variations in the copy

numbers of several hydrolytic enzymes were found but due to the lack of information on the specific function of the genes it is not possible to connect the presence of certain copies to the virulence of the species. However, it is known that gene duplications are also involved in the evolution of important virulence factors in other fungal pathogens like *C. albicans* (Jackson et al, 2009). The search for virulence-associated traits and the corresponding genes of other pathogenic organisms in *Lichtheimia* species did not reveal any specific factors in the two clinical species. Based on these results it seems like most factors involved in pathogenicity are present in all *Lichtheimia* species and may just be differentially expressed. Accordingly, clinically not relevant *Lichtheimia* species are attenuated but not avirulent in the embryonated hen egg model and comparable in virulence to clinically relevant species in a wax moth larvae infection model (Schwartze et al, 2012; Schwartze et al, 2014a; Schwartze et al, 2016). However, the high amount of duplications and the lack of functional annotation limit the specificity of these analyses. In addition, putative virulence factors might not be identified if no functional domains are recognised. Despite the high genome similarity, *Lichtheimia* species differ profoundly in morphology but most importantly in their physiology. This may contribute to the higher virulence of the clinically relevant *Lichtheimia* species as discussed in the next section in more detail. The results of this study give first insights into the changes of genome structure between closely-related sibling species and show that small changes in the genetic make-up have a strong impact on the physiology and virulence of mucoralean species.

The lower heat resistance of clinically non-relevant *Lichtheimia* species is linked to the accumulation of misfolded proteins and can be modified by combinatory stresses.

The growth at elevated temperatures is a prerequisite for pathogens to cause infections in warm-blooded animals. Reduced ability to grow at body temperature or above correlates with the reduced virulence of human fungal pathogens (McCormick et al, 2010; Verghese et al, 2012; Leach and Cowen, 2013; Brown et al, 2014). Significant differences in the growth of clinically relevant and clinically non-relevant *Lichtheimia* species were detected at elevated temperatures, which correlated with the virulence of the species (Alastruey-Izquierdo et al, 2010b; Schwartze et al, 2012; Schwartze et al, 2016). Stress experiments showed that the differences are not only based

on the slower growth of the strains but are due to a lower survival of the fungi at high temperatures (Schwartze et al, 2016). In addition, clinically non-relevant *L. hyalospora* was unable to utilise certain nutrients as carbon or nitrogen sources on minimal medium at elevated temperatures. This study focused on the identification of possible reasons for the differences, in order to understand the mechanisms involved in the high thermotolerance of clinically relevant *Lichtheimia* species. As discussed previously, the genome of *L. hyalospora* was similar in structure and gene content compared to clinically relevant *L. corymbifera* and *L. ramosa* (Schwartze et al, 2016). Searches for genes which are known to be involved in heat adaptation of other fungi did not reveal obvious differences indicating that the basal elements of the heat shock response are present in all species. Elevated temperatures affect a variety of cellular structures including the cell membrane, cell wall and proteins (Verghese et al, 2012; Leach and Cowen, 2013). Physiological assays showed no specific synergistic effects between heat and chemical stressors in *L. hyalospora* except for dithiothreitol (Schwartze et al, 2016). This substance is a reducing agent and destroys disulphide bridges in proteins resulting in the misfolding of proteins. Increased accumulation of misfolded proteins is a typical consequence of high temperatures and leads to a loss of function and aggregation of proteins inside the cell (Richie et al, 2009; Verghese et al, 2012; Leach and Cowen, 2013). Other inhibitors blocking the chaperone Hsp90 and the function of the proteasome showed similar effects in *L. hyalospora* supporting the hypothesis that differences in the accumulation or degradation of misfolded proteins are likely explanations for the higher sensitivity of clinically non-relevant species towards heat stress (Schwartze et al, 2016). The accumulation of misfolded proteins is normally recognised in the lumen of the endoplasmatic reticulum and induces the unfolded protein response (UPR) as discussed previously. A lack of proper UPR function is associated with a reduced ability to grow at elevated temperatures and decreased virulence in fungal pathogens (Wimalasena et al, 2008; Richie et al, 2009; Askew, 2014; Glazier and Panepinto, 2014; Krishnan and Askew, 2014; Miyazaki and Kohno, 2014). The proper function of the ER is also important for the ability to secrete proteins which play an important role in cell wall synthesis or the degradation of complex nutrients (Richie et al, 2009; Feng et al, 2011; Guillemette et al, 2014). Since *L. hyalospora* was still able to grow on complex nutrients and in the presence of cell wall stress, it is unlikely that it suffers from a general dysfunction of the ER or the UPR (Schwartze et al, 2016). In addition, comparative transcriptome analyses of *L. hyalospora* and two clinically relevant

Lichtheimia species showed that the response to ER stress appears to be similar between the species (Schwartze et al, 2016). However, besides defects in the degradation of misfolded proteins, a general higher level of protein misfolding could be an alternative explanation for the differences in heat sensitivity. Hsp90 function was essential for the growth of *L. hyalospora* at 42°C but not for the two clinically relevant species. Transcriptome analyses revealed a strong response to Hsp90 inhibitors even at 37°C in *L. hyalospora*, while the other two strains showed almost no transcriptional changes (Schwartze et al, 2016). Based on these results it appears most likely that the clinically non-relevant species suffer from decreased protein stability even at 37°C compared to the clinically relevant species. Previous studies could show that even slight increases in the growth temperature can have massive effects on the stability of the proteome (Ghosh and Dill, 2010) and several factors define the thermostability of proteins including the amino acid composition and secondary structure (Hart et al, 2014; Panja et al, 2015) but also the production of different osmolytes (Singer and Lindquist, 1998; Perlmutter, 2002; Abad et al, 2010; Rajan et al, 2011; Mayer et al, 2012). Osmolytes had positive effects on the growth of *L. hyalospora* in this study supporting the hypothesis that reduced growth was caused by increased misfolding of proteins (Schwartze et al, 2016). Thermoadaptation of eukaryotic proteins have been associated with higher frequencies of arginine, glutamic acid, isoleucine, leucine, tryptophan, tyrosine and valine in the proteins (van Noort et al, 2013). Only small overall differences between the *Lichtheimia* species were found indicating that specific changes of the protein structure do not play a major role in the thermoadaptation of *Lichtheimia* species (Schwartze et al, 2016). However, such global approaches may miss the changes in the thermostability of single genes, which could alter the response to thermal stress such as transcription factors.

