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Research article

Homolog identification of heat shock proteins in eight fungi belonging to Pezizomycetes and copy number comparisons

Elisa Zampieri[1], Antonietta Mello[2]

[1]Istituto per la Protezione Sostenibile delle Piante - CNR, Strada delle Cacce 73, 10135 Torino, Italy
[2]Istituto per la Protezione Sostenibile delle Piante - CNR, Viale P.A. Mattioli 25, 10125 - Torino, Italy

Corresponding author e-mail: antonietta.mello@cnr.it

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Abstract
Heat shock proteins are ubiquitous, highly conserved and play a role in the fungal adaptation to the environment. In the present study of comparative genomics, we used gene models, identified as the stress-response related gene families in the genome of *Tuber melanosporum*, to find the homologs in the genome of seven other Pezizomycetes having different lifestyles and in the new genome assembly of *T. melanosporum*. The produced list of homologs was also used to search for a correlation: 1) between heat shock protein copy number and lifestyle (saprotrophic and symbiotic) and 2) heat shock protein copy number and genome size. No significant correlation was found between copies and size, as well as between copies and lifestyle within the limited set of considered fungal species. The utility of a list of genes homologs in Pezizomycetes coding for heat shock proteins or other chaperones is discussed.

Keywords
Pezizomycetes, comparative genomics, blastp, heat shock proteins, chaperones, stress

Introduction

Stress is a critical factor influencing the survival and performance of all organisms, including fungi, which are considered among the most successful eukaryotes due to their ability to cope with diverse environmental challenges (Muggia et al., 2020). Fungi, as all organisms, respond to stress conditions by activating genes encoding heat shock proteins (HSPs), which play a key role in cellular stress tolerance and environmental adaptation (Hartl et al., 2011; Sagini and Ligabue-Braun, 2024). HSPs can prevent or reduce the aggregation of proteins damaged by heat or other stressors and assist in their refolding or degradation, thereby functioning as molecular chaperones (Chen et al., 2018 and reference therein). Generally, when cells are subjected to heat shock and other stress conditions (e.g., pH changes, drought, high or low temperatures, etc.), HSP levels increase, whereas under non-stress conditions a basal level of expression is maintained, which is required for processes such as protein

folding, signal transduction and development (Tiwari et al., 2015; Chen et al., 2018 and reference therein). The HSPs can be activated by different types of stress, and they are classified based on the size, which ranges from 15 to 110 kDa, and on the function, which includes chaperone and catalytic activity (Tiwari et al., 2015). Zampieri et al. (2011, 2014) have demonstrated HSP expression in response to cold stress in *Tuber melanosporum* Vittad. mycelium and in *T. magnatum* Picco fruiting bodies, while Leonardi et al. (2017) have assessed the mycelial growth of *T. borchii* Vittad. under high temperatures and the ability of this species to colonize the host roots. Gabella et al. (2005) proposed a member of HSP9/12 family as molecular marker for fruiting body development in *T. borchii*. It has been then demonstrated that the expression of HSP9 increased during fruiting body in a differentiation-dependent manner in *Grifola frondosa* (Dicks.) Gray*, Pleurotus eryngii* (DC.) Quél.*, Hypsizygus marmoreus* (Peck) H.E. Bigelow, and *Lentinula edodes* (Berk.) Pegler*,* suggesting that HSP9 may be used as an industrially useful marker for monitoring fruiting body differentiation during the cultivation process (Kurahashi et al., 2014).

