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From phylogeny to biotechnology:
integrative genomic studies on *Pleurotus eryngii* and other fungal taxa

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Abstract

Fungal biodiversity represents a key component of terrestrial ecosystems and a promising source of biotechnological innovation. Yet, the genomic forces shaping diversity, adaptation, and functional potential within and among fungal species remain only partially understood. This doctoral thesis combines multigene phylogenetics, population genomics, and functional assays to investigate fungal variability across three taxa: *Peziza* spp., *Tuber borchii* Vittad., and *Pleurotus eryngii* (DC.) Quél. In the first part, we explored fine-scale genetic variation in a nivicolous *Peziza* species inhabiting a spatially restricted and environmentally extreme Alpine habitat. Using multigene analyses and sequence-based diversity metrics, we reconstructed intraspecific relationships and assessed whether micro-geographical isolation and selective pressures promote cryptic diversification. The second research axis focused on the Mediterranean truffle *T. borchii*, a species of high ecological and commercial relevance. Whole-genome resequencing of Sardinian and continental isolates enabled single-nucleotide polymorphism (SNP) discovery, principal component analysis (PCA), and F_{ST} -based genomic scans. Results revealed very low overall divergence (mean $F_{ST} \approx 0.008$), supporting This finding may therefore reflect historical connectivity. The final part of the thesis concentrated on the edible and medicinal mushroom *P. eryngii*. We generated a genome-wide characterization of commercial strains, integrating SNP calling, Gene Ontology analysis, and the bioaccumulation assays of mycelia and fruiting bodies under heavy-metal exposure. Ethanol extracts of *P. eryngii* were also tested on hepatocarcinoma cell lines (Huh7 and HepG2), revealing antioxidant capacity and potential cytoprotective effects, supporting emerging nutraceutical applications. Taken together, this work provides an integrated view of fungal diversity, spanning evolutionary scales, biological and ecological contexts, and applied biotechnology highlighting how genomics can bridge knowledge gaps in fungal research and can improve sustainable exploitation of fungal resources.

Chapter 1

Introduction

Thesis aim and objectives

Mushrooms are becoming an increasingly important resource for human health and nutrition that goes beyond their crucial role in ecosystem functioning. The development of knowledge on genetics and ecology of mushroom species as well as their biological responses to environmental changes is fundamental for valorization, conservation and biotechnological exploitation of these fungi. The overall aim of this PhD thesis was to explore fungal diversity, genomic variation, and functional traits across different ecological and biological systems, using three complementary models: a nivicolous ascomycete (*Peziza spp.*), an ectomycorrhizal truffle (*Tuber borchii*), and an economically important edible mushroom cultivated worldwide at industrial scale (*Pleurotus eryngii*). These three systems were selected to represent distinct ecological strategies and levels of biological organization, allowing the investigation of fungal diversity from multiple perspectives: (i) biodiversity and environmental adaptation in extreme alpine habitats, (ii) population genomics and spatial genetic structure in ectomycorrhizal fungi, and (iii) comparative strain genomics and biotechnological potential in cultivated edible mushrooms, including their functional properties and interaction with heavy metals. These research axes were addressed through an integrative experimental framework combining multi-gene phylogenetic analyses, comparative genomics, in vitro cellular assays, and analytical quantification of heavy metal uptake and bioaccumulation. This first chapter of the thesis provides an overview of the relevance of edible mushrooms in nutrition and health, bioremediation and heavy metal uptake and describes the molecular approaches applied so far for their genetic characterization, with particular attention paid to *Pleurotus* genus and *P. eryngii*. Chapters two to four investigate the genetic diversity of three mushroom species across different environmental and spatial contexts, integrating different genetic approaches (multi-gene analysis and comparative genomics) with ecologic data to address questions such as biological variability, adaptive traits, and biotechnological applications. Cultivated strains employed in commercial production systems (chapter 4) are investigated within a broader genomic framework that includes a wild-type reference enabling the exploration of evolutionary relationships, and signatures of divergence linked to domestication and breeding activities. In chapter 5, antioxidant, anti-inflammatory and anti-proliferative potential of a *P. eryngii* commercial strain are evaluated in in vitro experimental systems through a comparative analysis of mycelial and fruiting body ethanolic extracts. Finally, the last two chapters address the capacity of *P. eryngii* to interact with heavy metals both in vivo and in vitro with particular emphasis

on cadmium uptake and bioaccumulation. By integrating experimental analyses conducted on fruiting bodies, cultivation substrates, and mycelia, in these chapters we describe patterns of metal biosorption and accumulation and discuss implications about food safety, and fungal physiology.

