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Thermotolerance and post-fire growth in *Rhizina undulata* is associated with the expansion of heat stress-related protein families

Andi M. Wilson[1,2*], Michael J. Wingfield[1], Tuan A. Duong[1] and Brenda D. Wingfield[1]

Abstract

Background *Rhizina undulata* is an important tree pathogen, infecting a wide variety of conifer species, including those in the genus *Pinus*. The fungus relies on heat shock-mediated activation of its ascospores, which require high temperatures to initiate germination. Consequently, disease due to *R. undulata* often occurs after fire events in both natural and managed forests. The genetic mechanisms contributing to the pyrophilous nature of *R. undulata* have not been investigated. We sought to identify key genes that may be responsible for thermotolerance in the fungus. A comparative genomics approach was used, by sequencing the genome of *R. undulata* for the first time and comparing its predicted proteome to those from other Pezizomycetes, both with and without known associations with fire.

Results Three protein families were shown to be expanded in *R. undulata*; heat shock protein 20 (HSP20), glutathione-S transferases (GST), and aromatic compound dioxygenases (ACD). While HSP20 was uniquely over-represented in *R. undulata*, the expansions of the GST and ACD families were also identified in other fire-associated species.

Conclusion HSP20s are known to protect cells against heat stress, GSTs are involved in the detoxification of reactive oxygen species, and ACDs play a role in the metabolism of recalcitrant compounds present in post-fire environments. The expansion of these families thus suggests that they may play an important role in protecting and stimulating *R. undulata* ascospores during and after fire-induced heat shock, activating the fungus, and enabling it to colonise the root systems of conifers.

Keywords Thermotolerance, Heat shock proteins, Glutathione S-transferase, Aromatic compound dioxygenases, *Rhizina undulata*

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Introduction

Rhizina undulata (*Pezizomycetes, Pezizales, Rhizinaceae*) is an important pathogen of conifers, particularly in southern Africa, as well as parts of North America, Asia and Europe [1–5]. It infects the roots of various *Pinus* spp. and other conifers, causing serious disease events. The fungus is notable in being pyrophilous, with its ascospores relying on heat shock (HS)-mediated activation at temperatures of between 35 and 45 °C [6]. Upon activation, the ascospores germinate and colonize the root systems of susceptible hosts, thereby causing disease.

In naturally occurring forests, *R. undulata* causes "patch death" or "group dying" after ascospore germination and subsequent radial mycelia growth, resulting in expanding infection centres. The number of trees in these centres continues to increase, with new trees succumbing to root disease each year [7]. Because disease typically emerges after a heat-generating event such as a wild fire [4], the pathogen has been colloquially referred to as the "coffee fire fungus" due to the infection of trees near camp fires made to heat coffee on forest trails [3].

In plantation forestry settings, such as in southern Africa, the disease occurs in newly established plantations, particularly after intentional burning of forest waste after clear-felling [8]. Slash burning provides an effective means to reduce populations of bark beetles, other insect pests, and rodents that feed on newly established seedlings [9]. However, due to its pyrophilous nature, the presence of *R. undulata* in the soil can result in serious root disease problems after fire events and thus, the risks posed by slashburning may exceed the benefits thereof [8]. Nonethess, accidental wild fires still occur in these plantations and disease due to *R. undulata* remains a serious constraint to forestry operations [9].

The genetic and physiological mechanisms that underly thermotolerance in *R. undulata* have not been investigated. However, research conducted on a wide variety of other pyrophilous fungi has shown that many of the mechanisms and pathways responsible for survival during HS are conserved. An example of one such conserved protective mechanism is the expression of heat shock proteins (HSPs), a wide variety of proteins that are typically up-regulated in response to HS [10]. The presence of HSPs is widespread across all domains of life, including bacteria [11, 12], archaea [13], plants [14, 15] and animals [16], suggesting that they are important for the HS-response in most organisms.

HSPs are molecular chaperones that act by disaggregating and then either refolding or degrading stress-denatured proteins [reviewed by 17]. They also contribute to the folding of newly synthesized proteins, ensuring correct 3D structure despite unfavourable cellular conditions. These proteins are named and classified based on their size, and examples include HSP70 and HSP90. The smallest HSPs are those within the HSP20 family and are also referred to as small HSPs (sHSPs). The different HSP families do not share evolutionary history and as such, are variably present across the domains of life [10]. For example, while HSP20 and HSP70 homologs have been identified in the genomes of archaea, bacteria and eukaryotes, HSP100 homologs are found only in bacteria, plants and fungi [10]. Nonetheless, many of these proteins are essential across all living organisms and provide protection against stressors including HS, oxidate stress [18], and osmotic stress [19].

Other protective mechanisms against HS include the production of reactive oxygen species (ROS) and enzymatic antioxidants, as well as the synthesis of sugars. ROS are generated in response to heat shock and act as signalling molecules, initiating stress-responsive cascades [20, 21]. However, excessive ROS accumulation can lead to DNA damage, mitochondrial dysfunction, and apoptosis. To counteract this ROS-associated damage, antioxidant enzymes such as superoxide dismutases and glutathione S-transferases are also upregulated during HS [22, 23]. Additionally, certain sugars may be synthesized at elevated levels during exposure to elevated temperatures. Trehalose, for example, plays a multifaceted protective role; it stabilizes the cellular membrane, prevents protein denaturation or aggregation, and acts as a compatible solute, protecting the cell against HS-associated dehydration [24–26].

The aim of this study was to identify genetic factors that contribute to the thermotolerance of the pyrophillic fungus, *R. undulata*. To this end, the genome of *R. undulata* was sequenced and is reported here for the first time. The draft genome was compared to genomes of other species within the Pezizomycetes, including those with and without known fire associations (Fig. 1). This comparison was used to investigate the ability of *R. undulata* to withstand the extreme temperatures associated with fire-induced germination.

