PROTEOMIC ANALYSIS OF *Pseudogymnoascus* spp. FROM DIFFERENT GEOGRAPHICAL REGIONS IN RESPONSE TO TEMPERATURE VARIATION

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Nurlizah binti Abu Bakar

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Name of Degree: Doctor of Philosophy

Title of Thesis:

Proteomic analysis of *Pseudogymnoascus* spp. from different geographical regions in response to temperature variation.

Field of Study: Biochemistry; fungal physiology; mass spectrometry.

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PROTEOMIC ANALYSIS OF PSEUDOGYMNOASCUS SPP. FROM DIFFERENT GEOGRAPHICAL REGIONS IN RESPONSE TO TEMPERATURE VARIATION

ABSTRACT

The need for understanding the detrimental effects of environmental stress for soil microorganisms is becoming more significant with current global warming issues. Temperature may alter the abundance of species in soil ecosystems, leading to consequential changes in microbial communities. Under temperature stress, fungi undergo numerous physiological changes in their proteome in order to survive. Understanding the changes in fungal proteomes can give insights into the complex protein responses that occur under high and low temperature stress. Mass spectrometry (MS)-based proteomics is a powerful tool that has helped researchers to identify and quantify complex protein mixtures in various cell systems. *Pseudogymnoascus*, a soil fungal genus that occurs in polar and temperate regions, is also a known producer of many extracellular hydrolase enzymes that contribute to soil decomposition. It is not known whether the mechanisms of temperature stress response of *Pseudogymnoascus* spp. differ in strains isolated from different regions and exposed to different environmental conditions. In this study, *Pseudogymnoascus* was chosen as a model taxon to characterise changes in fungal proteomic profiles in response to temperature stress. Analyses of the thermal tolerance and sensitivity of six isolates of *Pseudogymnoascus* spp. were carried out using temperature-dependent growth studies and colony morphological changes. Description of proteome profiles of *Pseudogymnoacus* spp. cultured at a non-stressful temperature (15°C) was carried out using liquid chromatography tandem mass spectrometry (LC MS/MS) to provide baseline information and knowledge of the phenotypic diversity of all six isolates. Bioinformatic analyses of differentially expressed proteins and Gene Ontology (GO) enrichment were used to identify the pathways that were significantly enriched in response to temperature variation (cold and heat stress studies). All six

isolates were characterised as psychrotolerant fungi with lower and upper temperature limits for growth of 5°C and 25°C, respectively. The proteome profiles of all six isolates showed that the majority of proteins identified were clustered into groups representing metabolic functions and catalytic activities. Temperature stress response of *Pseudogymnoascus* spp. involved a wide range of pathways being enriched, with no suggestion of response mechanisms following specific geographical patterns. The data obtained in this study provide new information on how *Pseudogymnoascus* spp. respond to temperature variation in their environment and increase our understanding of how these temperature stress responses in the context of global climate change may affect decomposition processes in soil ecosystems.

Keywords: Soil microfungi, temperature-stress response, proteomic approach

ANALISIS PROTEOMIK TERHADAP TINDAKBALAS KEPELBAGAIAN SUHU PADA KULAT *PSEUDOGYMNOASCUS* SPP. YANG DIPENCIL DARI PELBAGAI KAWASAN GEOGRAFIK

ABSTRAK

Keperluan untuk memahami kesan buruk terhadap mikroorganisma tanah akibat dari tekanan persekitaran menjadi penting dalam isu pemanasan global semasa. Perubahan suhu merupakan faktor utama yang mempengaruhi komuniti mikroorganisma di dalam ekosistem tanah. Di bawah pengaruh suhu, kulat mengalami perubahan fisiologi proteom untuk kekal hidup. Pemahaman berkaitan dengan perubahan proteom kulat dapat memberi maklumat tentang tindakbalas kompleks protin di bawah tekanan suhu yang berbeza. Analysis proteomik berasaskan spektrometri massa (MS) merupakan teknik terkini yang membantu penyelidik mengenal pasti dan mengukur kompleks protin dalam pelbagai sel. *Pseudogymnoascus* merupakan kulat tanah yang terdapat di kawasan polar dan beriklim sederhana. Ia juga merupakan penghasil kebanyakan enzim hidrolisis ekstraselular yang membantu dalam proses penguraian. Mekanisme tindak balas terhadap suhu dan persekitaran yang berbeza oleh *Pseudogymnoascus* spp. dari strain dan kawasan yang berbeza masih tidak dapat dipastikan. Dalam kajian penyelidikan ini, *Pseudogymnoascus* dipilih sebagai model organisma untuk mencirikan tindak balas perubahan profil proteomik kulat terhadap tekanan suhu. Analisis toleransi dan kepekaan terhadap perubahan suhu oleh enam *Pseudogymnoascus* spp. telah dijalankan berdasarkan kajian kebergantungan pertumbuhan kulat terhadap suhu dan perubahan morfologi koloni. Perincian profil proteom *Pseudogymnoascus* spp. yang dikultur pada suhu tanpa-tekanan (15°C) dilakukan dengan menggunakan tandem spektrometri massa kromatografi cecair (LC MS/MS) dapat memberikan maklumat dan pengetahuan asas mengenai kepelbagaian fenotip keenam-enam kulat berkenaan. Analisis bioinformatik terhadap perubahan protin yang diekspresikan dan pengkayaan jaringan Gene Ontology

(GO) telah digunakan untuk mengenalpasti laluan tapak yang ketara dalam proses tindak balas perubahan suhu (kajian suhu rendah dan tinggi). Keenam-enam kulat telah dikategorikan sebagai kulat psikrotoleran dengan had untuk pertumbuhan bagi suhu bawah dan atas masing-masing ialah 5°C dan 25°C. Profil proteom dari keenam-enam kulat menunjukkan bahawa sebahagian besar protin yang dikenalpasti adalah dikategorikan di bawah kelompok fungsi metabolik dan aktiviti pemangkinan. Tindak balas terhadap perubahan suhu oleh *Pseudogymnoascus* spp. adalah melibatkan pelbagai tapak yang diperkaya. Namun begitu, mekanisma tindak balas tidak mengikuti sebarang corak pembahagian geografi yang tertentu. Data yang diperolehi dalam kajian ini memberikan maklumat baru tentang bagaimana *Pseudogymnoascus* spp. bertindak balas terhadap perubahan suhu di persekitaran mereka, sekaligus meningkatkan pemahaman kita tentang bagaimana tindak balas perubahan suhu ini memainkan peranan dalam konteks perubahan iklim global yang akhirnya mempengaruhi proses penguraian dalam ekosistem tanah.

Kata kunci: Kulat tanah, tindakbalas perubahan suhu, pendekatan proteomik

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I finally made it. Cheers.

For **Ummar and Sarah**,
I pulled through only for you guys.
Love always,
Ibu

Nurlizah Abu Bakar

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2021

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

1D-PAGE	:	One-dimensional polyacrylamide gel electrophoresis
ACN	:	Acetonitrile
ATP	:	Adenosine triphosphate
cAMP	:	Cyclic adenosine monophosphate
CS	:	cold stress
DEP	:	differentially expressed protein
E_a	:	Activation energy
ESI	:	Electron spray ionization
ETC	:	electron transport chain
FA	:	Formic acid
FDR	:	False discovery rate
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
GO	:	Gene Ontology
HS	:	heat stress
HSP	:	Heat shock protein
HSR	:	Heat stress response
IPCC	:	Intergovernmental Panel on Climate Change
IPCC	:	Intergovernmental Panel on Climate Change
i-TRAQ	:	Isobaric tag for relative and absolute quantitation
kDa	:	KiloDalton
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
KOBAS	:	KEGG Orthology Based Annotation System
LC MS/MS	:	Liquid chromatography tandem mass spectrometry
LSU	:	28S nuclear ribosomal large subunit rRNA gene

MA	:	microarray
MALDI-TOF	:	Matrix-assisted laser desorption/ionization – time of flight
MANOVA	:	multivariate analysis of variance
MAPK	:	Mitogen-activated protein kinase
MFS	:	Major facilitator superfamily
mRNA	:	messenger ribonucleic acid
NCBI	:	National Center for Biotechnology Information
nLC-ESI-MS/MS	:	nano liquid chromatography electrospray ionisation tandem mass spectrometry
NMD	:	Nucleoside-mediated decay
PAH	:	polycyclic aromatic hydrocarbon
PCA	:	Principal Component Analysis
PKA	:	protein kinase A
SMD	:	Spliceosome-mediated decay
TCA	:	trichloroacetic acid
TCA cycle	:	Tricarboxylic acid cycle
TER	:	transitional endoplasmic reticulum
T_{max}	:	Maximum growth temperature
T_{opt}	:	Optimum growth temperature
UPGMA	:	unweighted pair group method with arithmetic mean
UPR	:	Unfolded protein response
v/v	:	volume/volume
vol	:	Volume
w/v	:	weight/volume
WNS	:	White-nose syndrome

CHAPTER 1: INTRODUCTION

1.1 Research background

Global warming and associated environmental changes impact living organisms in various ways. The recent Intergovernmental Panel on Climate Change (IPCC, 2018) Special Report: Global Warming of 1.5°C, predicted that extreme hot day events in mid-latitudes may warm by up to ~3°C with global warming of 1.5°C. It is also predicted that extreme night-time low temperatures at high latitudes will warm by ~4.5°C (IPCC, 2018). If current trends persist, global ice loss will continue, the temperate regions will experience more frequent extreme high and low temperature events (Francis & Skific, 2015), and different parts of the tropics will experience longer drought and wet seasons (Overland *et al.*, 2015). This can potentially lead to the breakdown of ecosystem structure and functions, making them yet more sensitive to future climate changes. There have already been some drastic changes in rainfall patterns and patterns of temperature variation (Kundzewicz *et al.*, 2005), already leading to significant reductions in land surface area suitable for crop cultivation (Lesk *et al.*, 2016). Several reviews have addressed research approaches for the development of improved crops in low-quality soils, and of soil management systems to improve resilience to current climate change challenges (Fan *et al.*, 2012; Zhang *et al.*, 2013; Rao *et al.*, 2016b). Major outcomes of these include the use of better-adapted phenotypes (Rao *et al.*, 2016a; Simova-Stoilova *et al.*, 2016), integrated soil-crop system management (Zhang *et al.*, 2013) and maintenance of functional diversity in the soil fungal community by retaining a capacity for symbiosis-driven recycling of organic nutrient pools (Kyaschenko *et al.*, 2017).

The fundamental importance of including mycological studies in efforts to sustain agricultural industries and underpin economic growth is recognised in the European Union's common research 2014-2020 program, known as *Horizon 2020* (Lange *et al.*, 2012). Fungi are generally the most important agent of primary decomposition and

nutrient cycling in soil microbial communities (A'Bear *et al.*, 2014b). In boreal forest management and long-term forest production, the functional diversity of the ectomycorrhizal fungal community plays an important role in facilitating symbiosis-driven recycling of organic nutrient pools (Kyaschenko *et al.*, 2017). The key role of fungi identified in such studies highlights the importance of understanding fungal responses to temperature stress. Active fungi initiate stress tolerance strategies at the molecular level and this can be observed in the physiological changes they undergo. Under temperature stress conditions, the tertiary structure of molecules such as enzymes and other functional proteins can be damaged; they will not function properly and may be degraded. Fungi may produce heat or cold marker molecules such as heat shock proteins and chaperones to assist in the repair of functional structure (Bai *et al.*, 2015; Tiwari *et al.*, 2015). There will be substantial physiological changes that can be understood from their molecular-level responses. Such physiological changes can include the production of anti-freeze proteins coating the cell wall (Bagwell & Ricker, 2019), the production of extracellular hydrolases such as chitinolytic enzymes (Fenice, 2016) and increased production of heat shock proteins to minimize protein misfolding (Kroll *et al.*, 2014; Miteva-Staleva *et al.*, 2017). Such stress tolerance strategies improve cell and organism survival in stressed and sub-optimal environments (Williams *et al.*, 2011).

Changes in protein contents generate a signature that can be identified and characterized. Potentially, these signatures can help understand organismal responses to stress, and be used as biomarkers for monitoring environmental changes and their effects on the ecosystem. Comparative analyses of fungal proteomic profiles can be used to understand changes in protein abundance that may serve as important biomarkers of temperature stress (Kroll *et al.*, 2014). To date, most studies of fungal proteomic response towards temperature stress have been conducted on pathogenic species. Examples include the black yeast, *Exophiala dermatitidis*, that can cause serious skin infections in humans

(Tesei *et al.*, 2015); *Aspergillus flavus*, a producer of lethal aflatoxins (Bai *et al.*, 2015; Delgado *et al.*, 2015); *Aspergillus fumigatus*, a thermo-tolerant human pathogen (Albrecht *et al.*, 2010); *Ustilago maydis*, a basidiomycete that causes corn smut disease (Salmeron-Santiago *et al.*, 2011); and *Metarhizium acridum*, an entomopathogenic species that can be used as an alternative to chemical insecticides (Barros *et al.*, 2010). Several studies have also been carried out on extremophiles such as *Cryomyces antarcticus* in order to understand extreme stress tolerance from the proteomic perspective (Isola *et al.*, 2011; Tesei *et al.*, 2012; Zakharova *et al.*, 2014).

From the foregoing, it is well understood that temperature increase, as has been recorded throughout the globe, will bring negative impacts to microbial communities. The central challenge is to understand how fungi may respond to these temperature changes and ultimately be able to survive the current global warming phenomenon. *Pseudogymnoascus* spp. is one potential genus to be studied as a representative of the group of soil microfungi, with a wide geographic distribution that covers both poles and the temperate regions. *Pseudogymnoascus* is known to play an important role in soil environments, and is a producer of many extracellular hydrolases that contribute to decomposition processes in the soil, such as ureases (Zhang *et al.*, 2015), proteases (Pannkuk *et al.*, 2015), endopeptidases (O'Donoghue *et al.*, 2015) and laccases (Kolomytseva *et al.*, 2017). However, it is not known whether *Pseudogymnoascus* spp. responses towards cold and heat stress differs as a result of the varied environmental conditions they have evolved under in different regions of the planet.

1.2 Research questions

Following the premise as stated above, the problem statements for this study were:

a) What are *Pseudogymnoascus* spp. protein abundances under non-stressful temperature condition (baseline)?

b) Under cold or heat stress, are there any proteins up- or down-regulated in all isolates?

c) What are the heat stress response mechanisms of *Pseudogymnoascus* spp.?

d) Are there any underlying geographical patterns in the cold and heat stress responses of *Pseudogymnoascus* spp.?

Thus, the objectives of this study are as follows:

1.3 General research objectives

i. To determine the thermal tolerance and sensitivity of *Pseudogymnoascus* spp. from different geographical regions.

ii. To investigate changes in protein abundance of polar and temperate region isolates of *Pseudogymnoascus* in response to changes in temperature.

Specific Objectives

i. To compare the thermal tolerance and sensitivity of *Pseudogymnoascus* spp. using temperature-dependent growth curves and colony morphological changes.

ii. To identify protein(s) that can potentially serve as temperature stress biomarker(s) for *Pseudogymnoascus* spp. from temperate and polar regions.

iii. To identify potential pathway(s) involved in response to temperature stress of *Pseudogymnoascus* spp. from temperate and polar regions.

CHAPTER 2: LITERATURE REVIEW

2.1 The evolution of proteomics

In the last 10 years an increasing number of studies have analysed fungal temperature stress responses using proteomic approaches (Albrecht *et al.*, 2010; Salmeron-Santiago *et al.*, 2011; Zakharova *et al.*, 2014; Bai *et al.*, 2015; Tesei *et al.*, 2015). Some have also used 'multi-omic' approaches that combine the simultaneous analysis of different molecules (proteins, mRNA, metabolites) to better understand complex biological responses (Bai *et al.*, 2015; Su *et al.*, 2016). Since the late 1990s, proteomic methodologies and technologies have evolved from an approach that relied on two-dimensional gel electrophoresis to gel-free analyses. The protein extraction method is a fundamentally important stage in any proteomic analysis. Optimisation of protein extraction is key in maximising yield and resolution, followed by the selection of proteomic profiling methods (Isola *et al.*, 2011; Bianco & Perrotta, 2015; Daim *et al.*, 2015). Label-free or labelled proteomic approaches, such as isotope and fluorescent labelling, are now used extensively because of their many advantages and provide useful information on differential expression of proteins in complex biological samples (Fricker, 2018). However, proteomics remains a very costly field, and interpreting the data obtained requires advanced knowledge of the entire technical process involved, as well as understanding of appropriate statistical principles for estimation and inference (Karpievitch *et al.*, 2010).

2.2 High temperature stress

In general, definitions of high temperature stress have primarily been made based on the optimum growth temperature (T_{opt}) and maximum growth temperature (T_{max}) (Tesei *et al.*, 2012; Su *et al.*, 2016). However, this is not always the case. For example, in the study of pathogenic fungi, experimental temperature selection is often based on the host's

body temperature (Tesei *et al.*, 2015). Temperature can also be selected based on the induction or optimisation of mycotoxin production (Salmeron-Santiago *et al.*, 2011; Bai *et al.*, 2015). Deviations from the optimum temperature are expected to trigger a response at the molecular level, with the fungal proteome altering to prevent damage to the cell. Another important parameter in high temperature stress studies is the total duration or interval of heat exposure. Shorter exposures of seconds or minutes can lead to different proteomic changes compared to longer, or repeat, exposures (Albrecht *et al.*, 2010; Tesei *et al.*, 2015). This is because native proteins take time to undergo the processes of denaturing, unfolding or refolding, or are eventually degraded under high temperature conditions (Aragno, 1981). There are two important stages in understanding the impact of high temperature stress on cells: the onset of any stress response, and its upper thermal limit. Together, these often give an indication of specific temperature stress response pathways. With an increase in environmental temperature, a higher metabolic rate is generally anticipated in cells. Thus, heat response pathways will be initiated to utilize the extra energy that is being produced from an increase in metabolic rate, and proteomic profiling can be used to understand the physiological changes taking place (Richter *et al.*, 2010). However, on average, cells are only able to tolerate an increase in temperature of a few degrees above T_{opt} (Aragno, 1981). Further increase in temperature beyond this limit causes irreversible damage and ultimately leads to cell death. Table 2.1 summarises significant findings on fungal proteome profiling in response to high temperature stress, including the identification of proteins and suggestion of pathways that are potentially related to the identified proteins.

A recent integrated -omics study of *Mrakia psychrophila* provided insight into the adaptation mechanisms of psychrophilic (low temperature adapted) fungi (Su *et al.*, 2016). At 4°C and 20°C, *M. psychrophila* produced an increased amount of major facilitator superfamily (MFS) proteins that are involved mainly in energy metabolism,

compared to the level at 12°C (optimal growth temperature range 12°C-15°C). However, heat shock proteins were upregulated only at 20°C, which likely leads to the activation of the unfolded protein response (UPR). Consistent with this, at 20°C, there was a negative correlation between the protein level change and transcript level change. The proteome abundances of another psychrophilic fungus, *Friedmanniomyces endolithicus* (optimal growth temperature range 10°C-15°C), assessed using a classical 2D gel electrophoresis (non-comparative) approach, showed a reduction from 284 (at 28°C) to 224 (comparison between 15°C and 28°C) protein spots (Tesei *et al.*, 2012). The authors hypothesized that, with exposure to high temperature, the basic set of proteins necessary to survive is relatively stable without the help of various chaperones. It is also likely that other non-protein protective metabolites and molecules are involved in the response pathways (Keller, 2019).

For human pathogenic fungi, their pathogenic characteristics become significant at body temperature. These have been explored in many species, such as *Penicillium marneffei* and *A. flavus*. Gauthier (2017) reviewed the molecular strategies used by thermally dimorphic fungi, focusing on their ability to adapt to core body temperature (37°C) and transition to yeast morphology for virulence capabilities. These fungi used various strategies, such as upregulation of virulence factors, to promote cell adhesion to host, lysis of macrophages, avert cytokine responses, and impair host immunity. *Penicillium marneffei* is the only dimorphic species in its genus and forms its secondary cellular development, which is a uninucleate yeast, at 37°C (Chandler *et al.*, 2008). Increased levels of RanA expression, a GTP-binding nuclear protein that plays a role in nucleocytoplasmic transport, suggest that there is an additional signalling mechanism involved during phase transition in *P. marneffei*. Comparative work on extracellular proteomes of *P. marneffei* in yeast and mycelial phases showed upregulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heat shock protein 60

(HSP60), respectively, that may play an important role in cell-host adherence (Lau *et al.*, 2013). Bai et al. (2015) used an integrated transcriptomic and proteomic approach to study the response towards temperature changes in *A. flavus*, a species whose growth optimum is 37°C, but that produces highest levels of mycotoxin is at 28°C. The data obtained showed that a subset of 664 proteins involved in translation-related pathways, metabolic pathways and biosynthesis of secondary metabolites were differentially expressed at the two temperatures. At 28°C, the expression pattern of proteins and transcripts related to aflatoxin biosynthesis was upregulated, and the authors suggested that change in the *aflR* transcript level was a better marker for the activation of aflatoxin biosynthesis. However, they also noted that there was a low correlation between overall transcript level and protein concentration in *A. flavus*, suggesting that the post-transcription modification processes may play a critical role in the regulation of the final protein expression level.

The plant pathogen, *U. maydis* is a well-studied causative agent for corn smut disease, but there are also reports of *Ustilago* spp. infections in humans (Teo & Tay, 2006; McNeil & Palazzi, 2012). Under various stress conditions, fungi often accumulate trehalose and initiate trehalose biosynthesis pathways (Nwaka & Holzer, 1997; Al-Bader *et al.*, 2010). In a study of the effect of temperature stress on *U. maydis*, increased production of 11 proteins, commonly up-regulated in response to osmotic and sorbitol stress, was observed (Salmeron-Santiago *et al.*, 2011). However, there were no changes in the trehalose concentration, thus suggesting that the up-regulated proteins are common proteins in the general stress response of *U. maydis*, non-specific to trehalose metabolism pathways. A more recent review (Perez-Nadales *et al.*, 2014) highlighted the overall mechanisms of pathogenesis and some unifying themes among various fungal model organisms, emphasising the importance of conserved signalling pathways such as the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A and mitogen-activated protein (MAP) kinases, and the central roles of secondary metabolic pathways in a very

wide range of pathogenic fungi. However, comparison of the effect of temperature on the pathogenicity of fungal model organisms was not a focus of that study.

Mesophilic fungi, which have T_{opt} of 25-30°C, are generally able to tolerate a wider range of temperatures and associated stress, having a relatively wider range of temperature-dependent growth curves (Dix & Webster, 1995). In a non-comparative experiment on three mesophilic fungi (*Exophiala jeanselmei, Coniosporium perforans* and *Penicillium chrysogenum*), high temperature exposure led to variation in their proteomic expression patterns (Tesei *et al.*, 2012). Exposure to 40°C increased the number of expressed proteins detected in *P. chrysogenum* but decreased those of *E. jeanselmei* and *C. perforans*. A comparison was also made between species at 40°C by selecting *P. chrysogenum* as the reference strain for mesophilic black fungi, which revealed 50 and 62 common proteins spots in *E. jeanselmei* and *C. perforans*, respectively. The study suggested that there was a lack of a heat-shock protein response in *E. jeanselmei* and *C. perforans*. Furthermore, the disappearance of spots from the same pI and molecular mass range (respectively 5-7 and 30-90 kDa) suggested that both strains probably downregulated similar sets of proteins. Tesei et al. (2012) hypothesized that either the basic set of proteins that is important in high temperature exposure is stable without the help of heat-shock proteins or that other non-protein protective metabolites are involved. It is important to understand the limitations of such non-comparative experiments conducted through gel-based methods, where technical variations between replicates are expected. Quantification and identification of protein spots can be a challenge if differences in staining occur between gels with no normalization.

An integrated analysis of transcriptomic and proteomic responses of *A. fumigatus* exposed to high temperature stress improved understanding of the thermotolerant characteristics responsible for the pathogenicity of this fungus (Albrecht *et al.*, 2010). The

study identified 91 differentially regulated protein spots representing 64 different proteins using two-dimensional difference gel electrophoresis (2D-DIGE) and tandem mass spectrometry (MS/MS) identification methods. These included a number of previously undescribed putative targets for the heat shock regulator Hsf1, providing evidence for Hsf1-dependent regulation of mannitol biosynthesis, translation, cytoskeletal dynamics, and cell division in *A. fumigatus*. Albrecht et al. (2010) also demonstrated a negative correlation between protein expression and gene transcription levels in *A. fumigatus* when exposed to supra-optimal temperatures (from 30°C to 48°C). The synthesis of most proteins was delayed by 60 to 90 minutes (medium delay) and up to 105 minutes (strong delay) after gene transcription by exposure to supra-optimal temperatures.

High temperature exposure causes specific stress response mechanisms to be activated that can be further explored using fungal proteomic profiles. Most fungi respond to high temperature stress by increasing the production of heat-shock proteins and chaperones and initiating alternative metabolic pathways (Albrecht *et al.*, 2010; Bai *et al.*, 2015; Su *et al.*, 2016). However, some species show no significant changes in their proteome profiles, suggesting that the basic set of proteins needed for survival are highly thermotolerant (Tesei *et al.*, 2012; Tesei *et al.*, 2015). This is explained by the production of secondary metabolites and other non-protein protective metabolites that cannot be determined through proteomic approaches (Zhang *et al.*, 2016). In addition, high temperature stress may induce a negative correlation between gene transcription and protein production in fungal cells due to post-translational modification and the initiation of protein degradation pathways, such as the ubiquitin-mediated protein degradation pathway and spliceosome-mediated decay (SMD) pathway (Albrecht *et al.*, 2010; Bai *et al.*, 2015).

Table 2.1: Summary of fungal proteomic profiling analysis when exposed to high temperature stress (work published from 2008-2020)

T_{exp} (°C)	T_{opt} (°C)	Time	Species	Origin	# no. of proteins		Proteins identified	Classification/Pathways involved	Refs.
20°C	12°C	1 month	*Mrakia psychrophila* (1)	Qinghai-Tibet, China	1673	Down	Citrate synthase, DLST, LSC2 MTCP1 CLD1	TCA cycle Oxidative phosphorylation Glycerophospholipid metabolism	(Su *et al.*, 2016)
						Up	DNAJ MPSY4181, MPSY2821	Heat shock proteins MFS transporters	
28°C	15°C	7 days	*Friedmanniomyces endolithicus* (2)	Antarctica	284	Down	Reduced no. of protein spots (141)	*Reduction in total number of protein spots, indicating a lack of a heat shock response	(Tesei *et al.*, 2012)
37°C	25°C	1 day	*Penicillium marneffei* (2)	Chiang Mai, Thailand	270	Up	Hsp 30, Hsp 70, antigenic mitochondrial protein HSP60 hypothetical protein (activator of HSP90 ATPase) succinyl-CoA synthetase alpha subunit, beta subunit of ATP synthase, NAD-dependent formate dehydrogenase adenylate kinase, phosphoglycerate kinase Ran GTPase spi1 Cdc48p UDP-N-acetylglucosamine pyrophosphorylase	Heat shock proteins Energy production and metabolism Regulate mitotic activities Cell cycle and division Synthesis of N-acetylglucosamine (monomeric component of cell-wall chitin)	(Chandler *et al.*, 2008)
37°C	28°C	1.5 days	*Aspergillus flavus* (1)	Data not available	3886	Down	2-heptaprenyl-1,4-naphthoquinone methyltransferase, esterase/lipase, predicted O-methyltransferase, aminotransferase GliI-like, and aflE	Translation and biosynthetic pathways	(Bai *et al.*, 2015)
37°C	28°C	2 hours	*Ustilago maydis* (2)	United States of America	-	Up	Enolase, Phosphoglycerate kinase Mitochondrial HSP 70, HSP 70, HSP 60 β-succinil CoA synthetase Glutathione S-transferase V/A- ATPase-A Oxidoreductase (AKR's)	Carbohydrate metabolism Protein folding Amino acid metabolism Redox regulation Ion homeostasis Other	(Salmeron-Santiago *et al.*, 2011)

40°C	28°C	7 days	*Exophiala jeanselmei* *Coniosporium perforans* [2]	Vienna, Austria	174 255	Down Down	Reduced no. of protein spots (208) Reduced no. of protein spots (70)	*Spots from the same pI (5-7) and molecular weight range (30-90 kDa) were extinct after heat stress, suggesting that both probably downregulated similar sets of proteins.	(Tesei *et al.*, 2012)
			Penicillium chrysogenum [2]	Vienna, Austria	601	Up	Increased no. of protein spots (220)	*Over-expression of proteins, interpreted as the synthesis of HSPs	
40°C	28°C	48 hours	*Pleurotus ostreatus* [1]	China	61	Up	Calmodulin 2-Methylcitrate synthase mitochondrial, Type 1 phosphatases regulator YPI1 Putative aryl-alcohol dehydrogenase AAD6, Phenylalanine ammonia-lyase (fragment), Peptidyl-prolyl cis-trans isomerase ATP-dependent RNA helicase ded1, Elongation factor 1-alpha, Enolase 2, Fructose-2,6-bisphosphatase HSP 60 mitochondrial, HSP SSA2, HSP82, hsp98	Developmental process Organelle part Catalytic activity Binding proteins Heat shock proteins	(Zou et al. 2018)
					70	Down	Subtilisin-like protease CPC735_066880 ATP-dependent RNA helicase DHH1, Myosin-1, Serine/threonine-protein kinase ppk8, Tubulin beta chain	Catalytic activity Binding proteins	
48°C	30°C	0-4 minutes, Time interval (heat shock)	*Aspergillus fumigatus* [1]	England, United Kingdom	1886	Up	HSP 70 chaperone HSP88 Tubulin alpha-1 subunit, actin-bundling protein Sac6 Nuclear movement protein NudC Allergen Asp F3 Transketolase TktA, hexokinase Kxk, Adenosylhomocysteinase, Nitrite reductase NiiA Cdc48	Heat shock protein Cell wall and cytoskeleton Transport protein Defence against oxidative and nitrosative stress Carbohydrate and nitrogen metabolism Cell cycle and division	(Albrecht *et al.*, 2010)
						Down	Mitochondrial co-chaperone GrpE CRAL/TRIO domain protein	Chaperone Transport protein	

							Cytochrome c oxidase polypeptide vib	Energy generation	
50°C	30°C	24 hours	*Aspergillus niger* 3.316[(1)]	China	481	Up	phosphatidylinositol 4-kinase type II subunit alpha	Cellular signalling	(Deng et al. 2020)
							β-galactosidase, carboxypeptidase	Carbohydrate metabolism	
							β-1,6-glucanase, extracellular α-glucosidase		
							rhamnogalacturonate lyase A	Cell wall organisation	

Key and abbreviations:

T_{exp} – Temperature of exposure, T_{opt} – Temperature of optimal growth
Proteomic profiling approaches.
(1) – Gel- free methods
(2) – Gel-based method
* – Non-conclusive remarks (no protein identification involved)

2.3 Low temperature stress

The study of low temperature stress resistance in microorganisms and other organisms has led to many beneficial findings. Proteins, such as cold-adapted enzymes, have been applied in many biotechnology industries such as in the manufacture of food and beverages, detergents, textiles and in industrial molecular biology (Sarmiento *et al.*, 2015). Research on cold-adapted proteins often involves psychrophilic or psychrotolerant microorganisms from the polar regions or deep oceans (Cavicchioli *et al.*, 2000). Psychrophiles produce cold-adapted proteins with unique characteristics that have weak protein interactions, low thermal stability and increased specific activity, in order to achieve higher protein activity and flexibility at low temperatures (Reed *et al.*, 2013). Protein structures are modified with subtle changes in the amino acid composition, thus remaining functional under extremely cold conditions. Wang et al. (2017) reviewed fungal adaptation to cold stress, providing an overview of life history strategies and highlighting the importance of cold-adapted fungi in the discovery of novel secondary metabolites and enzymes. Studies on fungal proteome profiles in response to low temperature stress are summarised in Table 2.2.

In a study of black rock-inhabiting fungi exposed to low temperature stress, their proteomic profiles generally showed an increase in the total number of spots, except for *P. chrysogenum* (Tesei *et al.*, 2012). *Friedmanniomyces endolithicus* and *C. perforans* proteome profiles exhibited changes in high molecular mass protein spots (70 – 170 kDa), while that of *E. jeanselmei* showed pattern changes at slightly lower molecular mass (25 – 100 kDa). *Penicillium chrysogenum* exhibited a decrease in the total protein spots, interpreted as being due to lower metabolic activity under cold stress. *Exophiala dermatitidis*, a mesophilic fungus that is pathogenic in humans, was experimentally exposed to low temperature in order to understand the relationship between its thermotolerance properties and pathogenicity (Tesei *et al.*, 2015). The strain was exposed

to 1°C (low temperature stress) for one week and comparison was then made with proteome profiles obtained at 37°C (optimum temperature for growth) and 45°C (high temperature stress). Using 2D-DIGE and nano-scale liquid-chromatography electrospray-ionisation, tandem mass spectrometry (nLC-ESI-MS/MS), the study showed an average of 1700 protein spots detected in *E. dermatitidis*. Exposure to low temperature stress led to a reduction in proteins associated with metabolic activity, mostly relating to general carbon metabolism. A large set of proteins involved in energy metabolism pathways were down-regulated, such as malate synthase, malate dehydrogenase, acetyl-coenzyme A synthetase and glyceraldehyde-3-phosphate dehydrogenase. Decreased levels of proteins involved in the response to heat stress such as Hsp70s, elongation factor 1α and Hsp30 might also be related to the reduction in metabolic rate and associated reduced energy consumption. Despite utilising alternative metabolic pathways, *E. dermatitidis* undergoes downregulation of metabolic pathways under exposure to non-optimal temperatures, resulted in a much slower growth rate. Comparison of the proteome profiles of *E. dermatitidis* under high and low temperature stress demonstrated the contribution to its success as a pathogen made by regulating the expression of basic thermotolerance proteins and modulating the production of proteins in major metabolic pathways.

Mrakia psychrophila, another psychrophilic yeast, showed a slightly different response to low temperature stress compared to *E. dermatitidis*, but more similar to other cold-adapted fungi such as *Flammulina velutipes* (Liu *et al.*, 2017b) and *Mortierella isabellina* (Hu *et al.*, 2016). The responses included desaturation of fatty acids and accumulation of glycerol (Su *et al.*, 2016). At 4°C, two proteins were up-regulated (glutamine synthetase, GLNA and MFS transporter protein, MPSY), and four proteins were down-regulated (citrate synthase, succinyl-CoA ligase, pyruvate decarboxylase and ribosomal protein). Comparative transcriptomic analysis showed that genes involved in ribosome production and energy metabolism were also up-regulated at 4°C. Proteomic

analysis indicated that protein levels were positively correlated with transcription levels in some pathways under low temperature stress, suggesting the up-regulation of chaperones and energy metabolism pathways. A study of the white rot fungus, *F. velutipes*, showed a similar change in protein profile in response to short-term and long-term exposure to low temperature (Liu *et al.*, 2017b). Of 63 differentially expressed proteins, 31 were up-regulated, 24 of which were involved in energy metabolic pathways, amino acid biosynthesis and metabolism, signalling pathways, transport and translation. Four up-regulated proteins were involved in energy metabolic pathways such as starch and sucrose metabolism (catalase, glucose-6-phosphate isomerase, trehalase and beta-glucosidase). However, catalase was up-regulated only in the short term after exposure to low temperature, and its levels returned to normal after longer exposure to low temperature. Eleven differentially expressed proteins were involved in the biosynthesis of nine amino acids, indicating a role played in cold stress response by modifying nitrogen-containing molecule storage. Signalling molecules and processes involved in protein degradation were also up-regulated, thus suggesting the importance of these molecules in controlling *F. velutipes* mycelium growth and fruiting body formation under cold stress. Proteomic profiling of *M. isabellina* (a soil fungus involved in many biotechnological applications) under low temperature stress identified 44 differentially expressed proteins under cold stress (Hu *et al.*, 2016). These proteins were mainly involved in the regulation of ATP synthesis (ATP synthase subunit beta, ATP synthase subunit δ), glycolytic pathways (fructose-bisphosphate aldolase), protein modification and electron transport (cytochrome c oxidase polypeptide 5B). The responses identified in *M. isabellina* also supported protein degradation pathways playing an important role in cold stress responses in fungi. Proteins such as HSPs and transitional endoplasmic reticulum ATPase (TER ATPase), that are essential in proteasome pathways, were also up-regulated under cold stress.

Integrating the findings of these different studies has led to deeper understanding of cold-adaptation mechanisms in fungi. They respond to low temperature stress by modifying their energy metabolic pathways, increasing the production of chaperones to minimise misfolded protein production, and regulating the synthesis of amino acids that are important in nitrogen storage. In contrast with studies of high temperature stress, the overall response of many fungal species indicates that low temperature does not cause irreversible damage to cells, with responses acting by modifying molecular content rather than wholesale transformation of complex protein networks. The huge amount of information that can be generated in fungal proteomic studies examining low temperature stress can be utilised for more specific approaches, for example in process optimization and purification of cold-adapted biomolecules. Future studies on cold adaptation mechanisms in fungi will benefit from a focus on identifying common proteins that act as cold stress biomarkers and exploiting the advantages of cold-adapted proteins in biotechnological applications.