Mushrooms and nutrition

Food systems today are at the center of an increasingly complex crisis, exacerbated by rapid climate change, rising hunger and malnutrition, and a growing social divide (Fanzo et al., 2022). This situation is further worsened by the unsustainability of many current production models, which cause intense exploitation of natural resources and significant environmental impacts. To effectively address this emergency, a profound and systemic transformation of the food sector will be necessary. The global food system, through its entire production chain, significantly contributes to greenhouse gas emissions. The resulting environmental effects, such as global warming, ozone layer depletion, soil and water acidification, desertification, deforestation, soil degradation, and biodiversity loss, demonstrate how the current pressure exerted by human activities is surpassing the planet's regenerative capacity. This scenario highlights the urgency to steer production and consumption models towards greater sustainability (Holden et al., 2018). From this perspective, the United Nations Food and Agriculture Organization (FAO) defines sustainable diets as “diets with low environmental impact that contribute to food security and nutrition and to a healthy life for present and future generations.” Compared to diets rich in animal-source products, plant-based diets are more sustainable as they require less use of natural resources and produce a lower environmental impact (Sabate and Soret, 2014). Among foods that best meet these criteria, mushrooms represent a promising choice. They not only present a favorable ecological and economic profile but also are a high-quality nutritional food, thus constituting a valid ally in promoting more sustainable eating habits (Das et al., 2021). The use of mushrooms for food and therapeutic purposes was already known to ancient Greek, Egyptian, Roman, and Chinese civilizations (Raghavendra et al., 2017). Global mushroom production in 2018-2019 was estimated at 43 million tons with *Lentinula edodes* (Berk.) Pegler (Shiitake) contributing 26%, *Auricularia* spp. 21%, *Pleurotus ostreatus* (Jacq.) P. Kumm. 16%, *Agaricus bisporus* (J.E. Lange) Imbach 11% (Singh et al., 2020). Based on their use, mushrooms can be divided into three main categories: edible (54%), medicinal (38%), and wild (8%) (Royse et al., 2017).

Nutritional and therapeutic value of mushrooms

Mushrooms represent not only a valuable dietary component but also a promising class of functional foods, thanks to their combined nutritional, physiological, and therapeutic properties (Teniou et al., 2022). While conventional foods primarily supply essential nutrients, functional foods provide