Methods

Genome data used

The genome assemblies from 22 fungal species were downloaded from the National Centre for Biotechnology Information (NCBI) Genome repository (Table S1). This represented a subset of the 60 genome assemblies that were available as of September 2022 using the search term "Pezizomycetes". They were chosen because they had been identified to species level. In subsequent analyses, the genomes for *Ascobolus immersus and Tuber magnatum* were excluded due to their low BUSCO completeness values. The genome of *Orbilia oligospora* (Pezizomycotina; Orbiliomycetes) was used as an outgroup in the various analyses. Thus, including the genome of *R. undulata* that was sequenced for this study,

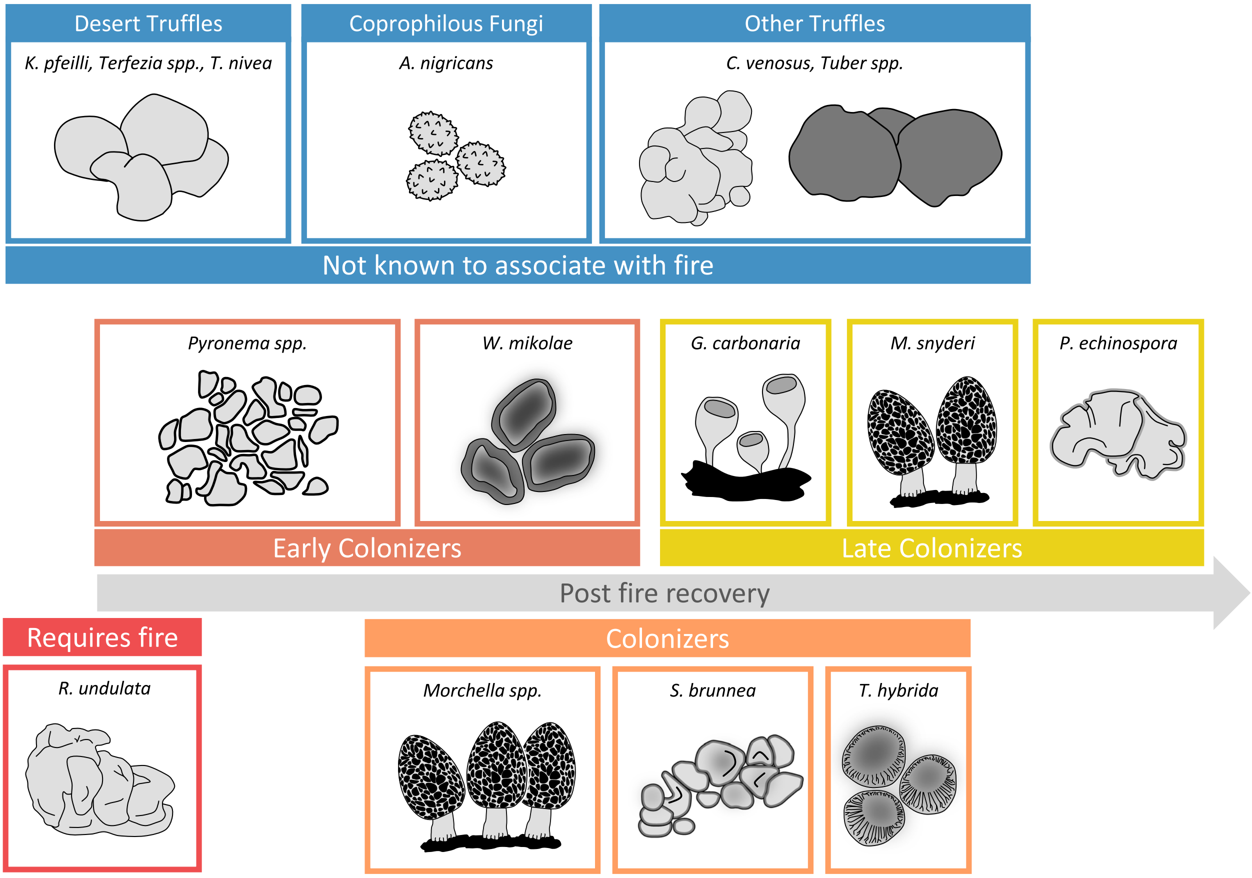

Fig. 1 Ecological niches of fungi included in this study, with particular reference to their known associations with fire. Pezizomycetes not known to be associated with fire or other heat shock events include a variety of truffles as well as a coprophilous fungus. The remaining species are known to have some association with fire and include morels, cup fungi, and others. Those for which data relating to the time of colonization exist are categorized as either "early colonizers" or "late colonizers", based on the classification system of [57]. Those for which no such data exists are categorized as "colonizers". Published literature suggests that *R. undulata* is the only species dependent on a heat shock, such as fire, for the completion of its life cycle

a total of 21 Pezizomycete genomes and one outgroup genome were analysed.

DNA extraction and genome sequencing

R. undulata isolate CMW 412 (culture collection of the Forestry & Agricultural Biotechnology Institute [FABI] at the University of Pretoria, South Africa) was grown in malt yeast broth (2% malt extract, 0.5% yeast extract; Biolab, Midrand, South Africa). The mycelium was harvested, freeze-dried and used for genomic DNA extraction using the Qiagen DNeasy Plant Mini Kit as per the manufacturer's instructions. This involved the mechanical disruption of the freeze-dried material, lysis with the relevant buffer and a column-based purification process. Genome sequencing was carried out by Macrogen Inc. (Seoul, South Korea) where a library with a 350 bp median insert size was constructed using the TruSeq PCR-Free kit (Illumina, CA, USA). The library was sequenced on the NovaSeq 6000 S4 (Illumina), producing paired-end reads of 151 bp.

Genome assembly, quality and completeness

Illumina sequencing of the *R. undulata* isolate produced just over of 43 million raw reads (Table S2), which were processed through FastQC v0.11.5 (Babraham Bioinformatics, Babraham Institute, Cambridge, UK) and Trimmomatic v0.39 [27] to assess data quality and to remove poor quality data, respectively. The resulting 35 million high quality paired-end reads and 6 million high quality single-end reads were assembled using SPAdes v3.13.0 [28], with contigs of 1 000 bp or more being retained in the final assembly. Genome assembly statistics were assessed using QUAST v5.1 [29].

BUSCO v5.3.2 [30] was used to assess the completeness of the *R. undulata* genome as well as for the 21 genomes downloaded from the NCBI. The *ascomycota_odb10* and *fungi_odb10* datasets were used with default settings. BUSCO v5.3.2 was also used to assess the assemblies for bacterial contamination using the *bacteria_odb10* dataset with default settings. All of the scripts and parameters used for the genome assembly and its assessment are available in Additional File 1.