Table 2.2: Studies of fungal proteomic profiling in response to low temperature stress (published from 2008-2020)

T_{exp} (°C)	T_{opt} (°C)	Time	Species	Origin	# no. of proteins		Proteins identified	Pathways/functions involved	Refs.
1°C	**15°C**	1 week	*F. endolithicus*	Antarctica	466	-	Increased no. of protein spots (41)	*Increased in high molecular weight spots (range from 70 to 170 kDa), suggesting production of cold-acclimation proteins	(Tesei *et al.*, 2012)
1°C	**28°C**	1 week	*E. jeanselmei*	Vienna, Austria	387	-	Increased no. of protein spots (5)	*Changes in expression patterns (spots with MW of 25 – 100 kDa)	
			C. perforans		494		Increased no. of protein spots (169)	*exhibited high molecular weight spots (range from 70 to 170 kDa), suggesting *production of cold-acclimation proteins	
			P. chrysogenum		358		Decreased no. of protein spots (23)	slight decreased indicates downregulation of metabolic activity	
1°C	**37°C**	1 week	*E. dermatitidis*	Data not available	1700	Up	14-3-3 family proteins Minor allergen Alt a7 Nucleoside diphosphate kinase Glyceraldehyde-3-phosphate dehydrogenase	Signalling proteins Small allergen molecules ATP production pathway Glycolytic pathway	(Tesei *et al.*, 2015)
						Down	Acetyl-coenzyme A synthetase Alcohol oxidase Aldehyde dehydrogenase Phosphoenol pyruvate carboxykinase [ATP] Malate synthase, glyoxysomal	Acetate metabolism Alcohol metabolism Aldehyde metabolism Gluconeogenesis Pyruvate metabolism	
1°C	**45°C****	1 week	*E. dermatitidis*	Data not available	1700	Up	14-3-3 family proteins Acetyl-coenzyme A synthetase Minor allergen Alt a7 Nucleoside diphosphate kinase Glyceraldehyde-3-phosphate dehydrogenase Hsp30	Signalling proteins Acetate metabolism Small allergen molecules ATP production pathway Glycolytic pathway Heat shock proteins	
						Down	Alcohol oxidase Aldehyde dehydrogenase Phosphoenol pyruvate carboxykinase [ATP] Beta-lactamase Hsp70-like protein	Alcohol metabolism Aldehyde metabolism Gluconeogenesis Antibiotic resistance proteins Heat shock proteins	
4°C	**12°C**	NA	*M. psychrophila*		1673	Up	GLNA MPSY protein	Amino acid metabolism MFS transporter	

				Qinghai-Tibet, China		Down	citrate synthase and LSC2 proteins PDC	TCA cycle Glycolysis	(Su *et al.*, 2016)
12-15°C	**23-25°C**	2 weeks	*Flammulina velutipes*	Data not available	1198	Up	Trehalase (Fragment) Proteasome subunit β type	Trehalose metabolism Proteosome pathway	(Liu *et al.*, 2017b)
						Down	60s ribosomal protein Acetylornithine aminotransferase Glutathione-disulfide reductase Adenylosuccinate synthetase 1	Heat shock proteins Urea cycle Oxidative stress pathway Purine biosynthesis	
12-15°C	**23-25°C**	3 days	*Flammulina velutipes*	Data not available	1198	Up	Heat shock cognate 70 Catalase	Heat shock proteins Catalytic enzymes	
						Down	Mitochondrial cytochrome T-complex protein eta subunit (Tcp-1-eta) Serine hydroxymethyltransferase Transketolase Methylmalonate-semialdehyde dehydrogenase	Electron transport chain Chaperonins Serine metabolism Pentose phosphate pathway Amino acid metabolism	
15°C	**30°C**	24 hours	*Mortierella isabellina M6-22*	China	1800	Up	fructose-bisphosphate aldolase cytochrome c oxidase polypeptide 5B ATP synthase subunit β, ATP synthase δ subunit E3 ubiquitinprotein ligase BRE1, and histone acetyltransferase GCN5	Glycolytic pathway Electron transport ATP production Ubiquitin–proteasome pathway	(Hu *et al.*, 2016)

* Non-conclusive remarks (no protein identification involved)

** 45°C is not the optimal growth temperature for *E. dermatitidis*, rather an experimental high temperature stress

2.4 Effects of temperature stress on fungal proteomes

Fungi respond to temperature stress through regulation of various proteins, that can be visualised using proteomic profiling techniques within the limitations noted above. Changes in fungal proteomes exposed to various temperature stresses (Tables 1, 2) demonstrate that many cellular functions are affected, such as the TCA cycle and energy production and metabolism, oxidative phosphorylation, regulation of mitotic activities, cell cycle and division, transcriptional and translational stages of biosynthetic pathways, cell wall and cytoskeleton reorganisation, transport protein remodelling, defence against oxidative and other stresses, and cellular signalling mechanisms. Undeniably, these proteins and their roles in cellular functions are highly significant, with the complex cellular protein network maintained through protein homeostasis (Mühlhofer *et al.*, 2019; O' Neill *et al.*, 2020). It is currently extremely challenging to identify any specific set of proteins as being the most affected by or vulnerable to temperature stress, or being used as potential temperature stress biomarkers. Metabolic pathways and HSPs are considered a primary focus and elaborated below. These two components are crucial in energy regulation and protein turnover, and hence for cell survival (Yan *et al.*, 2020). More widely, biosynthesis and regulation of metabolic pathways and HSPs are of importance in many applied fields such as agriculture, food biotechnology and medicine (Ene *et al.*, 2014; Sarmiento *et al.*, 2015; Lamoth *et al.*, 2016).

2.4.1 Effects of temperature stress on metabolic pathways

Fungal adaptation to temperature stress involves alterations in the utilisation of many metabolic pathways in trade-offs to compensate for the amount of energy needed or the extra energy to be utilised in delivering the stress response. As temperature increases, the rate of metabolism (of a non-endothermic organism) increases as a purely physical consequence, and then rapidly declines at higher temperatures as the metabolic systems start to fail – eventually leading to cell death. Metabolic regulation is one strategy

employed by organisms to adapt to temperature stress, which is controlled by factors such as concentration of substrates, products or allosteric effectors (Suarez & Moyes, 2012; Clarke, 2018). Understanding thermal adaptation in fungi requires an overall understanding of the effects of temperature on pathway flux, including such things as the roles and limitations of enzymes involved, concentration of substrates and products, and other protein and non-protein components (Schulte, 2015). Advances in proteomic technologies have made it possible to determine many of the proteins involved in the complex network of metabolic pathways, as low as in zeptomole-scale mixtures (Swaminathan *et al.*, 2018), but it remains challenging to accurately identify and allocate all protein molecules involved into their various pathways (Timp & Timp, 2020). However, many studies have developed illustrative diagrams of complex protein-protein interaction networks, and it is accepted that temperature stress causes quantifiable changes in these metabolic pathways compared to non-stress conditions (Kostadinova *et al.*, 2011; Bai *et al.*, 2015).

Glycolysis is an important metabolic pathway in most organisms, including fungi. Many fungi produce enzymes to break down complex polysaccharides such as cellulose, hemicellulose, pectin and starch to produce glucose (Krishnan *et al.*, 2016; Xiong *et al.*, 2017). Studies have confirmed that fungi produce a range of extracellular hydrolases to help them utilise different carbon and nitrogen sources under exposure to temperature stress (Krishnan *et al.*, 2017; Tajuddin *et al.*, 2018). The production of these enzymes responds to changing environmental temperature, in order to maintain metabolic requirements and protein turnover. As noted earlier, trehalose production is also important for fungi under temperature stress. Trehalose is known to be important in the acquisition of thermotolerance and desiccation tolerance in many fungal species (and much more widely across multiple groups of biota) (Gancedo & Flores, 2004; Tereshina, 2005; Everatt *et al.*, 2015; Liu *et al.*, 2019). In *Saccharomyces cerevisiae*, trehalose and

intracellular water stabilise the membrane structure and other intracellular networks under temperature stress conditions (Piper, 1993). Various studies document increased activity of trehalases, such as trehalose synthase and fructose-1,6-biphosphatase, in response to heat stress (Bonini *et al.*, 1995; Cai *et al.*, 2009). In addition, enzymes such as cAMP-dependent protein kinase and plasma membrane ATPase also play significant roles in fungal thermotolerance determination (Piper, 1993; Jurick II *et al.*, 2004). Enolase, an enzyme that converts 2-phosphoglyceric acid to phosphoenolpyruvic acid in glycolysis, is also crucial in the heat stress response of many fungal species and yeasts (Ji *et al.*, 2016). It has been suggested that enolase (ENO1) is closely involved in heat stress responses, and ENO1 of yeasts and streptococcal strains isolated from rats has high thermal stability (Iida & Yahara, 1985; Kustrzeba-Wójcicka & Golczak, 2000; Cuéllar-Cruz *et al.*, 2013).

2.4.2 Effects of temperature stress on heat shock proteins (HSPs)

In the event of heat stress, HSPs are produced in cells to protect proteins from aggregation, unfold or refold aggregated proteins, or target them for the degradation pathway. Although initially named following discovery in heat stress experiments, HSPs are a part of general stress responses, produced when cells and organisms are exposed to multiple types of stressors. Most HSPs are molecular chaperones produced constitutively or induced upon cell stress, which can be triggered by a temperature change of just a few degrees (Richter *et al.*, 2010). Heat shock proteins are primarily categorised and named according to their molecular mass, which varies from 10 to 100 kDa. Their multifunctional properties have made them an important and reliable target biomarker in various fields such as crop management, plant and microbe adaptation towards environmental stress and cancer related studies (Jee, 2016). HSPs are known to play a role in temperature-stressed environments (Tiwari *et al.*, 2015), but understanding the complexity of the HSP network as a defence mechanism in the cellular environment

requires much further research. The cellular functions of the various HSPs that are involved as part of the heat shock response network in fungi, using *S. cerevisiae* as a model, have been discussed in depth (Verghese *et al.*, 2012). Evidence suggests that HSPs are involved in cell cycle arrest (Vergés *et al.*, 2007), metabolic reprogramming (Elliott *et al.*, 1996), modulating cell wall and membrane dynamics (Winkler *et al.*, 2002; Truman *et al.*, 2007; Shaner *et al.*, 2008) and protein aggregation (Nathan *et al.*, 1997).

Heat shock protein 60 (HSP60) is a highly conserved molecule in many organisms and has been found to respond to various stress conditions in fungi. This HSP is up-regulated in response to increased temperature in species such as *A. fumigatus*, *Aspergillus terreus*, *P. chrysogenum*, *Scedosporium apiospermum*, *Trichophyton mentagrophytes*, *Candida albicans* and *S. cerevisiae* (Raggam *et al.*, 2011). HSP60 has also been proposed to play a role in the assembly of precursor polypeptides into oligomeric complexes following incorporation into the mitochondrial matrix (Patriarca & Maresca, 1990). Galello *et al.* (2014) recently described the interaction of HSP60 and Ira2 with Bcy1, a regulatory subunit of protein kinase A (PKA) in *S. cerevisiae*. Using an MS-based proteomic approach, this study demonstrated that HSP60 localized the entire PKA-Ras complex to mitochondria under the regulation of the cAMP-PKA-signalling pathway. There are also reports of HSP70 having a role in enhancing fungal resistance to heat stress and other abiotic stresses (Montero-Barrientos *et al.*, 2008; 2010). The overexpression of HSP0 genes in *Trichoderma harzianum* T34 resulted in an increase in biomass and enhanced tolerance to other abiotic stresses after heat shock treatment (Montero-Barrientos *et al.*, 2008). Subsequently, the function of the HSP70 gene from *T. harzianum* T34 was further studied in the transgenic plant, *Arabidopsis thaliana*, showing that the transgenic plants exhibited higher tolerance towards heat stress (Montero-Barrientos *et al.*, 2010). HSP90, one of the most ubiquitous chaperones in yeasts (Nathan *et al.*, 1997), was found to interact with calcineurin (Imai & Yahara, 2000) and respond to heat stress indirectly

through facilitating the activation of the MAPK complex (Truman *et al.*, 2007). More recent work has shown that HSP90 plays a central role in heat stress responses in *Fusarium graminearum*, in addition to its crucial roles in fungal vegetative growth, reproduction and virulence (Bui *et al.*, 2016). In *A. fumigatus*, HSP90 has been implicated in drug resistance, as disruption of the HSP90 circuitry leads to activation of the antifungal agent caspofungin (Lamoth *et al.*, 2016).

2.5 *Pseudogymnoascus* spp.

Pseudogymnoascus is a fungal genus belonging to the family Pseudeurotiaceae and phylum Ascomycota. This genus is characterised by considerable variation in morphology and the structures of peridial hyphae and ascospores (Tsuneda, 1982; Rice & Currah, 2006; Chaturvedi *et al.*, 2010). Psychrotolerant fungi have been defined as species that can grow close to 0°C, have the optimum growth temperature > 15°C, and the maximum growth temperature of > 20°C (Morita, 1975). In general, *Pseudogymnoascus* spp. are psychrotolerant (cold tolerant) fungi commonly identified to be anamorphic based on the absence of evidence of sexual reproduction (Kochkina *et al.*, 2007; Hayes, 2012). Previously known as *Geomyces*, many species were reclassified to *Pseudogymnoascus* based on molecular phylogenetic analysis (Minnis & Lindner, 2013). Since then, many studies have been carried out to further identify and reclassify *Pseudogymnoascus* spp. based on phylogenetic and genomic analyses and assembly (Shuey *et al.*, 2014; Leushkin *et al.*, 2015; Palmer *et al.*, 2018). Two species, *P. destructans* (Blehert & Gargas) Minnis & D.L. Lindner and *P. pannorum* (Link) Minnis & D.L. Lindner, have gained particular interest among researchers worldwide. *Pseudogymnoascus destructans* is a pathogenic fungus associated with white-nose syndrome (WNS) in bats (Blehert *et al.*, 2009; Gargas *et al.*, 2009), while *P. pannorum* is found in soils and is widely distributed globally, but is only rarely associated with human and canine diseases (Christen-Zaech *et al.*, 2008; Chaturvedi *et al.*, 2018).

Pseudogymnoascus spp. are known producers of many extracellular hydrolases that contribute to soil decomposition processes, including ureases (Zhang *et al.*, 2015), proteases (Pannkuk *et al.*, 2015), endopeptidases (O'Donoghue *et al.*, 2015) and laccases (Kolomytseva *et al.*, 2017). Furthermore, *Pseudogymnoascus* spp. have been isolated from crude-oil contaminated soils in the Ecuadorian Amazon rainforest (Maddela *et al.*, 2015) and linked to the biodegradation of diesel oil in Antarctic soils (Donovan *et al.*, 2018). A recent study of fungal isolates from lake sediments in the Antarctic Peninsula reported that *Pseudogymnoascus* spp. demonstrate selective trypanocidal and herbicidal activities (Ogaki *et al.*, 2020). Moreover, *Pseudogymnoascus* spp. have also been suggested for use in various biotechnological applications, such as the potential use of its lipase in detergent formulations (Sahay & Chouhan, 2018) and promising applications in the elimination of polycyclic aromatic hydrocarbons (PAHs) and phenols from hyperalkalophile industrial effluents (Batista-García *et al.*, 2017).

CHAPTER 3: THERMAL TOLERANCE AND SENSITIVITY OF PSEUDOGYMNOASCUS SPP. FROM DIFFERENT GEOGRAPHICAL REGIONS

3.1 Introduction

Temperature changes alter the relative abundance of species that regulate soil ecosystem processes, thus leading to further changes in community composition (Ladau *et al.*, 2017). To humans, these changes in diversity can indirectly reduce the quality and quantity of food sources, if the ecosystems involved include agricultural areas used in crop production (Chapin *et al.*, 2000). In the soil ecosystem, fungi are considered to be very successful and key community members because of their high plasticity and ability to adopt various forms in response to adverse or unfavorable conditions (A'Bear *et al.*, 2014c). In the present study, the genus *Pseudogymnoascus* was chosen as a model organism to understand soil fungal response to temperature stress. Other than high abundance and easy to culture, members of *Pseudogymnoascus* are also well known to play important roles in the soil environment (Maddela *et al.*, 2015; Donovan *et al.*, 2018). They are widely reported from polar and temperate regions, where they are involved in various soil decomposition processes and play major roles in bioremediation of many pollutants (Zhang *et al.*, 2015; Donovan *et al.*, 2018). The phenotypic diversity of *Pseudogymnoascus* spp. is of great interest in understanding underlying adaptation characteristics of fungi towards temperature stress. The objective of this study was to determine the thermal tolerance and sensitivity of *Pseudogymnoascus* spp. originating from different global geographical regions.

3.2 Literature review

Fungal adaptation towards any stress is as achieved through ‘tolerance mechanisms’. Other than producing spores, fungi also initiate stress tolerance strategies at the molecular level. Fungi undergo biochemical changes such as an increased production of trehalose

in the mycelial structures (Al-Bader *et al.*, 2010), the production of various pigments (Sumathy *et al.*, 2007), the production of anti-freeze proteins coating the cell wall, the production of extracellular hydrolases such as chitinolytic enzymes (Fenice, 2016) and increased production of heat shock proteins to minimize protein misfolding (Kroll *et al.*, 2014; Miteva-Staleva *et al.*, 2017). All of these are strategies that assist in tolerance of environmental stress and increase the chances of reproduction in non-optimal environments (Williams *et al.*, 2011). The study of low temperature stress, or cold stress, in microorganisms has led to many beneficial findings worldwide. Proteins, such as cold-adapted enzymes, have been applied in many biotechnological applications such as in manufacturing of food and beverages, detergents, textiles and industrial molecular biology (Sarmiento *et al.*, 2015). Research on cold-adapted proteins often involves psychrophilic microorganisms from the polar regions and deep oceans (Cavicchioli *et al.*, 2000). Psychrophiles produce cold-adapted proteins with unique characteristics that have weak protein interactions, low thermal stability and increased specific activity, in order to achieve higher protein activity and flexibility at low temperatures (Reed *et al.*, 2013). However, in comparison with high temperature stress, the overall response of many fungal species showed that low temperature did not cause serious damage to cells, thus implying the effect of modifying biomolecular content rather than large-scale transformation of complex protein networks. A recent review of fungal adaptation to cold environments provides an overview of their life history strategies and highlights the importance of cold-adapted fungi in the discovery of novel secondary metabolites and enzymes (Wang *et al.*, 2017).

The diversity and abundance of *Pseudogymnoascus* spp. in soil environments have been reported globally. Various research works have been carried out to understand their thermal tolerance or growth rates under temperature variation (Krishnan *et al.*, 2017; Misiak *et al.*, 2021). Previous studies showed that the optimum growth rate for strain

HND16 R2-1 sp.2 and another *Pseudogymnoascus* strain from the Antarctic (AK07KGI503 R2-1 sp. 3) is achieved at 20°C, consistent with them being psychrotolerant (Tajuddin *et al.*, 2018; Tajuddin *et al.*, 2019). In this study, a comparison of growth rates and morphological changes of six isolates of *Pseudogymnoascus* spp. originating from different geographical regions was made in order to characterise the thermal tolerance of this genus. The data obtained provide baseline information on *Pseudogymnoascus* spp. responses to temperature stress.

3.3 Methodology

3.3.1 Selection of study strains and maintenance of cultures

Four isolates of *Pseudogymnoascus* spp. from the Arctic (*Sp1* and HND16 R2-1 sp.2) and Antarctic (AK07KGI1202 R1-1 sp.3 and AK07KGI1202 R1-1 sp.4) regions were obtained from the culture collection of the National Antarctic Research Centre (NARC), Universiti Malaya, Malaysia. Isolation, identification and phylogenetic analysis of these isolates is described by (Wong, 2019). All four isolates cluster within an undescribed group of *Pseudogymnoascus* strains (Wong, 2019). Two further isolates of *P. pannorum* (CBS 106.13 and CBS 107.65) from the temperate region (Switzerland and Germany) were purchased from the Centraalbureau voor Schimmelcultures (CBS-KNAW) Fungal Biodiversity Centre (Utrecht, The Netherlands). All isolates were grown on Czapek-Dox agar and kept at 5°C for culture maintenance purposes.

Table 3.1 *Pseudogymnoascus* isolates with information on their origin and identification codes.

Taxon name	Isolate code	Code used in text	Sampling location	Region	Mean annual temperature (approximate)	Mean annual precipitation (approximate)	Collection centre	GenBank Accession number
Pseudogymnoascus sp.	HND16 R4-1 sp.1	*sp1*	Hornsund, Spitsbergen	Arctic	3 – 12°C	15 mm w.e	NARC, Malaysia	MK443476
Pseudogymnoascus sp.	HND16 R2-1 sp.2	*sp2*	Hornsund, Spitsbergen	Arctic	3 – 12°C	15 mm w.e	NARC, Malaysia	MK443477
Pseudogymnoascus sp.	AK07KGI12 02 R1-1 sp.3	*sp3*	Fildes Peninsula, King George Island	Antarctic	-2 – 8°C	50 mm w.e	NARC, Malaysia	MK443474
Pseudogymnoascus sp.	AK07KGI12 02 R1-1 sp.4	*sp4*	Fildes Peninsula, King George Island	Antarctic	-2 – 8°C	50 mm w.e	NARC, Malaysia	MK443475
P. pannorum	CBS 106.13	*C106*	Sainte-Croix, Switzerland	Temperate	9°C	1800 mm w.e	CBS-KNAW Fungal Biodiversity Centre	MH854616
P. pannorum	CBS 107.65	*C107*	Schleswig-Holstein, Kiel-Kitzeberg, Germany	Temperate	8.7°	853 mm w.e	CBS-KNAW Fungal Biodiversity Centre	MH858505

* mm w.e : mm water equivalent

3.3.2 Temperature-dependent growth curve studies

Fungal colony plugs (diameter 5 mm) were inoculated onto fresh Czapek-Dox agar at the center of prepared Petri dishes. Fungal colony diameter was measured every 24 h over a period of 10 d. The inoculations were done in a laminar flow hood with off-airflow to minimise contamination from stray spores, as described by Misiak *et al.* (2021). Morphological changes were recorded, specifically relating to colony pigmentation and mycelial elevation. A time-course experiment was performed where plates of each fungal strain were exposed to one of a selected temperature range (5°C, 10°C, 15°C, 20°C, 25°C or 30°C) and growth (colony diameter) was recorded daily over 10 d. Colony diameters were measured using a Vernier caliper (±0.1mm) every 24 h. Fungal colony morphology assessment was performed on day 10 of growth. Temperature-dependent growth curves were plotted of radial growth measurements (mm), analysed using Gompertz and Richards' logistics growth models (Annadurai *et al.*, 2000). All experiments were conducted in triplicate.

3.4 Results

All six isolates showed significant changes in colony appearances and marginal structures with increasing temperature (Figures 3.1a-3.6a). Three isolates - *sp1*, *C106*, and *C107* - appeared as translucent colonies when exposed to 5°C and 10°C, but turned opaque with slightly yellowish pigmentation when exposed to 20°C and 25°C (Figures 3.1a, 3.5a, 3.6a). For isolates *sp2* and *sp3*, colony morphology showed an increase of yellow pigmentation with increasing temperatures and changes in marginal structures from filiform to an undulating margin (Figures 3.2a, 3.3a). For isolate *sp4*, an increase in temperature caused a change of colony pigmentation from slightly pink at 10°C and 15°C, to yellowish at 20°C and 25°C (Figure 3.4a).

Colony extension rates of each strain were plotted for all six temperature exposures to identify the optimum temperature for growth (Figures 3.1b-3.6b). Calculated values of colony diameter at each time point were analyzed using multivariate analysis of variance (MANOVA), using Bonferroni's test to make *a posteriori* comparison. As shown in Figures 3.1b-3.6b, colony diameter extension increased with increasing temperature, with the greatest colony diameters recorded at 15°C or 20°C, depending on the strain. Analysis of variance showed a significant multivariate effect for the colony diameter of different strains as a group in relation to the temperature, $F_{10,241} = 15.110$, $P < 0.001$, with a small effect size, $\eta 2 = 0.562$ and observed power 1.0. The Arctic strains (isolates s*p1* and *sp2*) and the temperate strains (isolates *C106* and *C107*), showed optimum growth at 20°C after 10 d. For the Antarctic strains (isolates *sp3* and *sp4*) the optimum temperature for growth was 15°C.

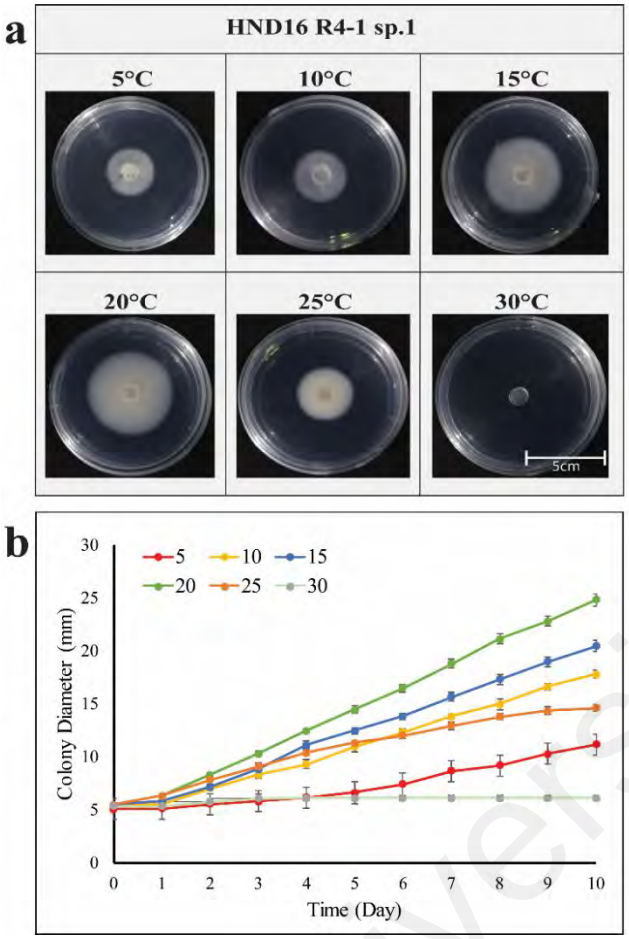

Figure 3.1: Colony morphological changes of isolate *sp1* after 10 d exposure to six different temperatures (a). Isolate appeared as translucent colony under cold stress (5°C and 10°C) and increased in yellowish pigmentation under heat stress (20°C and 25°C). Calculated mean and standard deviation values of colony extension of isolate recorded during 10 d growth experiments at six different temperatures (b).

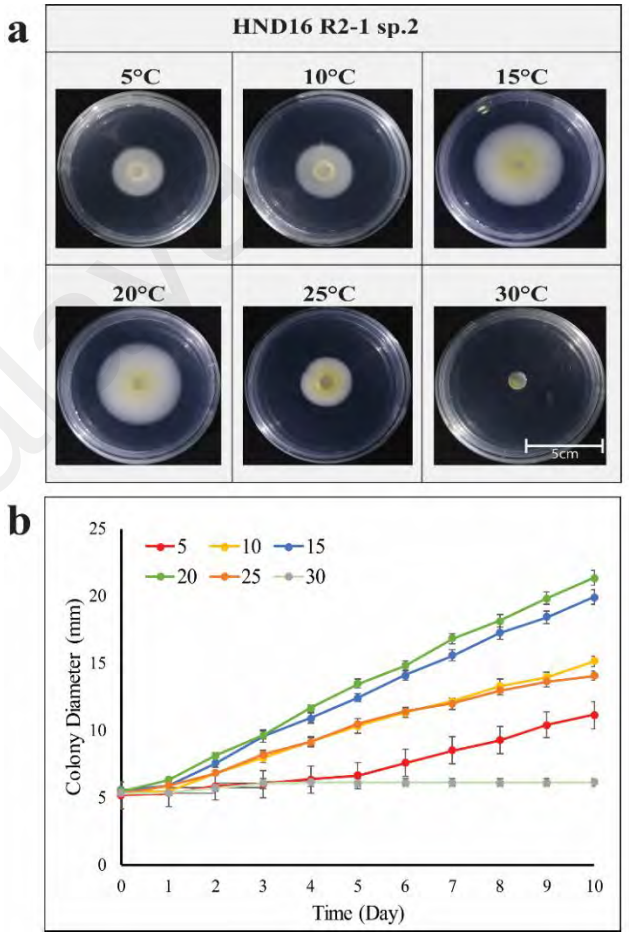

Figure 3.2: Colony morphological changes of isolate *sp2* after 10 d exposure to six different temperatures (a). Increasing heat induced pigmentation and changes in marginal structure of isolate. Calculated mean and standard deviation values of colony extension of isolate recorded during 10 d growth experiments at six different temperatures (b).

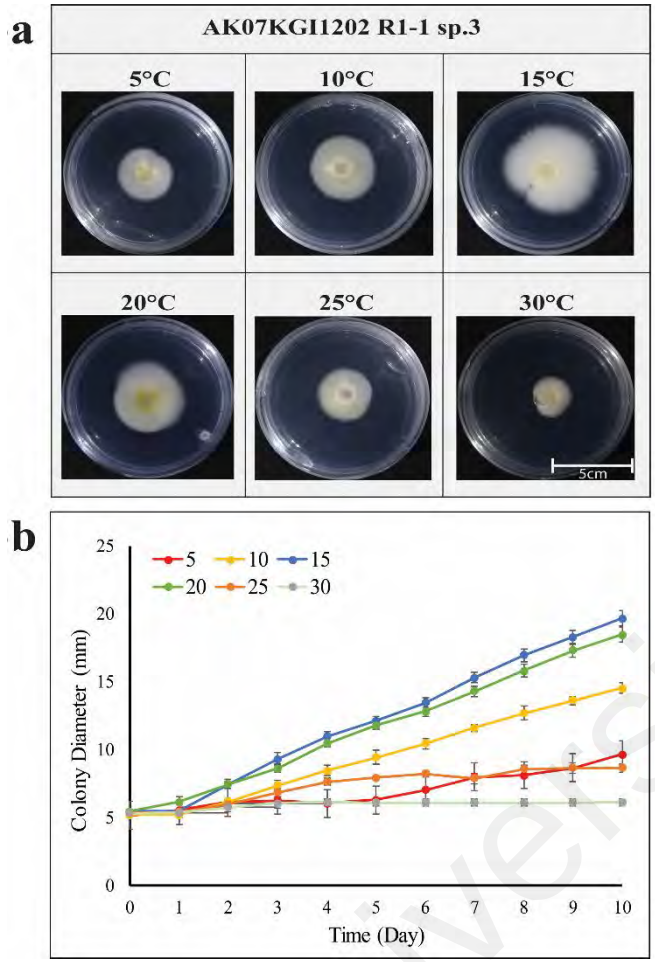

Figure 3.3: Colony morphological changes of isolate *sp3* after 10 d exposure to six different temperatures (a). Heat stress induced pigmentation and changes in marginal structure of isolate (20°C, 25°C and 30°C). Calculated mean and standard deviation values of colony extension of isolate recorded during 10 d growth experiments at six different temperatures (b).

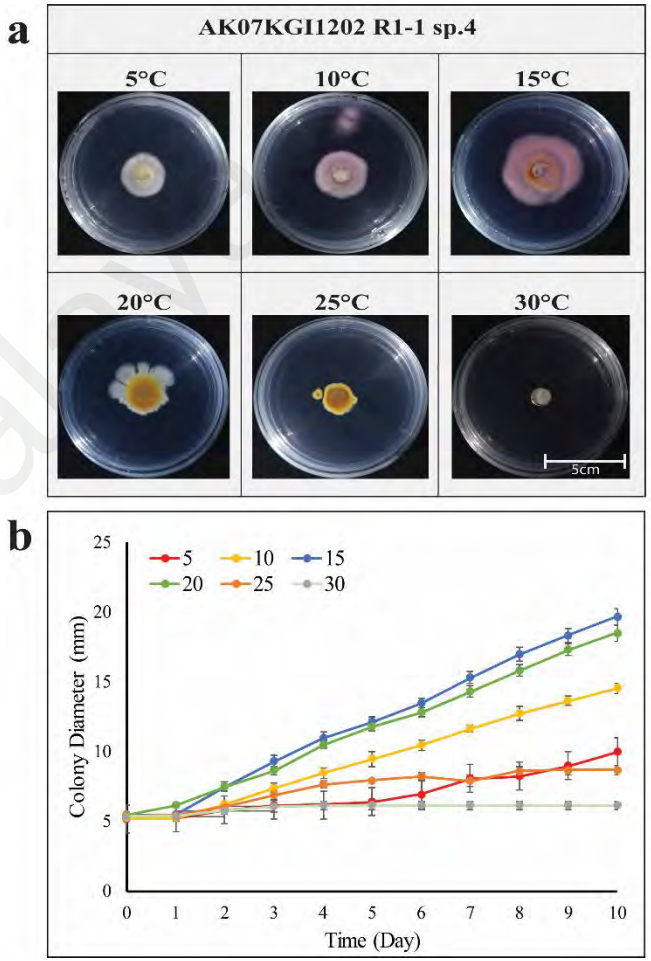

Figure 3.4: Colony morphological changes of isolate *sp4* after 10 d exposure to six different temperatures (a). Isolate appeared as slightly pink colony at 10°C and 15°C, but became yellowish at 20°C and 25°C. Calculated mean and standard deviation values of colony extension of isolate recorded during 10 d growth experiments at six different temperatures (b).

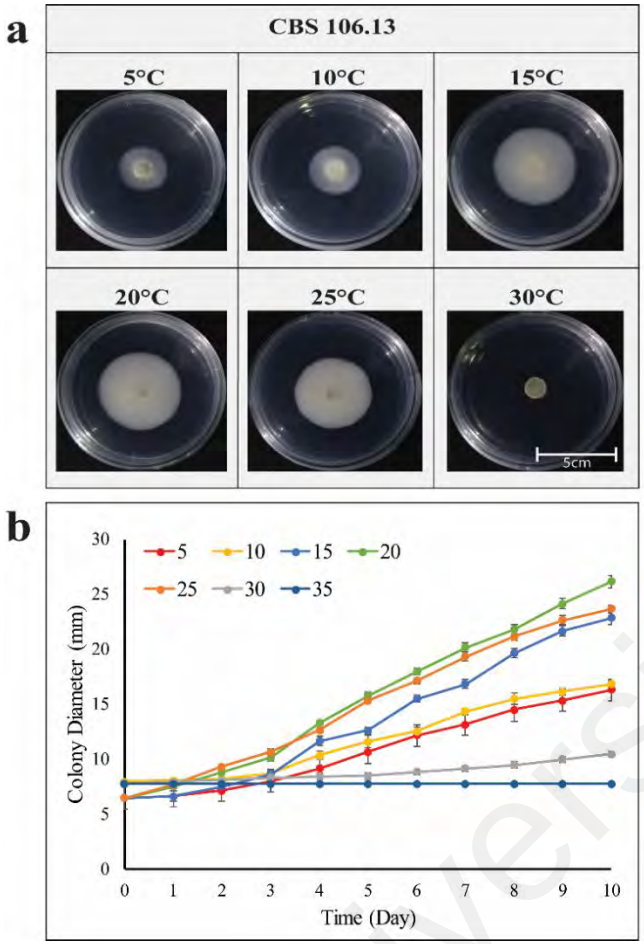

Figure 3.5: Colony morphological changes of isolate *C106* after 10 d exposure to six different temperatures (a). Isolate appeared as translucent colony under cold stress (5°C and 10°C), but turned opaque with increasing temperatures (20°C and 25°C). Calculated mean and standard deviation values of colony extension of isolate recorded during 10 d growth experiments at six different temperatures (b).

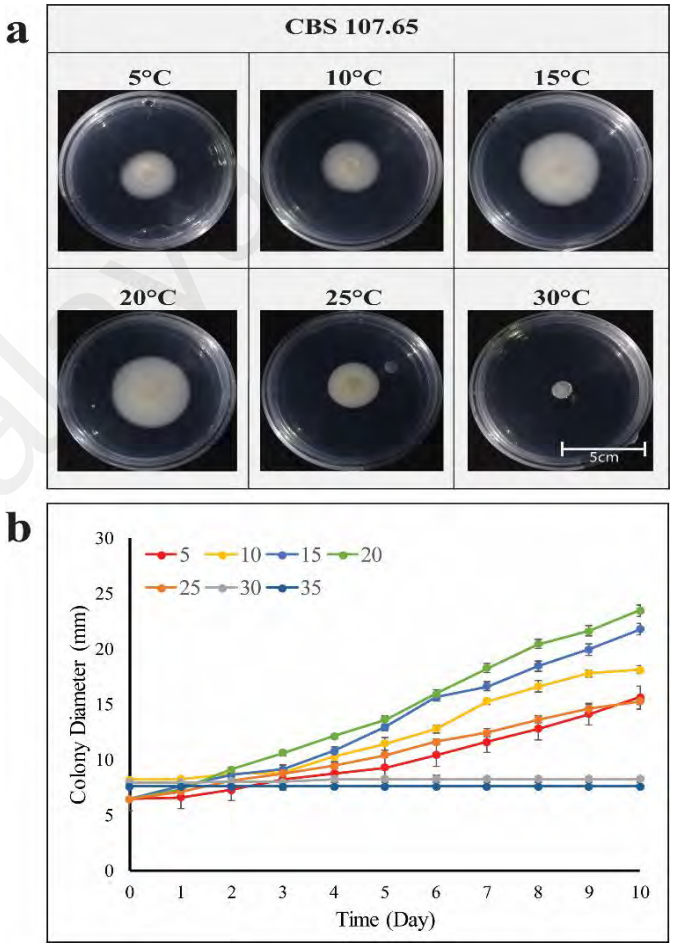

Figure 3.6: Colony morphological changes of isolate *C107* after 10 d exposure to six different temperatures (a). Isolate appeared as translucent colony under cold stress (5°C and 10°C), but turned opaque with yellowish pigmentation under heat stress (25°C). Calculated mean and standard deviation values of colony extension of isolate recorded during 10 d growth experiments at six different temperatures (b).