In accordance with the strong growth inhibition at elevated temperatures, transcriptome analyses revealed a more pronounced response of *L. hyalospora* at 42°C compared to the two clinical species (Schwartze et al, 2016). The results also showed that regulation and expression levels of major heat stress response genes were conserved among the species. However, minor differences in the expression levels of certain heat shock-related proteins were found in *L. hyalospora*, which may influence the protein stability at elevated temperatures (Schwartze et al, 2016). No specific

transcriptional changes of the two clinically relevant species were found indicating that they are less affected by heat stress rather than possessing specific adaptations in the heat shock response.

Many genes were specifically regulated in *L. hyalospora* and an enrichment of amino acid synthesis genes was found (Schwartze et al, 2016). A possible explanation could be a higher demand for amino acids due to the synthesis of new proteins. However, supplementation of the medium with amino acids did not increase growth but reduced growth further, pointing to a defect in the sensing, uptake or utilisation of amino acids under heat stress conditions. Several links between amino acid metabolism and the response to stresses have been found in eukaryotic cells. Amino acid deprivation was shown to reduce the DNA binding activity of HSF1 in mammalian cells and reduces the expression of important cellular chaperones (Hensen et al, 2012). While no significant reduction of chaperone expression could be found in *L. hyalospora*, other targets of Hsf1 might be affected contributing to the reduced thermotolerance of the species. In *S. cerevisiae*, the transcription factor Gcn4 is a main transcriptional regulator involved in the transcriptional response to amino acid starvation but was also found to play an important role in other stress responses including the unfolded protein response (Patil et al, 2004; Herzog et al, 2013). No orthologue of GCN4 was found in *Lichtheimia* species but other transcription factors may be involved in the cross talk between the stress pathways. The increased expression of genes involved in the amino acid metabolism was not observed under ER stress conditions and the effect seems to be specific for the heat stress response of *L. hyalospora*. Since nothing is known about the stress response pathways in closely-related species or basal fungi in general, specific annotation of gene function is often lacking and further research is necessary to understand the specific role of the genes during stress response. The results of the transcriptomic analyses give first insights into genes which may play a role during stress response and can be used as a working base for detailed analyses on the stress response of basal fungi.

Interestingly, the growth defect of *L. hyalospora* at elevated temperatures could be alleviated by the parallel application of heat and osmotic stress conditions. The effect was not restricted to a higher growth rate on complete media but also restored growth on minimal media with lipids as sole carbon source (Schwartze et al, 2016). A similar phenomenon was described in yeast species, where the prior application of subinhibitory stresses results in increased stress resistance of the

cells (Leach et al, 2012; Brown et al, 2014). In contrast, the effect in *L. hyalospora* depends on the parallel application of both stresses, as subsequent application of osmotic and heat stress did not increase the survival (Schwartze et al, 2016). Defects in the ER-stress response of *A. fumigatus* also resulted in reduced growth at elevated temperatures, which could be restored by the addition of sorbitol or potassium chloride to the medium (Richie et al, 2009). However, the effect in the study resulted from the osmotic stabilisation of the cells since they exhibited defects in cell wall integrity. No defects in the cell wall integrity of *L. hyalospora* were found even under heat stress conditions. In addition, heat tolerance was not reduced in hypo-osmotic buffers supporting the conclusion that osmotic stabilisation does not play a major role (Schwartze et al, 2016). In a recent publication a similar effect could be observed in a deletion mutant of the small heat shock protein Hsp21 in *C. albicans* (Mayer et al, 2012). The deletion mutant showed strongly reduced growth at 42°C, which could be reverted in the presence of osmotic stress. The reduced growth of the mutant was most likely due the reduced production of osmolytes like trehalose (Mayer et al, 2012). Application of osmotic stress activated additional stress response pathways bypassing the lack of Hsp21 (Mayer et al, 2012). Combinatorial stresses were shown to profoundly change the transcriptional response of fungi and even changes in expression of components of the normal stress response pathways were found (Kaloriti et al, 2014). In contrast, transcriptome data of *L. hyalospora* revealed only small changes in the gene expression under combinatorial osmotic and heat stress compared to heat stress alone (Schwartze et al, 2016). Despite the increased thermotolerance of *L. hyalospora* under combinatory stress, no major changes in the expression of chaperones or other heat-induced genes, which help to explain the increased stability of the *L. hyalospora* proteins, could be observed (Schwartze et al, 2016). Several genes which were lower expressed in *L. hyalospora* compared to the two clinically relevant species were increased in expression under combinatory stress. Only few of these genes could be linked to the folding or stabilisation of proteins. Thus, the adaptation to growth at elevated temperatures seems to require only small changes in the gene expression of chaperones and other genes involved in protein folding. Accordingly, all main components of the heat shock response were present and up-regulated under heat stress conditions in *Lichtheimia* species, independent of the ability to grow at elevated temperatures. However, several expressional changes were found in genes which are involved in the uptake or synthesis of osmolytes such as glycerol and proline (Schwartze et al, 2016). These substances are