The sequencing of fungal genomes has allowed deepened comparisons among several species having different lifestyles, as performed by Murat et al. (2018) who compared Pezizomycetes able to form ectomycorrhizae [*T. magnatum*, *T. melanosporum*, *T. aestivum* Vittad., *Terfezia boudieri* Chatin and *Choiromyces venosus* (Fr.) Th. Fr.] with those that are saprotrophic (*Morchella importuna* M. Kuo, O'Donnell & T.J. Volk, *Ascobolus immersus* Pers., and *Pyronema confluens* Tul. & C. Tul.). Murat et al. (2018) demonstrated that species belonging to *Tuber* clustered together close to *C. venosus* and that *Tuber* species genomic features were very similar, characterized by a high transposon content that increases the genome size, few genes coding lignocellulose degrading enzymes, and lineage-specific fruiting body upregulated genes. Recently, a comparative genomic analysis by Martelossi et al. (2025) investigated the interplay between transposable elements (TEs) and genome evolution in the Tuberaceae. In this study, we aim to provide a homology-based inventory of HSP-related genes in the fungal genomes analyzed by Murat et al. (2018). Using a comparative genomics approach, our goal is to identify these genes and to investigate whether there is a correlation between fungal genome size or different lifestyles and the number of HSP family members, taking into account that symbiotic and saprotrophic fungi share genetic similarities and that the ectomycorrhizal lifestyle can rely on different molecular symbiotic toolkits depending on the involved fungal species (Lebreton et al., 2021).

Materials and Methods

Based on a comparative search through Blastp, gene models of *T. melanosporum*, previously assigned to different protein families and listed in the supplementary table 19 of Martin et al. (2010), were used to find the homolog in the genome of seven other Pezizomycetes and in the new assembly of *T. melanosporum*. The gene models include several stress-response-related gene families (Supplementary Tables S1 and S2). The Supplementary Table S1 includes all regions in Pezizomycetes that showed homology with the gene models reported in Martin et al. (2010), while the Supplementary Table S2 contains only the top homologous sequence for each case. In this study, we focused on the following HSP families: HSP90, HSP20, HSP9/12, DNAJ, CPN60-TPC1, and HSP70. We also examined co-chaperones of the HSP90 family (Cdc37, Sti1, cns1, wos2), the BAG

family, which modulates the chaperone activity of HSP70 (Takayama et al., 1999), and Cyclophilin, known to cooperate with HSPs (Andreeva et al., 1999).

Based on Blastp results, the absence of start and stop codons was noted and documented in Supplementary Table S1. The presence of a signal peptide and the expected protein domain was assessed by examining each protein ID on the corresponding Pezizomycetes genome website, and missing domains were likewise documented in Supplementary Tables S1.

Genome assemblies and corresponding gene annotations were retrieved from JGI MycoCosm platform at the following URLs: https://genome.jgi.doe.gov/Ascim1 (*A. immersus* v1.0); https://genome.jgi.doe.gov/Chove1 (*C. venosus* v1.0); https://genome.jgi.doe.gov/Morco1 (*M. importuna* v1.0); https://genome.jgi.doe.gov/Pyrco1 (*P. confluens* v1.0); https://mycocosm.jgi.doe.gov/Terbo1/Terbo1.home.html (*T. boudieri* v1.1); https://genome.jgi.doe.gov/Tubae1 (*T. aestivum* v1.0); https://genome.jgi.doe.gov/Tubma1 (*T. magnatum* v1.0); https://genome.jgi.doe.gov/Tubme1v2 (*T. melanosporum* v1.2). The blastp was performed in 2015/2016 using a dedicated portal on the MycorWebsite (http://mycor.nancy.inra.fr/IMGC/Pezizomycetes/ where the e-value parameter was set to e^{-10} by default). This platform allowed us to search across all genomes without having to query NCBI directly.

The Maximum likelihood tree was built by Mega 6 (Tamura et al., 2013) using Jones-Taylor-Thornton (JTT) model, on the base of the full protein sequence aligning performed with CLUSTAL omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Clade stability was assessed using bootstrap analysis with a 1000 replicate dataset. To explore the relationship between the genome size and the HSP numbers, a Pearson's correlation was performed. The comparisons between groups were performed using the Wilcoxon rank-sum test, in R v4.1.2 (base stats::wilcox.test), using a two-sided alternative and unpaired samples (default settings). Species were assigned to the categorical variable "trophic mode" with two levels ("Symbiont" and "Saprotroph") based on information from the literature and lifestyle annotations available in the MycoCosm portal (JGI). This test allowed to compare the fungal lifestyle with the number of HSP members.