Figure 3.7 shows the temperature-dependent growth curves of all six isolates based on mean fungal colony diameter on day 10 of incubation with individual best-fitting curves generated using Gompertz and Richards' logistics growth models. The four polar isolates had optimum growth temperature between 15°C and 20°C with no significant differences between the two temperatures (Figures 3.7a, c). There were also no significant differences in mean fungal colony diameters achieved between 25°C and 10°C for these four isolates. The two temperate isolates had optimum growth temperatures of 20°C (Figure 3.7b). The growth characteristics of all six isolates are consistent with them being psychrotolerant fungi.

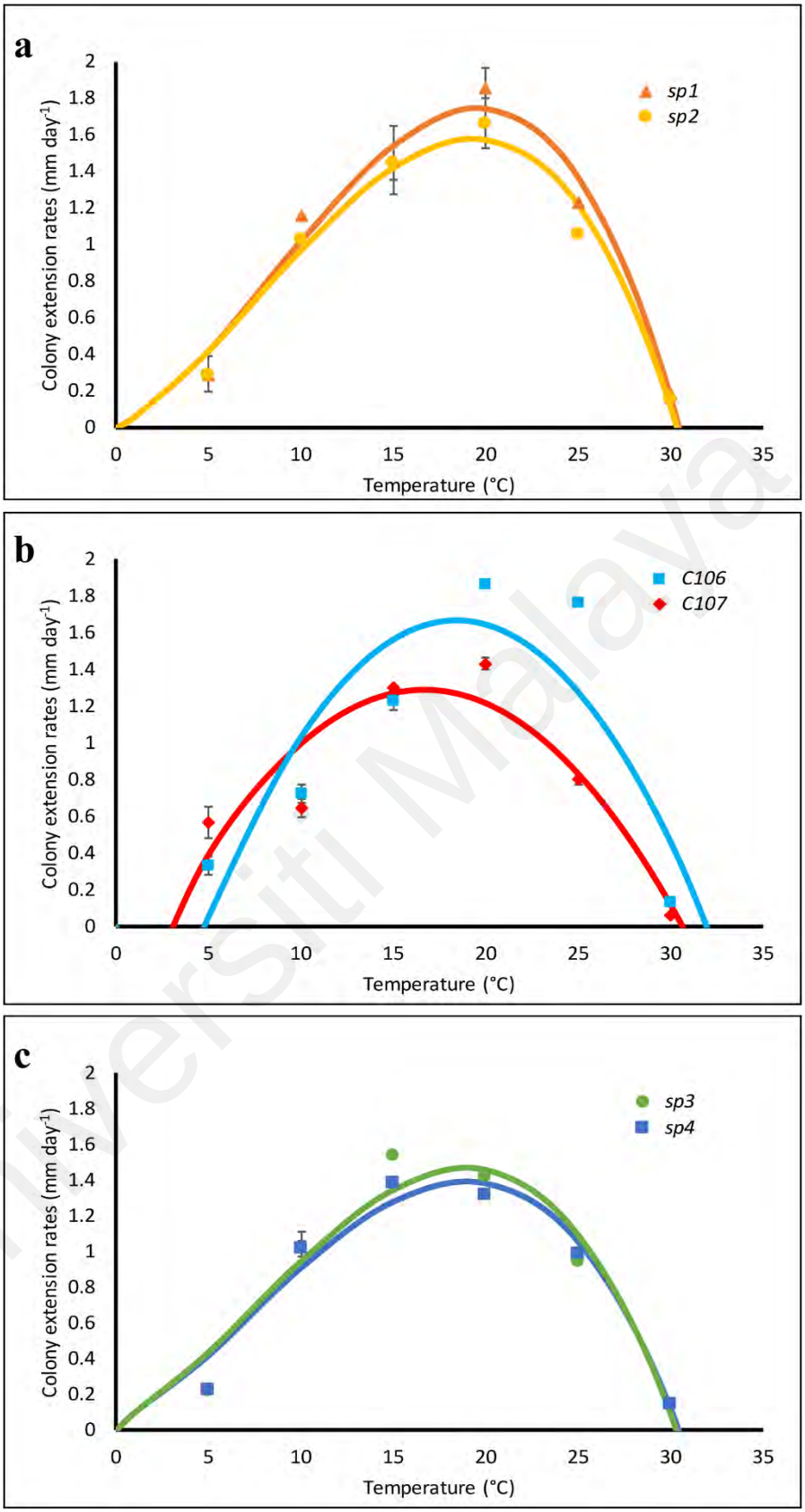

Figure 3.7: Temperature-dependent growth curves of six *Pseudogymnoascus* spp. isolates originating from different global geographical regions. a) Arctic; b) Temperate; c) Antarctic. There were no significant differences between growth at 15°C and 20°C, and 10°C and 25°C, for the four polar isolates. There was also no significant difference between 5°C and 10°C for the temperate isolate *C107*.

3.5 Discussion

The concept of environmental stress has been discussed and reviewed by many researchers (Hoffman & Parsons, 1994; Parsons, 2005; Nevo, 2011). Variation in stress may be represented in a continuum of fitness – stress, where fitness is inversely related to stress levels (Nevo, 2011). Temperature stress is a physiological stress that can hinder function and cause injury or death due to excessive heat or cold. Different biological systems vary in their range of temperature tolerance, which influences their ability to survive sudden changes in their environment. Under temperature stress, pigment production has been suggested to be one of the mechanisms of heat tolerance activated by fungi (Sumathy *et al.*, 2007). Changes in colony morphology are also regarded as a stress indicator in fungi (Sterflinger & Krumbein, 1995).

The six *Pseudogymnoascus* spp. isolates studied here, originating from different global geographical regions, showed a wide range of phenotypic variation and differing patterns of thermal tolerance. *Pseudogymnoascus* spp. isolated originating from polar and temperate regions had optimum growth temperatures of between 15°C and 20°C, and 20°C, respectively.

Phylogenetic analysis of the four isolates from the polar regions were inconclusive, only being able to identify them as *Pseudogymnoascus* sp. (Wong, 2019). Initial BLAST results of 28S nuclear ribosomal large subunit rRNA gene (LSU) sequences obtained from these isolates showed 99-100% identity scores with *Geomyces pannorum*/*P. pannorum* (Wong, 2019). *Pseudogymnoascus* spp. are ubiquitous fungi in the soil ecosystem, often associated with cold environments (Barratt *et al.*, 2003; Arenz *et al.*, 2011; Hayes, 2012). They have a wide range of growth temperature (4°C to 25°C) with many species classified as psychrotolerant (Ali *et al.*, 2013; Krishnan *et al.*, 2016; Kolomytseva *et al.*, 2017). A recent study of one of the isolates used here, *sp2*, together

with another strain of *Pseudogymnoascus* sp. from the Antarctic (AK07KGI503 R2-1 sp. 3), showed both had optimum growth temperature of 20°C (Tajuddin *et al.*, 2018). Other factors should be taken into consideration in such growth studies, such as the relationship of the activation energy (E_a) and Q_{10} values of the isolate with temperature (Tajuddin *et al.*, 2018; Tajuddin *et al.*, 2019). Although the data obtained here identified the temperature for optimum growth to be between 15°C and 20°C, with no induction of stress-related morphology at these temperatures, *Pseudogymnoascus* spp. have a wide range of temperature tolerance. No growth was observed in any of the six isolates at temperature below 5°C or above 30°C. In situ analysis conducted on Alexander Island in the southern maritime Antarctic using *Pseudogymnoascus roseus* showed that the growth and extracellular enzymes activities of the fungi were inhibited when exposed to temperatures above 20°C (Misiak *et al.*, 2021). In this work, only minimal growth was observed after 10 d incubation at 5°C and 25°C. The temperature-growth characteristics of all six isolates confirm that they are psychrotolerant and it is unlikely for these isolates to grow at temperatures of more than 25°C in their natural environment (Misiak *et al.*, 2021).

3.6 Conclusions

Pseudogymnoascus is a genus of filamentous fungi widely distributed in soil ecosystems of the polar and temperate regions. The key role of *Pseudogymnoascus* spp. in decomposition processes in the soil make members of the genus ideal candidates for use as model organisms in soil environmental studies. The data obtained in this study confirm that the six *Pseudogymnoascus* spp. isolates examined have wide phenotypic variation and optimum growth temperatures of 15-20°C and a growth temperature range of 5-25°C, defining them as psychrotolerant. The temperature-related growth characteristics documented here provide baseline criteria for the studies of proteomic responses of these isolates to cold and heat stress described in later chapters.

CHAPTER 4: PROTEOMIC CHARACTERISATION OF PSEUDOGYMNOASCUS SPP. USING TANDEM MASS SPECTROMETRY

4.1 Introduction

Proteomic approaches are increasingly being used in the field of mycology. The objective is mainly to aid in understanding the complex network of protein-protein interactions that have been implicated in certain molecular functions of fungi (Bhadauria *et al.*, 2007). For soil fungi, the majority of proteomic research to date has been focused on fungal-plant interaction and how fungal proteomes may affect the quality of plants and soil (Kroll *et al.*, 2014). However, it is also a useful tool to compare metabolic differences within a genus (Pigosso *et al.*, 2013). *Pseudogymnoascus* is a widespread fungal genus with representatives found in many soil ecosystems, playing important roles in decomposition through the production of many extracellular hydrolase enzymes (Maddela *et al.*, 2015; Krishnan *et al.*, 2016). Some studies have determined specific hydrolase enzymes produced by *Pseudogymnoascus* spp. isolated from polar soils (Tajuddin *et al.*, 2018; Tajuddin *et al.*, 2019). However, despite the acknowledged importance of *Pseudogymnoascus* spp. in soil ecosystems, few research studies have addressed molecular functions within the genus as yet. Thus, the objectives of this study were, 1) to optimize a protein extraction method for *Pseudogymnoascus* spp., and 2) to characterise *Pseudogymnoascus* spp. isolated from different regions globally through proteomic profiling. The data generated are intended to contribute to the baseline information available about *Pseudogymnoascus* spp. proteomes and support research into the roles *Pseudogymnoascus* spp. in soil ecosystems.

4.2 Literature review

The use of various '-omic' approaches that analyse different molecules (e.g. proteins, mRNA, metabolites) benefits research aiming to understand the complex biological networks within an organism (Bai *et al.*, 2015; Donovan *et al.*, 2018; Fricker, 2018). In

the field of soil biology and biochemistry, proteomics have been used to link between the phylogeny and functionality of soil microorganisms and advance understanding of the effects on deforestation, contamination and agricultural management regimes (Bastida & Jehmlich, 2016; Liu *et al.*, 2017a). For instance, investigation of microbial community functions in contaminated soils using a proteomic approach provided baseline information about the molecular functions that contribute to *Fusarium* wilt in infected tomatoes (Manikandan *et al.*, 2017). Proteomics have also been used to identify strategies used by various human pathogens such as *Paracoccidioides* spp. (Parente-Rocha *et al.*, 2018) and *Exophiala dermatitidis* (Tesei *et al.*, 2015) to establish infection in their host. Proteomic profiling is a very useful approach to give basic information on the spectrum of protein interactions in cells (Zakharova *et al.*, 2014). Information generated in such studies also enables researchers to explore the potential for use of proteins in various fields and biotechnological applications.

Considerable effort has been invested in fungal proteomics to identify specific biological or molecular functions that are affected in certain experimental conditions. Proteomic approaches have also been used to identify proteins responsible for dimorphism characteristics in dimorphic fungi, such as *Paracoccidioides brasiliensis*, *P. lutzii* and *Penicillium marneffei* (Chandler *et al.*, 2008; Gauthier, 2017). Detailed proteomic characterisation of four *Paracoccidioides* species identified significant metabolic differences between them as well as proposing the use of particular biochemical criteria as markers to distinguish between members of the genus (Pigosso *et al.*, 2013). To date, studies of *Pseudogymnoascus* spp. have mainly been carried out on *P. destructans* and *P. pannorum* (Barratt *et al.*, 2003; Verant *et al.*, 2012; Zhang *et al.*, 2015; Verant *et al.*, 2018). Limited data are available for other species in the genus, with most studies only relating to isolation work from various sources (Kochkina *et al.*, 2007; Ali *et al.*, 2013; Krishnan *et al.*, 2016; Godinho *et al.*, 2019). However, an extensive

comparative genomic analysis carried out on 14 species of *Pseudogymnoascus* isolated from various geographical regions provides a very useful database for *Pseudogymnoascus* spp. (Leushkin *et al.*, 2015). The study showed high nucleotide diversity among the 14 species, and that species from permafrost regions have close relatives from temperate environments.

Protein extraction is a very important element of any proteomic analysis. In mass spectrometry analysis, problems can occur through achieving a low depth of protein coverage that results in relatively high false negative findings or the under-representation of important biological processes (Mathieson & Thomas, 2013; Callister *et al.*, 2018). Capturing and validating total protein content can only be done in relation to the total protein extracted within the specific extraction method used. Thus, optimisation of protein extraction methods is a key element important in achieving high protein yield and better resolution, distinct from the selection of proteomic profiling methods (Isola *et al.*, 2011; Bianco & Perrotta, 2015; Daim *et al.*, 2015; Seong *et al.*, 2017). To date, very data are available for this genus, hampering understanding of the diversity of *Pseudogymnoascus* spp. isolated from soils and their complex network of proteins that contribute to their ecological roles and adaptation strategies. To address this, this study investigated six isolates obtained from three different global geographical regions - the Arctic, Antarctica and temperate regions. The objective of this chapter was to characterize these isolates through proteomic profiling, thereby providing baseline information and characterisation of their proteomes and protein profiles. The data obtained will potentially assist in species differentiation and improve understanding of their contribution to decomposition processes in soil ecosystems.

4.3 Methodology

4.3.1 Total protein extraction methods

Three methods of protein extraction were conducted to optimise the quality and total protein yield achieved from fungal biomass. Mycelia of *sp2* (from 10 day cultures) were carefully scraped from culture plates using a sterile spatula. An average of 5 g of fungi mycelia (initial wet mass) were inoculated into 300 mL of Czapek-Dox liquid cultures in three replicates and were grown for 5 d at the experimentally non-stress temperature (15°C). On day 5, biomass was harvested using a 0.45µm filter paper and transferred to sterile tubes to be weighed. Biomass was immediately flash-frozen and ground into fine powder in liquid nitrogen. Approximately 1 g of ground mycelia was used in each extraction. Three independent extraction replicates were performed to assess the reproducibility of each method. Total protein was extracted from the ground tissues using the following protocols.

4.3.1.1 TCA-acetone extraction

Total proteins were extracted using a TCA–acetone method as described by Daim *et al.* (2015). Briefly, ground biomass was precipitated in 5 mL of cold acetone/20% TCA/0.2 % DTT at -20°C and incubated overnight. Then, the mixture was centrifuged at 10,000 × g for 30 min. The pellet was collected and rinsed twice in cold acetone/0.2% DTT after the supernatant was discarded. At each rinsing step, the mixture was incubated for a minimum of 1 h at -20°C after which it was centrifuged at 10,000 × g for 10 min. The resulting pellet was air-dried and analysed further for total protein content. Remaining samples were kept at -20°C for further use.

4.3.1.2 TCA-acetone-phenol extraction

For this extraction, the initial step was carried out as described by Tesei *et al.* (2015). The resulting pellet was further incubated in lysis buffer (7 M urea, 2 M thiourea, 4%

CHAPS, 30 mM Tris HCl pH 8.5) for 1 h. The mixture was bath-sonicated for 15 min at 20°C, 5 mL of Tris-buffered phenol solution pH 8.0 (Sigma Aldrich) was added to the cell lysate and the phenolic phase was collected after centrifugation (3300 × g for 20 min). Proteins were precipitated overnight at -20°C by addition of 5 vol of 0.1 M ammonium acetate in methanol (w/v). After centrifugation at 3300 × g for 30 min, the precipitate was washed with ice-cold methanol (absolute) and ice-cold acetone (80%, v/v). The resulting pellet was air-dried and analysed further for total protein content.

4.3.1.3 Phenol-guanidine isothiocyanate extraction

The third protein extraction method used a modified phenol-guanidine isothiocyanate method (Daim *et al.*, 2015). Initially, 5 mL of TRI Reagent (Molecular Research Centre, Inc.) was added to the fine ground biomass. This was followed by vigorous vortexing and incubated at room temperature for 15 min. The mixture was centrifuged at 12,000 × g for 10 min and the clear phenol extract (protein extract) was transferred into a new tube. A total of 200 µL of chloroform was added into the transferred extract. It was vortexed and centrifuged at 12,000 × g for 10 min to remove insoluble materials. The aqueous phase (which contains RNA) was carefully discarded and 300 µL of cold absolute ethanol was added to precipitate the nucleic acids. The tube was inverted to mix the contents and centrifuged at 2000 × g for 5 min, after which the phenol–ethanol supernatant (protein extract) was transferred into a new tube. The protein extract was precipitated with 1.5 mL of cold isopropanol. After incubation for 30 min at room temperature, the mixture was centrifuged at 12,000 × g for 10 min. The resulting protein pellet was washed three times in 1.5 mL cold 0.3 M guanidine hydrochloride in 95% ethanol and then centrifuged at 7500 × g for 5 min. After the final wash, the protein pellet was vortexed in 1.5 mL absolute ethanol. After incubation for 20 min, the pellet was centrifuged at 7500 × g for 5 min and air-dried.

4.3.2 Determination of protein content

The Bradford protein assay (Bradford, 1976) was used to determine the concentration of proteins in fungal extracts. Reactions were carried out in 96-well microtiter plates. A standard curve was established using serial dilutions from 0.8 µg/mL to 100 µg/mL of bovine serum albumin (BSA, Pierce, USA). The resulting optical density (OD) at 595 nm was measured with a plate reader (Tecan Austria, Austria). The mean protein concentrations of triplicate samples were calculated using the standard curve obtained.

4.3.3 One-Dimensional Polyacrylamide Gel Electrophoresis (1D-PAGE)

Preliminary evaluation of the quality of protein extracts was carried out using 1D-PAGE. For each extract, 5 µg of protein was suspended in 2.5 µL loading buffer (0.25 M Tris, 1.92 M glycine, 1 % SDS, 20 mM DTT) to give a total volume of 10 µL. The solution was heated at 95°C for 10 min and then separated through 5 % stacking gels followed by 12.5 % resolving gels. Electrophoresis was performed at 90 V for 1.5 h using a Mini-PROTEAN 3 Cell (Bio-Rad) with 1.0 mm thick combs to separate proteins based on their mass. The gels were stained using Coomassie blue.

4.3.4 In-solution peptide digestion

In-solution peptide digestion was carried out following Lau and Othman (2019). Extracted proteins (50 µg) were re-suspended in 100 µL of 50 mM ammonium bicarbonate and 1 M urea. The proteins were reduced and alkylated using 100 mM Tris buffer and 200 mM iodoacetamide, respectively. Sodium deoxycholate in 5 mM ammonium bicarbonate [1% (w/v)] was added to the reduced and alkylated proteins to enhance the tryptic digestion at 37°C for 10 min. Tryptic digestion using 1 µg of sequencing grade bovine trypsin (Promega, Madison, WI, USA) per 50 µg protein was performed at 37°C for 17 h. The resulting peptide mixture was then acidified with 0.5% formic acid, and sodium deoxycholate precipitate was obtained through centrifugation at

14 000 × g (Eppendorf, Thermo Scientific) at room temperature for 15 min. The remaining solvents and acids were removed using a centrifugal evaporator (CentriVap Concentrator, Labconco, MO, USA). The desiccated peptides were re-suspended in 100 µL of 0.1% formic acid and gently mixed before peptide purification. An Empore solid phase extraction disk (3M Purification, Inc., MN, USA), conditioned with acetonitrile and methanol, was added into the peptide resuspension and incubated at room temperature for 3 h to bind the peptides. Elution of the peptides from the disk was done twice using 50% ACN in 0.1% FA for 30 min, each. This solvent was then removed.

4.3.5 Liquid chromatography-mass spectrometry analysis

Peptides were reconstituted in 30 µL of 0.1% FA and 5% ACN. Then, 2 µL of the digest was loaded onto an Acclaim PepMap 100 C18 column (3 µm, 0.075 × 150 mm) (Thermo Scientific, MA, USA). The reverse phase column was equilibrated with 0.1% FA (mobile phase A) and 80% ACN in 0.1% FA (mobile phase B). A gradient of 5-35% mobile phase B over a total of 70 min, at a flow rate of 300 nL min^{-1}, was applied to elute the peptides. Separation of the peptides was achieved using EASY-nano liquid chromatography (EASY-nLC) 1200 System (Thermo Scientific, MA, USA). An online Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer system (Thermo Scientific, MA, USA) was used to generate the peptide ions with a spray voltage of 1800 V in positive mode. The precursor ion scan was conducted with a resolution of 70,000 and a mass range of m/z 310-1800. Precursors containing charge states from 2+ to 8+ were fragmented further. The fragmentation was done via collision-induced and high-energy collision-induced at a normalized energy of 28%. The resolution, isolation window and ion injection time were set at 17,500, 0.7 Da and 60 ms, respectively. Scanned precursor mass range was set at m/z 110-1800.

4.3.6 Protein identification and bioinformatic analysis

Mass spectra of the peptides were acquired using Xcalibur (Ver. 4.1.31.9) (Thermo Scientific, MA, USA) and deconvoluted with Proteome Discoverer (Ver. 2.4) (Thermo Scientific, MA, USA) to create the peptide mass list. The SEQUEST HT search engine, incorporated in the Proteome Discoverer, was used to match the generated mass list against *Pseudogymnoascus destructans* (Taxonomy ID is 655981, 82,900 sequences). Mass tolerance for the proteins and their fragments were fixed at 10 ppm and 0.02 Da, respectively. Trypsin was indicated as the digestion enzyme used, with up to two missed cleavages allowed during the search. Carbamidomethylation modification on cysteine residues was set as a static modification while variable amino acid modifications included deamidation (asparagine and glutamine residues) and oxidation (methionine residues). The mass list was also searched against a decoy database generated from randomised protein sequences of the taxonomy mentioned earlier. Only proteins having at least the Rank 1 peptide and a false discovery rate of 1% were accepted. Spectra that matched the sequences were further validated using the Percolator algorithm (Ver. 2.04) with q-value set at 1% false discovery rate. The sequences were clustered using Principal Component Analysis (PCA) and heat map generation was done with the statistical analysis component in Proteome Discoverer. The clustering method used was a simple agglomerative hierarchical clustering method (UPGMA). The distance measure applied was Euclidean in logarithmic scale for rows. Distance between clusters was computed with Ward's method. Venn diagrams were generated using the web-based software available at https://bioinfogp.cnb.csic.es/tools/venny/index.html (Oliveros, 2007-2015)

4.3.7 Statistical analysis

Total protein content from three extraction methods were determined from a standard curve plotted using bovine serum albumin. Value represents mean of three biological replicates.

4.4 Results

4.4.1 Optimisation of extraction methods

Three extraction methods were compared to identify the method giving the highest protein yield with good quality proteins extracted for proteomic application. Figure 4.1 shows the total protein yield from each extraction method applied to *sp2* (µg proteins per g biomass). The TCA-acetone-phenol extraction method generated the highest protein yield (1445 ± 79 µg g^{-1}) compared to phenol-guanidine isothiocyanate method (1274 ± 81 µg g^{-1}) and the TCA-acetone method (1121 ± 35 µg g^{-1}). The combination of TCA-acetone and phenol-based precipitation resulted in more efficient protein extraction, as compared to the use of phenol-guanidine isothiocyanate or TCA-acetone only. The quality of protein extracted from the three different methods was evaluated using 1D-PAGE stained with standard Coomassie staining protocol (Figure 4.2). The quality of protein extract was greatest with TCA-acetone-phenol, with the highest number of distinct bands appearing in all replicates. TCA-acetone protein extracts showed a slightly lower number of distinct bands when compared to TCA-acetone-phenol, where proteins with molecular weight less than 25 kDa and between 30-40 kDa were missing in all replicates. Phenol-guanidine isothiocyanate extracts showed the lowest quality of proteins separated on 1D-PAGE even though the quantity of proteins was higher than that of TCA-acetone.

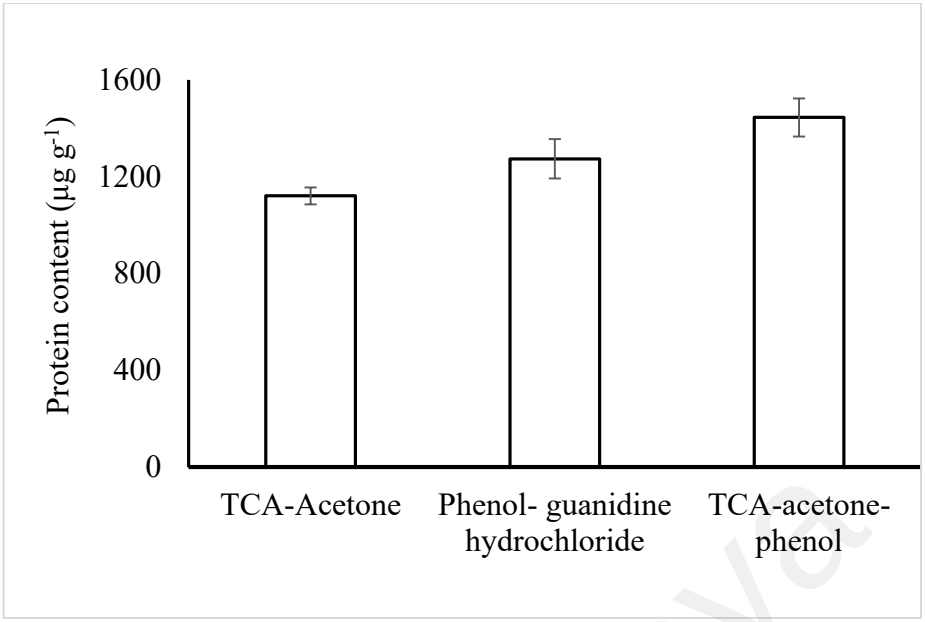

Figure 4.1 Total proteins yielded from three different extraction methods (amount of proteins expressed in µg proteins per g fungal biomass; ± standard deviation of three independent replicates).

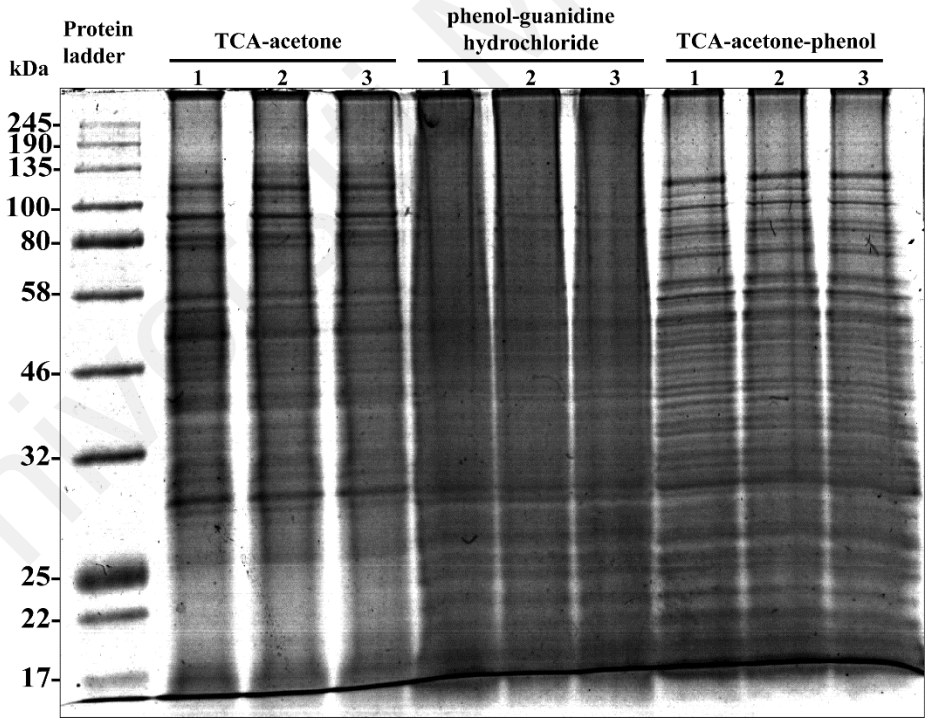

Figure 4.2: Visual comparison between protein extraction methods using 1D-PAGE of *sp2* (numbers on each lane represents individual replicates from three extraction protocols). Five micrograms of protein from each replicate were loaded into the well, and the gel was stained using the Coomassie staining protocol.

4.4.2 Whole proteome profiling of *Pseudogymnoascus* spp. strains from various geographical regions

Proteomic profiling of *Pseudogymnoascus* spp. isolates was carried out using LC MS/MS analysis. The isolates originated from three different geographical regions (two isolates per region), representing Arctic, Antarctic and temperate regions.

In total, 2,153 proteins were identified from all six *Pseudogymnoascus* isolates (Supplementary 1). Protein function was determined by inputting each assigned protein identification (NCBI accession number) into the UniProtKB database (http://www.uniprot.org/blast/) and detecting the respective Gene Ontology (GO) terms and annotations. UniProt functional annotation was used to classify all identified proteins according to biological processes (Figure 4.3a) and molecular functions (Figure 4.3b). Overall, 2,003 proteins were successfully mapped to the UniProtIDs database. The majority of these were involved in cellular (44%) and metabolic (41%) processes. Smaller proportions were involved in localization processes (7%) and biological regulation (5%) (Figure 4.3a). Proteins classified under localization processes were mainly involved in the establishment of process and macromolecular localization. Moreover, 2% of proteins were classified under response to stimulus, which included responses towards stress, abiotic stimulus and chemicals. Finally, 1% of classified proteins were involved in developmental processes, conidia formation and cellular detoxification. Classification based on molecular functions (Figure 4.3b) showed that 48% of proteins were involved in catalytic activities. The majority of these were involved in hydrolase and transferase activities. This was followed by proteins involved in molecular binding functions (39%), such as those involved in the binding of heterocyclic and organic compounds, small molecules and ions. The remaining proteins were classified under structural molecules (4%), translation factors (2%), proteins with antioxidant activities (2%) and other molecular functions (1%).

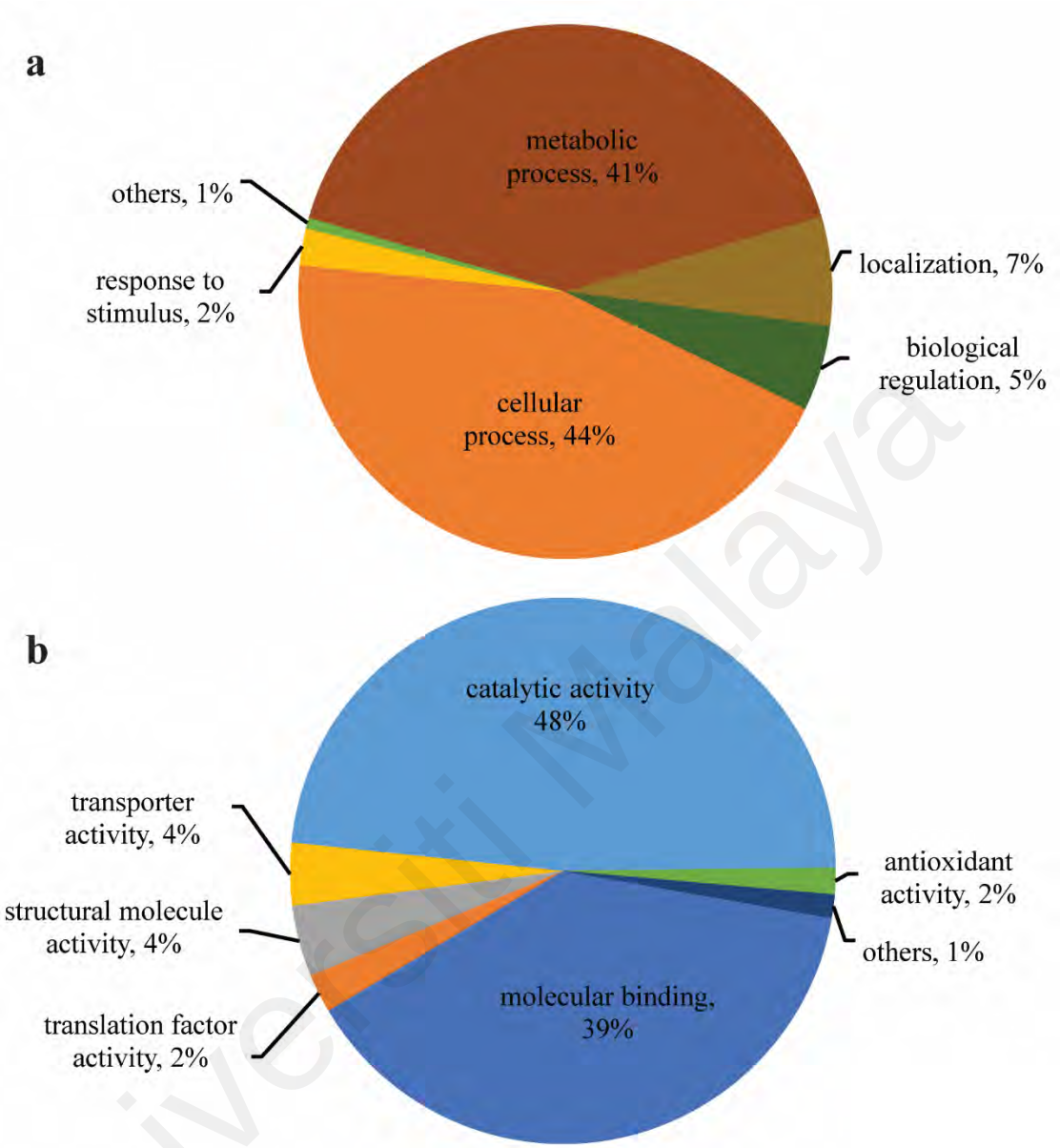

Figure 4.3: Pie charts representing the whole proteome identified in *Pseudogymnoascus* spp. The identified proteins were classified according to (a) biological processes and (b) molecular functions following UniProt functional annotation. The identified proteins were mainly involved in biological processes such as cellular and metabolic processes, with a high percentage of catalytic and molecular binding activities.

The proteomic profiles from each isolate were further analysed using a Venn diagram to determine protein overlaps between geographical regions (Figure 4.4). Overall, 1,201 proteins (55.8% of the total proteome) were present in all six isolates. The remaining proportion were restricted to two regions or unique to a single region. The overlap between two regions ranged between 131-154 proteins (6.1-7.2% of the total proteome),

with the Arctic and temperate regions having the highest number of shared proteins whereas the Arctic and Antarctica the lowest. The number of proteins unique to a single region was considerably greater, 113-263 proteins (5.2 – 12.2% of proteome). The temperate region had the highest number of unique proteins (263 proteins), while the Antarctic and Arctic regions had 146 and 113 unique proteins, respectively.

Figure 4.4: Venn diagram showing the numbers of overlapping and unique identified proteins (% of proteins identified) for isolates clustered according to their geographical regions of isolation, a) the Arctic; b) the Antarctic; and c) the temperate region.

The unique proteins were further classified according to their GO annotations, to provide an overview of their profiles for each geographical region (Figure 4.5) and identify any possible differences in functions/roles. The majority of the unique proteins were involved in catalytic activity (53–102 proteins), with transferase being the most numerous in the proteome of isolates from the Arctic and temperate regions, and

hydrolase in the Antarctic region. The list of unique proteins that were classified under transferase and hydrolase activities is given in Table 4.1. It is important to note that numerous proteins were uncharacterised, highlighting the limited state of current knowledge and the need for baseline information and more detailed characterisation of *Pseudogymnoascus* spp. proteomes. The data in Table 4.1 demonstrate that some proteins shared similar catalytic functions, even though they were identified as unique for a particular region. This suggests that, in different geographical regions, the same catalytic processes may potentially be performed by different proteins. For example, under the classification of transferase activity, serine/threonine protein kinase was found in all three geographical regions. Similarly, amongst proteins classified under hydrolase activity, chitinase were also found in all regions. On the other hand, malate synthase and methyltransf_2 domain-containing protein were found only in the Arctic and temperate regions. Guanine deaminase and peptidase domain-containing protein were found only in the Antarctic and temperate regions. Detailed information on the unique proteins classified under hydrolase and transferase activities (see Table 4.1) provides basic proteomic profiles for *Pseudogymnoascus* spp. from each region.