known to increase the thermostability of proteins in cells (Singer and Lindquist, 1998; Rajan et al, 2011) and may also contribute to the increased thermotolerance of *L. hyalospora*. In addition, genes involved in the lipid metabolism were up-regulated in the presence of combinatory stresses and also growth on lipids as sole carbon source was restored in the presence of sodium chloride (Schwartze et al, 2016). ER stress has been shown to induce the expansion of the ER membrane, which alleviates ER stress in *S. cerevisiae* (Schuck et al, 2009). This process is linked to the lipid metabolism and the increased synthesis of membranes. While heat stress did not induce a general defect of cell membranes in *L. hyalospora*, increased synthesis of lipids may contribute to the function of the ER and help to alleviate the ER stress at elevated temperatures. In addition, the expression of membrane-associated proteins and proteins involved in the ER function and the secretory pathway were mainly affected under combinatory stress (Schwartze et al, 2016). The specific up-regulation of genes involved in amino acid biosynthesis in *L. hyalospora* was not reverted by the presence of osmotic stress suggesting that the fungus still requires high amounts of amino acids. In accordance with this theory, growth was strongly promoted on medium containing amino acids and sodium chloride suggesting that the initial low growth of *L. hyalospora* on amino acids at elevated temperatures was most likely due to defects in the utilisation or uptake (Schwartze et al, 2016).

It is unclear if the observed changes are a response to the presence of osmotic stressors and coincidentally also support the growth at elevated temperatures. Alternatively, the combination of osmotic stress and heat stress activates genes which are usually required for the heat stress adaptation but are not activated under heat stress alone due to defects in the sensing of thermal stress or in the signal transduction.

Pathogenicity evolved multiple times independently in thermotolerant mucoralean fungi and requires conserved and lineage-specific adaptations.

To date more than 20 mucoralean fungi are known to cause infections in humans. These species belong to six families of the Mucorales, which diverged from each other several hundred million years ago (Zhou et al, 2014; Schwartze et al, 2016). Increased thermotolerance in mucoralean

fungi often coincides with the ability to cause infections in humans. Several species show growth up to human body temperature (37°C) but most of them were never observed in clinical cases. In contrast, species which can still grow above 40°C often also play a role in mucormycosis (Fig. 5.1; Walther et al, 2012). While thermotolerance is an important prerequisite to cause infections in warm-blooded animals, many additional factors are involved in the infection process of fungal pathogens such as stress resistance, metabolic flexibility and surface proteins.

A recent study showed that the evolution of higher virulence and human pathogenicity coincides with higher heat stress resistance in *Rhizopus* species (Kaerger et al, 2015). However, mesophilic species showed also reduced virulence in infection experiments performed at temperatures which were supporting the growth of these species. Thus, additional adaptations are required for the pathogenicity of *Rhizopus* spp. and small differences in the utilisation of amino acids were found in the pathogenic species, which may explain their higher virulence potential (Kaerger et al, 2015). All thermotolerant *Rhizopus* species form a monophyletic group and show similar virulence potential despite differences in the frequency in human infections (Kaerger et al, 2015). Based on these results, pathogenicity most likely evolved once in *Rhizopus* species and coincides with higher thermotolerance and additional metabolic adaptations.

Only three *Lichtheimia* species are known to cause infections in humans. While all species can grow at 37°C and above, only species which show well growth above 40°C are clinically relevant (Alastruey-Izquierdo et al, 2010b, Schwartze et al, 2016). In contrast to the situation in *Rhizopus* species, phylogenomic analyses of *Lichtheimia* species suggest that the clinically relevant species do not form a monophyletic group (Schwartze et al, 2016). It is not clear whether higher thermotolerance evolved multiple times in this genus or if *Lichtheimia* species derived from a highly thermotolerant ancestor and thermotolerance got lost multiple times during evolution. However, the results of the transcriptome analyses indicate that subtle changes in expression of some genes may be sufficient to explain the differences in thermotolerance. Physiological screenings did not reveal substantial differences in the metabolic flexibility and stress tolerance of *Lichtheimia* species and the adaptation to higher temperatures may be sufficient to explain the increased virulence (Schwartze et al, 2012; Schwartze et al, 2016).