Results

Blasting the *T. melanosporum* sequences against the seven Pezizomycetes and the new version of *T. melanosporum* all the stress-response related gene families were represented in their genomes (Supplementary Tables S1 and S2). Among all the stress-response related gene families, we report the results for the HSP families (HSP90, HSP20, HSP9/12, DNAJ, CPN60-TPC1, and HSP70), as well as for the HSP90 co-chaperones (Cdc37, Sti1, cns1, wos2). We also include BAG family and Cyclophilin, as they co-operate with HSPs.

Concerning HSP90 family, in all the Pezizomycetes a single HSP90 homolog was detected in all analyzed species. Moving to co-chaperones of this family, a single Cdc37 homolog was detected in all the Pezizomycetes, except for *C. venosus,* where six homologs were present. Homologs of Sti1 (stress-inducible protein) are present in the three *Tuber* species, in *C. venosus,* in *A. immersus* and in *P. confluens,* but it is absent in the other fungi (*M. conica* and *T. bouderi*). A homolog of cns1 (the alternative name is Cyclophilin seven suppressor 1) (https://www.uniprot.org/uniprot/P33313) is present in all the Pezizomycetes as well as a homolog of protein wos2 (a protein with significant homology to human p23, a HSP90-associated co-chaperone; Felts and Toft, 2003).

HSP20 family is characterized by a conserved C-terminal domain, alpha-crystalline domain, of about 100 residues (https://pfam.xfam.org/family/PF00011). While three homologs are present in *T. melanosporum*, four are in *T. magnatum*, *A. immersus* and *P. confluens*, three in *T. aestivum*, two in *T. bouderi*, five in *M. conica* and six in *C. venosus*. In the phylogenetic tree, although the overall bootstrap values were generally low, the three *T. melanosporum* proteins clustered with *T. aestivum*, *T. magnatum* and *Choiromyces,* supported by bootstrap values above 55 (Fig. 1).

HSP9/12 family shows two homologs to *T. melanosporum* in *T. aestivum*, in *C. venosus* and in *T. bouderi*. By contrast one homolog has been found in *T. magnatum*, four in *A. immersus* and in *M. conica*, eight in *P. confluens*.

DNAJ family is characterized by a region of 70 amino acid building blocks known as the J-domain, which is the site of interaction between DNAJ proteins and DNAK proteins (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR036869/). DNAJ proteins are also known as HSP40s, while DNAK proteins are also referred to HSP70 (https://pfam.xfam.org/family/PF00226). Starting from 14 gene models of the DNAJ family found in *T. melanosporum* and published in Martin et al. (2010) further four homologs were found in this species, 16 in *T. magnatum*, 17 in *T. aestivum*, 18 in *C. venosus*, 20 in *M. conica*, 17 in *P. confluens*, 22 in *A. immersus* and 17 in *Te. bouderi*.

The CPN60-TCP1 family includes molecular chaperones belonging to the octameric complex TCP1 (CCT). Genes coding for eight of the known TCP1 subunits (alpha, beta, gamma, delta, epsilon, zeta, eta, theta) and one gene coding for an HSP60 were identified in *T. melanosporum* (*Tmelcct1, cct2, cct3, cct4, cct5, cct6, cct7, cct8,* and *TmelHsp60*) (Martin et al., 2010). The protein similarity search in the Pezizomycetes has given 11 homologs in *T. melanosporum* v1.2, 14 in *T. aestivum*, 13 in *T. magnatum*, 11 in *C. venosus* and 10 in *Te. bouderi* as well as in *A. immersus*, in *P. confluens* and in *M. conica*. According to the blast analysis, each of the Pezizomycetes presented the eight subunits of TCP1 (although the subunit zeta cct6 was not found in *T. aestivum*), and the homolog of HSP60.