Phylogenetic analysis

To ensure that the newly sequenced genome represented *R. undulata*, a phylogeny was generated using the internal transcribed spacers (ITS). For this analysis, publicly available ITS region sequences were downloaded for a variety of Rhizinaceae species, including other *R. undulata* isolates (Table S3). The homologous region was extracted from the newly sequenced genome in CLC MainWorkbench v22 (QIAGEN, Aarhus, Denmark), combined with the publicly available data, and aligned using the online version of MAFFT v7 [31, 32]. The resulting alignment was subjected to model testing using MrModelTest2 v2.4 [33], after which MrBayes v3.2.7 [34] was used to perform Bayesian inference analyses. The analysis was run for one million generations, with 10 parallel runs, each with four chains. Trees were sampled every 100 generations, with 25% of the sampled trees being discarded as burn-in and posterior probabilities being calculated from the remaining trees. The tree was visualized and annotated in FigTree v1.4.4 [35] and Affinity Designer v1.10. The alignments and parameters used for this analysis can be found in Additional File 2.

Phylogenomic analysis

In order to establish the relationships between the 22 species considered in this study, a phylogenomic tree was generated using the 783 shared BUSCO proteins from these species. These proteins were selected from the list of complete, single copy BUSCO proteins identified using the *ascomycota_odb10* dataset. Each BUSCO dataset was aligned using MUSCLE v3.8.31 [36] and the resulting alignments were trimmed using trimAl v1.4.rev22 [37]. Individual BUSCO trees were generated using IQ-TREE v1.6.12 [38, 39] with 1 000 bootstrap replicates. A species tree was subsequently generated using ASTRAL v5.6.3 [40], using the individual BUSCO trees. All of the scripts and parameters used for the phylogenomic analyses are available in Additional File 3.

Gene prediction and functional annotation

Funannotate [41] was used to predict genes and provide functional annotations in the 22 genomes used in this study. Funannotate uses *ab initio* predictors, including Augustus [42], SNAP [43] and GeneMark-ES/ET [44] and can incorporate additional RNA or protein evidence to improve gene prediction. While many of the genomes had previously generated annotations, this was done to ensure that comparable annotations were generated for all the species included in this study. Gene prediction was conducted using the *funannotate predict* command with additional protein evidence from either the *Rhizina undulata* (Project ID: 1,042,903) or *Tuber borchii* (Project ID: 1,034,997, [45]) proteomes available from the Joint Genome Institute (Table S1). The predicted proteomes for each genome were assessed for completeness using BUSCO v5.3.2 with the *ascomycota_odb10* and *fungi_odb10* datasets. Functional annotation was conducted using the *funannotate annotate* command, using input from InterProScan v5.52–86.0 [46], AntiSmash v6.1.1 [47], eggNOG-Mapper v2.1.11 [48], and Phobius v1.01 [49]. All of the scripts and parameters used for the gene prediction and functional annotation are available in Additional File 4.

Identification of domains associated with the heat shock response

Because the expression of heat shock proteins is a common response to heat stress and thus may be important for thermotolerance in *R. undulata*, the presence and/or absence of numerous heat shock domains was assessed in all 22 genomes. The search team "HSP" was used to search for the PFAM domains associated with heat shock. This yielded a list of 48 domains (Table S4), including domains like HSP20, HSP33, HSP70, and HSP90 as well as HSP-interacting proteins (HipN and CHIP_TPR_N) and other proteins known to be important for the heat shock response (GST_C and GST_N). The functional annotation files generated by Funannotate were used to determine the number of the HSP-associated domains present in each genome. To compare the number of each HSP-associated domains in each genome, a heatmap was generated using *pheatmap* from the R Tidyverse library. The script used to generate this heatmap can be found in Additional File 5. tm

Protein family expansions

OrthoFinder v2.5.5 [50] was used to identify orthologs across the 22 genomes and to determine whether specific protein families had undergone significant expansion or contraction. The complete protein lists as predicted by Funannotate were used for these analyses. OrthoFinder was run using default settings and generated both orthologous groups and phylogenies. Significant differences in the size of these protein families in fire-associated compared with non-fire associated species was assessed using a Wilcoxon rank sum test with continuity correction in the R package *multcomp*. This test was chosen as Shapiro–Wilk normality tests showed that the datasets were not normally distributed. All of the scripts and parameters used for the statistical analyses are available in Additional File 6.

Results

Genome assembly of *Rhizina undulata* CMW 412

The *R. undulata* genome was assembled into 7 923 contigs of 1 000 bp or more (Tables S1 and S2). Despite the discontiguous nature of the assembly, genome completeness was not compromised. The genome was shown to be highly complete with regards to the presence of BUSCO genes from both the *fungi_odb10* and *ascomycota_odb10* datasets, with BUSCO completeness values of 98.8% and 95.3%, respectively (Fig. 2, Table S1). Notably, the values for duplicated and fragmented BUSCO genes were negligible. The assembly was estimated to be approximately 77.5 Mb in size, had a GC content of 41.5% and encoded a predicted 11 213 proteins (Fig. 2, Table S2). The predicted proteome was 98.7% and 96.7% complete when assessed against the *fungi_odb10* and *ascomycota_odb10* datasets, respectively.

The assembly statistics for the *R. undulata* genome were comparable to those of the other Pezizomycete genomes assessed in this study (Fig. 2, Table S1). These genomes ranged in size from approximately 27 to 192 Mb and GC content of between 40 and 51%, and they encoded between 7 800 and 24 000 proteins. These genomes were also considered complete, with BUSCO values of at least 90% with respect to both the *ascomycota_odb10* and *fungi_odb10* datasets. Similarly, proteomes from all the species except *Geopyxis carbonaria* had completeness values of at least 85% with respect to the same two datasets.

The phylogenetic analyses including publicly available *R. undulata* sequences, sequences from the newly sequenced genome, as well sequences from other Rhizinaceae species showed that the isolate selected for genome sequencing clustered most closely with other *R. undulata* isolates (Figure S1). This confirmed the identity of the isolate chosen for genome sequencing. Furthermore, phylogenomic analyses using complete, single copy BUSCO proteins produced the expected relationships between the Pezizomycete species included here, with *R. undulata* grouping with species in the genera *Morchella*, *Tuber* and *Choiromyces* (Fig. 2, Figure S2).

Heat shock and heat shock-associated proteins are common in the genomes of Pezizomycete fungi

Of the 48 PFAM domains associated with the search term "HSP", 29 were present in at least a single copy in one of the 22 genomes considered (Fig. 3, Table S5). Many of these domains were present in multiple copies in the genomes, including heat shock protein domains such as PF00011 (HSP20/alpha crystallin family), PF00012 (HSP70), PF02518 (Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase), and PF13589 (Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase), as well as molecular chaperones such as PF00118 (TCP-1/cpn60 chaperonin family), PF00226 (DnaJ domain), PF01556 (DnaJ C-terminal domain). Additionally, domains involved in glutathione S-transferase activity were also present as multiple copies and included PF00043 (GST C-terminal) and PF02798 (GST N-terminal).