additional bioactive components that can support or enhance human health and well-being (Teniou et al., 2022; Espinosa-Páez et al., 2021). Functional foods may occur naturally or result from the modification, enrichment, or removal of specific components that can improve the bioavailability of beneficial molecules or combine multiple such beneficial effects within a single product (Gupta & Pragya, 2021; Silveira et al., 2003). Functional foods and mushroom supplements are gaining popularity for their ability to prevent various nutritional disorders and are considered part of a new generation of foods that promote health and well-being, both as nutraceuticals and pharmaceuticals (Bell et al., 2022). Biomolecules contained in mushrooms offer a wide range of health benefits, including immunomodulatory, antiviral, antibacterial, antifungal, antioxidant, anti-inflammatory, anti-tumor, anti-diabetic, and anti-arthritic functions (Singh et al., 2022). From an energy standpoint, 100 g of fresh mushrooms provide about 22 calories, a value significantly lower than other protein-rich foods such as veal (172 kcal), chicken (239 kcal), and lentils (116 kcal) (Pashaei et al., 2024). Proteins are essential for humans, accounting for about 17% of total caloric requirements. In edible mushrooms, protein content ranges from 6.6 to 36.9 g per 100 g dry weight, with an average value of about 23.8 g/100 g, confirming their role as a valid protein source in the diet (Zhou et al., 2020). On and Bassey (2021) observed that mushrooms contain all nine essential amino acids, which the human body cannot synthesize and must obtain through diet. Mushrooms are also a unique natural, vegan source of vitamin D, generated from ergosterol when exposed to UV light. This distinguishes them from many other plant protein sources, which often lack one or more essential amino acids. Furthermore, mushrooms are rich in branched-chain amino acids, typically found in higher quantities in animal proteins. Essential amino acids, such as arginine, glutamine, and glutamic acid, as well as vitamins and minerals, are common in *Pleurotus* species (Da Silva et al., 2012). Among the polysaccharides, both homopolysaccharides (such as glucans, chitin, and glycogen) and heteropolysaccharides like xylomannan, α -(1→4)-D-glucopyranosyl, and β -(1→6)-D-galactopyranosyl have been found (Villares et al., 2012). The main indigestible polysaccharides are glucans and chitin; the latter, together with its derivative chitosan, has antimicrobial properties and promotes wound healing (Buliyaminu et al., 2023). These compounds are classified as dietary fibers and are found both in the fruiting bodies and in the fungal thallus during the vegetative and reproductive stages. They have a crucial role in the formation and maintenance of cell wall integrity (Cerletti et al., 2021). β -glucans have attracted particular interest for their immunomodulatory properties, as they stimulate macrophages and leukocyte activity (Brown and Gordon, 2001). Rop et al. (2009) highlighted that water-soluble β -glucans exhibit a more pronounced immunostimulatory activity compared to insoluble forms. These characteristics make these compounds particularly interesting as adjuvants in chemotherapy. The dual role of mushrooms as both a source of nutrients

and functional food, makes them a promising component of more sustainable and health-oriented food systems. Species in the genera *Ganoderma*, *Pleurotus*, *Boletus*, *Inonotus*, *Grifola*, and *Armillaria* have long been used for their medicinal activities, such as antimicrobial, antiviral, antidiabetic, anti-inflammatory, hypocholesterolemic, and antitumor activities (Liu et al., 2020). Mushrooms have been used as prebiotics and dietary supplements to improve the overall health of consumers (Carlson et al., 2018). A notable example is *L. edodes* [syn. *Lentinus edodes* (Berk.) Singer], which has multiple health benefits, including antimicrobial, antitumor, antiviral, anti-inflammatory, and antioxidant effects. In addition, Shiitake is rich in protein, fiber, B vitamins, vitamin D, antioxidants, potassium, and selenium, making it a food with extraordinary nutritional properties (Ahmad et al., 2023). Combined studies on Shiitake and Astragalus have explored their synergy in promoting health and fighting cancer, through innovative production processes that integrate metabolites from both species (Balakrishnan et al., 2021). These insights reaffirm the relevance of mushrooms as biofunctional organisms at the crossroads between food and medicine. Their remarkable capacity to provide both nutritional value and therapeutic potential makes them increasingly significant in the development of innovative strategies for human health and sustainable nutrition.

Fungi in bioremediation: bioaccumulation of heavy metals

Heavy metals, defined as elements with high density and potential toxicity, are persistent in the environment and represent a serious threat to both ecosystems and human health (Duffus, 2002; Tchounwou et al., 2012). Their accumulation in soil and water systems is of particular concern due to their non-degradable nature and the risk of biomagnification through the food chain (Ali et al., 2019). Mushrooms, especially saprotrophic species, are known to absorb nutrients and other molecules from the growth substrate, including heavy metals, making them both valuable bioindicators of environmental pollution and potential sources of toxicity when consumed (Kalac & Svoboda, 1999; Riederer et al., 2014; Chen et al., 2022). Recent studies have highlighted significant variations in metal accumulation depending on mushroom species, substrate composition, environmental pH, and developmental stage (Dong et al., 2024). This variability needs careful monitoring of edible mushrooms, particularly those cultivated for food or nutraceutical purposes. Their bioaccumulation capacity can represent a potential risk to human health when fruiting bodies grown on contaminated substrates are consumed (Das et al., 2023). At the same time, the ability of mushrooms to accumulate and tolerate contaminants opens valuable opportunities for bioremediation strategies. It has been widely demonstrated that microorganisms such as fungi and bacteria represent a strategic resource for environmental decontamination. For example, Liu et al. (2015) studied the