Relating HSP 70 family, in *T. melanosporum* genome (Martin et al., 2010), twelve HSP70-like genes were identified: in detail, seven coded for HSP70 protein, one for Hsp88, one for Hsp SSB, one for Hsp SSC1, one for 78 KDa glucose-regulated protein and one for ribosome-associated complex subunit SSZ1. The similarity search across Pezizomycetes has allowed to find five additional homologous genes in *T. melanosporum* v1.2, in addition to the 12 previously identified by Martin et al. (2010), 23 in *T. magnatum,* 22 in *Te. bouderi*, 24 in *C. venosus*, 20 in *A. immersus* and in *M. conica*, 18 in *T. aestivum* and 16 in *P. confluens*.

BAG family protein is characterized by BAG domain, which is located at the C terminus and binds the ATPase domain of Hsc70/Hsp70 (https://pfam.xfam.org/family/PF02179). In all the Pezizomycetes a single BAG homolog was detected, except for *T. aestivum,* where no *T. melanosporum* homolog was found. In *P. confluens,* the BAG protein contains an ubiquitin domain (PF00240), which suggests that the protein may be targeted to the proteasome.

The search for cyclophilins proteins has allowed the identification of three additional homologs in *T. melanosporum* v1.2, 12 in *T. magnatum, T. aestivum*, *P. confluens, T. bouderi*, and in *M. conica*, 10 in *A. immersus* and 11 in *C. venosus*. Some sequences contain the TPR (tetratricopeptide repeat) domain, which forms scaffolds to mediate protein–protein interactions and often the assembly of multiprotein complexes (https://pfam.xfam.org/family/PF00515), and the RRM domain, a motif that

is probably diagnostic of an RNA binding protein (https://pfam.xfam.org/family/PF00076). Summarising, the number of gene homologs for each family is shown in Fig. 2.