The Pezizomycete genomes had an average of 116 HSP-associated PFAM domains (Table S5), with *Kalaharituber pfeilii* having the fewest (n = 43) and *R. undulata* harbouring the greatest number of these domains (n = 146). The two most common PFAM domains across the 22 species were PF00226, encoding DnaJ/HSP40, a cochaperone of the HSP70 family proteins, and PF02518, encoding an HSP90-like protein.

Fig. 2 Genome comparisons across the Pezizomycetes. **A** An annotated phylogeny illustrating differences in genome statistics. The phylogeny was generated using 783 shared BUSCO proteins and represents the known relationships between these species. The full phylogeny is included in Figure S2 with additional information and node support data. Species known to associate with fire are in orange text and the *R. undulata* isolate sequenced for this study is in bold, red text (See Fig. 1 and Table S1 for details on fire associations). The Pezizomycete genomes ranged from ~ 27 to ~ 171 Mb and encoded between ~ 7 800 and ~ 24 000 genes. All of the genomes were considered complete (94.3 to 99.1% with respect to the fungal BUSCO dataset) and none were contaminated with bacterial sequences (5.6 to 11.3% with respect to the bacterial BUSCO dataset). **B** Plots comparing the genome size, GC content, and number of protein coding genes in the genomes of the 22 species. The *R. undulata* genome (represented by the red dots) is within the expected length, gene count and GC content statistics of the other Pezizomycetes (represented by the blue dots)

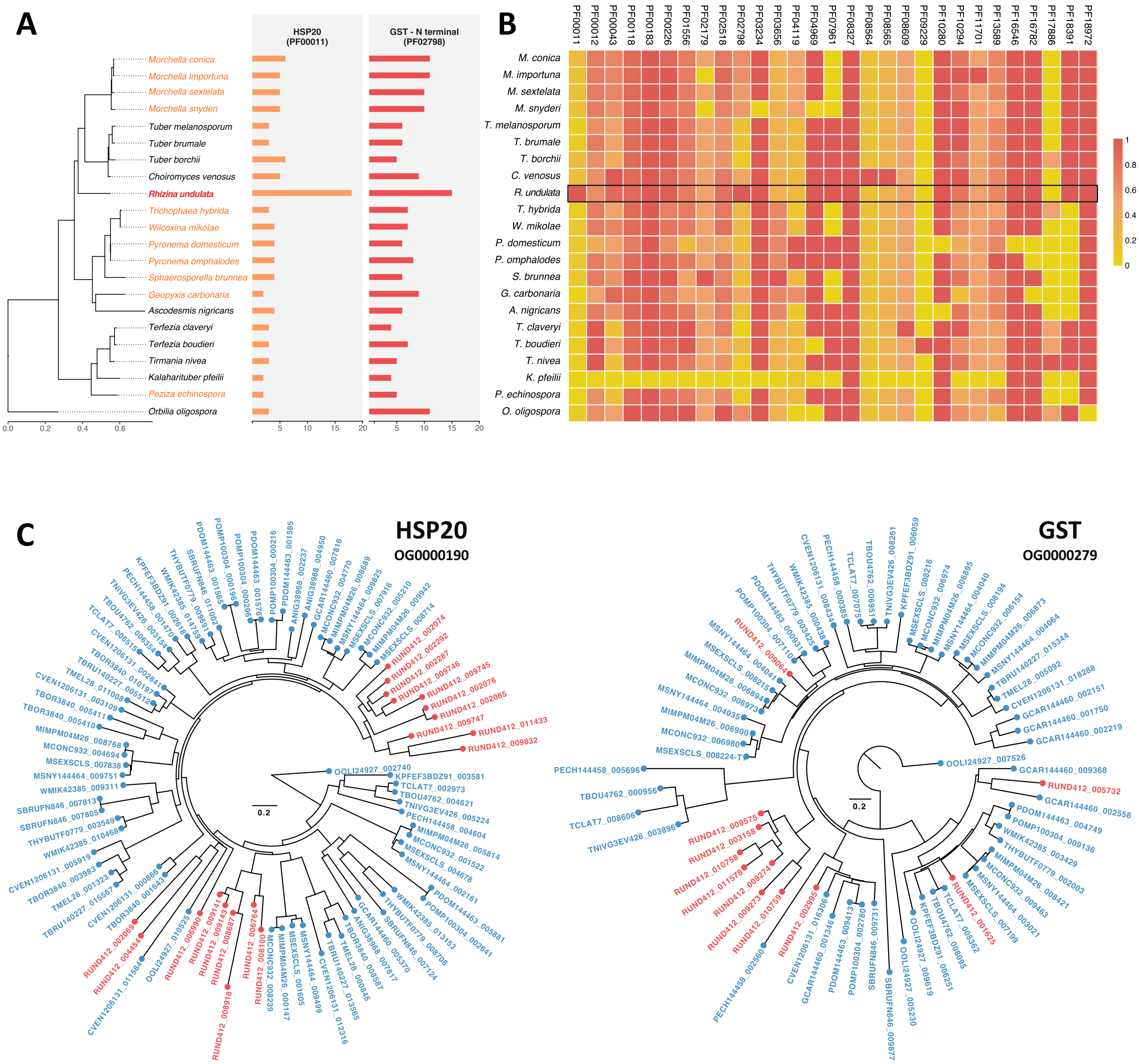

Fig. 3 The presence of HSP-associated PFAM domains across the Pezizomycetes. **A** An annotated phylogeny illustrating the numbers of HSP20 (PF00011) and GST N-Terminal (PF02798) proteins in each of the genomes. The full phylogeny is included in Figure S2 with additional information and node support data. The phylogeny was generated using 783 shared BUSCO proteins and represents the known relationships between these species. Species known to associate with fire are presented in orange text and the *R. undulata* isolate sequenced for this study is in bold, red text. The *R. undulata* genome harbours many more proteins in the HSP20 and GST families than the remaining Pezizomycetes. **B** A heatmap illustrating the number of HSP-associated PFAM domains encoded in each genome. *R. undulata* is highlighted in a black box. Note that each column is normalized individually, and the absolute values can be found in Table S5. **C** Phylogenies of the orthologues from two orthogroups harbouring protein families that are expanded in *R. undulata*. OG0000190 represents the HSP20 proteins and is comprised of 18 proteins with HSP20 PFAM domains and an additional protein identified by OrthoFinder. OG0000279 represents GST proteins and is comprised of nine proteins with GST-N PFAM domains and two additional proteins identified by OrthoFinder. The *R. undulata* orthologues are represented by red circles and red text, while those from the other 21 species are represented by blue circles and blue text. Note that each orthologue ID begins with four letters, made up of the first letter of the genus and the first three letters of the species epithet (e.g., *Rhizina undulata* = RUND)

The expansion of heat shock associated protein families in *R. undulata*

A total of 305 334 proteins were considered in the OrthoFinder analysis and 275 123 (90%) of these proteins were assigned into one of the 16 310 orthogroups. The species tree generated by OrthoFinder showed identical species relationships to the phylogenomic tree generated used the single copy BUSCO genes (Figure S2).