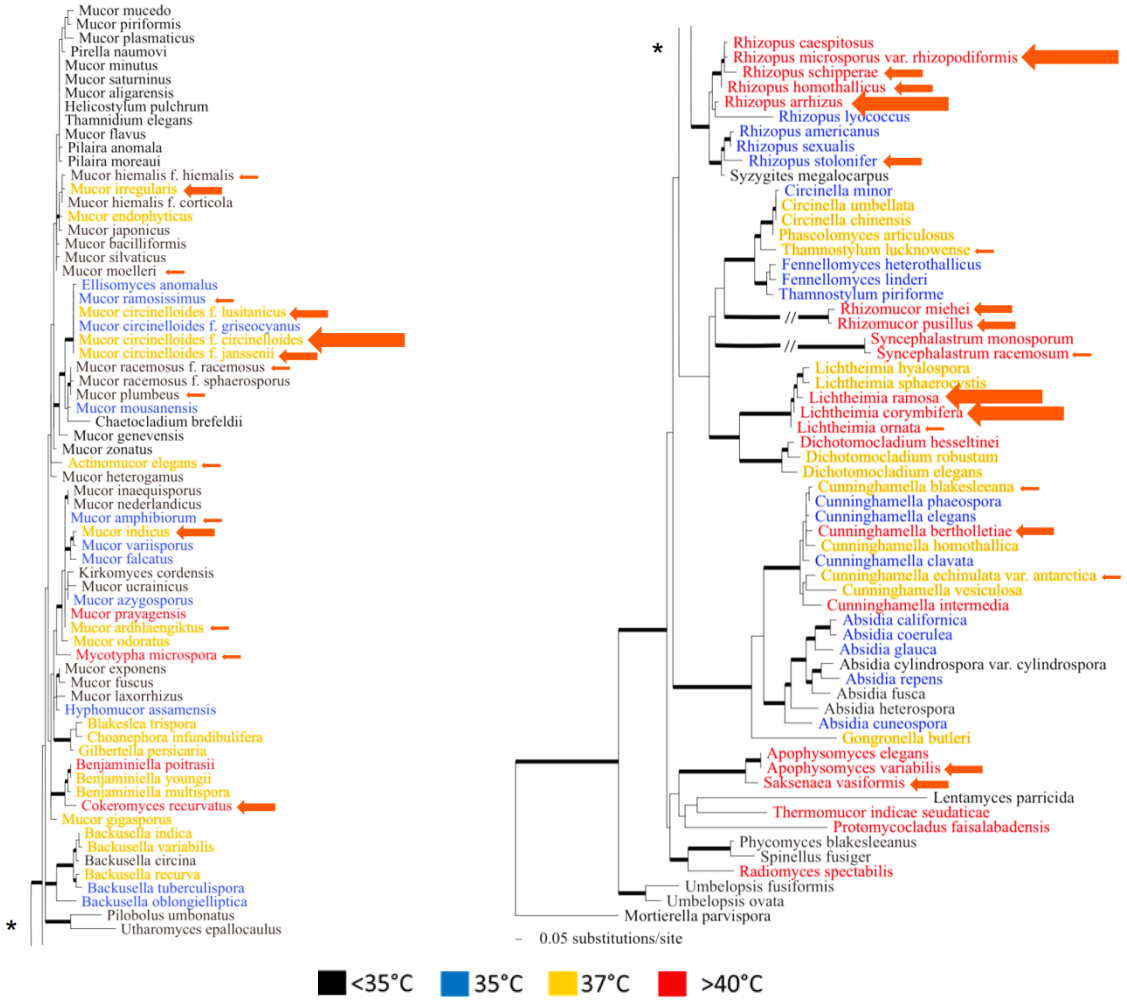


Figure 5.1: Phylogenetic distribution of thermotolerant and human pathogenic species in the Mucorales (Walther et al, 2012). Maximum growth temperatures of the different species are color-coded and the involvement in human infections is indicated as arrows. The sizes of the arrows represent the frequency of clinical cases. The connection points of the left and right part of the phylogenetic tree are marked by asterisks.

Such comparative studies of pathogenic and non-pathogenic species are lacking in other mucoralean genera. Thus, it is unclear how pathogenicity evolved in these groups and which adaptations are associated with the occurrence of pathogenicity. However, it has been shown that for example the morphological switch between yeast and hyphae may play a role in the pathogenicity of *M. circinelloides* (Cooper, 1987; Lee et al, 2013). Yeast stages have been also

observed in other human pathogenic *Mucor* species. To date, this seems to be a unique adaptation of *Mucor* species among mucoralean pathogens.

Despite the high dissimilarity of the mucoralean genomes, known virulence factors are well-conserved among mucoralean fungi such as high copy numbers of proteases, Ftr1, Fob1/2 and CotH (Rüchel et al, 2004; Ma et al, 2009; Gebremariam et al, 2014, Schwartze et al, 2014b; Liu et al, 2015; Schwartze et al, 2016). Since mucoralean fungi are primarily saprotrophic fungi, they are not specifically adapted to the human host and genes involved in virulence serve also functions during growth in the environment. The lifestyle of mucoralean fungi seems to support the retention of these virulence-associated traits. This fits to the observation that the adaptation to high temperatures coincides with the pathogenicity of the species and indicates that thermoadaptation is one of the main limiting factors in the evolution of pathogenicity of mucoralean fungi. In parallel, the high rate of gene duplication and retention results in a high genomic flexibility supporting the adaptation to new environments and the evolution of lineage-specific factors. Many duplications encompass genes which are involved in stress response but also in the signal transduction and transcriptional regulation. The results of this study also suggest that besides the presence and absence of genes, small differences in the expression levels and regulation contribute to the adaptation to new environments.

7 Summary

7.1 Summary

Despite the increasing recognition of life-threatening infections with members of the Mucorales (mucormycosis), little is known about genomics and the molecular basis of pathogenicity in these fungi. More than 20 mucoralean species are known to cause infections in humans. Although these species are only distantly related, all infections are described by the term mucormycosis and species-specific features are only rarely discussed. Moreover, research on virulence factors is mainly limited to the two derived mucoralean genera *Rhizopus* and *Mucor*. *Lichtheimia* species are basal mucoralean pathogens and represent the second most common cause of mucormycosis in Europe.

This work focused on species of the genus *Lichtheimia* as novel model organisms to study human pathogenic mucoralean fungi. A combination of genomic and transcriptomic approaches was used to get insights into general genomic features of *Lichtheimia* as one of the most basal human pathogenic genera among mucoralean fungi and to identify putative virulence factors.