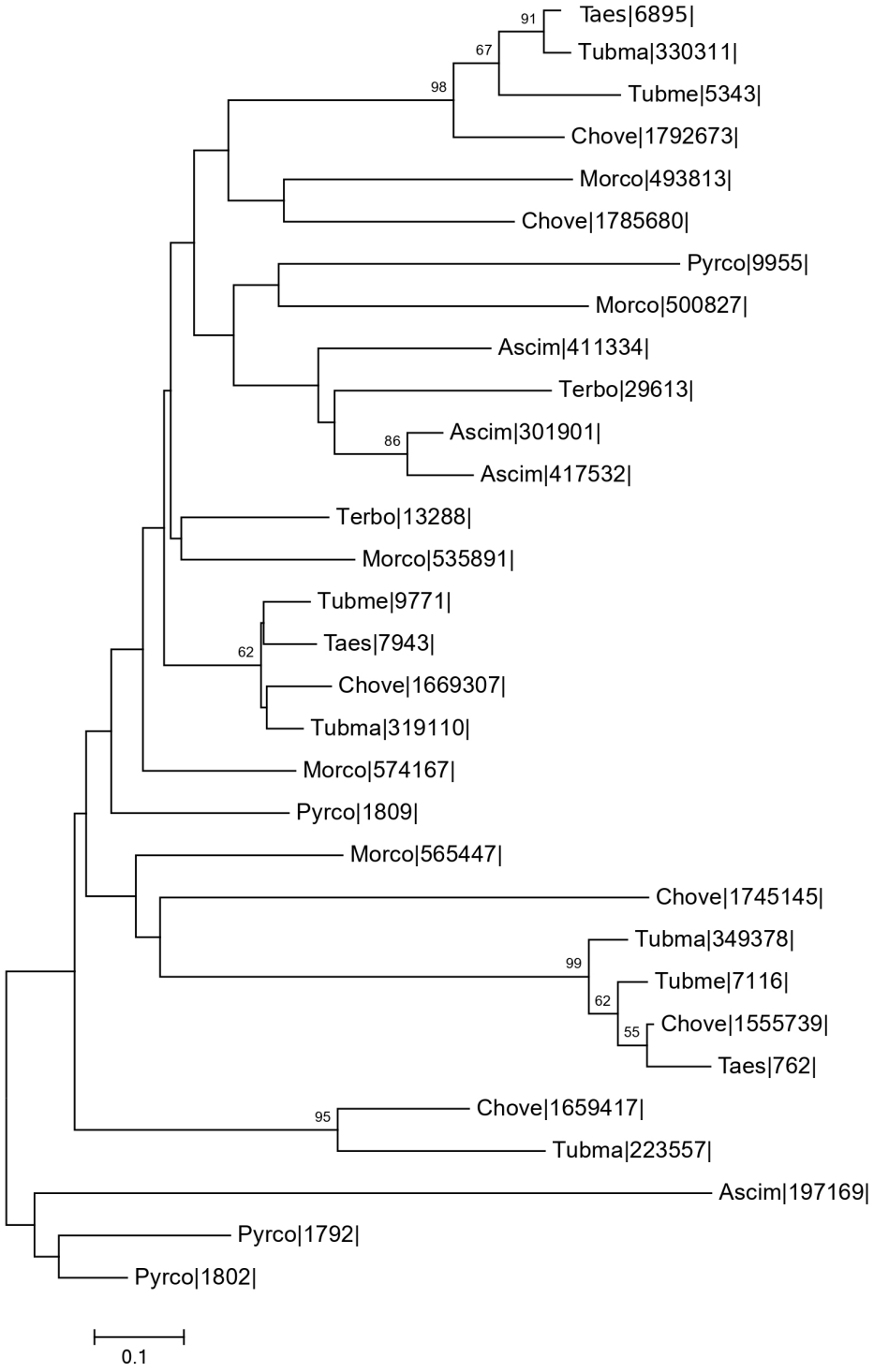

Fig. 1 - Maximum Likelihood tree based on the HSP20 protein sequence alignment from the eight fungi here considered. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Tubme= *T. melanosporum*; Tubma= *T. magnatum*; Taes= *T. aestivum*; Terbo= *Te. boudieri*; Ascim= *A. immersus;* Pyrco= *P. confluens;* Morco= *M. conica; Chove= C. venosus.*

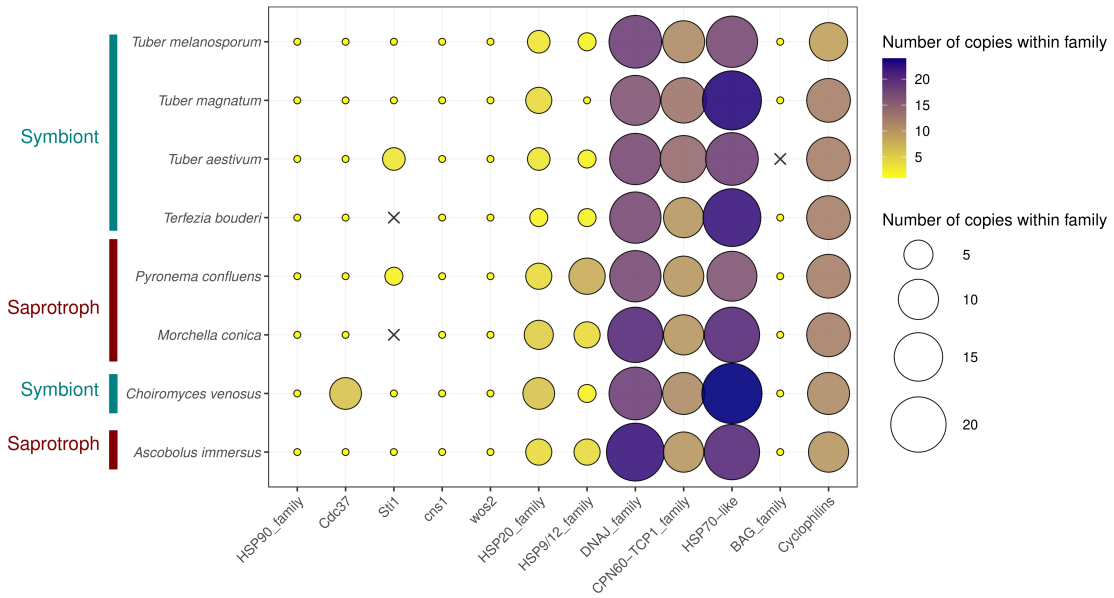

Fig. 2. – Bubble plot of the number of homologs for each family.