The HSP20 protein family

Functional annotation identified 18 genes encoding proteins with the PFAM domain for HSP20 proteins, PF00011, in the *R. undulata* genome (Fig. 3, Table S5). This was supported by the OrthoFinder results, which grouped these 18 genes together with an additional *R. undulata* gene into orthogroup OG0000190 (Table S6). In comparison, the other Pezizomycetes were predicted to encode between two and six HSP20 proteins, again supported by the presence of between two and six genes in orthogroup OG0000190 for the other species. Notably, the number of proteins in this family did not differ significantly between species known to be associated with fire, or not. (Fig. 4, Table S7). This suggests that the HSP20 protein family has been significantly expanded in *R. undulata* compared to all of the other Pezizomycetes and that it represents a unique heat stress-related adaptation.

The GST protein family

A total of 15 proteins were functionally annotated with the PFAM domain for GST N-Terminal proteins, PF02798, in the *R. undulata* genome (Fig. 3, Table S5). OrthoFinder grouped 9 of these proteins together with two additional proteins in orthogroup OG0000279 (Table S6). In contrast to *R. undulata*, the remainder of the Pezizomycetes harboured an average of 7 PF02798 proteins. A similar pattern is present when considering the genes in OG0000279, with the remainder of the Pezizomycetes harbouring 6 or less genes in this orthogroup. Notably, there is a significant association between the number of genes in this orthogroup and the species' association with fire (Fig. 4). While those having associations with fire have between 2 and 11 genes ($\bar{x} = 4.3$), those without a known fire association have between 0 and 3 genes ($\bar{x} = 1.8$).

Aromatic compound dioxygenases

The orthgroup OG0000266 encoded proteins with similarity to aromatic compound dioxygenases (ACD, Table S6). These are proteins capable of catabolising aromatic compounds, which are known to exist in post-fire material. The number of ACD proteins present in this orthogroup appeared to correlate with the ecology of the species included in our analyses (Fig. 4, Fig. 5). Those with known fire associations tended to have more proteins in this orthogroup ($\bar{x} = 4.7$) compared to those with fire-independent ecologies ($\bar{x} = 1.6$). In particular, this orthogroup was largest in *R. undulata*, which harboured 10 proteins identified as dioxygenases, suggesting that this protein family is expanded in *R. undulata* (Fig. 5).

Discussion

In this study, the draft genome of *R. undulata* was sequenced and annotated for the first time. *R. undulata* is a unique pathogen known for its dependence on a heat shock, such as that induced by fire, for ascospore germination [6]. This genomic resource was used to identify

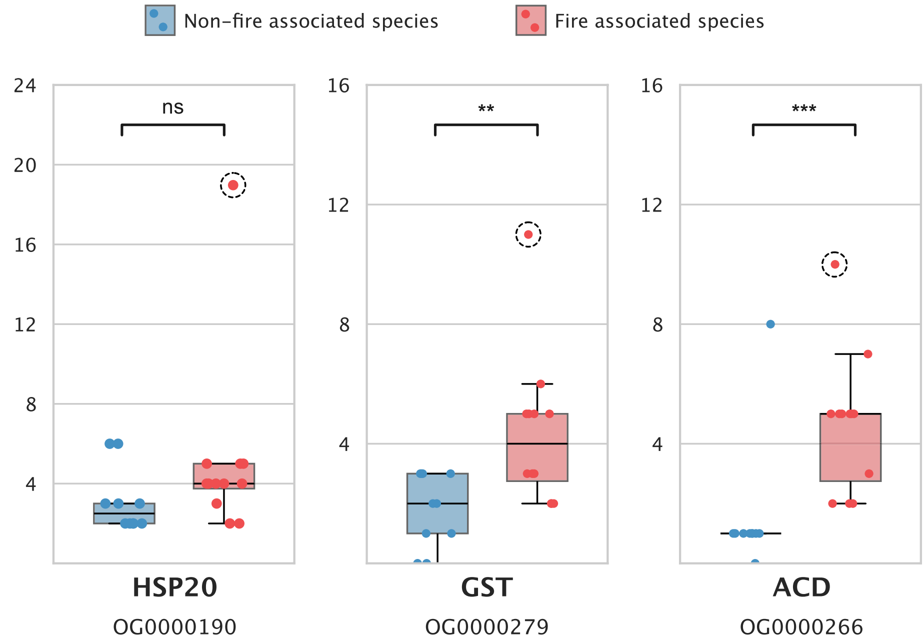

Fig. 4 Comparison in orthologue count between fire associated and non-fire associated species with respect to the expanded heat shock-associated protein families in *R. undulata*. In all three families, *R. undulata* is an outlier (red dots in dotted black circles), harbouring more orthologue copies than the other 21 species. In the case of the HSP20 protein family, there is not a significant difference in copy number between fire associated (red circles and boxes) and non-fire associated (blue circles and boxes) species. In contrast, the fire-associated fungi harbour more copies of the GST and ACD proteins than the non-fire associated species. This pattern holds true when *R. undulata* is removed from the analysis (Table S7). ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