Comparative genomics analyses revealed that the *L. corymbifera* genome is highly dissimilar from other mucoralean genomes in terms of gene content and genome structure, underlining the importance of additional genome projects in this group. A common feature of mucoralean genomes appears to be the high amount of duplicated genes, independent of genome size and phylogenetic position of the species. The phenomenon was first described in *R. arrhizus* and was shown to be caused by a recent whole genome duplication event in this species. In contrast, no similar whole genome duplication was found in *Lichtheimia* species and the results of phylogenomic analyses indicate that the duplicated genes result from at least one ancient whole genome duplication event at the base of all mucoralean fungi as well as additional lineage-specific gene duplications. The duplications and the resulting expansions include several putative virulence factors but also genes possibly involved in the resistance towards antifungals. Expression data show that different copies of genes are often differently regulated and may be involved in the adaptation to changing environments. In addition to the general genomic features, genome and transcriptome analyses under infection-associated conditions resulted in the identification of several conserved but also

novel putative virulence factors such as adhesins, iron uptake mechanisms and factors involved in stress resistance.

To get insights into the evolution of pathogenicity in mucoralean fungi, the genomes of the two clinically relevant species *L. corymbifera* and *L. ramosa* were compared with *L. hyalospora*, which has never been found to be involved in human infections. Interestingly, all three genomes are similar in genome structure, gene content and the pattern of duplicated genes. Virulence-associated traits are conserved between the species and known virulence factors are present in all three genomes. However, *Lichtheimia* species which are involved in human infections are more thermotolerant than other species of the genus. The higher sensitivity of *L. hyalospora* towards heat stress was linked to a higher accumulation of misfolded proteins but the effect seems to be specific for the growth under thermal stress and is not caused by a general dysfunction of the endoplasmatic reticulum. While genes involved in heat stress adaptation are conserved and show similar expression pattern in all *Lichtheimia* species, massive differences in the transcriptional response of *L. hyalospora* under heat stress conditions compared to more thermotolerant species were found. Additional experiments showed that the thermotolerance of *Lichtheimia* species can be modified by the osmolarity of the growth medium and the presence of osmolytes. Only small changes in the transcriptome of *L. hyalospora* were observed when it was grown under combinatory heat and osmotic stress conditions indicating that only minor adaptations are necessary to improve the survival and growth under host-like conditions. The data suggest that classical heat shock proteins like chaperones play no important role in the improved growth, while the uptake and production of osmolytes and proline may be responsible for the increased heat resistance. In addition, major changes in genes involved in the lipid metabolism and the synthesis of membrane components were detectable, which may contribute to the proper function of cellular components like the endoplasmatic reticulum under heat stress conditions. Phylome-based phylogenetic analyses revealed that species with higher thermotolerance do not form a monophyletic group and evolved several times independently in the genus *Lichtheimia*.

The comprehensive genome and transcriptome data produced during this study give first insights into the stress response of basal fungi and reveal severe differences compared to other fungi.

These data represent a valuable resource for future research and will help to identify virulence factors as well as targets for new antifungals.

7.2 Zusammenfassung

Obwohl lebensgefährliche Infektionen mit Pilzen aus der Ordnung Mucorales (Mucormykosen) zunehmend beobachtet werden, ist nur wenig über die Genome und die molekularen Faktoren bekannt, die zur Pathogenität dieser Pilze beitragen. Mehr als 20 Arten der Mucorales sind als Krankheitserreger in Menschen beschrieben. Obwohl diese Arten nur entfernt miteinander verwandt sind, werden alle Infektionen unter dem Begriff Mucormykose zusammengefasst. Die spezifischen Merkmale der unterschiedlichen Arten werden nur selten diskutiert und die Untersuchung von Virulenzfaktoren in Mucorales ist weitgehend auf die Gattungen *Mucor* und *Rhizopus* beschränkt, welche beide zu den abgeleiteten Vertretern der Mucorales gehören.

Pilze der Gattung *Lichtheimia* gehört zu den basalsten Mucorales und sind die zweithäufigste Ursache für Mucormykosen in Europa. In dieser Arbeit sollten Pilze der Gattung *Lichtheimia* als typische Vertreter von basalen Pathogenen der Ordnung Mucorales näher untersucht werden. Um erste Einsichten in die Genomstruktur von Pilzen dieser Gattung zu bekommen und potentielle Virulenzfaktoren zu identifizieren, wurden Ansätze der vergleichenden Genomanalysen und Expressionsanalysen vewendet.

Die Ergebnisse dieser Arbeit haben gezeigt, dass sich die Genomstruktur und die kodierten Gene von *L. corymbifera* stark von den Genomen anderer Pilze der Ordnung Mucorales unterscheiden. Neben diesen großen Unterschieden konnte sowohl in *L. corymbifera* als auch in den anderen untersuchten Genomen der Mucorales ein höherer Anteil an duplizierten Genen festgestellt werden als in Vertretern anderen Pilzgruppen. Dieses Phänomen wurde bereits bei *R. arrhizus* beschrieben und konnte auf eine kürzlich erfolgte Duplikation des Genoms (whole genome duplication) in dieser Art zurückgeführt werden. In *L. corymbifera* konnte keine solche spezifische Duplikation festgestellt werden. Stattdessen deuten die Ergebnisse von Phylomanalysen auf auf eine whole genome

duplication an der Basis der Mucorales und zusätzliche spezifische Duplikation als Ursache für den hohen Anteil von duplizierten Genen in den Mucorales hin. Genduplikationen und die, daraus resultierenden, Genexpansionen konnten bei vielen Genen, die als Virulenzfaktoren bekannt sind oder für die Resistenz gegenüber Antimykotika verantwortlich sein könnten, beobachtet werden. Basierend auf Transkriptomdaten konnte festgestellt werden, dass die verschiedenen Kopien unterschiedlich reguliert werden und somit eine Rolle bei der Anpassung an verschiedene Umweltbedingungen spielen könnten.