effects of 2,4,5-trichlorophenol (TCP) and heavy metals (Cu and Cd) on the growth of *Clitocybe maxima* (P. Gaertn., B. Mey. & Scherb.) P. Kumm., revealing the species' resilience and active role in soil purification. Among the most promising organisms for phytoremediation processes are fungi that establish mycorrhizal associations (Gadd, 2010). Their effectiveness depends on strain/species-specific tolerance, mycorrhizal status, and soil conditions (Meharg, 2003). Tullio et al. (2003) and Krupa & Kozdroj (2004) demonstrated that ectomycorrhizal fungi can immobilize toxic metals such as Cd and Pb, forming biological barriers that reduce translocation into plant tissues. Additional immobilization occurs through biomineralization, which contributes to long-term soil remediation. Lastly, a specialized bioremediation approach, exploits the vast repertoire of fungal enzymes, both intracellular and extracellular, to transform or degrade pollutants directly on polluted site (Thakur, 2014). All these properties make fungi promising agents for monitoring and actively restoring contaminated environments.

Molecular and genomic approaches in fungal characterization

Fungi are essential drivers of terrestrial and aquatic ecosystems, mediating nutrient cycling, organic matter decomposition, and symbiotic relationships with plants and microorganisms. Their extraordinary metabolic plasticity and enzymatic repertoire have also made them central to biotechnology and pharmaceutical innovation, particularly for the discovery of enzymes, secondary metabolites, and bioactive compounds (Hyde et al., 2019; Meyer et al., 2020). Historically, fungal taxonomy relied on the morphological and anatomical traits of reproductive structures, including the shape, texture, size, and color of fruiting bodies, as well as microscopic features of plectenchymas, hymenia, spores, and cystidia. However, morphology alone often proved insufficient due to environmental plasticity and the prevalence of cryptic or convergent lineages. These limitations prompted the development of molecular tools capable of revealing evolutionary relationships beyond visible traits. Early molecular mycology introduced biochemical and DNA-based markers such as isozyme polymorphisms, RAPD, and microsatellite loci (Amicucci et al., 1997; Bertault et al., 1998; Rossi et al., 2000). These approaches paved the way for standardized genetic barcoding, culminating in the adoption of the internal transcribed spacer (ITS) region of ribosomal DNA as the universal fungal barcode (Henrion et al., 1994; Schoch et al., 2012). Subsequently, multi-locus phylogenetic frameworks incorporating nuclear and protein-coding genes, including LSU, β -tubulin, elongation factor 1- α , and RNA polymerase II subunits (RPB1, RPB2), provided a more robust view of fungal relationships and evolution (Martin et al., 2010; Murat et al., 2018a,b). These methods helped uncover cryptic diversity and refine species boundaries, offering foundational insights into fungal evolution and biogeography (Belfiori et al., 2013; Kinoshita et al., 2018). Molecular methods have also

revolutionized the identification and characterization of fungi in food samples, offering unparalleled specificity, sensitivity, and accuracy (Borman et al., 2008). PCR-based techniques that target conserved DNA regions such as the ITS enable rapid amplification and detection of fungal DNA even in complex food matrices, while quantitative PCR (qPCR) allows the quantification of specific fungal species for risk assessment and monitoring (Ihrmark et al., 2012; Postollec et al., 2011). Accurate species and strain characterization is now essential for preventing food fraud, ensuring traceability, and promoting consumer awareness. At the same time, exploring fungal diversity continues to yield new enzymes, secondary metabolites, and bioactive compounds with nutritional, medical, industrial, and bioremediation applications (Meyer, 2020).