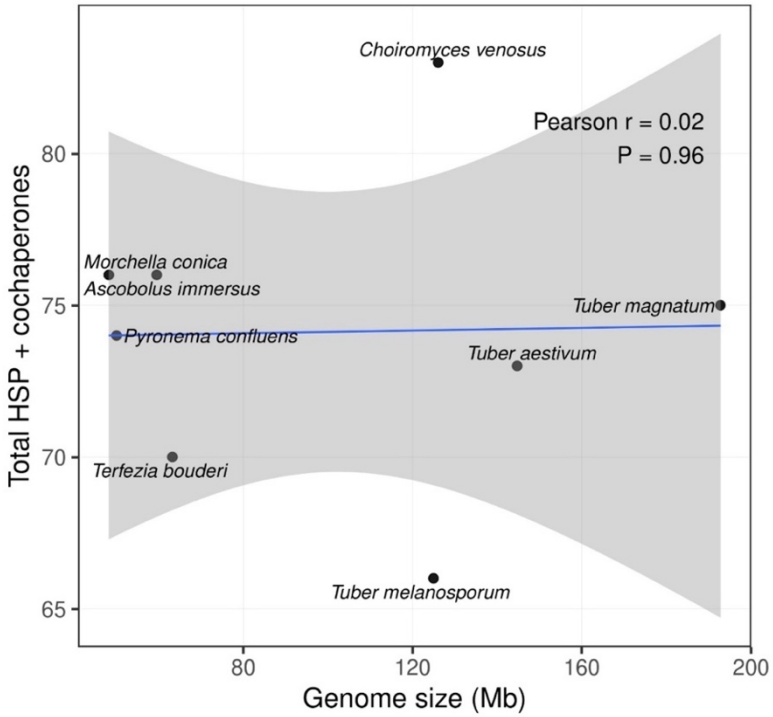

Fig. 3. – Correlation analysis between the HSP abundance and genome size of the eight Pezizomycetes.

Based on Wilcoxon test, performed on a small sample size and a limited number of fungal species, there was no significant difference in the total number of HSP and co-chaperones between symbiotic and saprotrophic fungal species (Wilcoxon rank-sum test, $p = 0.368$). A correlation analysis was then performed to verify the relationship between the HSP abundance and genome size of the eight Pezizomycetes obtained from Murat et al. (2018). Results show that, overall, there was not a

significant correlation between the number of homologs and genome size (p-value > 0.05). However, *P. confluens, T. magnatum* and *T. aestivum* appeared closer to the correlation line: the first exhibiting fewer HSPs and a smaller genome size, while the latter two displayed higher numbers of HSPs and larger genome sizes, compared with the other species (Fig. 3).