Neben diesen grundlegenden Merkmalen der Genomstruktur der Mucorales konnten auf Grundlage von vergleichenden Genomanalysen und Genexpressionsanalysen unter Stressbedingungen, welche während der Infektion auftreten, konservierte und neue potentielle Virulenzfaktoren identifiziert werden. Dabei wurden insbesondere Adhäsine, Aufnahmemechanismen für Eisen und Faktoren, die an der Stressantwort beteiligt sind, näher untersucht.

Auf Grundlage von vergleichenden Analysen der Genome von den beiden klinischen Arten *L. corymbifera* und *L. ramosa* mit *L. hyalospora*, welche bisher nie in Patienten beobachtet wurde, konnten erste Einblicke in die Evolution der Virulenz von basalen Pathogenen gewonnen werden. Alle drei Genome zeigen große Ähnlichkeiten in der Genomstruktur und den Protein-codierenden Genen, wobei auch potentielle Virulenzfaktoren zwischen den Arten konserviert sind. In physiologischen Untersuchungen konnte bestätigt werden, dass grundlegende Virulenz-assoziierte Fähigkeiten in allen drei Arten vorhanden sind. Die beiden klinischen Arten zeigten allerdings eine höhere Thermotoleranz als *L. hyalospora*, was auf eine größere Akkumulation von missgefalteten Proteinen bei hohen Wachstumstemperaturen zurückgeführt werden konnte. Da dieser Effekt nur bei höheren Wachstumstemperaturen beobachtet werden konnte und *L. hyalospora* auch sonst keine Anzeichen für eine grundlegende Störung in der Funktion des endoplasmatischen Retikulums zeigt, scheint der Effekt spezifisch bei thermalem Stress aufzutreten. Gene, die potentiell an der Stressantwort unter Hitzestressbedingungen beteiligt sind, scheinen in allen *Lichtheimia* Arten konserviert zu sein und zeigen eine vergleichbare Expression in diesen Arten. Die Hitzetoleranz von *L. hyalospora* kann durch die Präsenz von Osmolyten oder die parallele Applikation von osmotischem Stress verbessert werden. Die Analyse des Transkriptoms während des Wachstums unter einer Kombination beider Stresse konnte zeigen, dass geringe Änderungen in der

Genexpression ausreichen, um das Wachstum und das Überleben von *L. hyalospora* unter Hitzestress und somit potentiell auch während der Infektion zu verbessern. Dabei deuten die Ergebnisse darauf hin, dass klassische Hitzeschockproteine nur eine untergeordnete Rolle in der erhöhten Resistenz spielen. Stattdessen können große Veränderungen in der Regulation von Genen gefunden werden, welche eine Rolle in der Synthese oder Aufnahme von Osmolyten wie Prolin spielen oder in den Lipidmetabolismus involviert sind. Basierend auf den produzierten Phylom-Daten konnte festgestellt werden, dass thermotolerante Arten keine monophyletische Gruppe bilden und höhere Thermotoleranz sowie die daraus folgende höhere Virulenz mehrfach unabhängig in dieser Gattung entstanden sind.

Die umfangreichen Genom- und Transkriptomdaten, die in dieser Arbeit produziert wurden, geben zusätzlich erste Einsichten in die Stressantwort von basalen Pilzen. Diese Ergebnisse stellen somit eine wertvolle Basis für weitere Studien dar und können bei der Identifizierung und Untersuchung von Virulenzfaktoren sowie Angriffspunkten für neue Antimykotika hilfreich sein.

7.3 Outlook

This study represents a first step in the establishment of *Lichtheimia* species as a model for basal pathogens of the order Mucorales. The genomic and transcriptomic data produced during this study are a valuable resource for further research and the identification of virulence factors as well as potential drug targets of these fungal pathogens.

The current lack of efficient transformation methods in mucoralean fungi restricts the possibility to proof the exact function of the candidate genes. Thus, the establishment of a transformation system for *Lichtheimia* species is an important next step in order to understand their pathogenicity and the molecular factors involved. Based on the genome sequences of *Lichtheimia* species and the results of this thesis, first experiments to knock out genes in *L. corymbifera* are currently performed.

Besides the transformation of *Lichtheimia* species, proteomic and transcriptomic approaches are currently used to identify molecular factors which are involved in the interaction of *L. corymbifera*

with the immune system. The comparative analyses of the surface proteome of spores from strains with differences in the interaction with macrophages revealed several candidate genes that may play a role in the prevention of phagocytosis. To further characterise the proteins independent of a transformation system for *Lichtheimia*, heterologous expression in yeast is performed and purified proteins as well as antibodies for the proteins are produced.

In addition, further genomes of strains with reduced virulence and defects in physiological assays are currently analysed in order to investigate the genomic differences linked to the phenotypes. First results show that there is a wide genetic variation between different strains of the same species and signs of a heterokaryotic stage were found in one strain, which has never been described in the Mucorales so far.

Besides the infection biological aspect, the genomes of *Lichtheimia* species are also interesting in terms of genome structure and evolution of fungi. The high rate of gene duplication and the retention of the additional copies are a unique aspect of the mucoralean genomes. The results of this studie show, that the proportion of multi-gene families is similar in mucoralean fungi independent of the genome size and the occurrence of recent whole genome duplication events. However, the effect of the gene duplication on the biology of mucoralean fungi is still unclear and raises several questions. By which mechanisms are new gene copies generated and are certain gene families more prone to undergo duplication than others? Why do mucoralean fungi retain a high number of duplicated genes and which function do the new copies have in these fungi?