The genomic era revolutionized studies on taxonomic and functional diversity in fungi. Advances in high-throughput sequencing enabled the large-scale generation of fungal genomes, greatly expanding available phylogenetic and functional datasets (Grigoriev et al., 2014). Beyond gene discovery, these technologies opened the door to multi-omics integration, linking genomics, transcriptomics, proteomics, and metabolomics to dissect metabolic regulation, gene expression, and enzymatic pathways (Cao et al., 2023; De Rose et al., 2024; Xie et al., 2025). Whole-genome sequencing has also facilitated the identification of simple sequence repeats (SSR) and single nucleotide polymorphisms (SNPs), now widely used as high-resolution markers to assess population structure, genetic diversity, and adaptation (Murat et al., 2011, 2013; Payen et al., 2014). Comparative genomics further enables the study of ecological specialization, mating systems, and metabolic diversification across symbiotic and saprotrophic fungi (Stukenbrock, 2013; Benevenuto et al., 2018). Progress in sequencing technologies has been central to this transformation. Roche 454 pyrosequencing was the first NGS platform applied to genome sequencing and metagenomic studies (Buée et al., 2009; Öpik et al., 2009). Soon after, Illumina sequencing, based on the sequencing-by-synthesis (SBS) principle developed by Balasubramanian and Klennerman and commercialized in 2006, became dominant, providing short but highly accurate reads through cyclic reversible termination chemistry (Bentley et al., 2008; Shendure & Ji, 2008; Balasubramanian & Klennerman, 2009). Third-generation sequencing extended this precision to long reads: Pacific Biosciences' SMRT technology produces long and unbiased reads by observing single DNA polymerases in real time (Levene et al., 2003; Eid et al., 2009; Rhoads & Au, 2015), while Oxford Nanopore Technologies introduced portable platforms capable of reading ultra-long fragments and resolving repetitive regions (Deamer & Akeson, 2000; Deamer et al., 2016). Combined with improved genome assembly and annotation pipelines (Stanke et al., 2008; Emms & Kelly, 2019), these technologies have redefined the accuracy, scale, and accessibility of fungal genomics. Large-scale initiatives such as those of the U.S. Department of Energy Joint Genome Institute (JGI) have played a central role in the expansion of fungal genomic

resources. In particular, the 1000 Fungal Genomes Project represents a long-term effort aimed at sequencing fungal genomes across the tree of life. The genomic data generated within these initiatives are made accessible through dedicated platforms such as the MycoCosm portal, which provides a comprehensive and integrated framework for comparative genomic analyses. These efforts have produced hundreds of annotated fungal genomes, enabling integrative studies that combine phylogenomics, ecology, and biotechnology (Zampieri et al., 2023). These resources have revealed the genetic underpinnings of lignocellulose degradation, metal tolerance, and the biosynthesis of bioactive molecules, underscoring the ecological and industrial relevance of fungal metabolism (Priest et al., 2020; Meyer et al., 2020; Cao et al., 2023). Genomic and transcriptomic data also increasingly support conservation biology and environmental monitoring, given the role of fungi as decomposers, symbionts, and bioindicators of anthropogenic impact (Miyauchi et al., 2020; Lofgren & Stajich, 2021). In parallel, the field has expanded toward applied mycology and industrial biotechnology. Omics-based approaches provide deep insights into the physiology of edible fungi and guide breeding programs through the identification of genes and pathways associated with growth, yield, and quality (Cao et al., 2023). Integrated genomic, transcriptomic, and proteomic analyses are foundational for optimizing mushroom productivity and deciphering the molecular basis of metabolic and developmental regulation (Xie et al., 2025). Studies on high-yield-related genes are enabling precision breeding and gene-editing strategies to enhance industrial performance (Wang et al., 2024). Similarly, the integration of genomics with systems biology and metabolic engineering has accelerated the overproduction of bioactive metabolites in medicinal fungi, overcoming long-standing bottlenecks (Arshadi et al., 2023). Altogether, these advances have reshaped fungal biology into an integrative, data-driven discipline that bridges evolutionary genomics, ecology, and applied biotechnology.