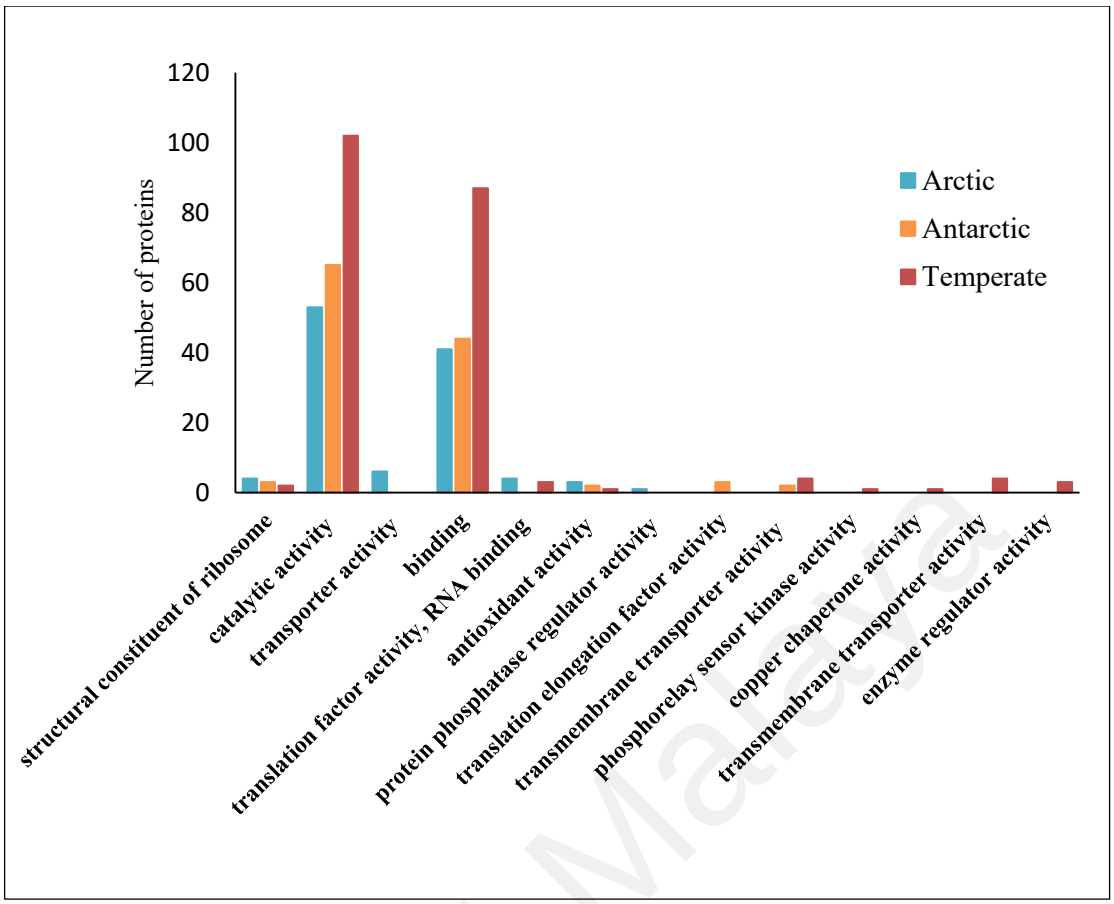

Figure 4.5: The molecular function clustering of unique proteins from each region according to their GO annotation. Blue) the Arctic region, Orange) the Antarctic region, and Red) the temperate region.

Table 4.1: List of unique proteins from each region classified under transferase and hydrolase activities.

Proteins classified under transferase activity		
Arctic	**GI accession number**	**UniProtID**
Atypical serine/threonine protein kinase BUD32	1040498399	A0A1B8D1Y3
Ribokinase	1040502300	A0A1B8EC27
Uridylate kinase	1040506245	A0A1B8DR90
SAM_MT_RSMB_NOP domain-containing protein	1040513629	A0A1B8DSB0
Phosphomevalonate kinase	1040515090	A0A1B8EBD0
Molybdopterin molybdenumtransferase	1040518253	A0A1B8CB98
1,3-beta-D-glucan-UDP glucosyltransferase	1040519743	A0A1B8CME2
Malate synthase	1040524492	A0A1B8C0B2
Adenylate kinase	1040524905	A0A1B8F6B1
Amidophosphoribosyltransferase	1040529445	A0A1B8F8J6
Uncharacterized protein	1040529530	A0A1B8F2A1
Glycylpeptide N-tetradecanoyltransferase	1040531617	A0A1B8EXE7
Uncharacterized protein	1040531866	A0A1B8E5C8
4-methyl-5-beta-hydroxyethylthiazole kinase	1040539535	A0A1B8D5I2
Uncharacterized protein	1040541242	A0A1B8DDP7
Methyltransf_2 domain-containing protein	1040542655	A0A1B8DI87

Antarctic		
Cobalamin-independent methionine synthase	1001840422	A0A126XZN6
CMGC/DYRK/DYRK2 protein kinase	1002620675	A0A1B8EG16
Histone-lysine N-methyltransferase	1026905232	A0A1B8DT25
Non-specific serine/threonine protein kinase	1040497495	A0A1B8EAH7
GPI ethanolamine phosphate transferase 1	1040501824	A0A1B8DPA1
1,3-beta-glucanosyltransferase	1040523627	A0A1B8E380
Protein kinase domain-containing protein	1040525014	A0A1B8C9Y6
Protein arginine N-methyltransferase 1	1040528592	A0A1B8BXW0
Protein kinase domain-containing protein	1040531330	A0A177A5B2
HMG-CoA synthase	1040533273	A0A1B8FEC7
Dolichyl-phosphate-mannose--protein mannosyltransferase	1040546007	A0A1B8GTY6
Temperate		
Trehalose-6-phosphate synthase	1026908464	A0A1B8D8P9
CMGC/SRPK protein kinase	1026909170	L8FQ38
N-acetyltransferase domain-containing protein	1026910215	A0A2P2SVQ8
ATP citrate synthase	1040499582	A0A1B8GEP5
Acetylglutamate kinase	1040502403	A0A1B8GPA3
Methyltransf_2 domain-containing protein	1040505877	A0A1B8EAS4
Mitogen-activated protein kinase	1040506813	A0A1B8E4N6
Atypical serine/threonine protein kinase BUD32	1040507339	A0A1B8DR08
Methyltransf_2 domain-containing protein	1040511525	A0A1B8DY10
Aminomethyltransferase	1040516209	A0A1B8E703
Gluconate kinase	1040524265	A0A1B8E0V4
Uracil phosphoribosyltransferase	1040526934	A0A1B8E3P5
CMGC/CDK/CDK5 protein kinase	1040527934	A0A1B8CBK0
Histidinol-phosphate aminotransferase	1040528918	A0A1B8C3L3
Mitogen-activated protein kinase	1040529256	A0A1B8CQN0
Imidazole glycerol phosphate synthase	1040530109	A0A1B8CP09
Histidine kinase	1040531430	A0A1B8CLC9
Methyltransf_11 domain-containing protein	1040536639	A0A1B8FPW7
Alpha-1,4 glucan phosphorylase	1040542046	A0A177AGY6
Nicotinate phosphoribosyltransferase	1040549942	A0A177AGW8
Homocysteine synthase	1040558827	A0A177AJZ8
Citrate synthase	1040561561	A0A1B8EP83
N-acetylglutamate synthase	1040562207	A0A1B8F4S1
Ornithine aminotransferase	1040565334	A0A1B8GY49
Methyltransf_11 domain-containing protein	1040565502	A0A1B8GMD9
GST C-terminal domain-containing protein	1069462591	L8GA92
Transketolase	1069463567	L8G8Z1
Malate synthase	1069469587	A0A1B8CX12

Proteins classified under hydrolase activity

Arctic	GI accession number	UniProtID
Uncharacterized protein	1040496820	A0A1B8DAY6
ATP-dependent RNA helicase	1040500406	L8FZ63
Elongation factor EF-3	1040504132	A0A1B8E3K3
Translation initiation factor	1040514867	A0A1B8EDG3
Endo-1,3(4)-beta-glucanase	1040517074	A0A1B8EAV4
Glucanase	1040518311	A0A1B8DV74

Chitinase	1040525950	A0A1B8BW26
Uncharacterized protein	1040528888	A0A1B8C5Y6
Uncharacterized protein	1040531469	A0A1B8CGF4
PfkB domain-containing protein	1040532371	A0A1B8F308
CN hydrolase domain-containing protein	1040541539	A0A1B8FTG7
DNA damage-inducible protein 1	1040551194	A0A1B8DEG3
Signal recognition particle 54 kDa protein	440635915	A0A1B8D4Z6
Antarctic		
Translation elongation factor EF-1a	1040498702	T2C6H6
Chitinase	1040499304	A0A1B8DIH4
Guanine deaminase	1040506005	A0A1B8GHB2
Uncharacterized protein	1040507045	A0A1B8ED83
Peptidase A1 domain-containing protein	1040515212	A0A1B8E764
Chitinase	1040517976	A0A1B8DUA7
Fn3 like domain-containing protein	1040519838	A0A1B8DYP9
Proteasome subunit alpha	1040523627	A0A1B8E7W8
ATP-dependent RNA helicase	1040525592	A0A1B8DUB0
GPI ethanolamine phosphate transferase	1040525616	A0A1B8DPA1
Chitinase	1040527072	A0A1B8C2U3
Lon protease homolog, mitochondrial	1040530173	A0A1B8CLR2
Peptidase A1 domain-containing protein	1040530224	A0A1B8C161
Elongation factor Tu	1040532270	A0A1B8CPP7
GH16 domain-containing protein	1040540068	L8G2Y5
1,4-alpha-D-glucan glucohydrolase	1040552623	A0A1B8FXC5
Alpha-glucuronidase	1040554007	A0A1B8G190
Glucoamylase	1040555923	A0A1B8G7C3
Uncharacterized protein	1040556213	A0A1B8G6J2
Adenosylhomocysteinase	1040559730	A0A2P2SXB0
Uncharacterized protein	1040562846	A0A1B8GR13
Endopolyphosphatase	1040566058	A0A1B8EZ27
Uncharacterized protein	1069462007	A0A1B8D5S2
Elongation factor 2	1069468175	A0A1B8DDA6
Temperate		
Lysophospholipase	1026903741	L8G2A9
Nudix hydrolase domain-containing protein	1026904489	L8G9X4
Guanine deaminase	1026905845	A0A1B8DV95
Uncharacterized protein	1040499521	A0A1B8DP18
Class I unconventional myosin (Myosin-1)	1040504779	A0A1B8EFQ3
Exo-1,4-beta-D-glucosaminidase	1040506813	A0A1B8DV84
Uncharacterized protein	1040517395	A0A1B8EEM6
Uncharacterized protein	1040517818	A0A1B8E4G4
Proteasome endopeptidase complex	1040523644	A0A1B8DS67
Uncharacterized protein	1040524860	A0A1B8DVT3
GH16 domain-containing protein	1040525961	A0A1B8DX20
Chitinase	1040525970	A0A1B8ECD6
Peptidase_S9 domain-containing protein	1040526153	A0A1B8C3G2
Imidazole glycerol phosphate synthase	1040526595	A0A1B8CP09
Glyco hydro 63 domain-containing protein	1040529036	A0A1B8CI96
Eukaryotic translation initiation factor 5B	1040531995	A0A1B8FX01
Uncharacterized protein	1040532786	A0A177A8K1
Arginase	1040532978	A0A177A333
Glycosidase	1040538520	A0A177A4T1
AAA domain-containing protein	1040541896	A0A1B8F438

Ubiquitinyl hydrolase 1	1040552482	A0A1B8G4N7
Histidine biosynthesis trifunctional protein	1040555230	A0A1B8EUJ8
Amidohydro-rel domain-containing protein	440636162	A0A1B8DD06
ANK_REP_REGION domain-containing protein	440638922	A0A1B8DBS7

4.5 Discussion

4.5.1 Evaluation of protein quality and yield based on different extraction methods

Selection of a method for protein extraction is a crucial step in order to obtain good coverage of the whole proteome. It is also necessary to optimise an extraction method to obtain high yield and quality of the extracted proteins. In general, psychrophilic and psychrotolerant fungi possess common adaptive characteristics of the plasma membrane bilayer, such as lipid or fatty acid content, compatible solutes such as polyols and use of disaccharides such as trehalose (Hassan *et al.*, 2016). *Pseudogymnoascus* spp. have relatively thick mycelial plasma membrane bilayers and contain high trehalose concentration (Hay & Ashbee, 2010; Wilson *et al.*, 2017). Therefore, a combination of physical forces, such as grinding in liquid nitrogen, and strong detergents are needed to ensure proper cell rupture and high quality of protein yield (Kramer *et al.*, 2015).

Chemicals such as trichloroacetic acid (TCA), acetone and phenol are mostly used to precipitate proteins, but a combination of these chemicals is necessary to produce high protein yield. TCA-acetone extraction is preferable because of its time efficiency, compared to phenol-based extraction methods. However, difficulties in resolubilization of protein pellets result in low reproducibility (Chen & Harmon, 2006; Bhadauria *et al.*, 2007). Both methods are efficient in successful precipitation of proteins but the phenol-based extraction shows significantly higher efficiency and solubility (Isola *et al.*, 2011). Other methods are available that use a combination of phenol, chloroform, isoamyl alcohol and guanidine isothiocyanate in total protein extraction that help inhibit RNAase activity, and separate DNA and RNA from the protein complexes (Chomczynski &

Sacchi, 2006). In the current study, the TCA-acetone-phenol method was found to be more efficient than phenol-guanidine isothiocyanate and TCA-acetone precipitation methods and yielded a higher amount of protein. 1D-PAGE confirmed that the combination of TCA-acetone and phenol precipitation was effective in removing impurities and ensuring good protein resolubilisation. In proteomic work, it is important to ensure the removal of nucleic acids and minimised proteolysis as it can interfere in protein separation in electrophoresis and chromatography processes. The use of phenol in the extraction process also helped to remove nucleic acids and minimized proteolysis (Isola *et al.*, 2011). It is also important to note that phenol acts as a solubilisation agent and helps to decrease the interactions of proteins with other compounds (Chatterjee *et al.*, 2012). This finding is consistent with previous optimisation work done on oil palm leaves (Daim *et al.*, 2015).

4.5.2 The importance of *Pseudogymnoascus* spp. in decomposition processes in the soil

A number of recent studies conducted in various geographical regions have consistently demonstrated that *Pseudogymnoascus* spp. play a major role in soil decomposition processes (Hayes, 2012; Krishnan *et al.*, 2017). The production of enzymes to support metabolic requirements and growth is a key requirement for fungal survival in both nutrient-poor and nutrient-rich environments. However, enzyme such as cellulase are not identified in all isolates in this work indicating that media used in experiments contribute to the differences of enzymes produced by *Pseudogymnoascus* spp. within each experiment. A recent microbial community study on permafrost in the Arctic showed that *Pseudogymnoascus* was one of the fungal taxa that increased in abundance with litter addition as a substrate (Adamczyk *et al.*, 2020). The proteomes of the six *Pseudogymnoascus* isolates characterised in the current study were consistent with

a general decomposition function in the soil environment (with high abundance of proteins with catalytic activities).

Almost 50% of the identified proteins in the proteomes were involved in metabolic processes, with the majority of these involved in catalytic functions such as transferase and hydrolase activities. Analyses carried out also indicated the presence of antimicrobial proteins (42 identified proteins, 2%), supporting the ability to thrive in mixed microbial communities. In soil ecosystems, many fungal taxa have developed defensive strategies against other competing microbes, producing antimicrobial compounds to effectively gain control over access to nutrients (Boer *et al.*, 2005; Bahram *et al.*, 2018).

Comparison of the proteomes of the six isolates studied here allows some inferences to be made about the proteins shared between and unique to the two polar and the temperate regions. The 1,201 identified proteins common to all six isolates were mostly related to basic cellular functions. Overall, the proteomes of all six isolates were relatively similar. Leushkin *et al.* (2015) reported similar results in a comparative genomic analysis of 14 strains of *Pseudogymnoascus* isolated from multiple locations, with all strains being very similar in functional abilities (synteny of genes was >0.9 between different clades). However, analyses carried out in this work also identified a number of proteins exclusive to each of the three global regions. Most of these regionally unique proteins were involved in various catalytic processes, with the majority again having transferase and hydrolase activities. Results showed that serine/threonine protein kinase and chitinase were found in all three regions (see Table 4.1). The basic function of serine/threonine (ser/thr) protein kinases is to phosphorylate the hydroxyl (-OH group) of serine or threonine. However, a more specific function in regulation and signal transduction was found in many fungal species (Kosti *et al.*, 2010). There are also reports on ser/thr protein kinase roles in thermal adaptation and pathogenicity of *P. destructans* (Parente-Rocha *et al.*, 2018;

Verant *et al.*, 2018; Fabri *et al.*, 2020). An extensive review of the molecular functions of ser/thr protein kinase discussed its pathological importance and potential application as immune response manipulator (Patel, 2017). Chitinase, another unique protein identified in this work, is generally known to be associated with degradation of exogenous chitin and cell wall remodeling during hyphal growth (Seidl, 2008). Chitinase has been used in various biotechnological applications such as the development of antifungal and insecticidal products for use in eco-friendly farming (Singh & Arya, 2019), and as an industrial biocatalyst (Tamadoni Jahromi & Barzkar, 2018). Data presented in this work suggest that the specific protein species present within each region can be further characterised to elucidate their potential use as molecular markers of the genus. However, more detailed studies are still necessary to recognize and develop the potential application of these proteins.

4.6 Conclusion

This study provides the first proteomic characterisation of *Pseudogymnoascus* spp. from soil habitats spanning a broad geographical range, including the Arctic, Antarctic and temperate regions. This was achieved by considering three separate protocols that achieved high efficiency of protein extraction both in terms of quality and quantity. The proteomic profiles of all six isolates showed that the majority of proteins identified were clustered into groups representing metabolic functions and catalytic activities. One particularly interesting observation was that, although a large number of proteins were uniquely identified in isolates from different regions, there were overlaps with regards to the biological functions governed by these proteins. Proteomic profiles such as those documented here provide invaluable insights into the potential roles of *Pseudogymnoascus* taxa in soil ecosystems.

CHAPTER 5: HEAT STRESS RESPONSE OF *PSEUDOGYMNOASCUS* SPP. ISOLATED FROM DIFFERENT GEOGRAPHICAL REGIONS

5.1 Introduction

In line with the challenges of climate change and global warming, elucidating heat stress responses of soil microfungi has become an important research topic in efforts to maintain soil quality and health. In the polar regions, soil microfungi have been implicated in many biological activities such as the bioremediation of hydrocarbons (Donovan *et al.*, 2018) and the production of herbicides (Ogaki *et al.*, 2020). Knowledge of heat stress responses may contribute to the understanding of the specific roles of fungi in extreme environments. Under heat stress, fungi undergo various physiological changes to maintain cell stability and use protective mechanisms to repair any damage caused. In general, fungi respond to heat stress using multiple protective mechanisms, including the modulation of protein homeostasis, regulation of energy production and activation of DNA damage and repair pathways. In Chapter 4, the effect of temperature on colony morphology and growth rates of six isolates of *Pseudogymnoascus* spp. originating from different geographical regions was discussed and baseline proteomic profiling of each isolate under non-stress conditions was documented. In this chapter, further proteomic analyses were carried out to clarify the heat stress responses of *Pseudogymnoascus* spp. The objective was to identify patterns of change in protein abundance of isolates of *Pseudogymnoascus* spp. originating from polar and temperate regions in response to heat stress.

5.2 Literature review

Proteomics has been utilized to increase understanding of mechanisms of adaptation in microorganisms (Dalluge & Connell, 2013; Vollmers *et al.*, 2013; Deng *et al.*, 2020). The tool has been used to study the expression patterns of various proteins in soil microfungi across latitudes when exposed to extreme temperatures (Wallenstein &

Weintraub, 2008; Zakharova *et al.*, 2014). Fungal stress responses to various abiotic stressors have been described based on proteomic approaches (Kroll *et al.*, 2014; Hassan *et al.*, 2016; Chadha *et al.*, 2019). Temperature is a key stress factor to be considered in studies of soil microfungal growth, reproduction and adaptation, as changes in temperature can alter fungal survival and interactions with other microorganisms in the soil (A'Bear *et al.*, 2014a). Studies have also assessed fungal heat stress response mechanisms in the development of tools for crop monitoring and production as well as in producing heat-tolerant species that are able to better adapt to climatic changes (Jagadish *et al.*, 2010; Simova-Stoilova *et al.*, 2016). Knowledge of fungal response mechanisms towards environmental stress factors such as heat stress is clearly relevant to various biotechnological and agricultural fields. By understanding the fungal proteome and specific protein expression and stress-related mechanisms, researchers will be able to fully exploit the potential of soil microfungi (Lange *et al.*, 2012; Classen *et al.*, 2015).

Geographical differences in soil microfungal populations can be characterized as a non-random distribution of biotic diversity in relation to environmental factors (Ansdell & Hanson, 2016). Therefore, fungal communities isolated from different geographical regions may have similar heat stress responses despite being separated by abiotic barriers. However, many other factors may confound attempts to exclude variation in heat stress response based on geographical patterns (Lennon & Jones, 2011; Talbot *et al.*, 2014). To overcome these limitations, understanding of fungal molecular characteristics can be developed through the use of various advanced -omics technologies. For instance, psychrophilic and psychrotolerant fungal strains isolated from the Arctic and Antarctic showed significant differences in their molecular characteristics and response mechanism towards heat stress, despite being isolated from the same geographical regions (Gocheva *et al.*, 2006; Tajuddin *et al.*, 2019). More generally, studies of geographical differentiation

in microorganisms remain limited, especially in the case of fungi (Ansdell & Hanson, 2016).

Exposure to heat stress often generates a cascade of molecular modifications in order to maintain cellular homeostasis and cell survival. Cells are programmed in their DNA to stabilise a basic set of cellular functions for survival and modulate changes in molecular interactions and networks through stress response mechanisms. In fungi, the heat stress response (HSR) mechanism has been studied in various model organisms such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Neurospora crassa* and *Candida albicans* (Arkowitz & Bassilana, 2015; Brandl & Andersen, 2017; Santiago *et al.*, 2020). In understanding fungal HSR, many proteins have been found to be important for cell survival (Albrecht *et al.*, 2010; Richter *et al.*, 2010; Tesei *et al.*, 2015). Enzymes and many functional proteins can be misfolded, or form aggregates when fungi are exposed to heat stress (Nillegoda *et al.*, 2018). Fungi also produced heat stress molecules such as heat shock proteins and chaperones to help misfolded proteins regain their functional structure (Dieterich *et al.*, 2015; Mühlhofer *et al.*, 2019). The production of proteins is also regulated either at the transcription, translation or post-translational level (Fang *et al.*, 2020; Santiago *et al.*, 2020). Overall, substantial physiological changes therefore underlie the ability of fungi to survive heat stress exposure. However, protein interactions and networks within the cell are extremely complex and an in-depth analysis of proteomic changes is needed to elucidate fungal response to heat stress (Bai *et al.*, 2015; Deng *et al.*, 2020). Single protein analysis helps in confirmation of specific function in cell, but may not represent the actual cellular mechanism of response (Li *et al.*, 2017). Thus, the application of proteomics may provide key baseline information helping to elucidate the HSR of *Pseudogymnoascus* spp. isolated from different geographical regions.

5.3 Methodology

5.3.1 Fungal cultivation and heat stress experimental design

All isolates used in this work (please refer Table 3.1 for isolates details) were grown for 5 d at 15°C or 25°C, representing non-stress (C) and heat stress (HS) conditions, respectively. Detailed description of the growth conditions and liquid media preparation is given in Section 3.3.2. All experiments were carried out in triplicates.

5.3.2 Peptide identification, quantification and bioinformatic analysis

Peptide identification, quantification and bioinformatics analysis were performed using LC MS/MS methodology and the statistical analysis component in Proteome Discoverer, described in detail in Section 4.3. Protein abundance values were used to calculate the $\log_2$ ratios of HS:C of each isolate. A microarray (MA) plot was constructed using $\log_2$ ratios against $-\log_{10}$ local FDR values, and this provided for each protein identified. A cut-off value of 1% FDR was applied to all data obtained from LC MS/MS and quantification before performing this analysis. Differentially expressed proteins (DEPs) were determined from the data with a minimum of ± 0.1-fold change. Venn diagrams were also constructed to compare DEPs of isolates within regions.

5.3.3 Gene Ontology enrichment analysis

KOBAS v2.0 (http://kobas.cbi.pku.edu.cn) was used to search for gene enrichment. The software uses gene-level statistics called overrepresentation analysis (Xie *et al.*, 2011). The analysis is based on the hypergeometric distribution/Fisher's exact test with the addition of Benjamini and Hochberg (1995) FDR correction. Data in the form of FASTA sequences were used to identify enriched pathways in the KEGG, BioCyc and Reactome databases based on the up- and down-regulated proteins. GO terms with p-value of ≤ 0.05 were considered significantly enriched. *Saccharomyces cerevisiae* was selected as the reference Ascomycota species. A simplified diagram was created from the

list of significantly enriched pathways to aid visualization of the HSR of *Pseudogymnoascus* spp. using Biorender.com.

5.4 Results

5.4.1 Proteomic profiles of *Pseudogymnoascus* spp. in response to heat stress

To determine changes in the *Pseudogymnoascus* spp. isolates proteomes in response to heat stress, determination and comparison of protein abundance profiles were carried out using LC MS/MS. A total of 2,122 proteins were identified with high confidence ($p<0.01$) in HS and C (Supplementary 1). A comparison of protein abundances identified in HS and C was carried out using microarray (MA) plot analysis (Figure 5.1). The fold change (log_2 ratio HS:C) of differentially expressed proteins (DEPs) showed 463 and 377 proteins were up- or down-regulated, respectively (with a minimum of ± 0.1-fold change). The plot also showed that the majority of proteins identified were clustered close to 0, with a minimum number of proteins having relatively high confidence values ($-log_{10}$ local FDR >800). This suggested that the proteomic profiles of the *Pseudogymnoascus* isolates from different geographical regions showed similar distribution patterns under heat stress.

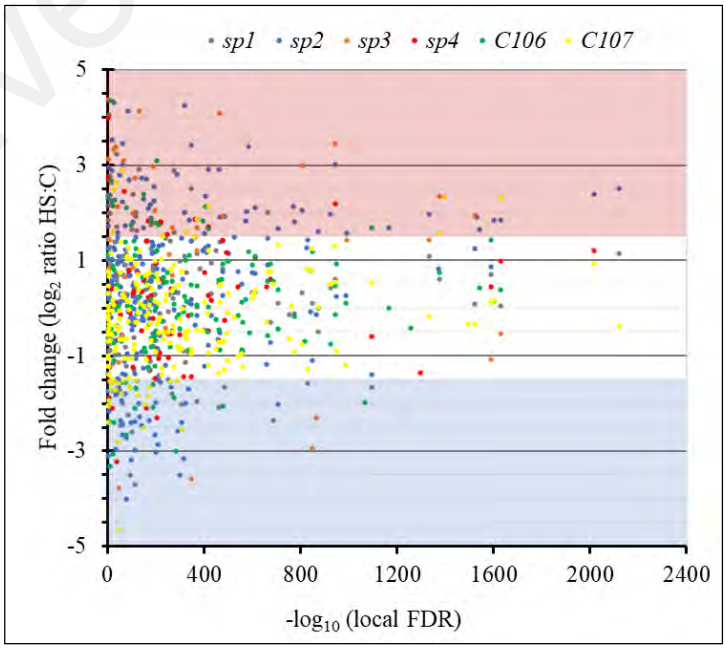

Figure 5.1 The MA plot representing the distribution profiles of proteins identified in all six isolates of *Pseudogymnoascus* spp. Different colours used to represent each isolate: *sp1*, grey; *sp2*, light blue; *sp3*, orange; *sp4*, red; *C106*, green; *C107*, yellow.

A comparison of DEPs for each isolate is presented in Figure 5.2, identifying up- and down-regulated proteins. The Arctic isolate *sp2* showed the highest number of up-regulated proteins (151 proteins) followed by *sp1* (95 proteins). The Antarctic isolate *sp3* had the lowest number of up-regulated proteins followed by *sp4* (31 and 38, respectively). In general, the numbers of down-regulated proteins were lower for all isolates with the exception of the temperate isolate, *C107*, which also had the highest number of down-regulated proteins (125 proteins). Both the Arctic isolates *sp1* and *sp2* had considerably more down-regulated proteins (60 and 93 proteins, respectively) compared to the Antarctic isolates *sp3* (11 proteins) and *sp4* (29 proteins). The relationship of DEPs from each isolate within regions is presented in a Venn diagram in Figure 5.3. Among the up-regulated proteins identified in each isolate, 4.5 – 13% of proteins were in common between isolates of the same region (Figure 5.3a-c). However, a slightly narrower range was found for down-regulated proteins, 7 – 10.1% (Figure 5.3d-f). The Antarctic isolates (Figure 5.3b, e) had relatively low numbers of common DEPs (6 proteins), whilst the Arctic (Figure 5.3a, d) and temperate isolates (Figure 5.3c, f) shared a higher number of common DEPs (29 proteins).

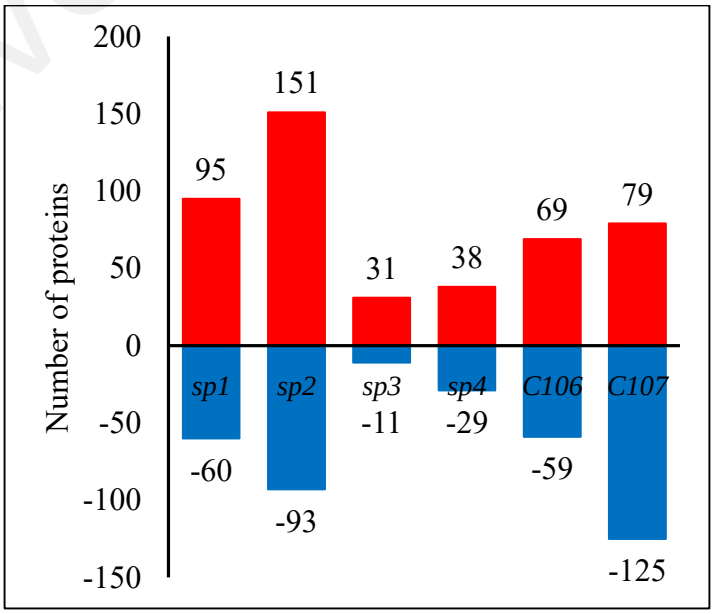

Figure 5.2 Bar graph representing number of proteins up- (red bars) and down-regulated (blue bars) in each isolate. The value on each bar represents the number of DEPs; (+) values, upregulated proteins; (–) values, downregulated proteins.

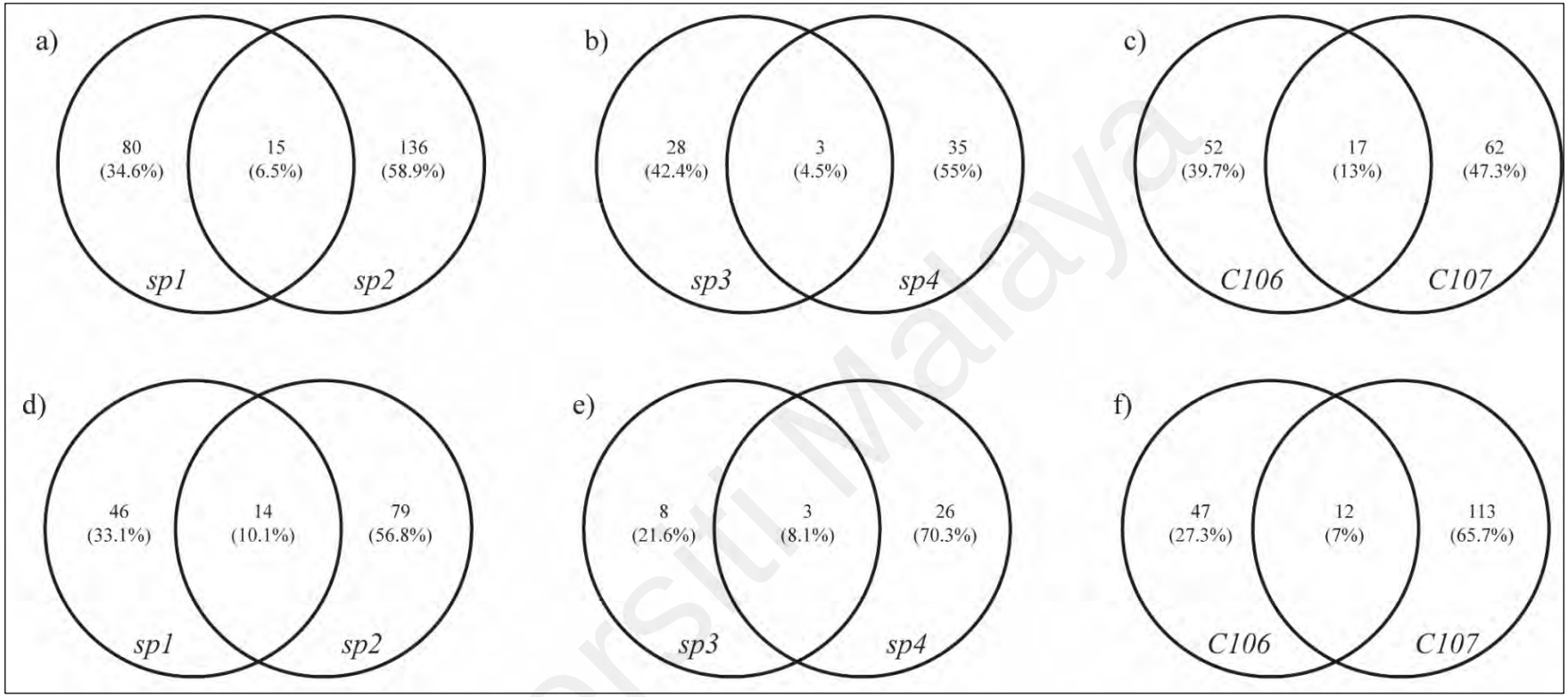

Figure 5.3 Venn diagram showing the relationship of up- and down-regulated proteins of each isolate within each region. a – c), upregulated proteins; d – f), downregulated proteins. a) and d), the Arctic isolates; b) and e), the Antarctic isolates; c) and f), the temperate isolates.

5.4.2 Gene Ontology enrichment analysis of significantly up-regulated proteins in *Pseudogymnoascus* spp.

Proteins were further analysed to identify significantly up-regulated proteins with fold change values of ≥ 1.5 in each isolate (Table 5.1). Knowledge of protein function was gained by inputting each assigned protein identification (NCBI accession number) into the UniProtKB database (http://www.uniprot.org/blast/) and detecting the respective Gene Ontology (GO) terms and annotations. In total, 143 proteins were significantly upregulated in *Pseudogymnoascus* spp., with 50% of these being hypothetical proteins. A large proportion of the proteins (61%) had fold change of > 2.0, with the highest being 5-fold. Most of the known proteins were enzymes, such as plasma membrane ATPase, malate dehydrogenase, glyceraldehyde 3-phosphate-dehydrogenase, argininosuccinate lyase, transketolase, enolase and catalase. There were also a significant number of heat shock proteins (hsp) or hsp-like proteins such as mitochondrial hsp60, hsp SSB1 and hsp70-like proteins. Two of the proteins identified were common to three isolates, malate dehydrogenase (GI: 1026904149) and hypothetical protein VE03_04396 (GI: 1040529249). Malate dehydrogenase was commonly upregulated in *sp2*, *sp3* and *sp4* isolates, whilst hypothetical protein VE03_04396 was commonly upregulated in *sp2*, *sp4* and *C106* isolates.

Table 5.1 List of significantly upregulated proteins under heat stress (fold change, log_2 ratios ≥ 1.5). Accession, protein names and species details are from the NCBI database.