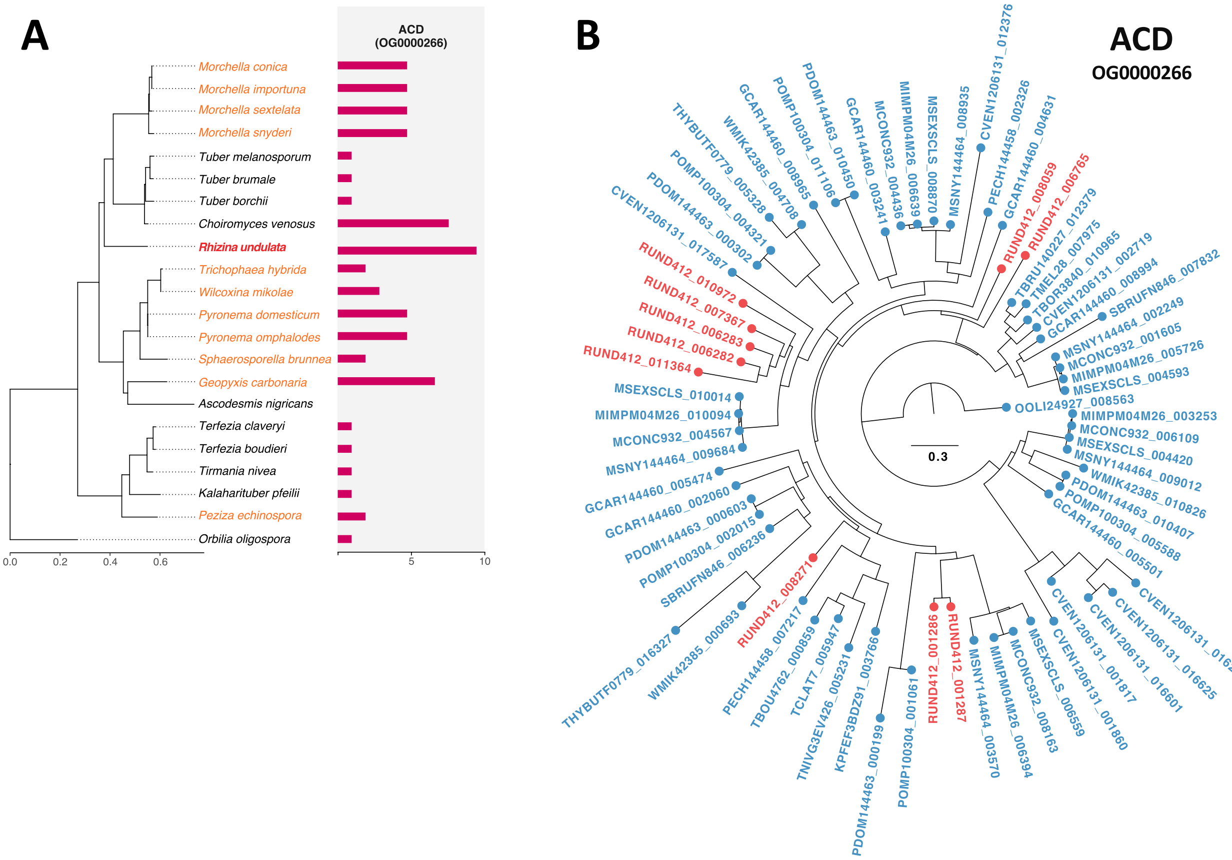

Fig. 5 The presence of orthologues from OG0000266 across the Pezizomycetes. The genes in this orthogroup encode aromatic compound dioxygenase (ACD) enzymes. **A** An annotated phylogeny illustrating the number of ACD proteins in each of the genomes. The full phylogeny is included in Figure S2 with additional information and node support data. The phylogeny was generated using 783 shared BUSCO proteins and represents the known relationships between these species. Species known to have fire associations are presented in orange text and the *R. undulata* isolate sequenced for this study is in bold, red text. The *R. undulata* genome harbours many more proteins in the ACD family than the remaining Pezizomycetes. **B** A phylogeny of the orthologues from OG0000266, a protein family that is expanded in *R. undulata*. The *R. undulata* orthologues are represented by red circles and red text, while those from the other 21 species are represented by blue circles and blue text. Note that each orthologue ID begins with four letters, made up of the first letter of the genus and the first three letters of the species epithet (e.g., *Rhizina undulata* = RUND)

potential mechanisms underlying the thermotolerance and post-fire recovery exhibited by *R. undulata* (Fig. 6). The analyses lead us to posit that expansions of the HSP20 and GST protein families have enabled the fungus to withstand the high temperatures associated with forest fires and to germinate shortly after the heat stress is removed. Furthermore, we hypothesize that expansions of the GST and ACD protein families enable the metabolism of recalcitrant materials in the post-fire environment, allowing the fungus to grow towards susceptible host roots. Because *R. undulata* requires a heat shock for ascospore germination and subsequent host infection, these proteins likely play a role in the life cycle of the pathogen and the manner in which it causes disease.

Fire is both a destructive and regenerative event, significantly impacting the diversity of fauna, flora, and fungi in the environment [51]. Fire can have a variety of positive effects on plants. For example, it can limit insect pests during post-fire growth seasons, thus allowing for increased seed production [52]. Fire is also known to stimulate flowering in a variety of terrestrial plants [53]. However, fire can also be required for the activity of plant pathogens. In the case of pyrophilic plant pathogens such as *R. undulata*, pathogenicity is entirely dependent on a fire-induced heat shock which enables ascospore germination [6]. Fire also decreases microbial diversity and therefore competition [51], improves spore dispersal by aerosolizing fungal spores [54], and weakens plant defences [55]. In the case of intensively managed forests, the advantages of using fire to remove forest waste and reduce insect infestation must be balanced with the risks associated with fire-associated pathogens, such as *R. undulata* [8, 9].

The Pezizomycetes considered in this study for comparative purposes included those with or without established connections to fire. Those not having a particular relationship with fire included species in the genus *Terfezia*, which typically form edible and highly sought-after