Discussion

By comparing the sequences of genomes of different fungal species, in addition to comprehend what discriminates them at the molecular level, it is possible to study evolutionary changes, identifying genes both conserved or common among species and unique (Touchman, 2010). One of the major goals of comparative genomics is to predict gene function. A comparative genomics approach within Pezizomycetes was used to identify heat shock proteins that are responsible for thermotolerance of the important tree pathogen and pyrophilous fungus *Rhizina undulata* Fr., which relies on heat shock-mediated activation in fire events for germination (Wilson et al., 2025). In the present investigation we have deepened the features of genes coding for HSPs, other chaperones, and proteins that bind HSPs, in eight fungal genomes belonging to Pezizomycetes with different life style (*T. magnatum, T. melanosporum, T. aestivum, T. boudieri* and *C. venosus* able to form ectomycorrhizae, and the saprotrophic *M. importuna, A. immerses, P. confluens*), in order to better characterize them and search for a correlation: 1) between HSP copy number and life style and 2) between HSP copy number and genome size. Considering that the study was conducted on a limited number of fungal species and with a relatively small sample size, no relationship was found between HSP copy number and lifestyle. However, we noticed that HSP9/12 family showed a number two to four times greater (eight copies) in *P. confluens* compared with the other fungi. *P. confluens* is a pyrophilous fungus (Seaver, 1909), and this expansion highlights a possible involvement of this family in the response of this fungus to postfire environments and in its adaptation to this particular ecological niche. It has been already observed that *P. confluens* contains nearly twice the number of protein-coding genes in comparison with *T. melanosporum*, suggesting a greater similarity to higher filamentous ascomycetes than to the black truffle genome (Traeger et al., 2013). Future studies could expand this analysis, by including additional Pezizomycetes, as well as a broader range of fungal species and lifestyle categories. Based on the phylogenetic tree constructed from HSP20 protein sequences, although the overall bootstrap values were generally low, *C. venosus* proteins are more closely related to those of the three *Tuber* species than to those of *Terfezia* or of saprotrophic fungi. This pattern can be explained by the fact that both *Choiromyces* and *Tuber* belong to the same family (i.e., Tuberaceae). It was not particularly surprising, as a phylogenetic reconstruction based on 2,093 concatenated conserved single-copy protein-coding genes from the eight Pezizomycetes had already shown a clustering between *Tuber* and *C. venosus,* and a pronounced level of gene co-linearity (microsynteny) across Tuberaceae genomes was observed (Murat et al., 2018).

The proteins associated to stress responses, in particular HSPs, were detected among the eight considered Pezizomycetes, as expected, since they are highly conserved and present in all organisms (Lindquist, 1986). Starting from the *T. melanosporum* gene models validated by bioinformatics tools in Martin et al. (2010) we found homologous sequences with two exceptions: the BAG family, for which no homologs are present in *T. aestivum*, and the HSP90 co-chaperone Sti1, for which no homologs are found in *M. conica* and *T. boudieri.* Dedicated experimental analyses would certainly be required to exclude potential assembly errors or the possibility that a homologous sequence is

present but diverges substantially from the predicted gene model. While Zhao et al. (2023) suggested that the abundance of HSP genes is associated with genome size in *L. edodes*, Turan (2023) reported that in *Aedes aegypti* the distribution of HSP genes is related to not only genome size but also to evolutionary and ecological factors. Although no correlation was found between copy number and Pezizomycetes genome size, our analysis highlighted gene duplication within HSP families. It has been widely hypothesized that gene duplication plays a significant role in the evolution and functional diversification of gene families (Chang and Duda, 2012), thus suggesting that the duplication and subsequent divergence of HSP genes may contribute to the ability of organisms to cope with diverse stress conditions (Powell et al., 2008; Wu et al., 2016; Liu et al., 2022; Cruz-Laufer et al., 2025). The wide variation in HSP composition and features may reflect environmental adaptation during the evolution of species, as observed in other organisms (Tercero and Place, 2020; Liu et al., 2022), playing a role in stress tolerance and environmental adaptation. Conversely, no duplication was detected in the HSP90 family, as previously observed in *Trematomus bernacchii* Boulenger, consistent with the specialized nature of this protein family. It has been hypothesized that an increase in gene copy number in HSP90 could have negative fitness consequences (Tercero and Place, 2020). Conversely, in other fungi, different from those here analysed, duplication events were observed inside HSP90 family (Pantzartzi et al., 2013).

In conclusion, producing a list of gene homologs in Pezizomycetes coding for HSP or other chaperones benefits researchers interested in studying genes that are typically expressed in different stress conditions or to validate RNA-seq experiments. In fact, the list of the stress gene sequences for the eight fungi here considered can be directly used to design primers for example for quantitative real time PCR (qRT-PCR). In addition, these sequences can be used as starting point to find homologs in other fungi and therefore the list can be considered a tool for other comparative genomics research. This was for example the approach used by Zampieri et al. (2014), who evaluated the gene expression changes during *T. magnatum* fruiting body storage starting from *T. melanosporum* genes.

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Data Availability Statement

The data supporting the results (supplementary Tables S1 and S2) is archived in Zenodo https://doi.org/10.5281/zenodo.19205839

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