An additional finding is the presence of a siderophore transporter in all basal terrestrial fungi, which is highly similar to the one identified in derived fungal groups (Dikarya). The absence of genes required for the production of the siderophores of derived fungi in the Mucorales and other basal fungi raises several questions regarding the evolution of the iron uptake in fungi. Did the siderophore uptake systems evolved prior to the ability to produce siderophores or was the ability lost in some fungal groups? Also, the question arises why ferritin, as an iron storage which is conserved from bacteria to mammals, was lost during the cause of evolution in fungi. Are the ferritins of basal fungi still functional and do they contribute to the pathogenicity of the species?

The results of this study represent a good starting point for studies on the different topics and add a new model system for the investigation of basal mucoralean pathogens. The analyses of additional mucoralean pathogens will help to understand the spectrum of pathogenicity mechanisms involved in the infection process of the distantly related species causing infections, collectively known as mucormycosis.

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10 Eigenständigkeitserklärung

Ich erkläre, dass ich die vorliegende Dissertation selbständig und nur unter der Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Sämtliche Stellen, die direkt oder indirekt anderen Werken entnommen sind, wurden unter Angabe der Quellen als Entlehnung kenntlich gemacht.

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Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät ist mir bekannt und wird von mir akzeptiert.

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Volker U. Schwartze

11 Curriculum vitae

11.1 Curriculum vitae

11.2 Contributions to peer-reviewed scientific journals

1. **Schwartze VU**, Klassert TE, Riege K, Marcet-Houben M, Nowrousian M, Binder U, Grigoriev I, Grøtli M, Gryganskyi A, Fleischauer M, Lipzen A, Park H, Salamov A, Stajich J, Tamas M, Tritt A, Winter S, Böcker S, Gabaldón T, Marz M, Lass-Flörl C, Slevogt H, Voigt K (2016) To the limit and beyond: Comparative genomic and transcriptomic analyses reveal stress adaptation determinants of human pathogenic *Lichtheimia* species. ***Manuscript in preparation***

2. Kaerger K, **Schwartze VU**, Dolatabadi S, Nyilasi I, Kovács SA, Binder U, Papp T, de Hoog S, Jacobsen ID, Voigt K (2015) Adaptation to thermotolerance in *Rhizopus* coincides with virulence as revealed by avian and invertebrate infection models, phylogeny, physiological and metabolic flexibility. ***Virulence*** 6(4): 395-403.

3. **Schwartze VU**, Santiago AL, Jacobsen ID, Voigt K (2014) The pathogenic potential of the *Lichtheimia* genus revisited: *Lichtheimia brasiliensis* is a novel, non-pathogenic species. ***Mycoses*** 57 (Suppl 3):128-131.

4. Linde J, **Schwartze VU**, Binder U, Lass-Flörl C, Voigt K, Horn F (2014) *De novo* whole-genome sequence and genome annotation of *Lichtheimia ramosa*. ***Genome Announcements*** 2(5):e00888-14.

5. **Schwartze VU**, Jacobsen ID (2014) Mucormycosis caused by *Lichtheimia* species. ***Mycoses*** 57 (Suppl 3): 73-78.

6. **Schwartze VU**, Winter S, Shelest E, Marcet-Houben M, Horn F, Wehner S; Linde J, Valiante V, Sammeth M, Riege K, Nowrousian M, Kaerger K, Jacobsen ID, Marz M, Brakhage AA, Gabaldón T, Böcker S, Voigt K (2014) Gene Expansion Shapes Genome Architecture in the Human Pathogen *Lichtheimia corymbifera*: An Evolutionary Genomics Analysis in the Ancient Terrestrial Mucorales (Mucoromycotina). ***PLoS Genetics*** 10 (8): e1004496.

7. **Schwartze VU**, Hoffmann K, Nyilasi I, Papp T, Vágvölgyi C, de Hoog S, Voigt K, Jacobsen ID (2012) *Lichtheimia* species exhibit differences in virulence potential. ***PLoS One*** 7 (7): e0040908.

8. Schrödl W, Heydel T, **Schwartze VU**, Hoffmann K, Große-Herrenthey A, Walther G, Alastruey-Izquierdo A, Rodriguez-Tudela JL, Olias P, Jacobsen ID, de Hoog GS, Voigt K (2011) Direct analysis and identification of pathogenic *Lichtheimia* species by Matrix Assisted Laser

Desorption Ionization (MALDI) Time-Of-Flight (TOF) analyzer-mediated mass spectrometry. ***Journal of Clinical Microbiology*** 50 (2): 419-427

11.3 Book chapters

1. **Schwartze VU**, Hoffmann K (2015) *Lichtheimia* (ex *Absidia*). In: *Robert Russell Monteith Paterson, Nelson Lima* (Eds.) ***Molecular Biology of Food and Water Borne Mycotoxigenic and Mycotic Fungi***. CRC Press Inc.

2. Rothhardt JE*, **Schwartze VU***, and Kerstin Voigt (2011) Entomophthorales. In: *Dongyou Liu* (Ed.) ***Molecular Detection of Human Fungal Pathogens***. CRC Press Inc. (*shared first author)

11.4 Contributions at scientific meetings

11.4.1 Oral presentations

1. **Schwartze VU**, Riege K, Klassert T, Marcet-Houben M, Linde J, Gabaldon T, Marz M, Slevogt H, Voigt K (2015) Comparative genomics and transcriptomics of human pathogenic *Lichtheimia* species. *6th European Conference on Prokaryotic and Fungal Genomics*, September 29 - October 2 2015, *Göttingen*, Germany.

2. Wagner L, **Schwartze VU**, Voigt K, de Hoog S, Kurzai O, Walther G (2015) A new species concept of the human pathogenic *Mucor circinelloides* complex. *49th Scientific Conference of the German Speaking Mycological Society and 1st International Symposium of the CRC/Transregio FungiNet*, September 16 - 19 2015, *Jena*, Germany.