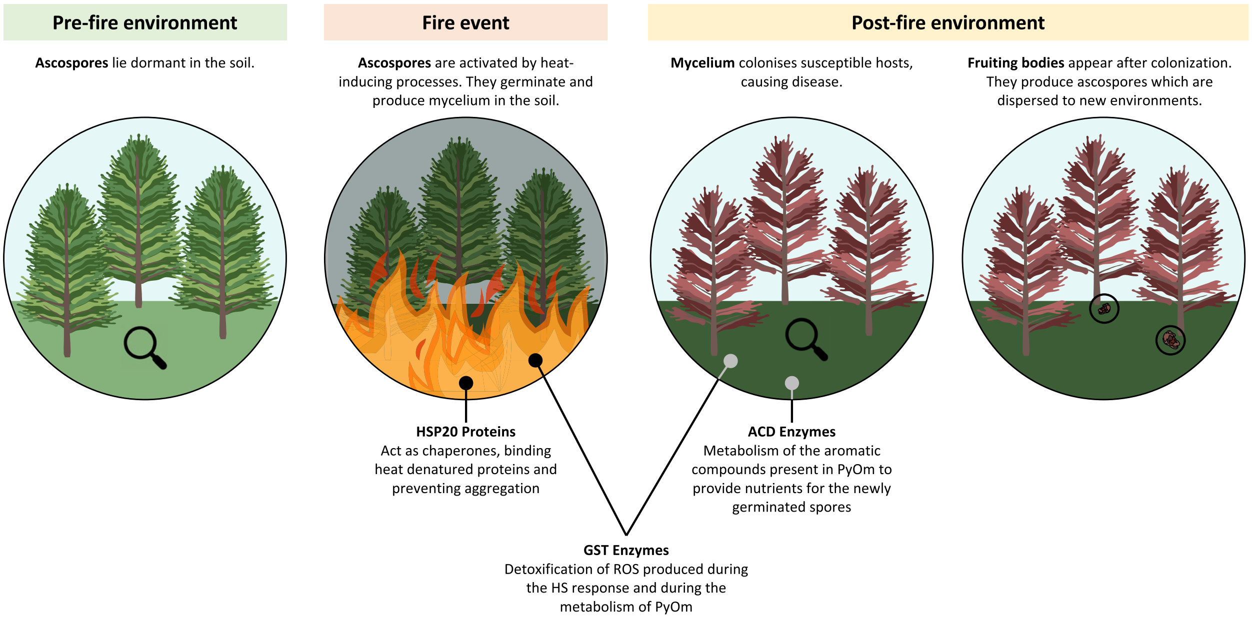

Fig. 6 The molecular mechanisms hypothesized to play a role in the thermotolerance and post-fire activation of *R. undulata*. Ascospores of *R. undulata* are airborne and remain dormant in the soil in areas where the fungus has previously sporulated. When a heat-inducing event, such as a fire, occurs in conifer plantations or forests, the spores are activated and germinate, after which they infect the roots of the trees, causing disease. In the presence of fire, HSP20 proteins likely protect the ascospores from heat-induced stress by binding to heat-denatured proteins, thereby preventing aggregation and further damage. GST enzymes detoxify the ROS molecules that are produced in response to the heat stress. In the post-fire environment, when the spores have germinated, nutrients may be provided through the ACD-facilitated metabolism of PyOm produced by the fire. Again, the GST enzymes would detoxify ROS molecules produced through this metabolism. The germinated ascospores subsequently give rise to mycelium that colonizes roots of the associated conifers in the post-fire environment, resulting in disease and producing apothecial fruiting bodies (ascocarps). These fruiting bodies produce large numbers of forcibly ejected ascospores, which are dispersed to new environments. The expansion of the HSP20 protein family is unique to *R. undulata*, whereas the expansions of the GST and ACD protein families, while particularly notable in *R. undulata*, are also found in the other heat-associated Pezizomycetes. Together, the expansion of these protein families in *R. undulata* likely enables the pathogen to be activated by fire events and to thrive in post-fire environments

desert truffles [56]. In contrast, many Pezizomycetes are known to rapidly colonize environments after the occurrence of fire. Early colonizers, such as *Wilcoxina mikolae,* typically emerge within the first few weeks or months, while others, such as *Peziza echinospora*, colonize much later [57]. These species are thought to be important in the recovery of post-fire ecosystems, where they are uniquely capable of withstanding heat stress during the fire but also have the enzymatic capacity to degrade organic matter that remains after fire events. Notably, however, very few fungi are known to rely on fire in the same way as *R. undulata*, where this source of heat plays an integral role in its life cycle. While it is hypothesized that some pyrophilic fungi may also display some level of heat-stimulated germination [58], *R. undulata* appears to be unique in its dependence on fire for ascospore germination. It is therefore, not surprising that it appears to have evolved in a manner different to that of other Pezizomycetes.

Microbes that are capable of withstanding the stress associated with extreme environments do so via many genetic, biochemical and physiological pathways [59]. The expression of HSPs in response to various types of abiotic stress is highly conserved across the major domains of life [60]. Consequently, it is not surprising that numerous HSPs were identified in the genomes of *R. undulata* and the other Pezizomycete species. For those species having no known association with fire, the presence of HSP-associated PFAM domains suggests that these proteins are involved in other stress responses not related to fire. For example, the species with the fewest HSP-associated PFAM domains was *K. pfeilii*, which is a desert truffle. Although this species does not need to withstand fire-induced stress, it does survive other environmental stressors associated with the extremes of a desert environment. Stressors such as water scarcity, UV exposure and elevated temperatures may thus account for the presence of various HSPs. In contrast, however, *R. undulata* had the greatest number of HSP-associated PFAM domains, with particularly large numbers of HSP20, HSP40 and HSP90, which is understandable given its fire-dependent life cycle.

Multiple HSP20 proteins were present in the genomes of all the species considered here, but the particularly large number of these proteins identified in the *R. undulata* genome indicates an expansion of the HSP20 family in the fungus. The presence of many sHSPs is common in plants (> 20 copies), but quite rare in fungi, which usually possess far fewer copies [14, 61, 62]. The elevated number of *R. undulata* HSP20 proteins is comparable with the 14 identified in *Coriolopsis trogii*, a mushroom-forming basidiomycete also specifically known for its thermotolerance [63]. It has been shown that many of the *C. trogii* HSP20 alleles are up regulated in response to incubation at elevated temperatures, indicating a direct role for these proteins in the heat shock repsonse (HSR, [63]). It is possible that a similar process occurs in *R. undulata*, with each HSP20 protein having a different role or being responsible for different parts of the HSR, particularly during the breaking of ascospore dormancy and subsequent germination. Transcriptomic studies that track the expression of the *R. undulata hsp20* genes throughout its life cycle and during exposure to heat and other abiotic stressors should thus be considered in the future.

In addition to their primary role of regulating the HSR, HSPs have also been implicated in various other cellular processes, including protein transport, transcriptional control, and apoptosis. Small heat shock proteins have been specifically linked to pathogenesis in the maize pathogen *Ustilago maydis* [64] and virulence in the insect-infecting *Beauveria bassian*a [62]. In contrast, the loss of virulence associated with successive subculturing in *Conidiobolus obscurus* involved the differential regulation of many *hsp20* genes [65]. Given the widespread importance of these genes in pathogenicity and virulence, their specific role in the stress response, and the notoriety of *R. undulata* as a post-fire pathogen, HSPs should be targeted for future study and functional characterization.