The expanding availability of fungal genomes continues to open new avenues for engineering industrial strains and addressing production bottlenecks (Meyer, 2020). Moreover, the relatively small size of fungal genomes makes these organisms efficient models for genetic research and microbial bioengineering (Gluck-Thaler et al., 2020). Overall, the integration of molecular taxonomy, functional genomics, and industrial applications underscores the strategic importance of rigorously characterizing fungal diversity. Each strain represents a potential biological novelty, a resource to be protected and valorized with applications across food, medical, environmental, and biotechnological sectors (Pouris et al., 2024). As highlighted by Meyer et al. (2020), fungal biotechnology can support the transition toward a bio-based and circular economy by enabling the sustainable production of foods, enzymes, and materials while enhancing environmental safety. In parallel, biodiversity conservation requires accurate species identification, genetic monitoring, and the management of

threatened populations. Genomics is crucial in this context, allowing researchers to assess genetic diversity, adaptive potential, and to integrate such information into conservation planning (Theissinger et al., 2023). Newly developed genomic resources and standardized protocols are now transforming conservation practices, providing effective tools to protect species and ecosystems in an era of accelerating environmental change (Theissinger et al., 2023).

The Pleurotus genus: nutritional, medicinal, and biotechnological perspectives

Overview of the genus *Pleurotus*

The genus *Pleurotus*, first described by Paul Kummer in 1871, belongs to the family Pleurotaceae and the order Agaricales (Basidiomycota). It includes numerous species commonly known as "oyster mushrooms", which are widely distributed in the wild and many of them are cultivated on a large-scale around all over the world (Villani et al., 2025). *Pleurotus* spp. are primarily white-rot saprotrophic fungi, thriving on decomposing lignocellulosic substrates and playing a crucial ecological role in the degradation and recycling of recalcitrant organic substrates (Zervakis et al., 2001). Some species have been occasionally described as weak pathogens (Raman et al., 2020). According to Index Fungorum (accessed on November 2025), *Pleurotus* includes 753 taxa, although only about 25 species are widely accepted as valid (He et al., 2019). The genus is characterized by remarkable morphological variability of basidiomata, which is strongly influenced by environmental factors and contributes to the taxonomic uncertainties of the group (Velázquez-De Lucio et al., 2022). Typically, *Pleurotus* fruiting bodies are shell- or oyster-shaped, with cap ranging from pink to cream, light brown, white, and grey (Irshad et al., 2023). Cultivation of *Pleurotus* species is especially prominent in Asia, particularly in China, Japan, South Korea, Taiwan, Thailand, Vietnam, and India, with China being the world's largest producer. Chinese production accounts for approximately 74% of global production (Jarial et al., 2024), and the country experienced a nearly 200% increase in output between 1997 and 2010 (Royse & Sanchez, 2017). Other major producers include Malaysia, India, and Australia (Villani et al., 2025). Due to their high perishability, *Pleurotus* fruiting bodies are frequently processed into dried or value-added products to enhance preservation and commercial availability (Rathore et al., 2017).

Nutritional and health properties

Both cultivated and other lesser-known species have long been investigated for their nutraceutical and medicinal potential (Carrasco-González et al., 2017; Raman, 2020; Juárez-Hernández et al., 2023). *Pleurotus* fruiting bodies are increasingly recognized as functional foods, owing to their rich nutritional composition and wide array of bioactive compounds. Extracts from *Pleurotus* mushrooms