Isolate	GI Accession	Protein name	Species	Coverage [%]	# Unique Peptides	# AAs	MW [kDa]	calc. pI	log_2
sp1	1040531100	plasma membrane ATPase	*Pseudogymnoascus* sp. 23342-1-I1	41	2	931	100.8	5.15	2.0
	1040532023	hypothetical protein VE03_01299	*Pseudogymnoascus* sp. 23342-1-I1	21	8	1015	105.6	5.01	2.5
	1040529276	hypothetical protein VE03_04321	*Pseudogymnoascus* sp. 23342-1-I1	54	6	411	45.8	5.43	2.9
	1040536341	hypothetical protein VF21_08040	*Pseudogymnoascus* sp. 05NY08	45	1	369	39.9	5.72	1.6
	1352886849	proteasome regulatory particle base subunit rpt5	*Pseudogymnoascus verrucosus*	47	16	462	51.6	5.01	1.8
	1040532080	26S protease regulatory subunit 6B	*Pseudogymnoascus* sp. 23342-1-I1	38	2	421	47.1	6	1.7

	1352887687	hypothetical protein VE01_04954	*Pseudogymnoascus verrucosus*	31	4	525	57.1	7.12	2.9
	1040555042	hypothetical protein VE02_00544	*Pseudogymnoascus* sp. 03VT05	32	8	275	29.8	4.83	1.6
	1040533074	hypothetical protein VE03_00632	*Pseudogymnoascus* sp. 23342-1-I1	42	11	219	22.9	5.27	1.9
	1040557715	putative Hybrid PKS-NRPS biosynthetic cluster	*Pseudogymnoascus verrucosus*	6	18	3957	433.1	5.92	2.0
	1040507187	NADH dehydrogenase Fe-S protein 3	*Pseudogymnoascus* sp. WSF 3629	44	11	280	31.9	7.94	2.4
	1040526950	6-phosphogluconolactonase	*Pseudogymnoascus* sp. 23342-1-I1	51	6	260	28	5.6	1.7
	1040523775	hypothetical protein VE03_10818	*Pseudogymnoascus* sp. 23342-1-I1	33	6	151	16	9.99	1.7
	1040524437	hypothetical protein VE03_08889	*Pseudogymnoascus* sp. 23342-1-I1	16	2	436	47	5.02	3.2
	1040537381	hypothetical protein VF21_08280	*Pseudogymnoascus* sp. 05NY08	1	2	5717	626	4.86	2.2
	1040528090	hypothetical protein VE03_04733	*Pseudogymnoascus* sp. 23342-1-I1	3	2	945	99.8	10.14	2.2
	1040528581	hypothetical protein VE03_05544	*Pseudogymnoascus* sp. 23342-1-I1	6	1	412	44	5.16	2.6
	1040527774	hypothetical protein VE03_06499	*Pseudogymnoascus* sp. 23342-1-I1	7	1	293	32	5.91	5.0
	1040523191	hypothetical protein VE03_10140	*Pseudogymnoascus* sp. 23342-1-I1	2	2	966	105	6.58	2.8
	1040536825	hypothetical protein VF21_07703	*Pseudogymnoascus* sp. 05NY08	4	2	842	95	6.01	2.0
	1040529653	hypothetical protein VE03_03956	*Pseudogymnoascus* sp. 23342-1-I1	4	2	322	33.8	4.63	2.0
	1040523594	hypothetical protein VE03_09822	*Pseudogymnoascus* sp. 23342-1-I1	1	1	895	101.9	8.54	2.7
	1040543257	hypothetical protein VF21_01377	*Pseudogymnoascus* sp. 05NY08	9	2	232	25.4	5.12	4.1
	1040500264	hypothetical protein VE00_06524	*Pseudogymnoascus* sp. WSF 3629	2	1	568	62.9	5.02	1.8
sp2	1040532488	hsp70-like protein	*Pseudogymnoascus* sp. 23342-1-I1	73	7	688	74.3	5.22	2.5
	1040547996	ATP synthase subunit beta, mitochondrial	*Pseudogymnoascus* sp. 03VT05	87	3	516	55.4	5.68	2.4
	1040524903	heat shock protein 60, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	75	6	737	78	5.63	1.8
	1040531119	glyceraldehyde 3-phosphate-dehydrogenase	*Pseudogymnoascus* sp. 23342-1-I1	86	3	339	36.5	6.95	1.9
	1040553812	heat shock protein SSB1	*Pseudogymnoascus* sp. 03VT05	48	2	767	84.1	8.43	1.7
	1040543863	heat shock protein 60, mitochondrial	*Pseudogymnoascus* sp. 05NY08	71	3	736	78	5.57	1.9
	1040502460	molecular chaperone HtpG	*Pseudogymnoascus* sp. WSF 3629	69	1	703	79.5	4.92	2.0
	440639856	tubulin beta chain	*Pseudogymnoascus destructans* 20631-21	76	37	446	49.6	4.93	1.7
	1040543410	elongation factor 1-alpha	*Pseudogymnoascus* sp. 05NY08	75	11	459	49.9	9.13	1.6
	1026904149	Malate dehydrogenase, cytoplasmic	*Pseudogymnoascus destructans*	80	6	339	35.2	8.92	3.0
	1040537109	ATP synthase subunit alpha, mitochondrial	*Pseudogymnoascus* sp. 05NY08	64	4	555	59.7	9.1	2.0
	1040529266	hypothetical protein VE03_04296	*Pseudogymnoascus* sp. 23342-1-I1	13	4	4080	451.6	6.43	1.6

1040529726	cell division control protein 48	*Pseudogymnoascus* sp. 23342-1-I1	60	53	823	89.9	5.05	2.0
1040506608	actin	*Pseudogymnoascus* sp. WSF 3629	77	29	375	41.5	5.69	1.8
541136033	translation elongation factor EF-1a, partial	*Pseudogymnoascus* sp. B AM-2013	82	1	283	30.7	8.84	2.1
1040530016	mitochondrial-processing peptidase subunit beta	*Pseudogymnoascus* sp. 23342-1-I1	62	27	478	52.5	5.74	1.7
1040537119	hypothetical protein VF21_07351	*Pseudogymnoascus* sp. 05NY08	72	1	496	53.4	5.8	2.1
1040500818	aldehyde dehydrogenase	*Pseudogymnoascus* sp. WSF 3629	67	1	496	53.4	5.95	3.4
1040563055	hypothetical protein VE01_03532	*Pseudogymnoascus verrucosus*	75	4	231	25.7	6.42	2.0
1040535944	transketolase	*Pseudogymnoascus* sp. 05NY08	45	3	685	74.6	6.13	1.8
1040566397	methionine adenosyltransferase sam2	*Pseudogymnoascus verrucosus*	54	4	394	43.1	6.47	1.9
1040538944	glucose-6-phosphate isomerase	*Pseudogymnoascus* sp. 05NY08	51	2	552	60.8	6.13	1.9
1040530832	hypothetical protein VE03_02453	*Pseudogymnoascus* sp. 23342-1-I1	45	6	462	48.7	8.29	2.9
1040529249	hypothetical protein VE03_04396	*Pseudogymnoascus* sp. 23342-1-I1	65	2	468	51.3	5.54	2.9
1026910166	NADH dehydrogenase (ubiquinone) 78K chain precursor, 5-prime end	*Pseudogymnoascus destructans*	51	2	745	81	7.08	2.3
1069480601	hypothetical protein VE01_10081	*Pseudogymnoascus verrucosus*	39	2	522	56.4	4.55	1.6
1040517350	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Pseudogymnoascus* sp. 24MN13	49	8	522	57.7	5.4	3.4
1040538463	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	*Pseudogymnoascus* sp. 05NY08	71	2	276	30.7	4.97	1.9
1026902306	hypothetical protein VC83_09257	*Pseudogymnoascus destructans*	50	4	545	60.5	6.11	2.0
1040504412	hypothetical protein VE00_02312	*Pseudogymnoascus* sp. WSF 3629	60	5	282	31.3	4.45	4.3
1069478965	hypothetical protein VE01_08888	*Pseudogymnoascus verrucosus*	57	2	298	33.5	7.02	2.8
1370880255	putrescine aminopropyltransferase	*Pseudogymnoascus destructans*	37	1	379	43	8.19	2.3
1026906371	hypothetical protein VC83_04944	*Pseudogymnoascus destructans*	68	2	154	17.4	7.88	2.6
1040525933	hypothetical protein VE03_08169	*Pseudogymnoascus* sp. 23342-1-I1	44	3	259	28	5.11	1.9
1352888288	ribosomal 40S subunit protein S27A	*Pseudogymnoascus verrucosus*	59	13	156	17.9	9.73	2.2
1040511267	hypothetical protein VE04_09537	*Pseudogymnoascus* sp. 24MN13	11	6	1822	202	6.47	2.1
1040524212	hypothetical protein VE03_09540	*Pseudogymnoascus* sp. 23342-1-I1	27	14	820	86.1	5.15	2.7
1040501360	40S ribosomal protein S17	*Pseudogymnoascus* sp. WSF 3629	39	3	148	17	9.8	1.7
1040526617	Fe-Mn family superoxide dismutase	*Pseudogymnoascus* sp. 23342-1-I1	51	8	229	25.1	8.68	2.3

	1040526348	dihydroxy-acid dehydratase	*Pseudogymnoascus* sp. 23342-1-I1	32	8	592	63.2	7.12	2.7
	1069461791	hydroxymethylglutaryl-CoA synthase	*Pseudogymnoascus verrucosus*	17	1	464	50.3	6.57	1.8
	1069478089	eukaryotic phosphomannomutase	*Pseudogymnoascus verrucosus*	37	8	268	30.5	5.14	2.7
	1370875018	hypothetical protein VC83_01337	*Pseudogymnoascus destructans*	25	6	603	64.3	6.73	4.1
	1040532311	1-pyrroline-5-carboxylate dehydrogenase	*Pseudogymnoascus* sp. 23342-1-I1	22	3	575	62.2	8.76	1.7
	1040514134	thioredoxin	*Pseudogymnoascus* sp. 24MN13	48	1	110	11.7	5.8	2.2
	1040529370	hypothetical protein VE03_04877	*Pseudogymnoascus* sp. 23342-1-I1	28	4	248	26.9	5.25	3.0
	1040530118	hypothetical protein VE03_04536	*Pseudogymnoascus* sp. 23342-1-I1	19	9	542	60.3	7.61	3.4
	1040529802	hypothetical protein VE03_04039	*Pseudogymnoascus* sp. 23342-1-I1	24	1	196	21.9	5.88	2.8
	1040543888	T-complex protein 1 subunit gamma	*Pseudogymnoascus* sp. 05NY08	17	1	541	59	5.99	5.0
	1040524989	hypothetical protein VE03_08992	*Pseudogymnoascus* sp. 23342-1-I1	17	5	503	54.1	5.67	1.6
	1040499575	hypothetical protein VE00_08055	*Pseudogymnoascus* sp. WSF 3629	8	3	481	50.2	7.58	4.3
	1040499304	hypothetical protein VE00_09058	*Pseudogymnoascus* sp. WSF 3629	5	1	478	51.1	8.84	2.9
	1352885771	hypothetical protein VC83_08421	*Pseudogymnoascus destructans*	9	2	256	27.1	9.79	2.2
	1040549091	hypothetical protein VE02_06915	*Pseudogymnoascus* sp. 03VT05	6	3	456	51.2	4.74	3.5
	1040527052	ATP-dependent RNA helicase DDX6/DHH1	*Pseudogymnoascus* sp. 23342-1-I1	9	5	545	60.4	8.97	2.4
	1040506083	orotidine 5'-phosphate decarboxylase	*Pseudogymnoascus* sp. WSF 3629	4	1	357	38.9	5.19	3.0
	1040536977	hypothetical protein VF21_07230	*Pseudogymnoascus* sp. 05NY08	7	1	178	18.9	5.52	2.1
	1040508251	hypothetical protein VE04_10213	*Pseudogymnoascus* sp. 24MN13	4	1	205	21.2	6.34	2.4
	1001840675	Polyribonucleotide nucleotidyltransferase	*Streptomyces albidoflavus*	1	1	735	78.9	5.12	1.7
	440640027	hypothetical protein GMDG_04422	*Pseudogymnoascus destructans* 20631-21	0	1	1866	208.6	7.2	2.3
	1001839554	argininosuccinate lyase	*Streptomyces albidoflavus*	2	1	419	44.9	5.19	2.3
sp3	1040506877	enolase	*Pseudogymnoascus* sp. WSF 3629	85	43	438	47.7	5.41	2.0
	1040523711	hsp70-like protein	*Pseudogymnoascus* sp. 23342-1-I1	65	10	676	73.5	5.74	2.4
	1026904149	Malate dehydrogenase, cytoplasmic	*Pseudogymnoascus destructans*	80	6	339	35.2	8.92	3.5
	1040529726	cell division control protein 48	*Pseudogymnoascus* sp. 23342-1-I1	60	53	823	89.9	5.05	3.0
	440634311	catalase	*Pseudogymnoascus destructans* 20631-21	60	2	505	57.4	7.3	1.9
	1040525455	hypothetical protein VE03_07380	*Pseudogymnoascus* sp. 23342-1-I1	63	4	231	25.6	6.42	4.1
	1040532321	translation elongation factor Tu	*Pseudogymnoascus* sp. 23342-1-I1	67	4	444	48.9	6.93	1.7
	1040533135	ATP synthase F1, gamma subunit	*Pseudogymnoascus* sp. 23342-1-I1	48	6	298	32.1	8.34	1.7
	1040499122	40S ribosomal protein S7	*Pseudogymnoascus* sp. WSF 3629	65	8	202	22.8	10.33	2.0

	1040523753	30S ribosomal protein S8e	*Pseudogymnoascus* sp. 23342-1-I1	55	2	202	22.9	11.24	3.0
	1040554705	hypothetical protein VE02_03336	*Pseudogymnoascus* sp. 03VT05	30	1	431	47.7	7.49	4.1
	440638868	GTP-binding protein ypt1	*Pseudogymnoascus destructans* 20631-21	47	8	201	22.2	5.44	2.7
	1026910022	hypothetical protein VC83_00976	*Pseudogymnoascus destructans*	53	9	183	20.9	6.58	2.0
	1040529367	hypothetical protein VE03_04870	*Pseudogymnoascus* sp. 23342-1-I1	21	5	485	52.8	5.6	3.1
	1040560425	hypothetical protein VE01_06071	*Pseudogymnoascus verrucosus*	5	2	1012	110.3	4.72	2.1
	440640832	hypothetical protein GMDG_05006	*Pseudogymnoascus destructans* 20631-21	7	2	598	62.9	5.6	3.4
	1040501120	hypothetical protein VE00_07309	*Pseudogymnoascus* sp. WSF 3629	4	2	1097	121.7	4.79	3.3
	1040524019	translation initiation factor eIF-4E	*Pseudogymnoascus* sp. 23342-1-I1	10	2	264	29	6.37	2.1
	1040516322	hypothetical protein VE04_04550	*Pseudogymnoascus* sp. 24MN13	4	1	232	26	5.45	4.4
	440636097	hypothetical protein GMDG_07727	*Pseudogymnoascus destructans* 20631-21	1	1	546	59.9	6	3.1
sp4	1026904149	Malate dehydrogenase, cytoplasmic	*Pseudogymnoascus destructans*	80	6	339	35.2	8.92	2.2
	1040529249	hypothetical protein VE03_04396	*Pseudogymnoascus* sp. 23342-1-I1	65	2	468	51.3	5.54	1.7
	1069465551	guanine nucleotide-binding protein subunit beta-like protein	*Pseudogymnoascus verrucosus*	66	3	316	35	7.03	1.9
	1040504790	argininosuccinate lyase	*Pseudogymnoascus* sp. WSF 3629	43	17	472	52.7	6.02	1.6
	1040526059	hypothetical protein VE03_07843	*Pseudogymnoascus* sp. 23342-1-I1	40	3	476	52.5	5.2	1.8
	1040531525	ubiquinol-cytochrome c reductase iron-sulfur subunit	*Pseudogymnoascus* sp. 23342-1-I1	38	6	239	25.8	8.43	1.8
	1040519866	T-complex protein 1 subunit epsilon	*Pseudogymnoascus* sp. 24MN13	33	12	548	59.7	5.5	1.8
	1040517617	hypothetical protein VE04_03781	*Pseudogymnoascus* sp. 24MN13	15	3	305	31.9	4.64	2.5
	1040563381	hypothetical protein VE01_03431	*Pseudogymnoascus verrucosus*	1	1	998	109.5	9.58	4.0
	1040553508	hypothetical protein VE02_01993	*Pseudogymnoascus* sp. 03VT05	4	1	234	24.9	5.77	2.7
C106	1040527559	NADP-specific glutamate dehydrogenase	*Pseudogymnoascus* sp. 23342-1-I1	60	3	449	48.8	6.06	1.7
	1040529249	hypothetical protein VE03_04396	*Pseudogymnoascus* sp. 23342-1-I1	65	2	468	51.3	5.54	1.7
	1040532273	ketol-acid reductoisomerase, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	69	2	400	44.5	7.05	2.1
	1040541217	transaldolase, variant	*Pseudogymnoascus* sp. 05NY08	60	6	325	35.8	6.44	1.8
	1040530717	NADH-ubiquinone oxidoreductase 51 kDa subunit, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	48	2	509	55.3	7.87	1.8
	1040504056	hypothetical protein VE00_03867	*Pseudogymnoascus* sp. WSF 3629	11	3	611	64.1	5.33	3.1
	1040506686	homocitrate synthase	*Pseudogymnoascus* sp. WSF 3629	42	15	421	45.9	5.76	1.8
	1040531923	dihydrolipoyllysine -residue	*Pseudogymnoascus* sp. 23342-1-I1	38	5	420	46.2	7.97	1.8

		succinyltransferase, E2 component							
	1040563295	isocitrate dehydrogenase (NAD(+)) idh1	*Pseudogymnoascus verrucosus*	45	3	381	41.5	9.04	2.4
	1040549859	RuvB-like helicase 1	*Pseudogymnoascus* sp. 03VT05	45	1	457	49.4	5.81	1.8
	1040526755	hypothetical protein VE03_06971	*Pseudogymnoascus* sp. 23342-1-I1	3	3	2518	275.9	5.55	2.1
	1040552965	hypothetical protein VE02_03946	*Pseudogymnoascus* sp. 03VT05	9	3	571	61.9	5.69	2.3
	1040526257	hypothetical protein VE03_07208	*Pseudogymnoascus* sp. 23342-1-I1	18	4	266	29.6	5.26	2.1
	1040504311	hypothetical protein VE00_02840	*Pseudogymnoascus* sp. WSF 3629	18	1	71	7.3	6.79	2.7
	1040499207	hypothetical protein VE00_10306	*Pseudogymnoascus* sp. WSF 3629	5	3	670	74	7.39	4.4
	1040565531	hypothetical protein VE01_00802	*Pseudogymnoascus verrucosus*	3	1	236	25.7	9.58	2.3
C107	1040524903	heat shock protein 60, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	75	6	737	78	5.63	2.3
	440640092	heat shock protein SSB1	*Pseudogymnoascus destructans* 20631-21	56	3	614	66.5	5.38	2.3
	1040523711	hsp70-like protein	*Pseudogymnoascus* sp. 23342-1-I1	65	10	676	73.5	5.74	1.6
	1026908832	hypothetical protein VC83_02633	*Pseudogymnoascus destructans*	60	3	313	33.8	9.79	2.1
	1040532321	translation elongation factor Tu	*Pseudogymnoascus* sp. 23342-1-I1	67	4	444	48.9	6.93	1.9
	1370871900	hypothetical protein VC83_00219	*Pseudogymnoascus destructans*	57	13	166	17.3	5.54	1.5
	1040532761	hypothetical protein VE03_00724	*Pseudogymnoascus* sp. 23342-1-I1	53	5	424	45.8	8.73	1.6
	1040523522	hypothetical protein VE03_09887	*Pseudogymnoascus* sp. 23342-1-I1	20	5	1063	118.3	6.61	1.5
	1040535961	hypothetical protein VF21_08654	*Pseudogymnoascus* sp. 05NY08	10	2	410	45.2	6.58	2.9
	1040499975	F-type H+transporting ATPase subunit F	*Pseudogymnoascus* sp. WSF 3629	25	2	101	11.1	10.49	2.6
	1352885345	hypothetical protein VC83_04296	*Pseudogymnoascus destructans*	13	2	294	32.2	9.48	2.5
	1040543138	hypothetical protein VF21_01332	*Pseudogymnoascus* sp. 05NY08	4	3	862	92.5	8.98	1.6

GO enrichment analysis was carried out for significantly up-regulated proteins using KOBAS v2.0 to search for over-represented categories of molecular pathways in the databases Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Panther, BioCyc and Reactome. A complete list of enriched pathways with p value ≤ 0.05 for each isolate is shown in Supplementary 2. The top 10 pathways and their respective p values were selected for each isolate and are presented in Figure 5.4. Each isolate showed a

different set of affected molecular pathways and no common pathway was enriched in all isolates. However, *Pseudogymnoascus* spp. showed enrichment of various metabolic pathways and cellular responses to stress in all six isolates from the different regions. In the Arctic isolates, significant differences between isolates were observed in the top 10 enriched pathways (Figure 5.4a, b). Up-regulated proteins in *sp1* included a high number of significant enrichment pathways related to DNA repair, such as the purine and other nucleotide salvage pathways (Figure 5.4a). However, the enriched pathways in *sp2* involved primarily metabolic pathways, general cellular response to heat stress, and activation of defense mechanism systems such as biosynthesis of secondary metabolites and antibiotics (Figure 5.4b). A different finding was observed in the Antarctic isolates, where protein homeostasis was commonly enriched in both isolates (Figure 5.4c, d). In *sp3*, up-regulated proteins were involved mainly in the metabolism of proteins, such as the ribosomal scanning and start codon recognition pathway, and the COPI-mediated anterograde and retrograde protein transport (Figure 5.4c). Protein homeostasis modulation, specifically the amino acid metabolism pathways, were observed in *sp4*, such as the urea cycle, arginine biosynthesis, aspartate degradation II, glutathione conjugation and gamma glutamyl cycle (Figure 5.4d). For temperate isolates, more diverse pathways were enriched in both isolates (Figure 5.4e, f). General metabolic pathways such as carbon metabolism, TCA cycle, and biosynthesis of amino acids were enriched in *C106* (Figure 5.4e). Other than amino acid metabolism, a cellular response to heat stress, specifically regulation of HSF1-mediated HSR, attenuation phase and RNA degradation were enriched in *C107* (Figure 5.4f).

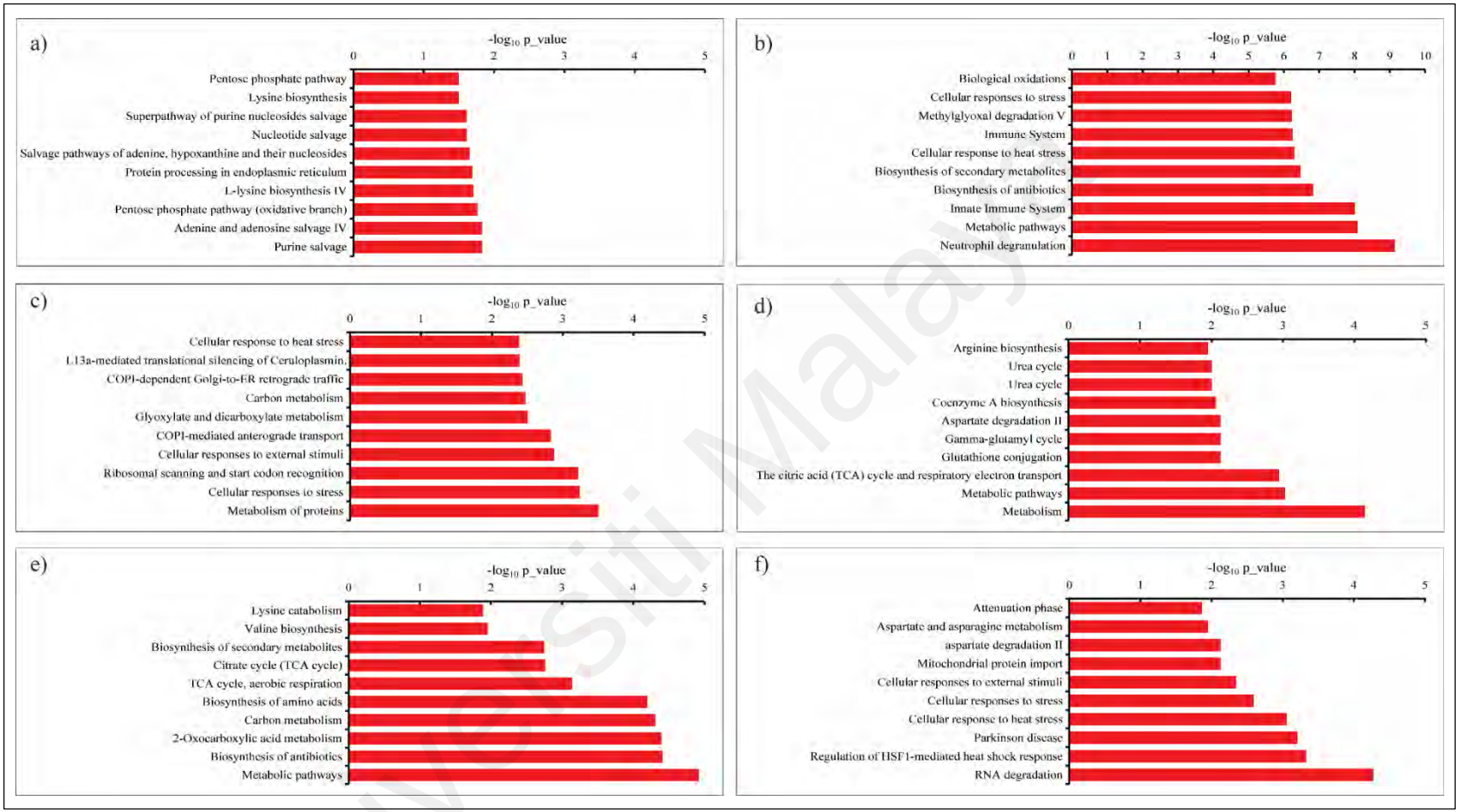

Figure 5.4 GO enrichment analysis of significantly up-regulated DEPs of *Pseudogymnoascus* spp. in response to heat stress (top 10 pathways). a) *sp1*; b) *sp2*; c) *sp3*; d) *sp4*; e) *C106*; f) *C107*.

5.4.3 Gene Ontology enrichment analysis of significantly down-regulated proteins in *Pseudogymnoascus* spp.

Heat stress response in *Pseudogymnoascus* spp. also involved the down-regulation of various proteins. From a total of 377 proteins that were down-regulated in all isolates, 37% were significantly down-regulated with fold change of ≤ -1.5 (Table 5.2), with 58 of these 104 proteins being hypothetical proteins. The variation in the number of significantly down-regulated proteins between isolates was wide, with *sp3* having the lowest number (4 proteins) and *sp2* the highest number of down-regulated proteins (36 proteins). All of the significantly down-regulated proteins identified were exclusive to a single isolate except for one common protein found in the two isolates from the Arctic, 60S ribosomal protein L16 (GI accession: 1040523417). Numerous species of ribosomal proteins were identified in all isolates. In *sp1*, small subunit (S2e, S3e, and S18) and large subunit ribosomal proteins (L5 and L16) were down-regulated with fold change between 1.6 - 3.5. In *sp2*, only large subunit ribosomal proteins were significantly down-regulated under heat stress (L2a, L3, L4e, L10a, L15, L16, L21e, L23, L26e, L27e and L37ae). The temperate isolates showed a pattern of ribosomal proteins identified similar to *sp1*, with both *C106* and C*107* showing down-regulation of various small and large subunit ribosomal proteins. There was also a significant number of down-regulated proteins involved in the translation pathway, such as alanine-tRNA ligase, translation initiation factor eIF5A, RNA export factor gle2 and alanyl-tRNA synthetase. In the Antarctic isolates, all significantly down-regulated proteins were identified as hypothetical proteins with the exception of porin por1, which was down-regulated in *sp3* with fold change of 2.9, hence it was not possible to infer which pathways were affected.

Table 5.2 List of significantly downregulated proteins under heat stress (fold change, log_2 ratios of ≤ -1.5)

Isolate	Accession	Description	Species	Coverage [%]	# Unique Peptides	# AAs	MW [kDa]	calc. pI	log_2
p1	1040527559	NADP-specific glutamate dehydrogenase	*Pseudogymnoascus* sp. 23342-1-I1	60	3	449	48.8	6.06	-1.7
	1040524347	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	*Pseudogymnoascus* sp. 23342-1-I1	63	4	768	86.2	6.48	-1.6
	1040544091	myo-inositol-1-phosphate synthase	*Pseudogymnoascus* sp. 05NY08	63	5	530	57.4	5.82	-2.4
	1040566397	methionine adenosyltransferase sam2	*Pseudogymnoascus verrucosus*	54	4	394	43.1	6.47	-1.7
	1040531987	serine hydroxymethyltransferase, cytosolic	*Pseudogymnoascus* sp. 23342-1-I1	51	1	484	53.3	7.78	-2.1
	440638352	hypothetical protein GMDG_03072	*Pseudogymnoascus destructans* 20631-21	51	6	386	43.5	7.66	-2.0
	1040537116	argininosuccinate synthase	*Pseudogymnoascus* sp. 05NY08	58	5	416	46.4	5.48	-2.0
	1040541506	small subunit ribosomal protein S3e	*Pseudogymnoascus* sp. 05NY08	70	21	262	28.5	9.11	-1.8
	1040550635	small subunit ribosomal protein S2e	*Pseudogymnoascus* sp. 03VT05	55	17	273	29.2	10.27	-1.6
	1040527701	NRPS-like protein	*Pseudogymnoascus* sp. 23342-1-I1	16	3	1172	130.6	5.78	-1.5
	1040525721	hypothetical protein VE03_07707	*Pseudogymnoascus* sp. 23342-1-I1	29	10	424	45.7	6.3	-3.7
	1040517415	40S ribosomal protein S18	*Pseudogymnoascus* sp. 24MN13	38	9	202	23	10.58	-2.6
	440639469	60S ribosomal protein L5	*Pseudogymnoascus destructans* 20631-21	33	1	299	34.4	8.46	-3.5
	1040532830	hypothetical protein VE03_00684	*Pseudogymnoascus* sp. 23342-1-I1	20	2	418	45.5	6.29	-2.2
	1040523417	60S ribosomal protein L16	*Pseudogymnoascus* sp. 23342-1-I1	37	1	202	23.1	10.51	-2.0
	1040525748	threonine synthase	*Pseudogymnoascus* sp. 23342-1-I1	14	6	567	61.6	5.33	-2.4
	440636994	hypothetical protein GMDG_02283	*Pseudogymnoascus destructans* 20631-21	16	2	515	53.7	5.06	-2.5
	1026905771	intracellular distribution of mitochondria	*Pseudogymnoascus destructans*	10	10	1291	142.5	5.72	-1.7
	1040525950	hypothetical protein VE03_08146	*Pseudogymnoascus* sp. 23342-1-I1	10	3	432	47.2	5.54	-1.8
	1040510207	hypothetical protein VE04_08323	*Pseudogymnoascus* sp. 24MN13	19	1	283	31.5	9.11	-1.7
sp2	1040536136	hypothetical protein VF21_08556	*Pseudogymnoascus* sp. 05NY08	33	3	529	56	6.19	-2.0
	1040561901	ribosomal protein L2A	*Pseudogymnoascus verrucosus*	59	21	335	36.7	11.09	-1.7
	1040536470	hypothetical protein VF21_07858	*Pseudogymnoascus* sp. 05NY08	36	1	613	68.4	6	-2.0
	1040514270	hypothetical protein VE04_07321	*Pseudogymnoascus* sp. 24MN13	22	4	775	81.3	6.1	-3.2
	1040525391	hypothetical protein VE03_07986	*Pseudogymnoascus* sp. 23342-1-I1	21	6	783	81.9	5.11	-2.4
	440632652	large subunit ribosomal protein L4e	*Pseudogymnoascus destructans* 20631-21	55	2	373	39.7	11.33	-3.5
	1040534050	catalase/peroxidase HPI	*Pseudogymnoascus* sp. 05NY08	26	4	790	86.4	6.01	-2.6
	1040525523	hypothetical protein VE03_08478	*Pseudogymnoascus* sp. 23342-1-I1	25	5	790	87.1	5.85	-1.7

	1026907381	ribosomal 60S subunit protein L3	*Pseudogymnoascus destructans*	28	2	626	68.4	9.86	-1.5
	1040501441	hypothetical protein VE00_06930	*Pseudogymnoascus* sp. WSF 3629	14	1	655	69.3	5.52	-2.9
	1040525559	protein transporter sec-23	*Pseudogymnoascus* sp. 23342-1-I1	24	20	955	106.6	7.78	-2.0
	1040519485	hypothetical protein VE04_01330	*Pseudogymnoascus* sp. 24MN13	25	3	598	64.8	5.9	-3.0
	1040533161	catalase/peroxidase HPI	*Pseudogymnoascus* sp. 23342-1-I1	23	5	795	87.3	5.5	-2.7
	1040501747	hypothetical protein VE00_06979	*Pseudogymnoascus* sp. WSF 3629	29	4	383	39.7	5.95	-2.4
	1040518813	60S ribosomal protein L15	*Pseudogymnoascus* sp. 24MN13	51	14	203	23.8	11.47	-2.5
	1040528493	hypothetical protein VE03_04938	*Pseudogymnoascus* sp. 23342-1-I1	48	6	336	37.4	9.09	-2.3
	1040539732	hypothetical protein VF21_05012	*Pseudogymnoascus* sp. 05NY08	12	2	568	58.4	5.34	-2.0
	440640816	60S ribosomal protein L10-A	*Pseudogymnoascus destructans* 20631-21	65	16	221	25.2	10.35	-2.4
	440632109	large subunit ribosomal protein L27e	*Pseudogymnoascus destructans* 20631-21	35	7	135	15.6	10.71	-3.0
	440635254	60S ribosomal protein L23	*Pseudogymnoascus destructans* 20631-21	45	8	139	14.6	10.21	-2.4
	1040523992	hypothetical protein VE03_10835	*Pseudogymnoascus* sp. 23342-1-I1	42	7	131	15	11.91	-3.2
	1040547240	hypothetical protein VE02_07770	*Pseudogymnoascus* sp. 03VT05	8	3	486	51.9	6.11	-4.0
	1040528549	large subunit ribosomal protein L26e	*Pseudogymnoascus* sp. 23342-1-I1	41	11	137	15.7	10.68	-2.7
	1069466243	large subunit ribosomal protein L21e	*Pseudogymnoascus verrucosus*	52	12	160	18.2	10.33	-1.8
	1040523417	60S ribosomal protein L16	*Pseudogymnoascus* sp. 23342-1-I1	37	1	202	23.1	10.51	-2.7
	1370889566	hypothetical protein VC83_08378	*Pseudogymnoascus destructans*	19	2	385	42.1	5.91	-2.4
	1040535171	hypothetical protein VF21_09623	*Pseudogymnoascus* sp. 05NY08	7	1	752	80.8	5.6	-1.6
	1040516362	hypothetical protein VE04_05577	*Pseudogymnoascus* sp. 24MN13	35	4	140	15.4	8.12	-1.9
	1040505794	hypothetical protein VE00_03008	*Pseudogymnoascus* sp. WSF 3629	28	8	186	21.4	11.18	-2.4
	1040551382	ribosomal protein L37ae	*Pseudogymnoascus* sp. 03VT05	47	4	92	10.2	10.55	-1.7
	1040496936	hypothetical protein VE00_10886	*Pseudogymnoascus* sp. WSF 3629	7	2	206	22.3	6.06	-1.8
	1026908888	hypothetical protein VC83_02735	*Pseudogymnoascus destructans*	15	1	370	39	7.36	-3.0
	1040500245	hypothetical protein VE00_06561	*Pseudogymnoascus* sp. WSF 3629	7	1	357	38	7.81	-2.1
	1040524966	hypothetical protein VE03_09214	*Pseudogymnoascus* sp. 23342-1-I1	3	2	1351	146.5	5.27	-2.2
	1040554267	hypothetical protein VE02_00857	*Pseudogymnoascus* sp. 03VT05	10	1	215	22.2	4.87	-1.7
	1040507457	hypothetical protein VE00_00106	*Pseudogymnoascus* sp. WSF 3629	1	1	799	85.4	6.64	-3.1
sp3	1040530398	hypothetical protein VE03_02988	*Pseudogymnoascus* sp. 23342-1-I1	73	8	514	55.1	6.27	-2.3
	1352887886	porin por1	*Pseudogymnoascus verrucosus*	94	6	283	30.3	8.98	-2.9