The GST protein family was also shown to be expanded in *R. undulata* and appeared to be over-represented in the genomes of the fire-associated Pezizomycetes compared to those with no connection to fire. In the context of HS and post-fire colonization, these GST proteins may play two important roles in the ecology of these fungi. Firstly, HS in fungi and other eukaryotes is associated with the production of ROS [20, 66–68]. While these small oxidative molecules act in signalling pathways which regulate the HSR, they can also be harmful to the cell if accumulated [20, 67]. GSTs are amongst the enzymes that are capable of ROS detoxification and have been associated with the HSR in diverse eukaryotes [69–71]. For example, genes encoding GST proteins are differentially regulated during HS in *Ganoderma lucidum* [72], *Laternulla elliptica* [68], and *Blastocladiella emersonii* [23], strongly supporting their role in mitigating the effects of heat stress. The increased number of GST proteins in *R. undulata* and the other fire-associated Pezizomycetes could thus enable a rapid recovery after a fire event.

Another role that GST proteins could play in fire-associated fungi is related to their capacity to acquire nutrients in post-fire environments. Fires turn easily available carbon present in soil into pyrogenic organic matter (PyOm, [73, 74]). This is a mixture of recalcitrant molecules and fungi that can utilize this nutrient source are able to recolonize much more rapidly than those lacking this capacity. For example, species in the genus *Pyronema* are capable of metabolizing PyOm via the expression of enzymes such as P450 monooxygenases [74]. However, during PyOM metabolism, significant levels of ROS are produced. The expansion of the various antioxidant GST enzymes in *R. undulata* and the other fire-associated fungi may thus enable safe nutrient acquisition after fire. In *R. undulata* specifically, this would ensure that freshly germinating spores could take advantage of many of the available nutrients before colonizing a host plant.

The expansion of the ACD enzymes in *R. undulata* and the greater number of these proteins in the other fire-associated fungi could also be related to the availability of PyOm as the predominant organic material in post-fire environments. PyOm is largely comprised of aromatic and polyaromatic compounds [74, 75], which are considered toxic to many organisms because the proteins that are capable of their metabolism are typically unique to bacteria and fungi [76, 77]. Dioxygenase enzymes catalyse a ring-opening step, transforming these aromatic compounds into a non-aromatic form that can be further broken down and eventually enter more common metabolic pathways [74]. The presence of genes encoding these proteins in the genomes of fire-associated fungi illustrates the precise role these fungi play in the post-fire recovery process, where they are uniquely able to cycle the carbon into more usable forms. As with the expansion of the GST proteins, the increased numbers of these proteins in *R. undulata* and the other fire-associated fungi supports their expansion as a response to the availability of PyOm in post-fire environments.

We have decribed the protein family expansions that are potentially involved in the ability of *R. undulata* to withstand and recover from stress associated with fire. The proteins and pathways that intiate germination only after exposure to heat are remain unknown, but would likely include those associated with dormancy breaking, growth, cell cycle regulation, and environmental sensing. Identification of these genes will require an investigation into gene expression patterns in key stages throughout the lifecycle of this fungus. Future research should thus focus on the generation of further NGS data for this enigmatic fungus.

Conclusions

The conifer pathogen *R. undulata* has long been known to be dependent on fire or other forms of heat stimulation to enable the germination of its ascospores, which in turn leads to infection of the roots of its hosts. The molecular mechanisms that facilitate this process and protect the fungus from the significant stressors induced by fire have not previously been investigated. In this study, we identified three protein families that are abundant in the *R. undulata* genome and likely play significant roles in the ability of this fungus to survive HS events and to thrive in post-fire environments. The sHSPs likely protect the spores from the heat stress, by protecting cellular proteins against denaturation and aggregation. The GSTs likely act to protect the fungal cells from the production of ROS, which occurs both during the HSR as well as during the metabolism of PyOm in post-fire environments. Lastly, the ACDs likely act to metabolise PyOm, providing *R. undulata* with the nutrients necessary to germinate and grow towards and infect its hosts.

Abbreviations

ACDs	Aromatic compound dioxygenases
BUSCO	Benchmarking Universal Single-Copy Orthologs
GSTs	Glutathione-S Transferases
HS	Heat Shock
HSPs	Heat Shock Proteins
HSP20	Heat Shock Protein 20
HSR	Heat Shock Response
ITS	Internal Transcribed Spacers
NCBI	National Centre for Biotechnology Information
QUAST	Quality Assessment Tool for Genome Assemblies
ROS	Reactive Oxygen Species
sHSPs	Small HSPs

Supplementary Information

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Supplementary Material 1. Genome assembly and assessment. This document contains the scripts and parameters used for the genome assembly and its assessment.

Supplementary Material 2. Phylogenetic analysis. This document contains the alignments and parameters used for the phylogenetic analyses.

Supplementary Material 3. Phylogenomic analyses. This document contains the scripts and parameters used for the phylogenomic analyses.

Supplementary Material 4. Gene prediction and functional annotations. This document contains the the scripts and parameters used for the gene prediction and functional annotation.

Supplementary Material 5. HeatMap. This docunment contains the script used to generate this heatmap in Fig. 3.

Supplementary Material 6. StatsForOG. This docunment contains the script used for the statistical analyses needed to generate Fig. 4.

Supplementary Material 7.

Supplementary Material 8.

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Authors' contributions

All authors were involved in the conceptualization, investigation and methodology, as well as the writing, reviewing and editing of the manuscript. AMW was responsible for the data curation, performing the analyses and presenting the results. AMW wrote the first draft of the manuscript. TAD assisted with the analyses and provided expertise in interpreting the results. MJW and BDW were responsible for project administration, supervision and funding acquisition. All authors have read and approved the final manuscript.

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Data availability

The genomes used in the study are all publicly available in the NCBI Genome repository, with the accession numbers and other relevant informations detailed in Table S1. The newly sequenced and fully annotated ***R. undulata*** genome is also available in this repository under the Accession number: JAWIKH000000000 and the filtered paired-end reads are available in the Sequence Read Archive respository. Both accessions are associated with BioProject and BioSamples numbers: PRJNA1026127 and SAMN37734914, respectively. The scripts used to perform all of the genomic, phylogenetic, phylogenomic, and statistical analyses detailed above are available as Additional Files (Files 1–6). The newly generated annotations for all 22 genomes, the Orthogroup counts generated by OrthoFinder, as well as the phylogenies generated using MrBayes, ASTRAL, and OrthoFinder can be found in the following FigShare folder, DOI: https://doi.org/10.6084/m9.figshare.28742765.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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