3. **Schwartze VU**, Klassert T, Riege K, Marcet-Houben M, Linde J, Nowrousian M, Gabaldon T, Marz M, Slevogt H, Voigt K (2015) Global Genome and Transcriptome Analyses of Human Pathogenic *Lichtheimia* Species. *49th Scientific Conference of the German Speaking Mycological Society and 1st International Symposium of the CRC/Transregio FungiNet*, September 16 - 19

2015, *Jena*, Germany.

4. **Schwartze VU**, Winter S, Shelest E, Horn F, Valiante V, Linde J, Sammeth M, Voigt M (2014) Genome analyses of human pathogenic *Lichtheimia* species. Microbiology and Infection 2014, *66th annual meeting of the German Society for Hygiene and Microbiology (DGHM) and Annual Meeting of the Association for General and Applied Microbiology (VAAM)*, October 5 - 8 2014, *Dresden*, Germany.

5. Jacobsen ID, **Schwartze VU**, Voigt K (2013) Infection models for *Lichtheimia* spp. *65th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM)*, September 22 - 25 2013, *Rostock*, Germany.

6. **Schwartze VU**, Winter S, Shelest E, Marcet-Houben M, Marz M, Valiante V, Gabaldón T, Böcker D, Brakhage AA, Voigt K (2013) Genome analyses of human pathogenic *Lichtheimia* species. *Emerging Zygomycetes, a new problem in the clinical lab. A workshop of the ECMM/ISHAM Working Group on Zygomycetes and Zygomycoses*, April 8 - 9 2013, *Utrecht*, The Netherlands.

7. Shelest E, **Schwartze VU**, Voigt K (2013) Predicting potential virulence factors in the *de novo* sequenced genome of *Conidiobolus coronatus*. *Emerging Zygomycetes, a new problem in the clinical lab. A workshop of the ECMM/ISHAM Working Group on Zygomycetes and Zygomycoses*, April 8 - 9 2013, *Utrecht*, The Netherlands.

8. **Schwartze VU**, Hoffmann K, Sammeth M, Winter S, Brakhage AA, Voigt K (2012) *De novo* sequenced genome from *Lichtheimia corymbifera*, an ancient human pathogenic basal lineage fungus causing mucormycoses. *18th Congress of the International Society for Human and Animal Mycology - ISHAM*, June 11 - 15 2012, *Berlin*, Germany.

9. **Schwartze VU** (2012) Comparative Gene Cluster Analysis in the Mucorales. *CeBiTec Colloquium of the University Bielefeld*, March 30 2012, *Bielefeld,* Germany.

10. **Schwartze VU**, Jacobsen ID, Voigt K (2011) Virulence of human pathogenic *Lichtheimia* species (Zygomycota). *Status workshop „Eukaryontische Krankheitserreger" of the German Society for Hygiene and Microbiology (DGHM)*, March 25 - 26 2011, *Düsseldorf*, Germany.

11. Jacobsen ID, **Schwartze VU**, Voigt K (2011) Embryonated eggs as alternative infection model to study the virulence of *Lichtheimia*. *The dynamics of zygomycete research in a changing world*, March 3 - 5 2011, *Utrecht*, The Netherlands.

12. Voigt K, Hoffmann K, Fliegerová K, Eckart M, Jacobsen ID, **Schwartze VU**, de Hoog GS, Ebersberger I, Gube M, Strauss S, Kupczok A, Kothe E, von Haeseler A (2010) Evolution of basal fungal lineages: linking phylogenies to pathogenicity and morphology. *The 9th International Mycological Congress (IMC 9)*, August 1 - 6 2010, *Edinburgh*, Scotland.

13. Jacobsen ID, **Schwartze VU**, Voigt K (2010) Embryonated eggs as alternative infection model to study the virulence of *Lichtheimia*. *The 9th International Mycological Congress (IMC 9)*, August 1 - 6 2010, *Edinburgh*, Scotland.

14. Eckart M, **Schwartze VU**, Rothhardt J, Hoffmann K, Voigt K (2009) Current concepts of phylogenetic relationships of basal fungi. *Botanikertagung 2009 (BT 09) "Plants for the Future"*, September 6 - 11 2009, *Leipzig*, Germany.

Note: Presenting author is the first author

11.4.2 Posters

1. **Schwartze VU**, Linde J, Klassert TE, Riege K, Marcet-Houben M, Gabaldon T, Roos S, Marz M, Slevogt H, Voigt K (2016) Identification of pathogenicity factors in basal fungi by comparative genomics and pathogenomics approaches. *Annual Meeting of the Association for General and Applied Microbiology (VAAM)*, March 13 -16 2016, *Jena*, Germany.

2. Jacobsen ID, **Schwartze VU**, Voigt K (2013) Infection models for mucormycosis caused by *Lichtheimia* spp. *47th Annual Meeting of the German-speaking Mycological Society (DMykG)*, September 5 - 7 2013, *Tübingen*, Germany.

3. Voigt K, **Schwartze VU**, Vogel H, Felder M, Müller S, Shelest E, Winter S, Nowrousian M, Platzer M, Vilcinskas A, Brakhage AA (2012) *De novo* sequenced genome from *Conidiobolus coronatus*, an ancient human pathogenic basal lineage fungus causing entomophthoromycoses. *18th Congress of the International Society for Human and Animal Mycology - ISHAM*, June 11 - 15 2012, *Berlin*, Germany.

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Note: Presenting author is the first author

11.5 Awards

medac-Forschungspreis 2014

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