	1040532023	hypothetical protein VE03_01299	*Pseudogymnoascus* sp. 23342-1-I1	21	8	1015	105.6	5.01	-3.6
	1040525910	hypothetical protein VE03_07646	*Pseudogymnoascus* sp. 23342-1-I1	21	7	441	48.7	7.3	-3.8
sp4	1040527838	hypothetical protein VE03_06433	*Pseudogymnoascus* sp. 23342-1-I1	22	4	811	91.2	6.44	-2.3
	1040512347	hypothetical protein VE04_08233, partial	*Pseudogymnoascus* sp. 24MN13	17	2	753	84.8	6.48	-2.1
	1040516152	hypothetical protein VE04_03958, partial	*Pseudogymnoascus* sp. 24MN13	8	5	760	85.3	5.47	-3.2
	1040529140	hypothetical protein VE03_05110	*Pseudogymnoascus* sp. 23342-1-I1	10	1	422	45.3	8.76	-2.1
	1040501128	hypothetical protein VE00_07165	*Pseudogymnoascus* sp. WSF 3629	2	1	651	71	4.64	-1.5
	1026906372	hypothetical protein VC83_04945	*Pseudogymnoascus destructans*	1	1	1316	147.2	6.23	-1.9
C106	1040528536	hypothetical protein VE03_05557	*Pseudogymnoascus* sp. 23342-1-I1	65	7	580	63.1	6.52	-2.0
	1352888949	phosphatidylinositol transfer protein csr1	*Pseudogymnoascus verrucosus*	63	1	221	24	9.39	-2.1
	1040561561	hypothetical protein VE01_04913	*Pseudogymnoascus verrucosus*	48	2	258	28	5.31	-3.0
	1026906159	Alanine--tRNA ligase	*Pseudogymnoascus destructans*	26	2	1016	113.9	5.83	-1.9
	1040557018	translation initiation factor eIF5A	*Pseudogymnoascus verrucosus*	40	9	166	18.1	5.08	-2.1
	1040541679	hypothetical protein VF21_02713	*Pseudogymnoascus* sp. 05NY08	51	1	323	34.1	6.58	-1.6
	1040539963	hypothetical protein VF21_03952	*Pseudogymnoascus* sp. 05NY08	41	9	294	31.9	8.85	-1.7
	440640816	60S ribosomal protein L10-A	*Pseudogymnoascus destructans* 20631-21	65	16	221	25.2	10.35	-2.1
	1370880779	RNA export factor gle2	*Pseudogymnoascus destructans*	33	8	359	39.2	8.1	-2.7
	1040527084	hypothetical protein VE03_06273	*Pseudogymnoascus* sp. 23342-1-I1	56	1	142	15.4	6.8	-1.6
	1026903843	60S ribosomal protein L31	*Pseudogymnoascus destructans*	43	6	124	13.9	10.35	-2.4
	1026906059	hypothetical protein VC83_06446	*Pseudogymnoascus destructans*	3	3	1096	118.3	8.87	-3.1
	1352888001	hypothetical protein VE01_06828	*Pseudogymnoascus verrucosus*	3	2	644	70.1	5.68	-3.3
	1040555230	hypothetical protein VE02_00448	*Pseudogymnoascus* sp. 03VT05	7	1	243	27.3	5	-2.4
C107	1370876424	hypothetical protein VC83_01837	*Pseudogymnoascus destructans*	44	3	259	28.1	5.06	-2.5
	1040532968	alanyl-tRNA synthetase	*Pseudogymnoascus* sp. 23342-1-I1	28	3	958	107.3	5.43	-1.5
	1069473919	40S ribosomal protein S1	*Pseudogymnoascus verrucosus*	75	22	256	29.3	10.07	-1.5
	1040528274	diphosphomevalonate decarboxylase	*Pseudogymnoascus* sp. 23342-1-I1	48	16	385	40.8	6.55	-1.7
	1069475329	hypothetical protein VE01_06151	*Pseudogymnoascus verrucosus*	64	11	137	14.8	10.13	-1.9
	1069463567	ornithine-oxo-acid transaminase	*Pseudogymnoascus verrucosus*	32	2	463	50.1	6.15	-2.0
	1040527436	large subunit ribosomal protein L7Ae	*Pseudogymnoascus* sp. 23342-1-I1	52	18	264	29.3	10.29	-2.5

1040560002	ABC transporter ATP-binding protein arb1	*Pseudogymnoascus verrucosus*	27	13	619	68.7	6.57	-1.8
1040505557	60S ribosomal protein L5	*Pseudogymnoascus* sp. WSF 3629	33	2	299	34.4	8.46	-1.7
1040506813	imidazole glycerol phosphate synthase hisHF	*Pseudogymnoascus* sp. WSF 3629	15	1	542	58.4	5.68	-1.6
1040550136	hypothetical protein VE02_06045	*Pseudogymnoascus* sp. 03VT05	17	3	275	29.5	6.04	-1.5
1026902878	60S ribosomal protein L22	*Pseudogymnoascus destructans*	38	5	125	14.3	9.26	-1.8
440637078	hypothetical protein GMDG_02319	*Pseudogymnoascus destructans* 20631-21	24	7	416	44.3	7.97	-1.6
1040505097	hypothetical protein VE00_01915	*Pseudogymnoascus* sp. WSF 3629	13	1	496	51.6	5.6	-4.6
1040531132	hypothetical protein VE03_02328	*Pseudogymnoascus* sp. 23342-1-I1	25	2	323	36.1	9.2	-2.8
1040544279	hypothetical protein VF21_00519	*Pseudogymnoascus* sp. 05NY08	30	1	206	22.7	4.93	-1.7
1040525004	E3 ubiquitin ligase complex SCF subunit sconC	*Pseudogymnoascus* sp. 23342-1-I1	44	3	166	18.9	4.55	-1.5
1040524033	hypothetical protein VE03_09559	*Pseudogymnoascus* sp. 23342-1-I1	13	1	160	18.1	6.35	-1.7
1040496816	hypothetical protein VE00_10639	*Pseudogymnoascus* sp. WSF 3629	9	3	343	37.3	10.46	-1.6
1040517844	hypothetical protein VE04_02723	*Pseudogymnoascus* sp. 24MN13	5	1	442	49	9.16	-1.5
1040528684	hypothetical protein VE03_03328	*Pseudogymnoascus* sp. 23342-1-I1	7	2	285	29.8	4.91	-1.9
1040517115	30S ribosomal protein S14p/S29e	*Pseudogymnoascus* sp. 24MN13	11	1	71	8.1	8.81	-2.0
1026909466	hypothetical protein VC83_01841	*Pseudogymnoascus destructans*	2	1	413	44.8	6.99	-2.4
1040553508	hypothetical protein VE02_01993	*Pseudogymnoascus* sp. 03VT05	4	1	234	24.9	5.77	-2.1

GO enrichment analysis was performed on the significantly down-regulated proteins and the top 10 enriched pathways for each isolates are presented in Figure 5.5, with the exception of the Antarctic isolates where the number of significantly enriched pathways was less than 10. Numerous common pathways were found to be enriched in all isolates except for the Antarctic isolates (Figure 5.5a-f). Seven common pathways were enriched in the Arctic isolates, including the formation of a pool of free 40S subunits, GTP hydrolysis and joining of the 60S ribosomal subunit, SRP-dependent co-translational protein targeting to membrane and various Nonsense Mediated Decay (NMD) pathways (Figure 5.5a, b). For the temperate isolates, pathways similar to the Arctic isolates were enriched with the addition of cap-dependent translation initiation (Figure 5.5c, d). For the

Antarctic isolates, *sp3* and *sp4* had only two and six significantly enriched pathways for down-regulated proteins, respectively. The significantly enriched pathways for *sp3* included mitophagy and MAPK signaling pathway, both pathways inferred from yeast (Figure 5.5c). mRNA decay, peroxisome-related pathways and RNA degradation were significantly down-regulated in *sp4* (Figure 5.5d).

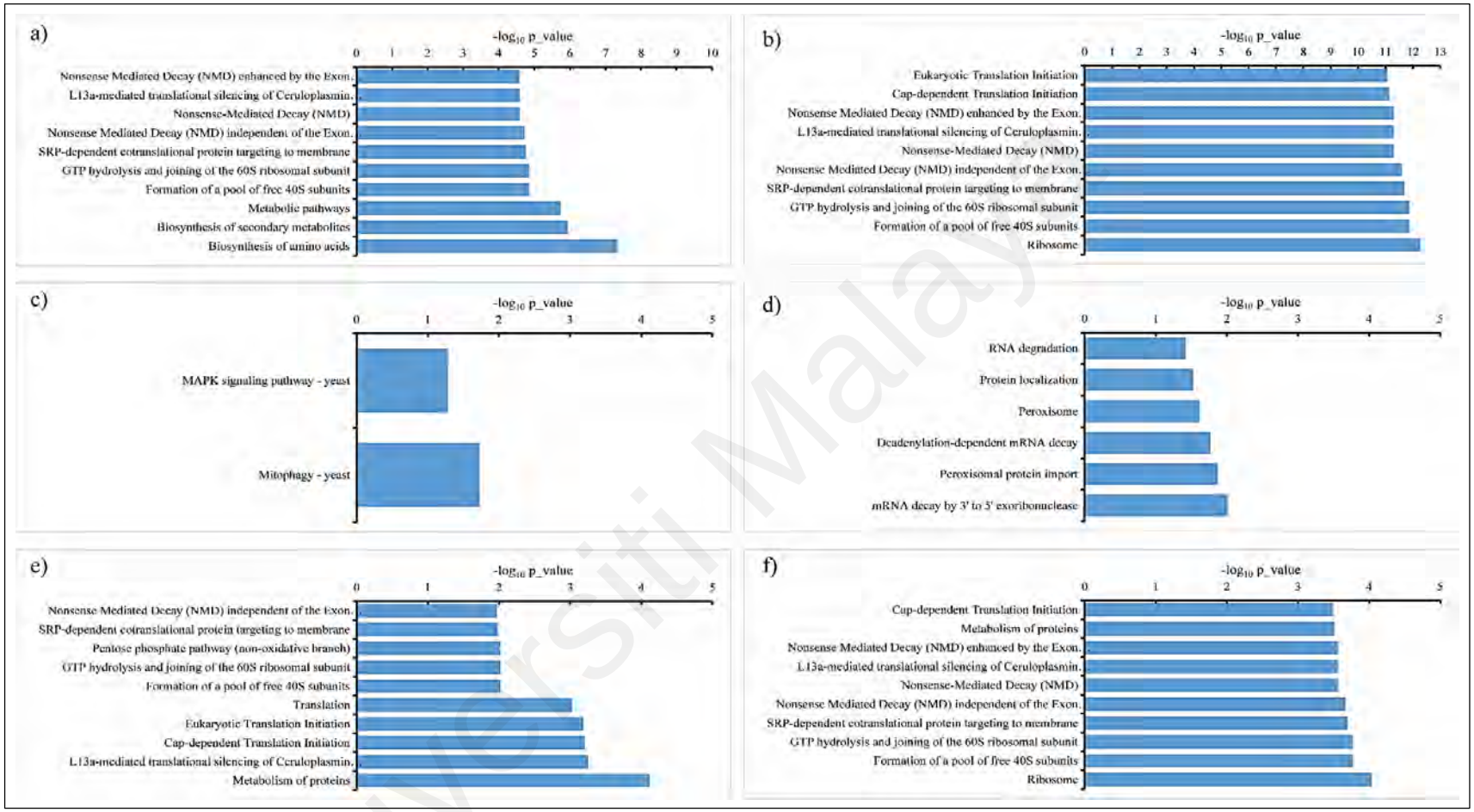

Figure 5.5 GO enrichment analysis of significantly down-regulated DEPs of *Pseudogymnoascus* spp. in response to heat stress (top 10 pathways, except for *sp3* and *sp4*). a) *sp1*; b) *sp2*; c) *sp3*; d) *sp4*; e) *C106*; f) *C107*.

5.5 Discussion

5.5.1 Variation of proteomic profiles of *Pseudogymnoascus* spp. isolates in response to heat stress

Using proteomics as an analytical tool, an overview of proteomic changes can provide general understanding of HSR in fungi. In Chapter 3, the upper threshold of temperature stress for the *Pseudogymnoascus* spp. isolates was identified as 25°C. In this chapter, proteomic changes in six isolates of *Pseudogymnoascus* spp. isolated from different geographical regions were compared based on relative peptide abundance changes between heat stress and control conditions (Figure 1). Overall, all isolates showed significant changes in their proteomic profiles. However, the Antarctic isolates *sp3* and *sp4* showed a relatively low number of DEPs, 42 and 67, respectively (Figure 2). The average numbers of DEPs for Antarctic isolates (54.5 DEPs), was approximately 3-4 times lower than seen in the temperate and Arctic isolates (166 and 199 DEPs, respectively).

In various fungal species, numbers of DEPs produced under heat stress vary greatly (Albrecht *et al.*, 2010; Bai *et al.*, 2015). Many technical factors can contribute to the occurrence of DEPs in the proteome. These include total intracellular protein extraction and the choice of peptide digestion methods (Isola *et al.*, 2011; Lau & Othman, 2019). However, the most significant factor is the choice of proteomic profiling approach used in the experimental design (Karpievitch *et al.*, 2010; Bianco & Perrotta, 2015; Fricker, 2018). For this reason, the number of DEPs identified in this study are lower than reported in studies of fungal HSR using a gel-free and labelled proteomic approach (the isobaric tags for relative and absolute quantitation, iTRAQ-based LC MS/MS) (Bai *et al.*, 2015; Deng *et al.*, 2020), with the exception of *Pleurotus ostreatus* proteomes (Zou *et al.*, 2018). Nevertheless, the data reported here remain comparable to those from gel-based

proteomic approaches such as 2-dimensional (2D) and 2D differential gel electrophoresis (2D-DIGE) (Chandler *et al.*, 2008; Salmeron-Santiago *et al.*, 2011; Tesei *et al.*, 2015).

5.5.2 Gene Ontology enrichment analysis of significantly up- and down-regulated proteins

Comparative proteomic studies of different species from the same genus have only been reported on *Paracoccidioides* sp. (Pigosso *et al.*, 2013). That study showed distinguishable proteomic profiles between species in the genus with unique metabolic features in each isolate, supported by biochemical assays. However, Pigosso *et al.* (2013) did not discuss proteomic similarities between the analysed isolates. In the current study, proteomic profiles at 25°C were analysed to understand HSR in *Pseudogymnoascus* spp. The data obtained showed that all isolates possessed distinct proteomic profiles when exposed to heat stress. Only a small percentage of common proteins were found between isolates from the same geographical regions (< 13%), highlighting a diversity of HSR among different isolates of *Pseudogymnoascus*.

Further comparison was carried out on significantly up-regulated and down-regulated proteins with fold change ≥ 1.5 and ≤ -1.5, respectively. Of the significantly up-regulated proteins, one common protein, hypothetical protein VE03_04396 (GI:1040529249) was identified in one isolate from all three regions (*sp2*, *sp4 and C106*). Homology search against the UniProt database showed 100% match to Rab GDP dissociation inhibitor (*Pseudogymnoascus* sp. 23342-1-I1, A0A1B8E4K9). This protein is involved in the dissociation of GDP from the small GTPase Rab, thereby preventing GTP from binding (Hutagalung & Novick, 2011). A recent study of this protein has shown that Rab GDP dissociation inhibitor plays an important role in cell wall chitin deposition in *Aspergillus niger* (van Leeuwe *et al.*, 2020). It is hypothesised that an increased abundance of this protein in *Pseudogymnoascus* spp. may be responsible for the mycelial growth inhibition

under heat stress condition (Dautt-Castro *et al.*, 2021). For significantly down-regulated proteins, only one common protein was identified in *sp1* and *sp2*, the 60S ribosomal protein L16. However, there were various species of small and large subunit ribosomal proteins, and proteins involved in the translation process that were downregulated in all six isolates. Ribosomal proteins are important in the translation process and are involved in the synthesis of new proteins in the cell, thus playing an important role in stress response (Lafontaine & Tollervey, 2001). The downregulation of various proteins involved in the translation process suggests that the translation process is negatively affected during heat stress.

The main HSR of *Pseudogymnoascus* spp. isolated from different geographical regions is visualized in Figure 5.6. GO enrichment analysis of the significantly up-regulated proteins showed a diversity of enriched pathways in each isolate. In general, *Pseudogymnoascus* spp. respond towards heat stress through activation of various pathways related to protein homeostasis, energy production and DNA repair. Various amino acid metabolism pathways are enriched such as those for lysine, valine, arginine, aspartate and asparagine. The urea cycle and protein processing in the endoplasmic reticulum (ER) were also enriched, showing significant activation of protein metabolism in the cytosol (Figure 5.6a).

Under stress conditions, amino acids are the key precursors for the syntheses of various cellular structure compounds and essential macromolecules to replenish damaged components in cells (Kroll *et al.*, 2014; McCarthy & Walsh, 2018). These amino acids are also the backbone components of various metabolic pathways such the TCA cycle and pentose phosphate pathways (Berg *et al.*, 2002). *Pseudogymnoascus* spp. also modulate energy production through the enrichment of the pentose phosphate pathway, citrate cycle (TCA cycle) and metabolism of carbon, methylglyoxyl, glyoxylate and

dicarboxylate (Figure 5.6b). The assorted products of these pathways can generate substantial amounts of substrates for fungal respiration, thus supporting survival and growth under stress conditions (Joseph-Horne *et al.*, 2001).

Under heat stress, *Pseudogymnoascus* spp. also appear to activate DNA repair mechanisms such as the nucleotide salvage pathways (adenine, adenosine and purine), and various defense mechanism pathways such as the innate immune system, biosynthesis of antibiotics and secondary metabolites (Figure 5.6c). In the enrichment analysis of down-regulated proteins, all isolates also showed significant down-regulation of various translation process pathways such as the formation of a pool of free 40S ribosome subunits and SRP-dependent co-translation protein targeting to membrane. Both profiles of up- and down-regulated proteins and their associated enriched pathways suggested that the primary processes taking place in *Pseudogymnoascus* spp. during heat stress were related to DNA damage and repair mechanisms.

The beneficial outcomes from cell death and DNA damage response in fungi triggered from environmental stressors such as heat stress and damaging UV radiation, have been studied in detail in various pathogenic fungi (Goldman *et al.*, 2002; Wong *et al.*, 2019). Fungi use programmed cell death to eliminate the toxic effects of hyphal cells that have undergone anastomosis, and knowledge of these processes has been exploited in the field of pharmacology and drug discovery (Kulkarni *et al.*, 2019; O'Connor *et al.*, 2020). Based on the results obtained from the proteomic analysis, we suggest that by using DNA damage and repair mechanisms, *Pseudogymnoascus* spp. modulate the salvaging of nucleotides, halt the translation processes involved in producing new polypeptide and focus on protein homeostasis through various aspects of amino acid metabolism.

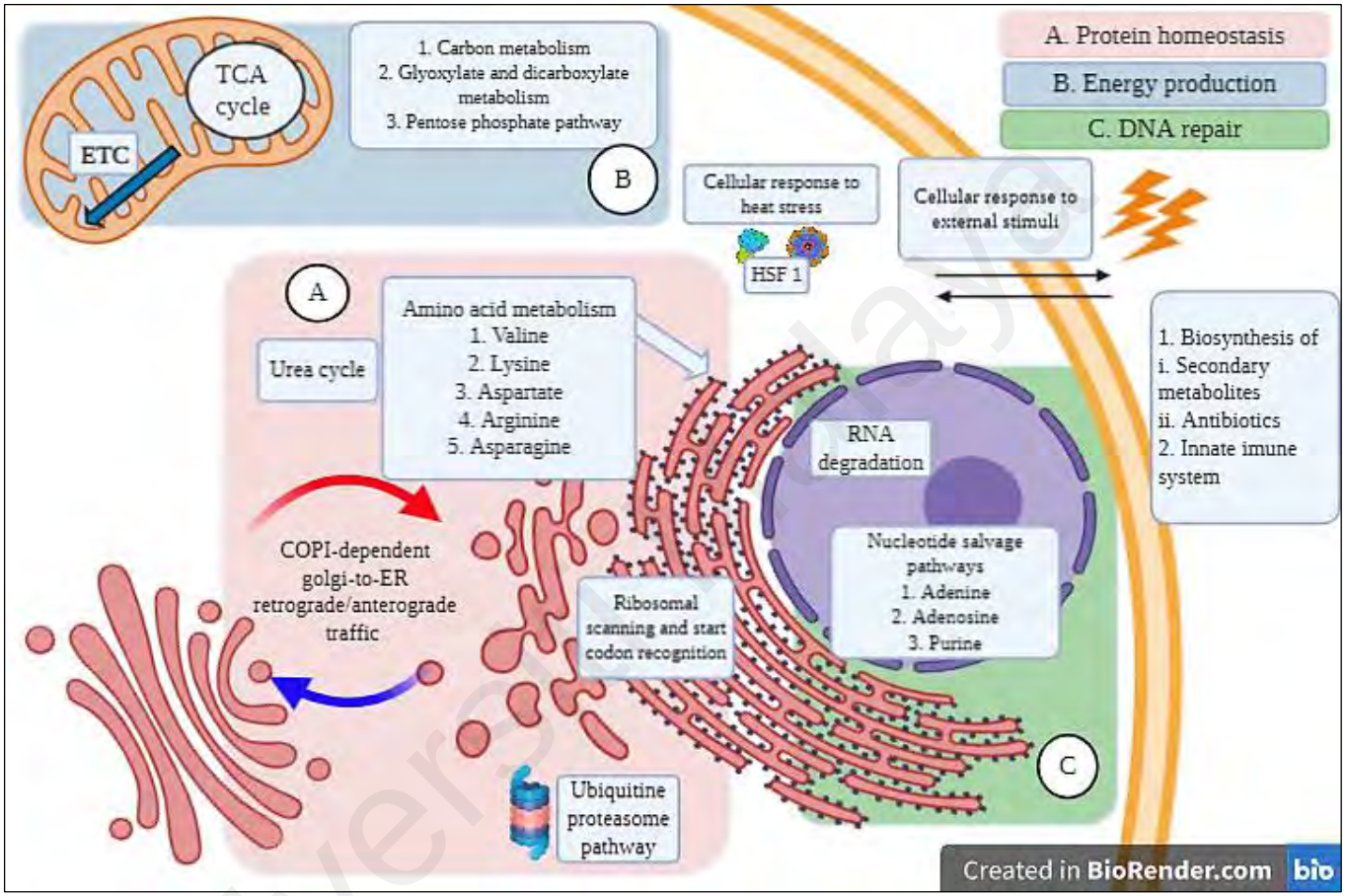

Figure 5.6 Simplified visualization of significantly enriched pathways in all isolates. A) Protein homeostasis - ribosomal scanning and start codon recognition, various amino acid metabolism pathways, urea cycle, protein transport through endoplasmic reticulum (ER) and Golgi and proteasome-mediated degradation pathway. B) Energy production pathways - carbon, glyoxylate and dicarboxylate metabolism, pentose phosphate pathway, citric acid cycle (TCA) and oxidative phosphorylation process through electron transport chain (ETC). C) DNA repair mechanisms - various nucleotide salvage pathways and RNA degradation. Various other pathways are enriched in *Pseudogymnoascus* spp. such as the biosynthesis of secondary metabolites, antibiotics, activation of innate immune system, and cellular response to heat stress and external stimuli.

5.6 Conclusions

In this study, the heat stress response of six isolates of *Pseudogymnoascus* spp. was investigated and characterized using proteomic profiling. The profiles of all isolates in response to heat stress varied widely in terms of the significantly up- and down-regulated protein species. All six isolates showed a wide range of significantly enriched pathways as identified through GO enrichment analysis. The heat stress response of the *Pseudogymnoascus* spp. isolates examined, while showing wide variation pathways enriched, did not show any obvious association with the geographical regions of origin of the isolates.

CHAPTER 6: EFFECTS OF COLD STRESS ON PSEUDOGYMNOASCUS SPP. PROTEOME

6.1 Introduction

In the understanding of stress response mechanisms in microorganisms, cold stress has received less attention than heat stress. Cold stress has largely been studied in pathogenic species in the fields of medical, agricultural and food technology. Various cosmopolitan model organisms such as *Saccharomyces cerevisiae* and *Aspergillus nidulans* have been a focus of research. Nonetheless, psychrophilic and psychrotolerant fungi have also been studied to provide specific details and information on their cold-adapted properties. In this chapter, *Pseudogymnoascus* spp. isolated from different global geographical regions were used as representatives of psychrotolerant species of soil microfungi and their proteomic profiles investigated in an attempt to understand their cold stress response mechanisms. The term "cold stress" used here refers to an experimentally-imposed low temperature condition compared to the optimal growth temperature, which is known to result in stress-related physiological change such as a reduction in growth rate. Information gathered from the proteomic response of *Pseudogymnoascus* spp. to cold stress will improve knowledge of the cold stress responses of soil microfungi.

6.2 Literature review

Cold-adapted or psychrotolerant fungi are defined as fungal species that have the ability to grow at temperatures close to 0°C and have optimum growth temperature of 15-20°C (Morita, 1975; Wang *et al.*, 2015). Psychrotolerant fungi are mostly found in the polar and temperate regions and in high altitude environments (Wang *et al.*, 2017). However, with their ability to grow at moderate temperatures, they are also present in man-made habitats such as in the refrigerated environment and this has led to research attention in medical and agricultural fields (Döğen *et al.*, 2013; Perez-Nadales *et al.*, 2014). Research on psychrotolerant soil microfungi has primarily related to their potential application in biotechnology, such as in their production of antibiotics and antifungal

properties, hydrocarbon bioremediation and enzyme technology (Barratt *et al.*, 2003; Gao *et al.*, 2016; Kulkarni *et al.*, 2019). With progressive advances in proteomic technologies, the cold-adapted properties of psychrotolerant fungi can be further understood and applied (Liu *et al.*, 2017b; Abd Latip *et al.*, 2019).

In the study of fungal stress-related properties, the term cold stress refers to temperatures that are lower than the optimum growth temperature. These conditions can lead to reduction in fungal growth and reproduction (Hallsworth, 2018; Rangel *et al.*, 2018). However, it should also be recognized that the natural micro-environments of many fungi are constantly changing due to various factors (Hallsworth, 2018). Therefore, experimentally-applied cold stress and non-stress conditions may be highly artificial and not closely replicate natural environmental variation (Antal *et al.*, 2020). Therefore, caution must be applied when interpreting data obtained from such studies. Improving understanding of the cold stress responses of psychrotolerant fungi is nevertheless an important research field.

From a proteomic perspective, cold stress responses are expected to involve a balanced production of protein networks within cells to eliminate the damaging effects of low temperature stress, while simultaneously supporting normal cell processes. Various mechanisms are proposed to underlie the overall complexity of fungal cold stress responses (Hassan *et al.*, 2016; Fabri *et al.*, 2020). Many of the proposed mechanisms involve a range of cold-adapted metabolic pathways (Kostadinova *et al.*, 2011; Fenice, 2016) and translation-related processes (Crawford & Pavitt, 2019; Bresson *et al.*, 2020). The cold stress responses of *Aspergillus flavus* and *Exophiala dermatitidis* showed increases use of metabolic pathways involved in amino acid and carbohydrate metabolism (Bai *et al.*, 2015; Tesei *et al.*, 2015). In other species such as *Flammulina velutipes* and *Mortierella isabellina*, the upregulation of energy metabolism pathways and ATP

production was reported (Hu *et al.*, 2016; Liu *et al.*, 2017b). Various lipid metabolic pathways are also involved in the cold stress response of fungi such as the metabolism of sphingolipids, phospholipids and unsaturated fatty acids (Su *et al.*, 2016; Fabri *et al.*, 2020). The modulation of various lipid metabolism pathways also contributes to the pathogenicity of fungi. Lipid modulation has been shown to be significantly related to the stability of fungal membrane structures and their integrity, allowing survival of the negative effects of freezing in low temperature environments (Pan *et al.*, 2018; Li *et al.*, 2019). The cold stress response of fungi also includes various translation-related processes such as the upregulation of SRP-dependent co-translational protein targeting to membrane pathway, various cold-adapted ribosomal protein biosynthesis, and translation elongation pathways (Bai *et al.*, 2015; Su *et al.*, 2016; Crawford & Pavitt, 2019). Various fungal cold stress response mechanisms have been shown to be useful in various biotechnological applications such as in the production of antibiotics, antifungal molecules, secondary metabolites and methane metabolism (Lenhart *et al.*, 2012; Wilson *et al.*, 2017).

6.3 Methodology

6.3.1 Fungal cultivation and cold stress experimental design

All isolates used in this work (please refer to Table 3.1 for isolates details) were grown for 5 d at 5°C or 15°C, representing non-stress (C) and cold stress (CS) conditions, respectively. Detailed description of the growth conditions and liquid media preparation is given in Section 3.3.2. All experiments were carried out in triplicates.

6.3.2 Peptide identification, quantification and bioinformatic analysis

Peptide identification, quantification and bioinformatics analysis were performed using LC MS/MS methodology and the statistical analysis component in Proteome Discoverer, described in detail in Section 4.3. Protein abundance values were used to

calculate the $\log_2$ ratios of HS:C of each isolate. A microarray (MA) plot was constructed using $\log_2$ ratios against $-\log_{10}$ local FDR values, and this provided for each protein identified. A cut-off value of 1% FDR was applied to all data obtained from LC MS/MS and quantification before performing this analysis. Differentially expressed proteins (DEPs) were determined from the data with a minimum of ± 0.1-fold change. Venn diagrams were also constructed to compare DEPs of isolates within regions.

6.3.3 Gene Ontology enrichment analysis

KOBAS v2.0 (http://kobas.cbi.pku.edu.cn) was used to search for gene enrichment. The software uses gene-level statistics called overrepresentation analysis (Xie *et al.*, 2011). The analysis is based on the hypergeometric distribution/Fisher's exact test with the addition of Benjamini and Hochberg (1995) FDR correction. Data in the form of FASTA sequences were used to identify enriched pathways in the KEGG, BioCyc and Reactome databases based on the up- and down-regulated proteins. GO terms with p-value of ≤ 0.05 were considered significantly enriched. *Saccharomyces cerevisiae* was selected as the reference Ascomycota species.

6.4 Results

6.4.1 Proteomic profiles of *Pseudogymnoascus* spp. in response to cold stress

The intracellular protein extracts of all six isolates of *Pseudogymnoascus* spp. were analysed using LC MS/MS. A total of 2,541 proteins were identified with high confidence ($p<0.01$) from all isolates in cold stress (CS) and control (C) conditions (Supplementary 1). The distributions of protein abundances under CS for all six isolates were determined using microarray (MA) plot analysis (Figure 6.1). The fold change ratios of differentially expressed proteins (DEPs) with a minimum of ± 0.1-fold were plotted against $-\log_{10}$ local FDR. The data indicated a total of 720 DEPs identified from all six isolates, with 383 and 337 proteins being up- and down-regulated, respectively. The majority of proteins

identified were clustered close to 0, with a significant number of proteins having relatively high confidence values (-$\log_{10}$ FDR > 800). All isolates showed a similar distribution pattern of DEPs under CS with no indication of differences related to geographical origin.

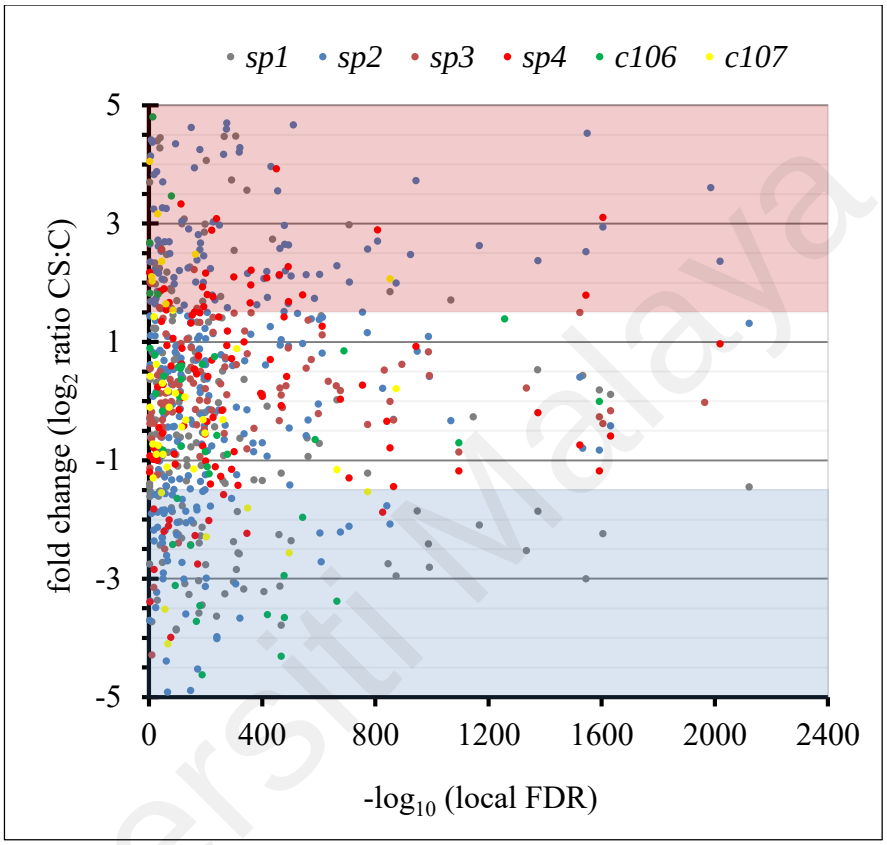

Figure 6.1 The MA plot representing the distribution profiles of proteins identified from all six isolates of *Pseudogymnoascus* spp. under cold stress. Different colours used to represent each isolate: *sp1*, grey; *sp2*, light blue; *sp3*, orange; *sp4*, red; *C106*, green; *C107*, yellow.

To understand the distribution patterns of DEPs for each isolate, a stacked bar graph was constructed (Figure 6.2). There was variation in the numbers of up- and down-regulated proteins between isolates in response to cold stress. One of the Arctic isolates, *sp2*, exhibited the highest numbers of up- and down-regulated proteins (161 and 110 proteins, respectively), while the other Arctic isolate, *sp1*, exhibited considerably lower numbers with only 70 up-regulated and 98 down-regulated proteins. Although the numbers of up-regulated proteins in these two isolates differed widely, the numbers of down-regulated proteins were more similar. Both Antarctic isolates, *sp3* and *sp4*,

produced very similar numbers of up-regulated proteins, 59 and 56, respectively. The production of down-regulated proteins by these isolates was 38 for *sp3* and 46 for *sp4*. In the temperate isolates, *C107* produced the lowest number of DEPs, with only 17 and 21 proteins up- and down-regulated. A similar number of DEPs was produced by *C106*, with 20 and 24 proteins being up- and down-regulated, respectively. Both temperate isolates and one of the Arctic isolates, *sp1* produced a lower number of upregulated proteins in response to cold stress compared to downregulated proteins. On the other hand, *sp2* and both Antarctic isolates produced a higher number of upregulated proteins compared to their downregulated ones. The individual plots of DEPs for each isolate (Figure 6.2) showed similar findings with respect to the distribution patterns of DEPs as observed in the overall MA plot (Figure 6.1), again with no indication of any influence of the geographical origin of the isolates.

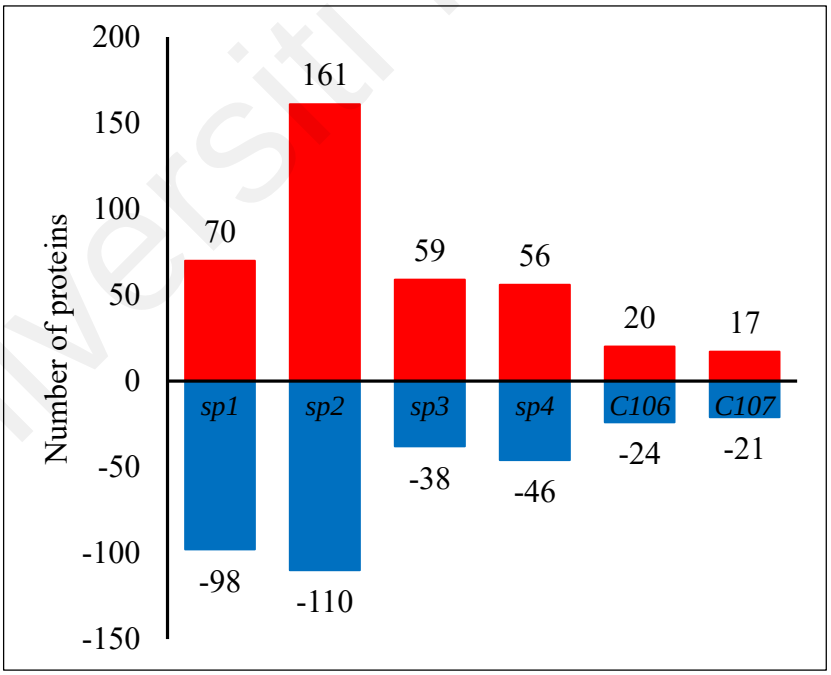

Figure 6.2 Bar graph showing the number of proteins up- (red bars) and down-regulated (blue bars) in each isolate in response to cold stress. The value on each bar shows the number of differentially expressed proteins; + values, up-regulated proteins; – values, down-regulated proteins.

A simple Venn diagram was used to illustrate common and unique proteins found in isolates of the same regions (Figure 6.3). This analysis is crucial to show the degree of similarity or differences among isolates within regions in order to better understand the relationship of DEPs between species of *Pseudogymnoascus* genus in response to cold stress. In the previous chapter, several proteins that were exclusively identified in each region were found to fall under the same molecular function - transferase and hydrolase activities. However, these proteins were identified under non-stress condition (baseline temperature of 15°C). In this work, proteins that were up- or down-regulated in isolates from the same region were compared to identify any common proteins that may play a major role during cold stress in *Pseudogymnoascus* spp. that the two Arctic isolates had only two proteins in common that were up-regulated under cold stress (Figure 6.3a), and both were hypothetical proteins with molecular weights (MW) of less than 30 kDa. However, the Arctic isolates had 10 down-regulated proteins in common (Figure 6.3d). These proteins were a mixture of enzymes (pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase, ATP synthase F1, and isocitrate dehydrogenase), small subunit ribosomal proteins (S3e, S13, S20 and S22), a structural protein (tubulin alpha-β chain), a transporter protein (protein transporter sec-23), and a degradation component protein (proteasome core particle subunit alpha 2). For the Antarctic isolates, a total of 16 common DEPs in both isolates were identified. An equal number of common proteins were up- and down-regulated (Figure 6.3b, e). Half of the common DEPs were hypothetical proteins with calculated MW of more than 30 kDa, with one exception (GI number 1040529802), hypothetical protein VE03_04039 with a MW of 21.9 kDa. Other than the hypothetical proteins, common proteins that were up-regulated in the Antarctic isolates were translation initiation factor eIF4, guanine nucleotide-binding protein subunit beta-like protein, isocitrate dehydrogenase and 60S ribosomal protein L20. The downregulated proteins in common were NADP-specific glutamate dehydrogenase,

mitochondrial heat shock protein 60, glucose-regulated protein and 60S ribosomal protein L11. Surprisingly, there were no common DEPs found in the temperate isolates in response to cold stress except for one down-regulated protein, the plasma membrane ATPase (Figure 6.3c, f).

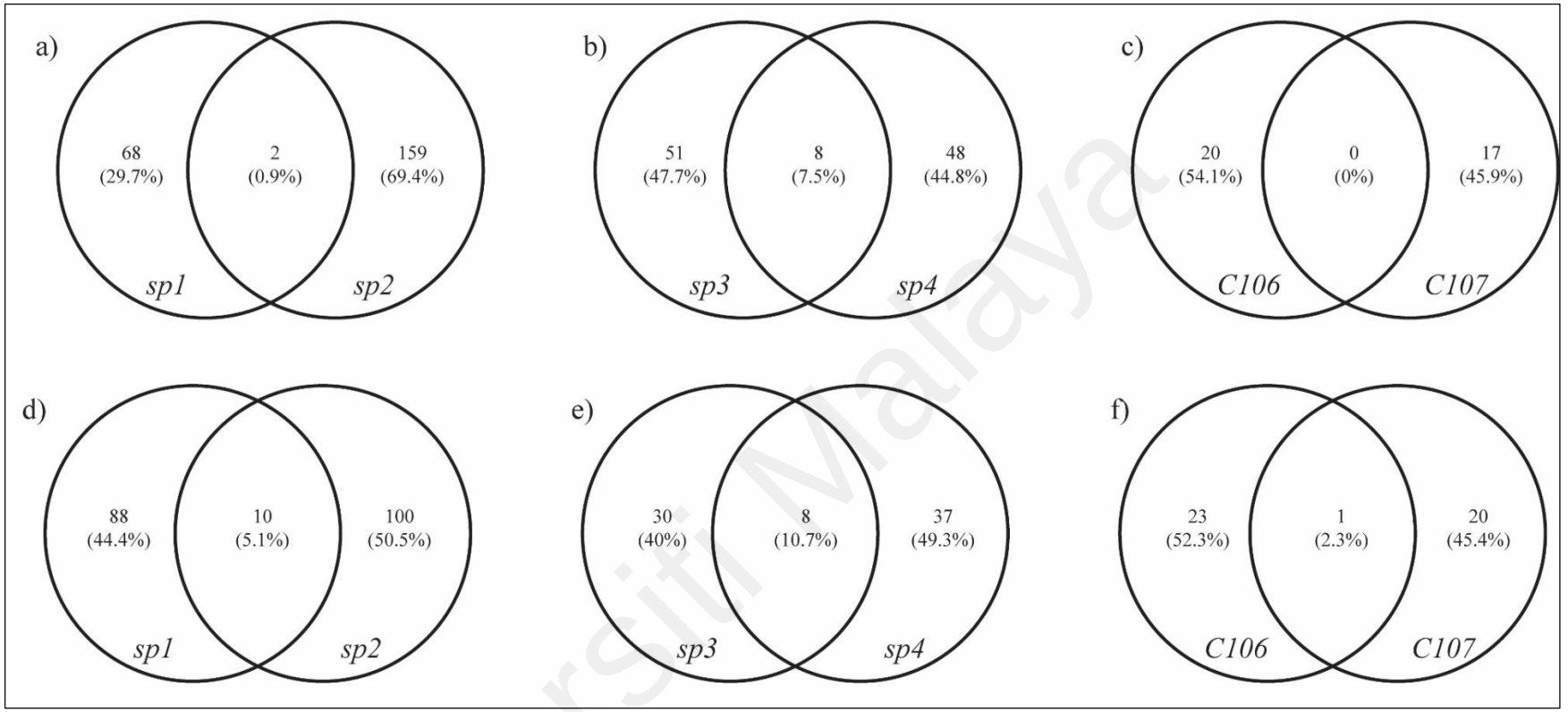

Figure 6.3 Venn diagrams showing the relationship of up- and down-regulated proteins for each isolate within geographical regions of origin. a – c), up-regulated proteins; d – f), down-regulated proteins. a) and d), the Arctic isolates; b) and e), the Antarctic isolates; c) and f), the temperate isolates.

6.4.2 Gene Ontology enrichment analysis of significantly up-regulated proteins in *Pseudogymnoascus* spp.

The differentially expressed proteins (DEPs) of *Pseudogymnoascus* spp. in response to cold stress were further analysed to identify significantly up-regulated proteins with a fold change of ≥ 1.5 (Table 6.1). A total of 176 proteins were significantly upregulated across all isolates, with 47% (84) of the proteins identified as hypothetical proteins. Amongst the six isolates, *sp2* and *C106* had the highest and the lowest numbers of significantly up-regulated proteins, 91 and 5, respectively. In general, all isolates significantly up-regulated various species of large ribosomal subunit proteins (L4e, L10a, L12, L16, L20, L21e, L26e, L35 and P0) and enzymes (catalase, pyruvate carboxylase, malate dehydrogenase, fatty acid synthase, transketolase, aldehyde dehydrogenase, enolase, etc). Furthermore, 45 proteins were highly up-regulated with a fold change of ≥ 3.0. Surprisingly, *sp2* and *sp4* were the only isolates that showed significant up-regulation of heat shock proteins and hsp-like protein species (heat shock protein SSB1 and hsp70-like proteins).

Table 6.1 List of significantly upregulated proteins under cold stress (fold change, log_2 ratios of ≥ 1.5)

Isolate	Accession	Description	Species	Coverage [%]	# Unique Peptides	# AAs	MW [kDa]	calc. pI	log_2
sp1	1352887886	porin por1	*Pseudogymnoascus verrucosus*	94	6	283	30.3	8.98	1.9
	1040536136	hypothetical protein VF21_08556	*Pseudogymnoascus* sp. 05NY08	33	3	529	56	6.19	3.0
	1040511155	hypothetical protein VE04_08019	*Pseudogymnoascus* sp. 24MN13	16	6	400	42.1	6.15	2.6
	1040523417	60S ribosomal protein L16	*Pseudogymnoascus* sp. 23342-1-I1	37	1	202	23.1	10.51	2.2
	1040533161	catalase/peroxidase HPI	*Pseudogymnoascus* sp. 23342-1-I1	23	5	795	87.3	5.5	3.0
	1040528549	large subunit ribosomal protein L26e	*Pseudogymnoascus* sp. 23342-1-I1	41	11	137	15.7	10.68	1.9
	1040533064	20S proteasome subunit alpha 7	*Pseudogymnoascus* sp. 23342-1-I1	43	12	295	31.7	4.91	1.7
	1040498553	hypothetical protein VE00_08699	*Pseudogymnoascus* sp. WSF 3629	31	3	583	63.3	5.72	2.9
	1040563505	translocase of outer mitochondrial membrane	*Pseudogymnoascus verrucosus*	48	1	356	38.5	6	1.8
	1040504933	hypothetical protein VE00_02597	*Pseudogymnoascus* sp. WSF 3629	35	4	323	34.1	5.87	2.7

	1352887735	hypothetical protein VE01_04516	*Pseudogymnoascus verrucosus*	13	4	541	60.3	7.08	1.9
	1040528536	hypothetical protein VE03_05557	*Pseudogymnoascus* sp. 23342-1-I1	65	7	580	63.1	6.52	1.7
	1040532023	hypothetical protein VE03_01299	*Pseudogymnoascus* sp. 23342-1-I1	21	8	1015	105.6	5.01	3.6
	1040505794	hypothetical protein VE00_03008	*Pseudogymnoascus* sp. WSF 3629	28	8	186	21.4	11.18	2.1
	1069466243	large subunit ribosomal protein L21e	*Pseudogymnoascus verrucosus*	52	12	160	18.2	10.33	1.9
	1040526528	hypothetical protein VE03_07310	*Pseudogymnoascus* sp. 23342-1-I1	25	12	1473	165.1	5.54	2.6
	1040525391	hypothetical protein VE03_07986	*Pseudogymnoascus* sp. 23342-1-I1	21	6	783	81.9	5.11	4.5
	1026905242	hypothetical protein VC83_04831	*Pseudogymnoascus destructans*	14	2	1231	128.4	5.34	3.0
	1040534050	catalase/peroxidase HPI	*Pseudogymnoascus* sp. 05NY08	26	4	790	86.4	6.01	3.7
	1040525523	hypothetical protein VE03_08478	*Pseudogymnoascus* sp. 23342-1-I1	25	5	790	87.1	5.85	4.5
	440636110	hypothetical protein GMDG_07740	*Pseudogymnoascus destructans* 20631-21	17	3	507	54	5.9	2.5
	1040502501	hypothetical protein VE00_05878	*Pseudogymnoascus* sp. WSF 3629	20	3	988	105.7	6.46	2.3
	1040530925	hypothetical protein VE03_02548	*Pseudogymnoascus* sp. 23342-1-I1	14	5	1009	110.2	5.43	1.7
	1040547240	hypothetical protein VE02_07770	*Pseudogymnoascus* sp. 03VT05	8	3	486	51.9	6.11	1.6
	1040530942	hypothetical protein VE03_02444	*Pseudogymnoascus* sp. 23342-1-I1	8	1	347	36.1	5.81	2.3
	1040504056	hypothetical protein VE00_03867	*Pseudogymnoascus* sp. WSF 3629	11	3	611	64.1	5.33	4.1
	1040520853	hypothetical protein VE04_00056	*Pseudogymnoascus* sp. 24MN13	31	3	505	54.2	8.24	3.1
	1040525910	hypothetical protein VE03_07646	*Pseudogymnoascus* sp. 23342-1-I1	21	7	441	48.7	7.3	2.5
	1069466961	hypothetical protein VE01_02450	*Pseudogymnoascus verrucosus*	2	4	2096	226.9	4.91	2.3
	1040544250	proteasome subunit beta type-2	*Pseudogymnoascus* sp. 05NY08	19	2	182	20.4	7.49	1.7
	1040525682	hypothetical protein VE03_07697	*Pseudogymnoascus* sp. 23342-1-I1	7	1	611	66.7	6.2	4.3
	1040520567	hypothetical protein VE04_00626	*Pseudogymnoascus* sp. 24MN13	9	4	646	71.5	7.3	4.5
	1026903545	Mitochondrial import inner membrane translocase subunit tim8	*Pseudogymnoascus destructans*	60	4	89	10.1	6.05	1.6
	1026909249	DASH complex subunit ask1	*Pseudogymnoascus destructans*	2	1	398	43.8	5.41	3.7
	1040507167	hypothetical protein VE00_00603	*Pseudogymnoascus* sp. WSF 3629	34	2	108	11.8	5.74	2.3
	1040503472	hypothetical protein VE00_03602	*Pseudogymnoascus* sp. WSF 3629	6	1	497	54	6.06	4.4
	1040531469	hypothetical protein VE03_02830	*Pseudogymnoascus* sp. 23342-1-I1	7	3	713	76.9	6.49	2.0
sp2	1040547996	ATP synthase subunit beta, mitochondrial	*Pseudogymnoascus* sp. 03VT05	87	3	516	55.4	5.68	2.4

1040531119	glyceraldehyde 3-phosphate-dehydrogenase	*Pseudogymnoascus* sp. 23342-1-I1	86	3	339	36.5	6.95	2.9
1040553812	heat shock protein SSB1	*Pseudogymnoascus* sp. 03VT05	48	2	767	84.1	8.43	2.5
1040537109	ATP synthase subunit alpha, mitochondrial	*Pseudogymnoascus* sp. 05NY08	64	4	555	59.7	9.1	2.5
1040529266	hypothetical protein VE03_04296	*Pseudogymnoascus* sp. 23342-1-I1	13	4	4080	451.6	6.43	2.0
1040523711	hsp70-like protein	*Pseudogymnoascus* sp. 23342-1-I1	65	10	676	73.5	5.74	2.4
440639856	tubulin beta chain	*Pseudogymnoascus destructans* 20631-21	76	37	446	49.6	4.93	2.6
1040524485	pyruvate carboxylase	*Pseudogymnoascus* sp. 23342-1-I1	47	3	1190	130.3	6.35	1.5
1040506608	actin	*Pseudogymnoascus* sp. WSF 3629	77	29	375	41.5	5.69	2.6
1026904149	Malate dehydrogenase, cytoplasmic	*Pseudogymnoascus destructans*	80	6	339	35.2	8.92	3.7
1040560294	translation initiation factor eIF4A	*Pseudogymnoascus verrucosus*	67	26	398	44.9	5.24	1.7
1040532273	ketol-acid reductoisomerase, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	69	2	400	44.5	7.05	2.1
1352888949	phosphatidylinositol transfer protein csr1	*Pseudogymnoascus verrucosus*	63	1	221	24	9.39	3.0
1040528425	hypothetical protein VE03_04962	*Pseudogymnoascus* sp. 23342-1-I1	23	6	514	53.8	4.94	4.6
1040530832	hypothetical protein VE03_02453	*Pseudogymnoascus* sp. 23342-1-I1	45	6	462	48.7	8.29	2.6
1040529726	cell division control protein 48	*Pseudogymnoascus* sp. 23342-1-I1	60	53	823	89.9	5.05	2.7
1040518845	fatty acid synthase subunit alpha	*Pseudogymnoascus* sp. 24MN13	30	1	1790	196.3	6.05	2.1
1040530016	mitochondrial-processing peptidase subunit beta	*Pseudogymnoascus* sp. 23342-1-I1	62	27	478	52.5	5.74	2.0
1040501521	heat shock protein SSB1	*Pseudogymnoascus* sp. WSF 3629	62	5	614	66.5	5.44	4.5
1040531120	NADH-ubiquinone oxidoreductase 78 kDa subunit, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	59	4	741	80.6	6.57	2.1
1040529066	hypothetical protein VE03_05085	*Pseudogymnoascus* sp. 23342-1-I1	66	28	441	50.4	6.71	2.7
1040530740	hypothetical protein VE03_03497	*Pseudogymnoascus* sp. 23342-1-I1	51	13	572	60.8	5.26	1.6
1040542063	hypothetical protein VF21_01051	*Pseudogymnoascus* sp. 05NY08	55	2	327	34.3	8.32	1.6
1026906053	erg10, acetyl-CoA C-acetyltransferase	*Pseudogymnoascus destructans*	79	5	399	41.2	6.8	2.1
1040531100	plasma membrane ATPase	*Pseudogymnoascus* sp. 23342-1-I1	41	2	931	100.8	5.15	2.3
1040525605	60S ribosomal protein L12	*Pseudogymnoascus* sp. 23342-1-I1	57	2	165	17.7	9.33	2.2
1040499942	hypothetical protein VE00_08276	*Pseudogymnoascus* sp. WSF 3629	26	8	473	49.1	5.82	2.7
1040524717	transketolase	*Pseudogymnoascus* sp. 23342-1-I1	35	7	685	74.8	5.97	2.6
1040511267	hypothetical protein VE04_09537	*Pseudogymnoascus* sp. 24MN13	11	6	1822	202	6.47	2.4
1040529249	hypothetical protein VE03_04396	*Pseudogymnoascus* sp. 23342-1-I1	65	2	468	51.3	5.54	2.2
1040525568	hypothetical protein VE03_07513	*Pseudogymnoascus* sp. 23342-1-I1	66	2	365	39.1	6.14	2.0
1069466751	saccharopine dehydrogenase	*Pseudogymnoascus verrucosus*	47	3	503	55.2	5.71	2.5

1040533483	hypothetical protein VE03_00182	*Pseudogymnoascus* sp. 23342-1-I1	51	3	253	27.4	7.12	2.0
1040528274	diphosphomevalonate decarboxylase	*Pseudogymnoascus* sp. 23342-1-I1	48	16	385	40.8	6.55	2.8
1040506765	glycine hydroxymethyltransferase	*Pseudogymnoascus* sp. WSF 3629	42	0	539	58.9	8.56	3.0
1040529880	hypothetical protein VE03_04169	*Pseudogymnoascus* sp. 23342-1-I1	30	10	1085	118.8	4.63	3.0
1040505261	succinate dehydrogenase flavoprotein subunit, mitochondrial	*Pseudogymnoascus* sp. WSF 3629	60	26	646	70.8	6.49	1.8
1040506012	hypothetical protein VE00_01435	*Pseudogymnoascus* sp. WSF 3629	16	25	2127	234.2	6.46	1.7
1040500818	aldehyde dehydrogenase	*Pseudogymnoascus* sp. WSF 3629	67	1	496	53.4	5.95	1.7
440634311	catalase	*Pseudogymnoascus destructans* 20631-21	60	2	505	57.4	7.3	1.5
1040517350	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Pseudogymnoascus* sp. 24MN13	49	8	522	57.7	5.4	2.2
1026902306	hypothetical protein VC83_09257	*Pseudogymnoascus destructans*	50	4	545	60.5	6.11	4.3
1040527086	N-acetyl-gamma-glutamyl-phosphate reductase/acetylglutamate kinase	*Pseudogymnoascus* sp. 23342-1-I1	27	2	880	96.3	7.17	4.3
1040503795	hypothetical protein VE00_03509	*Pseudogymnoascus* sp. WSF 3629	51	2	317	34.9	5.36	2.3
1040538418	clathrin, heavy polypeptide	*Pseudogymnoascus* sp. 05NY08	15	1	1682	190.1	5.4	2.5
1040543888	T-complex protein 1 subunit gamma	*Pseudogymnoascus* sp. 05NY08	17	1	541	59	5.99	3.7
1040504412	hypothetical protein VE00_02312	*Pseudogymnoascus* sp. WSF 3629	60	5	282	31.3	4.45	4.2
1040531360	hypothetical protein VE03_02918	*Pseudogymnoascus* sp. 23342-1-I1	32	6	210	22.8	4.87	2.0
1352888607	target of Sbf	*Pseudogymnoascus verrucosus*	12	1	448	46.1	5.5	4.4
1040530118	hypothetical protein VE03_04536	*Pseudogymnoascus* sp. 23342-1-I1	19	9	542	60.3	7.61	3.3
1040526016	plasma-membrane proton-efflux P-type ATPase	*Pseudogymnoascus* sp. 23342-1-I1	13	2	990	108.4	5.39	2.1
1040526348	dihydroxy-acid dehydratase	*Pseudogymnoascus* sp. 23342-1-I1	32	8	592	63.2	7.12	2.9
1040524212	hypothetical protein VE03_09540	*Pseudogymnoascus* sp. 23342-1-I1	27	14	820	86.1	5.15	3.9
1040501074	hypothetical protein VE00_07280	*Pseudogymnoascus* sp. WSF 3629	43	5	313	32.6	6.16	4.7
1040501360	40S ribosomal protein S17	*Pseudogymnoascus* sp. WSF 3629	39	3	148	17	9.8	4.6
1040515629	hypothetical protein VE04_03766	*Pseudogymnoascus* sp. 24MN13	51	5	403	44.9	5.35	4.2
1040501480	acetyl-CoA C-acetyltransferase	*Pseudogymnoascus* sp. WSF 3629	62	2	399	41.2	6.8	2.2
1069464671	guanine nucleotide-binding protein subunit beta	*Pseudogymnoascus verrucosus*	55	3	355	39.1	7.4	1.5
1352886849	proteasome regulatory particle base subunit rpt5	*Pseudogymnoascus verrucosus*	47	16	462	51.6	5.01	2.2
1040504856	ATP synthase F1, delta subunit	*Pseudogymnoascus* sp. WSF 3629	33	4	273	29	9.67	1.6
1040532080	26S protease regulatory subunit 6B	*Pseudogymnoascus* sp. 23342-1-I1	38	2	421	47.1	6	2.7

	1040499252	hsp70-like protein	*Pseudogymnoascus* sp. WSF 3629	63	4	682	73.9	5.19	3.6
	1040538336	hypothetical protein VF21_06756	*Pseudogymnoascus* sp. 05NY08	8	2	443	48.4	5.85	2.6
	440640697	hypothetical protein GMDG_04885	*Pseudogymnoascus destructans* 20631-21	10	2	666	70.3	5.26	2.7
	1040529285	hypothetical protein VE03_04902	*Pseudogymnoascus* sp. 23342-1-I1	31	4	198	21.3	6.79	3.9
	1040548610	hypothetical protein VE02_07270	*Pseudogymnoascus* sp. 03VT05	6	2	577	64	6.79	3.8
	1040530835	hypothetical protein VE03_02559	*Pseudogymnoascus* sp. 23342-1-I1	4	2	581	63.1	4.78	4.4
	440637926	hypothetical protein GMDG_00466	*Pseudogymnoascus destructans* 20631-21	18	2	334	35.7	5.6	1.5
	1040540357	hypothetical protein VF21_04798	*Pseudogymnoascus* sp. 05NY08	9	1	468	49.6	5.95	4.4
	1040526301	arginase	*Pseudogymnoascus* sp. 23342-1-I1	13	2	330	35.4	5.62	2.2
	1040520684	hypothetical protein VE04_00111	*Pseudogymnoascus* sp. 24MN13	41	4	462	48.6	8.9	1.8
	1040547997	NADH-ubiquinone oxidoreductase 78 kDa subunit, mitochondrial	*Pseudogymnoascus* sp. 03VT05	56	1	741	80.6	6.57	4.0
	1040529431	hypothetical protein VE03_03597	*Pseudogymnoascus* sp. 23342-1-I1	3	1	309	32.8	5.66	2.7
	1026904985	hypothetical protein VC83_06014	*Pseudogymnoascus destructans*	22	2	239	26.1	5.62	2.7
	1040526507	hypothetical protein VE03_07003	*Pseudogymnoascus* sp. 23342-1-I1	21	2	433	46.4	6.57	3.3
	1040518253	hypothetical protein VE04_03452	*Pseudogymnoascus* sp. 24MN13	9	2	282	31.3	5.54	2.2
	1001844792	Pyridoxal biosynthesis lyase pdxS	Streptomyces *albidoflavus*	4	1	306	32.1	5.33	2.4
	1040539921	hypothetical protein VF21_03606	*Pseudogymnoascus* sp. 05NY08	13	2	415	46.8	5.41	2.5
	1001842424	Inositol-1-phosphate synthase	Streptomyces *albidoflavus*	3	1	360	39.5	5.11	1.7
	1040532999	hypothetical protein VE03_00521	*Pseudogymnoascus* sp. 23342-1-I1	14	4	469	50.7	5.36	2.1
	1040553304	hypothetical protein VE02_01894	*Pseudogymnoascus* sp. 03VT05	9	2	319	34.6	5.99	1.7
	1040502810	hypothetical protein VE00_05825	*Pseudogymnoascus* sp. WSF 3629	20	3	811	91.3	6.51	2.6
	1040564605	Vacuolar protein 8	*Pseudogymnoascus verrucosus*	9	3	557	60.6	4.97	1.9
	1040539273	catalase	*Pseudogymnoascus* sp. 05NY08	54	1	505	57.3	7.47	4.7
	1040527824	catalase	*Pseudogymnoascus* sp. 23342-1-I1	56	2	505	57.2	7.08	3.6
	1040541436	hypothetical protein VF21_04301	*Pseudogymnoascus* sp. 05NY08	44	1	250	27.3	6.32	1.5
	1370888902	hypothetical protein VC83_07881	*Pseudogymnoascus destructans*	80	2	330	34.1	6.39	2.1
	1352888836	Protein disulfide-isomerase erp38	*Pseudogymnoascus verrucosus*	37	1	432	47.3	7.88	3.0
	1069467799	hypothetical protein VE01_03600	*Pseudogymnoascus verrucosus*	4	1	1126	123.7	6.21	1.6
	1040537170	urease accessory protein	*Pseudogymnoascus* sp. 05NY08	7	2	273	28.9	5.9	4.1
	1040532480	GTP-binding protein rho2	*Pseudogymnoascus* sp. 23342-1-I1	12	2	201	22.3	5.87	3.2
sp3	1040506877	enolase	*Pseudogymnoascus* sp. WSF 3629	85	43	438	47.7	5.41	1.5

	1040525877	60S acidic ribosomal protein P0	*Pseudogymnoascus* sp. 23342-1-I1	33	10	312	33.4	5.15	2.2
	1040513597	20S proteasome subunit alpha 4	*Pseudogymnoascus* sp. 24MN13	55	12	267	29.2	7.4	1.6
	1352886940	hypothetical protein VE01_00604	*Pseudogymnoascus verrucosus*	20	3	249	26.2	6.61	1.6
	1026907433	Cytochrome b-c1 complex subunit 7	*Pseudogymnoascus destructans*	25	4	123	14.4	9.07	2.6
	1352887810	hypothetical protein VE01_04771	*Pseudogymnoascus verrucosus*	8	3	329	36.1	6.33	2.0
	1040527667	hypothetical protein VE03_05893	*Pseudogymnoascus* sp. 23342-1-I1	3	1	351	38.8	9.17	2.0
sp4	1040531119	glyceraldehyde 3-phosphate-dehydrogenase	*Pseudogymnoascus* sp. 23342-1-I1	86	3	339	36.5	6.95	3.1
	1040553812	heat shock protein SSB1	*Pseudogymnoascus* sp. 03VT05	48	2	767	84.1	8.43	1.8
	1040560294	translation initiation factor eIF4A	*Pseudogymnoascus verrucosus*	67	26	398	44.9	5.24	2.3
	1040552218	40S ribosomal protein S14	*Pseudogymnoascus* sp. 03VT05	79	12	150	16	10.87	2.2
	440632652	large subunit ribosomal protein L4e	*Pseudogymnoascus destructans* 20631-21	55	2	373	39.7	11.33	2.1
	1040529726	cell division control protein 48	*Pseudogymnoascus* sp. 23342-1-I1	60	53	823	89.9	5.05	2.9
	1040526037	pyruvate kinase, variant	*Pseudogymnoascus* sp. 23342-1-I1	55	5	562	61.1	7.72	1.8
	1040550635	small subunit ribosomal protein S2e	*Pseudogymnoascus* sp. 03VT05	55	17	273	29.2	10.27	2.9
	1069468697	O-acetylhomoserine (thiol)-lyase	*Pseudogymnoascus verrucosus*	48	3	459	48.4	5.77	2.0
	1069465551	guanine nucleotide-binding protein subunit beta-like protein	*Pseudogymnoascus verrucosus*	66	3	316	35	7.03	2.2
	1040510730	hypothetical protein VE04_07723	*Pseudogymnoascus* sp. 24MN13	70	27	383	43.3	5.27	3.9
	1040560461	hypothetical protein VE01_06128	*Pseudogymnoascus verrucosus*	43	14	384	44.2	8.73	1.9
	1026906053	erg10, acetyl-CoA C-acetyltransferase	*Pseudogymnoascus destructans*	79	5	399	41.2	6.8	2.1
	1026905733	hypothetical protein VC83_05243	*Pseudogymnoascus destructans*	39	5	169	18.7	4.67	1.7
	1040524717	transketolase	*Pseudogymnoascus* sp. 23342-1-I1	35	7	685	74.8	5.97	1.7
	1040532244	hypothetical protein VE03_02026	*Pseudogymnoascus* sp. 23342-1-I1	49	5	346	37	5.52	3.1
	1040529249	hypothetical protein VE03_04396	*Pseudogymnoascus* sp. 23342-1-I1	65	2	468	51.3	5.54	2.1
	1069466751	saccharopine dehydrogenase	*Pseudogymnoascus verrucosus*	47	3	503	55.2	5.71	1.6
	1040502157	hypothetical protein VE00_04658	*Pseudogymnoascus* sp. WSF 3629	59	17	342	38.9	7.84	1.8
	1040529736	guanine nucleotide-binding protein subunit beta	*Pseudogymnoascus* sp. 23342-1-I1	46	2	355	39.1	7.4	1.5
	440638868	GTP-binding protein ypt1	*Pseudogymnoascus destructans* 20631-21	47	8	201	22.2	5.44	3.3
	1370882703	Delta(24)-sterol C-methyltransferase	*Pseudogymnoascus destructans*	22	2	377	42.5	6.38	1.9
	1069464671	guanine nucleotide-binding protein subunit beta	*Pseudogymnoascus verrucosus*	55	3	355	39.1	7.4	1.8
	1040526609	hypothetical protein VE03_06110	*Pseudogymnoascus* sp. 23342-1-I1	32	2	71	7.4	6.79	1.5
	1040499366	hypothetical protein VE00_09247	*Pseudogymnoascus* sp. WSF 3629	33	5	158	17.8	5.08	1.9

	1026909249	DASH complex subunit ask1	*Pseudogymnoascus destructans*	2	1	398	43.8	5.41	2.2
	1040530204	dTDP-glucose 4,6-dehydratase	*Pseudogymnoascus* sp. 23342-1-I1	64	2	423	47.4	6.18	1.7
C106	1026908689	60S ribosomal protein L35	*Pseudogymnoascus destructans*	46	1	125	14.4	11	1.8
	1040526755	hypothetical protein VE03_06971	*Pseudogymnoascus* sp. 23342-1-I1	3	3	2518	275.9	5.55	3.5
	1040537179	hypothetical protein VF21_06185	*Pseudogymnoascus* sp. 05NY08	1	1	1161	124	6.24	2.7
	1001843575	Catalase	*Streptomyces albidoflavus*	3	1	487	55.8	5.57	4.8
	1040511525	malate synthase, glyoxysomal	*Pseudogymnoascus* sp. 24MN13	1	1	542	60.5	7.72	1.8
C107	1352887886	porin por1	*Pseudogymnoascus verrucosus*	94	6	283	30.3	8.98	2.1
	1040557179	60S ribosomal protein L10a	*Pseudogymnoascus verrucosus*	47	11	218	24.2	9.83	2.5
	1040502912	60S ribosomal protein L20	*Pseudogymnoascus* sp. WSF 3629	35	1	184	22	10.76	1.5
	1040515250	hypothetical protein VE04_06692	*Pseudogymnoascus* sp. 24MN13	7	1	752	80.7	5.73	1.6
	1370880553	hypothetical protein VC83_03778	*Pseudogymnoascus destructans*	15	4	233	26.1	9.8	2.1
	1040560581	hypothetical protein VE01_06723	*Pseudogymnoascus verrucosus*	8	2	289	31	8.73	2.0
	1026909729	hypothetical protein VC83_01760	*Pseudogymnoascus destructans*	23	4	228	25.7	7.09	2.4
	1040503482	hypothetical protein VE00_03591	*Pseudogymnoascus* sp. WSF 3629	1	1	517	56.3	8.68	4.1
	1040502988	4-nitrophenyl phosphatase	*Pseudogymnoascus* sp. WSF 3629	10	4	306	33.4	5.31	3.2

GO enrichment analysis was carried out for significantly up-regulated proteins in response to cold stress using KOBAS v2.0 to search for over-represented categories of molecular pathways in the databases Kyoto Encyclopedia of Genes and Genomes (KEGG), Panther, BioCyc, and Reactome. A complete list of enriched pathways with p values ≤ 0.05 for each isolate is shown in Supplementary 3. The top 10 pathways and their respective p values were selected for each isolate and are presented in Figure 6.4. The upregulated proteins of *Pseudogymnoascus* spp. isolated from different geographical regions showed a variety of enriched pathways, but there was no common pathway shared between the pairs of isolates from the same region. For *sp1* and *C107*, the majority of enriched pathways were related to various translation processes such as the SRP-dependent co-translational protein targeting to membrane, cap-dependent translation initiation, eukaryotic translation initiation, various nonsense-mediated decay (NMD) processes, and ribosomal-related pathways such as the formation of a pool of free 40S

subunits, GTP hydrolysis and joining of the 60S ribosomal subunits (Figure 6.4a, f). Metabolic-related pathways were enriched in *sp2* and *C106*, such as the tryptophan, carbon, glyoxylate and dicarboxylate metabolism pathways, and biosynthesis of secondary metabolites (Figure 6.4b, e). In addition, *sp2* showed enrichment of cellular responses to stress, biosynthesis of antibiotics and activation of innate immune system (Figure 6.4b). *C106* also showed enrichment of additional pathways such as methane metabolism, peroxisomal protein import, longevity regulating pathway and detoxification of reactive oxygen species (ROS) (Figure 6.4e). A more distinct profile was observed in *sp3* with a majority of enriched pathways related to energy production, such as the glycolysis, gluconeogenesis and respiratory electron transport (ETC) and flavin/riboflavin metabolism pathways (Figure 6.4c). However, *sp3* had one pathway in common with *C106*, the methane metabolism pathway. In *sp4*, the enriched pathways showed similarities with both the Arctic and temperate isolates (Figure 6.4d). The up-regulated proteins showed enrichment of various metabolic pathways, mainly the biosynthesis of secondary metabolites, antibiotics and amino acids. *sp4* also showed enrichment of protein and carbon metabolism pathways and of various translation processes such as the ribosomal scanning and start codon recognition, cap-dependent and eukaryotic translation initiation pathways.

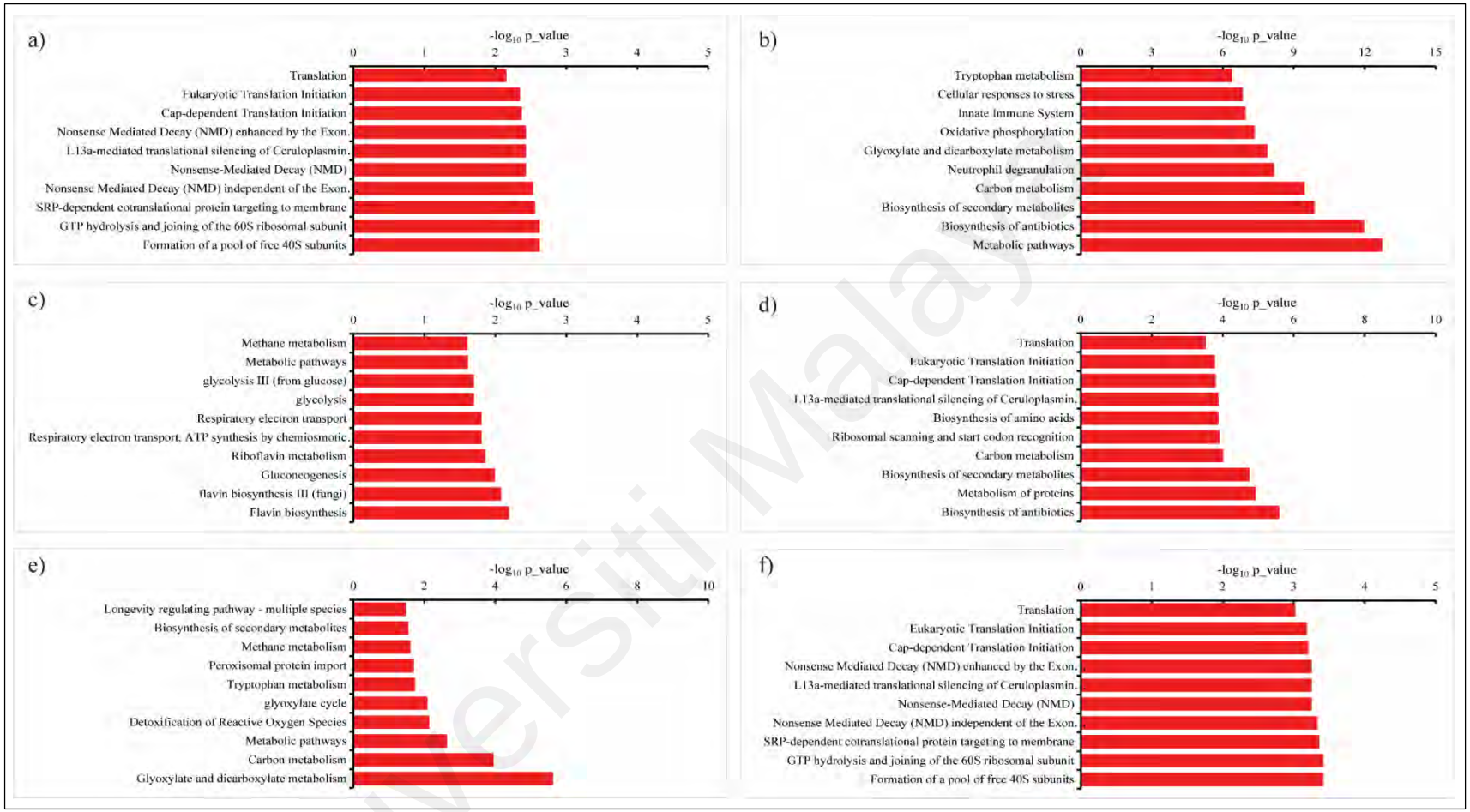

Figure 6.4 GO enrichment analysis of significantly up-regulated proteins of *Pseudogymnoascus* spp. in response to cold stress (top 10 pathways). a) *sp1*; b) *sp2*; c) *sp3*; d) *sp4*; e) *C106*; f) *C107*.

6.4.3 Gene Ontology enrichment analysis of significantly down-regulated proteins in *Pseudogymnoascus* spp.

A list of significantly down-regulated proteins (with fold change of ≤ -1.5) of *Pseudogymnoascus* spp. in response to cold stress, with detailed information on protein identification coverage (%), number of unique peptides, amino acids, MW (kDa), calculated pI, and fold change (log_2) is given in Table 6.2. One hundred and forty-eight proteins were significantly down-regulated across all isolates, with 77 identified as hypothetical proteins (46%). Forty-six of the proteins were highly down-regulated with fold change of at least 3.0-fold. Both of the Arctic isolates, *sp1* and *sp2*, showed a high number of significantly down-regulated proteins (54 and 55, respectively). In contrast, one of the Antarctic isolates, *sp3*, had the lowest number of significantly down-regulated proteins (5), all of which were identified as hypothetical proteins. In general, the significantly down-regulated proteins included various enzymes in energy production processes such the TCA cycle, glycolysis and gluconeogenesis, such as glyceraldehyde 3-phosphate-dehydrogenase, ATP-citrate synthase subunit 1, phosphoglycerate kinase, succinyl-CoA ligase subunit beta, isocitrate dehydrogenase, acetyl Co-A hydrolase, fatty acid synthase subunit beta and pyruvate kinase. Heat shock proteins or hsp-like proteins were only significantly down-regulated in *sp1* (heat shock protein SSB1 and hsp70-like protein).

Table 6.2 List of significantly downregulated proteins under cold stress (fold change, log_2 ratios of ≤ -1.5)

Isolate	Accession	Description	Species	Coverage [%]	# Unique Peptides	# AAs	MW [kDa]	calc. pI	log_2
sp1	1040543410	elongation factor 1-alpha	*Pseudogymnoascus* sp. 05NY08	75	11	459	49.9	9.13	-2.8
	1040531119	glyceraldehyde 3-phosphate-dehydrogenase	*Pseudogymnoascus* sp. 23342-1-I1	86	3	339	36.5	6.95	-2.2
	1040553812	heat shock protein SSB1	*Pseudogymnoascus* sp. 03VT05	48	2	767	84.1	8.43	-3.0
	1040525455	hypothetical protein VE03_07380	*Pseudogymnoascus* sp. 23342-1-I1	63	4	231	25.6	6.42	-3.8
	1040502460	molecular chaperone HtpG	*Pseudogymnoascus* sp. WSF 3629	69	1	703	79.5	4.92	-2.5
	1040529266	hypothetical protein VE03_04296	*Pseudogymnoascus* sp. 23342-1-I1	13	4	4080	451.6	6.43	-3.0

	1040523711	hsp70-like protein	*Pseudogymnoascus* sp. 23342-1-I1	65	10	676	73.5	5.74	-1.9
	440639856	tubulin beta chain	*Pseudogymnoascus destructans* 20631-21	76	37	446	49.6	4.93	-2.1
	1040499891	hypothetical protein VE00_07973	*Pseudogymnoascus* sp. WSF 3629	41	8	196	21.4	7.88	-3.0
	1040528567	ATP-citrate synthase subunit 1	*Pseudogymnoascus* sp. 23342-1-I1	74	8	668	72.4	8.34	-2.7
	1040540892	elongation factor EF-3	*Pseudogymnoascus* sp. 05NY08	58	3	1064	117.6	6.27	-2.4
	1040531987	serine hydroxymethyltransferase, cytosolic	*Pseudogymnoascus* sp. 23342-1-I1	51	1	484	53.3	7.78	-3.1
	1040532273	ketol-acid reductoisomerase, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	69	2	400	44.5	7.05	-3.2
	1040532121	fatty acid synthase subunit beta	*Pseudogymnoascus* sp. 23342-1-I1	43	7	2109	233.4	5.72	-1.8
	1040552218	40S ribosomal protein S14	*Pseudogymnoascus* sp. 03VT05	79	12	150	16	10.87	-2.8
	1352887002	hypothetical protein VE01_00786	*Pseudogymnoascus verrucosus*	41	1	292	31.6	8.92	-2.3
	1040537116	argininosuccinate synthase	*Pseudogymnoascus* sp. 05NY08	58	5	416	46.4	5.48	-2.8
	440637842	phosphoglycerate kinase	*Pseudogymnoascus destructans* 20631-21	62	1	417	44.4	6.47	-2.3
	1040531120	NADH-ubiquinone oxidoreductase 78 kDa subunit, mitochondrial	*Pseudogymnoascus* sp. 23342-1-I1	59	4	741	80.6	6.57	-2.4
	1069462575	small subunit ribosomal protein S12e	*Pseudogymnoascus verrucosus*	59	11	148	16.4	4.94	-2.4
	1040548810	succinyl-CoA ligase subunit beta, mitochondrial	*Pseudogymnoascus* sp. 03VT05	51	4	445	47.9	5.48	-2.6
	1040533135	ATP synthase F1, gamma subunit	*Pseudogymnoascus* sp. 23342-1-I1	48	6	298	32.1	8.34	-3.3
	1040504430	40S ribosomal protein S20	*Pseudogymnoascus* sp. WSF 3629	38	5	116	13.1	9.63	-1.5
	1040527945	triosephosphate isomerase	*Pseudogymnoascus* sp. 23342-1-I1	74	7	249	26.8	5.76	-3.2
	1040525856	eukaryotic translation initiation factor 3 subunit B	*Pseudogymnoascus* sp. 23342-1-I1	30	1	744	84.5	5.01	-3.6
	1040507376	40S ribosomal protein S22	*Pseudogymnoascus* sp. WSF 3629	58	8	130	14.5	9.89	-1.9
	440631821	hypothetical protein GMDG_00116	*Pseudogymnoascus destructans* 20631-21	56	1	231	25.4	4.48	-3.4
	1040532244	hypothetical protein VE03_02026	*Pseudogymnoascus* sp. 23342-1-I1	49	5	346	37	5.52	-3.6
	1026905771	intracellular distribution of mitochondria	*Pseudogymnoascus destructans*	10	10	1291	142.5	5.72	-1.5
	1040532770	V-type proton ATPase subunit B	*Pseudogymnoascus* sp. 23342-1-I1	60	24	516	57.5	5.74	-3.0
	1040526111	glutamine synthetase	*Pseudogymnoascus* sp. 23342-1-I1	64	19	366	40.6	5.8	-1.9
	1069477437	40S ribosomal protein S13	*Pseudogymnoascus verrucosus*	46	7	151	16.8	10.32	-1.6
	1040504427	T-complex protein 1, zeta subunit	*Pseudogymnoascus* sp. WSF 3629	29	12	541	59	6.49	-1.8
	1040497161	pyrABCN	*Pseudogymnoascus* sp. WSF 3629	10	4	2245	246.8	6	-2.3
	1040506854	glutamine-fructose-6-phosphate transaminase	*Pseudogymnoascus* sp. WSF 3629	31	11	1087	120.8	6.49	-2.4
	1040538646	isocitrate dehydrogenase, mitochondrial	*Pseudogymnoascus* sp. 05NY08	43	1	459	51.5	8.76	-2.1
	1040545371	hypothetical protein VE02_09611	*Pseudogymnoascus* sp. 03VT05	25	10	510	56	9.31	-3.1
	1040504491	hypothetical protein VE00_02345	*Pseudogymnoascus* sp. WSF 3629	34	1	357	39.5	5.49	-2.2

	1040532437	acetolactate synthase I/II/III large subunit	*Pseudogymnoascus* sp. 23342-1-I1	14	7	694	75.3	8.82	-3.9
	1040564667	Importin alpha subunit (Karyopherin alpha subunit) (Serine-rich RNA polymerase I suppressor protein)	*Pseudogymnoascus verrucosus*	25	11	552	60.2	5.11	-3.3
	1040504412	hypothetical protein VE00_02312	*Pseudogymnoascus* sp. WSF 3629	60	5	282	31.3	4.45	-2.6
	1040504451	hypothetical protein VE00_02372	*Pseudogymnoascus* sp. WSF 3629	4	1	271	29.1	6.55	-1.6
	1040533074	hypothetical protein VE03_00632	*Pseudogymnoascus* sp. 23342-1-I1	42	11	219	22.9	5.27	-1.8
	1040506454	phosphoribosylamine-glycine ligase/phosphoribosylformylglycinamidine cyclo-ligase	*Pseudogymnoascus* sp. WSF 3629	23	3	785	83.7	5.4	-2.1
	1040522863	hypothetical protein VE03_10908, partial	*Pseudogymnoascus* sp. 23342-1-I1	9	3	1176	126.6	6.89	-3.8
	1040528502	riboflavin synthase, alpha subunit	*Pseudogymnoascus* sp. 23342-1-I1	19	3	230	24.4	4.93	-3.4
	1040520742	T-complex protein 1 subunit alpha	*Pseudogymnoascus* sp. 24MN13	31	10	568	61.8	6.64	-2.0
	1352887691	hypothetical protein VE01_04967	*Pseudogymnoascus verrucosus*	15	6	415	44.5	7.11	-1.5
	1026908370	hypothetical protein VC83_03291	*Pseudogymnoascus destructans*	3	1	246	28.3	5.97	-2.7
	1040505097	hypothetical protein VE00_01915	*Pseudogymnoascus* sp. WSF 3629	13	1	496	51.6	5.6	-2.7
	1040526837	nitrite reductase	*Pseudogymnoascus* sp. 23342-1-I1	3	3	1111	123.1	6.62	-3.7
	1040516031	hypothetical protein VE04_03877	*Pseudogymnoascus* sp. 24MN13	19	1	326	35.7	4.98	-2.4
	1040533107	glutamine-fructose-6-phosphate transaminase	*Pseudogymnoascus* sp. 23342-1-I1	27	2	706	78.3	6.43	-2.3
	1040506083	orotidine 5'-phosphate decarboxylase	*Pseudogymnoascus* sp. WSF 3629	4	1	357	38.9	5.19	-1.5
sp2	1352887886	porin por1	*Pseudogymnoascus verrucosus*	94	6	283	30.3	8.98	-2.1
	1040506096	adenosylhomocysteinase	*Pseudogymnoascus* sp. WSF 3629	55	1	450	48.7	5.68	-1.8
	1040501747	hypothetical protein VE00_06979	*Pseudogymnoascus* sp. WSF 3629	29	4	383	39.7	5.95	-4.5
	1040547813	dihydrolipoyl dehydrogenase	*Pseudogymnoascus* sp. 03VT05	56	2	509	54	7.03	-2.2
	1040536136	hypothetical protein VF21_08556	*Pseudogymnoascus* sp. 05NY08	33	3	529	56	6.19	-2.1
	1040496401	60S ribosomal protein L11	*Pseudogymnoascus* sp. WSF 3629	40	8	172	19.8	10.17	-2.4
	1040530310	hypothetical protein VE03_03010	*Pseudogymnoascus* sp. 23342-1-I1	31	5	294	31.5	6.64	-3.0
	1040529999	60S ribosomal protein	*Pseudogymnoascus* sp. 23342-1-I1	57	6	109	11.8	9.95	-1.7
	1040541287	40S ribosomal protein S27	*Pseudogymnoascus* sp. 05NY08	27	3	82	8.8	9.26	-1.9
	1040528747	hypothetical protein VE03_03290	*Pseudogymnoascus* sp. 23342-1-I1	11	6	487	47.1	4.46	-1.8
	440640338	dihydrolipoyl dehydrogenase	*Pseudogymnoascus destructans* 20631-21	54	2	509	54.1	6.89	-2.7
	1040507376	40S ribosomal protein S22	*Pseudogymnoascus* sp. WSF 3629	58	8	130	14.5	9.89	-1.6
	1040539732	hypothetical protein VF21_05012	*Pseudogymnoascus* sp. 05NY08	12	2	568	58.4	5.34	-3.6

1040530977	hypothetical protein VE03_01549	*Pseudogymnoascus* sp. 23342-1-I1	53	5	315	32.7	9.31	-1.8
1069477643	vacuolar protease A	*Pseudogymnoascus verrucosus*	49	2	395	42.9	5.02	-2.2
1040533064	20S proteasome subunit alpha 7	*Pseudogymnoascus* sp. 23342-1-I1	43	12	295	31.7	4.91	-1.5
1040536470	hypothetical protein VF21_07858	*Pseudogymnoascus* sp. 05NY08	36	1	613	68.4	6	-3.7
440635254	60S ribosomal protein L23	*Pseudogymnoascus destructans* 20631-21	45	8	139	14.6	10.21	-2.0
1040517192	aspartate-semialdehyde dehydrogenase	*Pseudogymnoascus* sp. 24MN13	43	2	364	38.9	6.77	-1.8
1040513597	20S proteasome subunit alpha 4	*Pseudogymnoascus* sp. 24MN13	55	12	267	29.2	7.4	-1.8
1040527436	large subunit ribosomal protein L7Ae	*Pseudogymnoascus* sp. 23342-1-I1	52	18	264	29.3	10.29	-2.0
1040528493	hypothetical protein VE03_04938	*Pseudogymnoascus* sp. 23342-1-I1	48	6	336	37.4	9.09	-2.1
1040556291	proteasome subunit YC7alpha/Y8 (protease yscE subunit 7)	*Pseudogymnoascus verrucosus*	67	2	254	28	6.38	-1.9
1040525350	hypothetical protein VE03_08618	*Pseudogymnoascus* sp. 23342-1-I1	15	3	441	47.1	4.59	-1.9
1040519485	hypothetical protein VE04_01330	*Pseudogymnoascus* sp. 24MN13	25	3	598	64.8	5.9	-3.1
1040525391	hypothetical protein VE03_07986	*Pseudogymnoascus* sp. 23342-1-I1	21	6	783	81.9	5.11	-3.1
1026905242	hypothetical protein VC83_04831	*Pseudogymnoascus destructans*	14	2	1231	128.4	5.34	-2.7
1040534050	catalase/peroxidase HPI	*Pseudogymnoascus* sp. 05NY08	26	4	790	86.4	6.01	-1.6
1040504793	acetyl-CoA hydrolase	*Pseudogymnoascus* sp. WSF 3629	8	3	528	58.6	6.58	-1.9
1040530925	hypothetical protein VE03_02548	*Pseudogymnoascus* sp. 23342-1-I1	14	5	1009	110.2	5.43	-2.3
1040516362	hypothetical protein VE04_05577	*Pseudogymnoascus* sp. 24MN13	35	4	140	15.4	8.12	-2.2
1040510845	large subunit ribosomal protein L4e	*Pseudogymnoascus* sp. 24MN13	38	2	356	37.8	10.93	-4.0
1040547240	hypothetical protein VE02_07770	*Pseudogymnoascus* sp. 03VT05	8	3	486	51.9	6.11	-1.8
1040523481	hypothetical protein VE03_09986	*Pseudogymnoascus* sp. 23342-1-I1	26	1	300	32.3	6.44	-2.7
1040504056	hypothetical protein VE00_03867	*Pseudogymnoascus* sp. WSF 3629	11	3	611	64.1	5.33	-3.0
1040535243	hypothetical protein VF21_09386	*Pseudogymnoascus* sp. 05NY08	15	1	324	34.5	6.07	-3.2
1040531382	hypothetical protein VE03_02803	*Pseudogymnoascus* sp. 23342-1-I1	7	2	617	64.4	5.31	-3.0
1040539900	hypothetical protein VF21_03593	*Pseudogymnoascus* sp. 05NY08	9	1	116	13.1	11.09	-3.4
1352887341	hypothetical protein VE01_02784	*Pseudogymnoascus verrucosus*	47	8	211	23.5	9.55	-1.6
1040507457	hypothetical protein VE00_00106	*Pseudogymnoascus* sp. WSF 3629	1	1	799	85.4	6.64	-3.7
1040506170	hypothetical protein VE00_01618	*Pseudogymnoascus* sp. WSF 3629	4	4	1255	140.5	6.98	-2.1
1352885447	hypothetical protein VC83_06581	*Pseudogymnoascus destructans*	21	1	808	89.3	7.01	-4.0

	1040526014	hypothetical protein VE03_07417	*Pseudogymnoascus* sp. 23342-1-I1	9	1	650	69.1	5	-4.9
	1040515456	glucosamine-phosphate N-acetyltransferase	*Pseudogymnoascus* sp. 24MN13	21	3	180	20.1	5.87	-2.2
	1040528090	hypothetical protein VE03_04733	*Pseudogymnoascus* sp. 23342-1-I1	3	2	945	99.8	10.14	-2.4
	1040523420	hypothetical protein VE03_10013	*Pseudogymnoascus* sp. 23342-1-I1	9	2	507	56.2	6.04	-3.5
	1040529367	hypothetical protein VE03_04870	*Pseudogymnoascus* sp. 23342-1-I1	21	5	485	52.8	5.6	-4.9
	1040499918	hypothetical protein VE00_07980	*Pseudogymnoascus* sp. WSF 3629	11	4	209	23.2	5.24	-2.3
	1040503265	hypothetical protein VE00_05135	*Pseudogymnoascus* sp. WSF 3629	19	2	132	14.7	5.15	-1.6
	440636209	hypothetical protein GMDG_02002	*Pseudogymnoascus destructans* 20631-21	40	1	132	14.3	4.74	-2.9
	1370887271	Leucine aminopeptidase 1	*Pseudogymnoascus destructans*	14	1	434	47.7	5.54	-1.8
	1040539268	hypothetical protein VF21_05590	*Pseudogymnoascus* sp. 05NY08	7	2	528	54.5	5.55	-2.3
	1040503305	hypothetical protein VE00_05156	*Pseudogymnoascus* sp. WSF 3629	4	1	757	83.9	5.58	-1.8
	1040547051	hypothetical protein VE02_08445	*Pseudogymnoascus* sp. 03VT05	7	3	524	53.8	5.64	-2.6
	1040535582	endoribonuclease L-PSP	*Pseudogymnoascus* sp. 05NY08	49	1	128	13.8	6	-4.4
sp3	1040528747	hypothetical protein VE03_03290	*Pseudogymnoascus* sp. 23342-1-I1	11	6	487	47.1	4.46	-2.5
	1040530957	hypothetical protein VE03_01500	*Pseudogymnoascus* sp. 23342-1-I1	17	3	632	68.7	5.11	-2.4
	1040526014	hypothetical protein VE03_07417	*Pseudogymnoascus* sp. 23342-1-I1	9	1	650	69.1	5	-2.4
	1370880003	hypothetical protein VC83_03064	*Pseudogymnoascus destructans*	8	2	304	30.7	8.05	-3.1
	1040527834	hypothetical protein VE03_06437	*Pseudogymnoascus* sp. 23342-1-I1	2	2	727	77.8	6.99	-4.3
sp4	1040501747	hypothetical protein VE00_06979	*Pseudogymnoascus* sp. WSF 3629	29	4	383	39.7	5.95	-2.8
	1040554290	aminopeptidase 2	*Pseudogymnoascus* sp. 03VT05	56	2	891	99.5	5.4	-1.9
	1040532023	hypothetical protein VE03_01299	*Pseudogymnoascus* sp. 23342-1-I1	21	8	1015	105.6	5.01	-2.2
	1040543638	hypothetical protein VF21_01105	*Pseudogymnoascus* sp. 05NY08	19	2	627	67.7	8.37	-2.3
	1040530062	hypothetical protein VE03_04519	*Pseudogymnoascus* sp. 23342-1-I1	26	8	707	77.2	5.31	-2.0
	1040528450	hypothetical protein VE03_04986	*Pseudogymnoascus* sp. 23342-1-I1	17	2	375	40.3	5.81	-4.0
	1370872825	hypothetical protein VC83_00609	*Pseudogymnoascus destructans*	1	1	1423	161.5	7.93	-3.4
	1040517617	hypothetical protein VE04_03781	*Pseudogymnoascus* sp. 24MN13	15	3	305	31.9	4.64	-2.1
	1040536276	hypothetical protein VF21_08313	*Pseudogymnoascus* sp. 05NY08	7	3	575	60.8	4.83	-2.2
	1069469765	hypothetical protein VE01_04555	*Pseudogymnoascus verrucosus*	31	1	159	18.8	6.96	-2.0
	1370880003	hypothetical protein VC83_03064	*Pseudogymnoascus destructans*	8	2	304	30.7	8.05	-1.8

	1069462037	hypothetical protein VE01_00458	*Pseudogymnoascus verrucosus*	33	1	462	48.6	8.75	-1.6
	1040524869	hypothetical protein VE03_08655	*Pseudogymnoascus* sp. 23342-1-I1	7	2	415	46.9	6.67	-2.8
C106	1040525455	hypothetical protein VE03_07380	*Pseudogymnoascus* sp. 23342-1-I1	63	4	231	25.6	6.42	-4.3
	1352888949	phosphatidylinositol transfer protein csr1	*Pseudogymnoascus verrucosus*	63	1	221	24	9.39	-3.7
	1040526037	pyruvate kinase, variant	*Pseudogymnoascus* sp. 23342-1-I1	55	5	562	61.1	7.72	-2.0
	1040531100	plasma membrane ATPase	*Pseudogymnoascus* sp. 23342-1-I1	41	2	931	100.8	5.15	-3.4
	1040533135	ATP synthase F1, gamma subunit	*Pseudogymnoascus* sp. 23342-1-I1	48	6	298	32.1	8.34	-5.0
	1040526437	hypothetical protein VE03_08106	*Pseudogymnoascus* sp. 23342-1-I1	14	9	707	74.5	7.33	-2.4
	1040505261	succinate dehydrogenase flavoprotein subunit, mitochondrial	*Pseudogymnoascus* sp. WSF 3629	60	26	646	70.8	6.49	-3.6
	440634311	catalase	*Pseudogymnoascus destructans* 20631-21	60	2	505	57.4	7.3	-2.9
	1040527086	N-acetyl-gamma-glutamyl-phosphate reductase/acetylglutamate kinase	*Pseudogymnoascus* sp. 23342-1-I1	27	2	880	96.3	7.17	-3.5
	1040531151	hypothetical protein VE03_02408	*Pseudogymnoascus* sp. 23342-1-I1	56	9	103	11.4	11.36	-3.1
	1040519866	T-complex protein 1 subunit epsilon	*Pseudogymnoascus* sp. 24MN13	33	12	548	59.7	5.5	-3.7
	1040504839	hypothetical protein VE00_01830	*Pseudogymnoascus* sp. WSF 3629	48	9	192	20.7	8.62	-1.6
	1040541679	hypothetical protein VF21_02713	*Pseudogymnoascus* sp. 05NY08	51	1	323	34.1	6.58	-4.6
	1040501615	hypothetical protein VE00_06167	*Pseudogymnoascus* sp. WSF 3629	33	2	408	42.4	7.75	-2.4
C107	1040506608	actin	*Pseudogymnoascus* sp. WSF 3629	77	29	375	41.5	5.69	-1.5
	1370880945	methionine-synthesizing 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	*Pseudogymnoascus destructans*	49	1	768	86.2	6.58	-2.6
	1040517350	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Pseudogymnoascus* sp. 24MN13	49	8	522	57.7	5.4	-1.8
	1040530081	hypothetical protein VE03_04439	*Pseudogymnoascus* sp. 23342-1-I1	11	4	1819	201.3	6.27	-2.3
	1040538684	hypothetical protein VF21_06127	*Pseudogymnoascus* sp. 05NY08	17	1	474	49.9	8.06	-1.5
	1040550136	hypothetical protein VE02_06045	*Pseudogymnoascus* sp. 03VT05	17	3	275	29.5	6.04	-4.1
	1040524038	hypothetical protein VE03_09547	*Pseudogymnoascus* sp. 23342-1-I1	15	1	559	61.4	6.57	-3.5

GO enrichment analysis of the significantly down-regulated proteins showed a variety of metabolic and biosynthesis pathways enriched in all isolates (Figure 6.5). Metabolic

pathways related to protein homeostasis, such as protein metabolism and the biosynthesis of amino acids, were enriched in *sp1*, *sp4* and both of the temperate isolates (*C106* and *C107*) (Figure 6.5a, d-f). The biosynthesis of secondary metabolites was also enriched in *sp1*, *C106* and *C107* (Figure 6.5a, e-f). Carbon metabolism and biosynthesis of antibiotics were enriched in *sp1* and *C106* (Figure 6.5a, e), and glycogen degradation II in *sp4* and *C107* (Figure 6.5d, f). The down-regulated pathways in *sp4* also involved the wingless-related integration site (wnt) signaling pathway, neutrophil degranulation, respiratory electron transport (ETC), urea cycle and an activation of antigen pathway (Figure 6.5d). In *sp2*, the majority of down-regulated pathways were related to translation processes such as the eukaryotic and cap-dependent translation initiation, nonsense-mediated decay (NMD) processes and the formation of a pool of free 40S subunits (Figure 6.5b). Pyruvate metabolism was also down-regulated in *sp2*. In *sp3*, the majority of down-regulated pathways were related to phospholipid metabolism involving pathways such as the phospho-PLA2, hydrolysis of lysophosphatidylcholine (LPC), the acyl chain remodelling of cardiolipin (CL), phosphotidylcholine (PC) and phosphatidylinositol (PI). This isolate also showed down-regulation of starch and sucrose metabolism, COPI-independent Golgi-to-ER retrograde traffic and signal amplification pathways (Figure 6.5c).

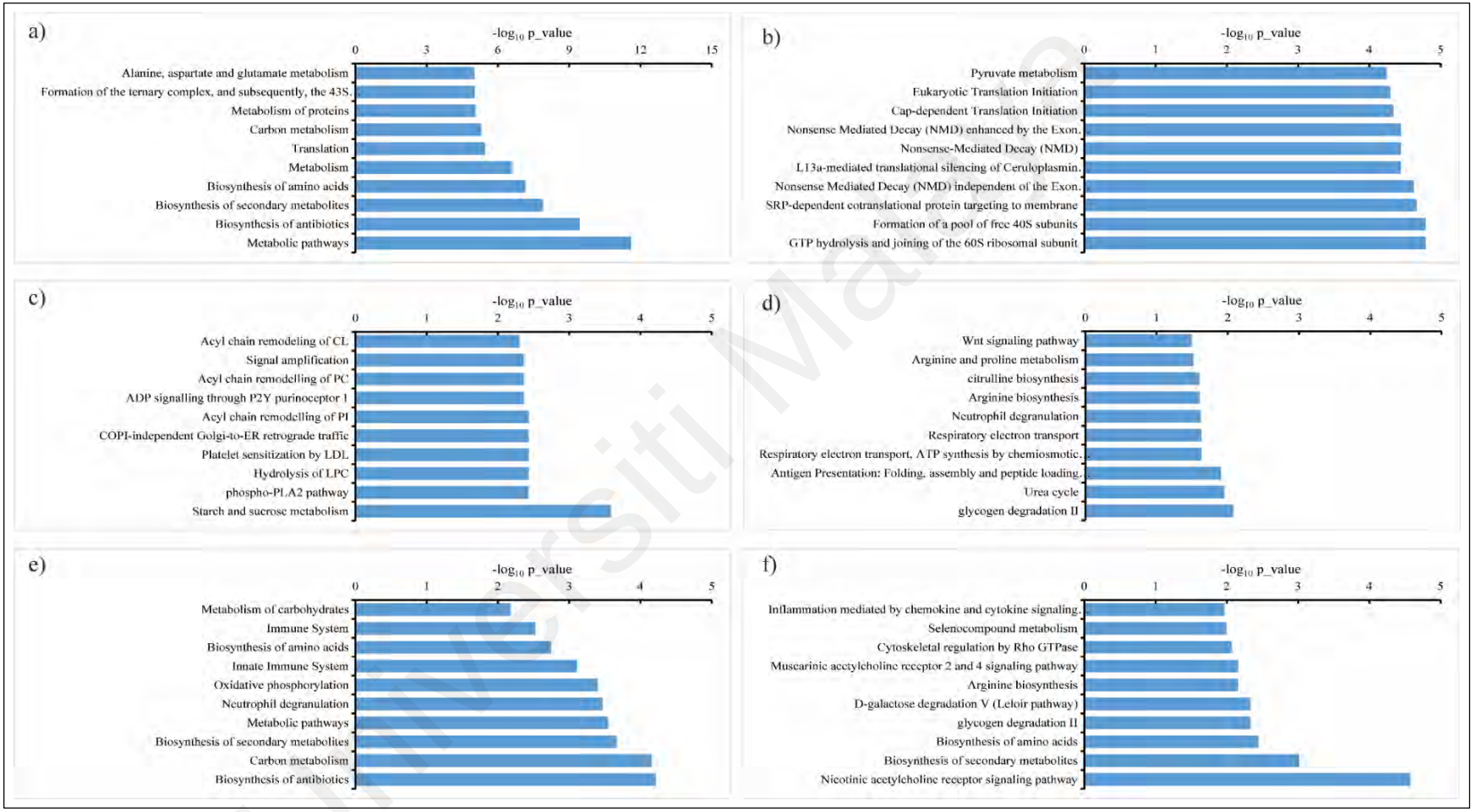

Figure 6.5. GO enrichment analysis of significantly down-regulated proteins of *Pseudogymnoascus* spp. isolates in response to cold stress (the top 10 pathways, a) *sp1*; b) *sp2*; c) *sp3*; d) *sp4*; e) *C106*; f) *C107*

6.5 Discussion

6.5.1 Variation in proteomic profiles of *Pseudogymnoascus* spp. isolates in response to cold stress

All of the polar and temperate isolates included in this study originate in environments that naturally experience cold temperatures (approximate temperature range is from -2°C to 12°C), although with a wide range of annual mean temperatures and distinct seasonal changes (Belda *et al.*, 2014; Convey *et al.*, 2018). Based on this, *Pseudogymnoascus* spp. isolated from the polar and temperate regions were hypothesized to share some common cold-adaptation mechanisms. The studies described in this chapter investigated the proteomic profiles of *Pseudogymnoascus* spp. isolates exposed to low temperature stress (5°C).

Comparing relative protein abundances between cold stress and control conditions (log_2 ratios CS:C), the distribution patterns were similar for all isolates, indicating no overall geographical influence on cold stress responses in these isolates. However, the individual plots of differentially expressed proteins (DEPs) of the six isolates showed wide variation in the number of proteins responding to cold stress. A very small number of DEPs were produced in common even by isolates originating from the same geographical region, with no common up-regulated proteins shared between isolates from the temperate region.

The numbers of significantly up- and down-regulated proteins varied greatly between isolates, within a range of 5-91 proteins. The cold stress responses showed a relatively low number of differentially up-regulated proteins. By comparison, *Flammulina velutipes*, a white-rot fungus that has a relatively low vegetative-growth temperature (20-24°C) produced a total of 31 differentially up-regulated proteins under cold stress (Liu *et al.*, 2017b). A psychrophilic fungus, *Mrakia psychrophila*, showed increases in 27

proteins when exposed to 4°C (Su *et al.*, 2016). Similar findings were also reported for the mesophilic fungi, *Mortierella isabellina* M6-22 and *Exophiala dermatitidis*, that showed up-regulation of 29 and 33 proteins, respectively, when exposed to cold stress (Tesei *et al.*, 2015; Hu *et al.*, 2016). The current study is the first to report the effect of cold stress on the proteome of multiple isolates of the same fungal genus isolated from different geographical regions.

6.5.2 Gene Ontology enrichment analysis of significantly up- and down-regulated proteins

Fungal stress response mechanisms have been proposed in various model organisms such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans*. These model organisms are useful cosmopolitan fungal species that have led to the discovery of various stress-related proteins (Brandl & Andersen, 2017; Santiago *et al.*, 2020). Under cold stress, fungi produce various cold-adapted enzymes and protective molecules to increase stability at low temperature (Wang *et al.*, 2017).

In the current study, the up-regulated proteins showed enrichment of various metabolic and translation-related pathways across all isolates. However, no common pathway was identified from isolates of the same region. Under cold stress, *Pseudogymnoascus* spp. isolated showed up-regulation of a variety of metabolic pathways such as those involved in carbon, glyoxylate and dicarboxylate, methane and amino acid metabolism. In addition, up-regulated pathways also included translation-related pathways such as the formation of a pool of free 40S subunits, nonsense-mediated decay (NMD), eukaryotic and cap-dependant translation initiation pathways. Only a limited number of reports are available of fungal cold stress response mechanisms studied using proteomic profiling, with the majority of these conducted on mesophilic fungi (Tesei *et al.*, 2015; Hu *et al.*, 2016; Liu *et al.*, 2017b). In *E. dermatitidis*, cold stress induced up-regulation of the beta-

oxidation of very long chain fatty acids, glycolysis/gluconeogenesis, peroxisomal lipid metabolism and cellular response to stress (Tesei *et al.*, 2015). *Flammulina velutipes* also showed up-regulation of amino acid biosynthesis, signalling pathways and various energy metabolism pathways such as the citrate cycle (TCA cycle), pentose phosphate pathway, glyoxylate and dicarboxylate metabolism (Liu *et al.*, 2017b). The psychrophilic fungus, *M. psychrophila*, showed up-regulation of energy metabolism and production of unsaturated fatty acids to regulate membrane fluidity under cold stress (Su *et al.*, 2016).

In general, many fungal studies have shown that low temperature does not cause irreversible damage to cells, and fungi respond by modifying molecular content in their complex protein networks. Comparing across all six isolates of *Pseudogymnoascus* spp. studied here, some of the down-regulated pathways identified in one isolate were up-regulated in another. This was particularly the case for various metabolic pathways such as carbon metabolism, biosynthesis of amino acids, secondary metabolites and antibiotics, and translation-related pathways. Su *et al.* (2016) reported that *M. psychrophila* also showed down-regulation of TCA cycle proteins, glycolysis and ribosomal proteins. Similarly, *E. dermatitidis* showed down-regulation of carbon and pyruvate metabolism and the pentose phosphate pathway (Tesei *et al.*, 2015). Across all isolates, only *sp3* showed a distinctive profile of cold stress response. The upregulated pathways of *sp3* involved mainly flavin/riboflavin biosynthesis, glycolysis/gluconeogenesis, respiratory electron transport (ETC) and methane metabolism. The production of biogenic methane is generally only associated with prokaryotic microorganisms such as methanogens and Archaea (Conrad, 2009). However, a more recent report has suggested that terrestrial vegetation and fungi are also involved in the production of methane gas in the environment (Lenhart *et al.*, 2012). Similarly, some down-regulated pathways were identified only in *sp3*, such as acyl chain remodelling of cardiolipin, phosphatidylcholine, phosphatidylinositol and hydrolysis of

lysophosphatidylcholine. Phospholipids are key molecules involved in the maintenance of membrane fluidity and are also involved in signalling pathways. The metabolism of fungal phospholipids has been studied in detailed in the model organism, *S. cerevisiae* (Pan *et al.*, 2018).

6.6 Conclusions

Responses towards cold stress showed variation in up- and down-regulated proteins between different isolates of *Pseudogymnoascus* spp. Several metabolic enzymes and ribosomal proteins were amongst differentially expressed proteins identified in all six isolates of *Pseudogymnoascus* spp. examined here when exposed to cold stress. GO enrichment analysis also showed diversity in the cold stress response pathways, with metabolic and translation-related processes being prominent in most isolates. There were no suggestions of the geographical region of origin of the isolates influencing the cold stress response of *Pseudogymnoascus* spp. However, one of the Antarctic isolates, *sp3*, showed a distinctive cold stress response profile that involved the up-regulation of flavin/riboflavin biosynthesis and methane metabolism. *sp3* is also the only isolate that showed down-regulation of elements of phospholipid metabolism when exposed to cold stress.

CHAPTER 7: CONCLUSIONS

This study's findings showed a diversity in temperature stress response mechanisms across the six isolates of *Pseudogymnoascus* spp. examined, even though they shared common thermal tolerance and sensitivity characteristics. All six isolates are psychrotolerant fungi with lower and upper temperature limits for growth of 5°C and 25°C, respectively. Using tandem mass spectrometry, proteomic characterisation showed that the majority of proteins identified in all isolates were clustered under metabolic function and catalytic activities. Serine/threonine protein kinase and chitinase, under the classifications of hydrolase and transferase activities, were two enzymes commonly found in all isolates. These enzymes hold a great potential that may act as inter-species biomarker for *Pseudogymnoascus* spp. Further research is required to elucidate the role and molecular function of these enzymes in *Pseudogymnoascus* spp. The use of comparative proteomics to understand heat stress response of isolates showed that the six isolates had wide variation pathways whose activity was enriched, with no indication of any influence of geographical origin of the isolates. Three major pathways were primarily enriched in response to heat stress, protein homeostasis, energy production and DNA repair. Similarly, a diversity in enriched response pathways was observed in all isolates under cold stress, with metabolic and translation-related processes are prominent in most isolates. Isolate *sp3* was the only isolate to have a distinctive proteomic profile under cold stress, showing up-regulation of flavin/riboflavin biosynthesis and methane metabolism, and down-regulation of various aspects of phospholipid metabolism.

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