and mycelia are also used into food supplements and cosmetics (Golak-Siwulska et al., 2018). Their medicinal properties are primarily attributed to primary and secondary metabolites found not only in the basidiomata, but also in the mycelium and in the culture medium resulting from mycelial growth (Salmones, 2017; Ogidi et al., 2020; Sen et al., 2021; Illuri et al., 2022). These compounds exhibit multiple pharmacological effects, including antibacterial, antiviral, antifungal, antitumor, anti-inflammatory, antiallergic, and immunomodulatory activities (Beltrán-Delgado et al., 2020; Torres-Martínez et al., 2022). The bioactive profile of the genus encompasses polysaccharides, protein-polysaccharide complexes, fatty acids, steroids, terpenes, saponins, vitamins, phenolic compounds, alkaloids, peptides, and a wide range of enzymes such as lectins, cellulases, xylanases, laccases, and invertases as well as essential minerals (Beltrán-Delgado et al., 2020; Cuzcano-Ruiz et al., 2020; Ogidi et al., 2020). They provide essential amino acids such as lysine, leucine, arginine, glutamine, and glutamic acid, while maintaining a low lipid content, predominantly composed of unsaturated fatty acids (Feeney et al., 2014; Da Silva et al., 2012). Compositional analyses have highlighted notable variability among different species and strains (Raman, 2020). Carbohydrates represent 25 to 76% of the dry weight. Ahmed et al. (2009) found carbohydrate content of 43% and 40% in *Pleurotus florida* Singer and *P. sajor-caju* (Fr.) Singer, respectively (2009). *Pleurotus tuber-regium* (Fr.) Singer has been reported to have a particularly high carbohydrate content and a low lipid content (Ijeh et al., 2009). A study carried out by Effiong (2024) evaluated the monosaccharide and disaccharide profile of *P. ostreatus* revealing the presence of glucose (55.08 g/100 g), xylose (7.19 g/100 g), fructose (19.70 g/100 g), galactose (17.47 g/100 g), trehalose (7.37 g/100 g), maltose (29.21 g/100 g), sucrose (51.60 g/100 g) and low amounts of cellobiose (0.01 g/100 g), erythrose (0.48 g/100 g) and other unidentified sugars. Protein content varies widely depending on species and agroclimatic factors, ranging from 9.29 to 37.4 g/100 g dry weight (Raman, 2020). For example, Tolera and Abera (2017) found 28.85% protein in fresh samples of *P. ostreatus*. Fatty acids account for approximately 30–70% of total lipids, and their composition differs significantly between species. Bioactive compounds from several *Pleurotus* spp., such as steroids and fatty acids, have been found to be effective nutraceuticals (Illuri et al., 2022). Furthermore, as the other edible mushrooms, oyster mushrooms can provide up to 25% of the recommended dietary fibre that amounts to 14 g/1000 kcal (Anderson et al., 2009). Indeed, the presence of fibre, and in particular β -glucans, gives these mushrooms significant biological properties, including hepatoprotective, cholesterol-lowering, antidiabetic, antioxidant, antimicrobial, and immunomodulatory effects (Juárez-Hernández et al., 2023). Among *Pleurotus* spp. genus, those with the highest fiber content include *P. eryngii* (28.29%), *P. flabellatus* Sacc. (11.9%), *P. florida* (11.5%), *P. sajor-caju* (10.9%), *P. ostreatus* (15.91%), *P. djamor* var. *roseus* Corner (14.60%), and *P. tuber-regium* (10.86%).

Regular consumption of *Pleurotus* basidiomata may help lower blood sugar and cholesterol levels, thus decreasing the risk of cancer and other diseases, making them an attractive and novel food source (Raman, 2020). Table 1.1 shows that the highest lipid content (7.50%) was observed in *P. eryngii*, while the lowest (1.06%) was observed in *P. tuber-regium*. Intermediate values were found in *P. ostreatus* (4.73%) and *P. citrinopileatus* Singer (3.9%) (Mehmet and Sevda, 2010; Singh, 2011). This variability can be partly attributed to the different lignocellulosic matrices used as cultivation substrates (Raman, 2020). The genus is also rich in water- and fat-soluble vitamins, as well as essential minerals, supporting its inclusion in both dietary and nutraceutical contexts. Thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, nicotinamide, folic acid (B9), cobalamin, vitamins C, D, and E are just some of the major vitamins documented in the genus (Feeney et al., 2014; Proserpio et al., 2019; Raman, 2020). For example, *P. ostreatus* has thiamine and riboflavin levels of 2.23 and 8.97 mg/100 g, respectively (Bano et al., 1986), while *P. flabellatus* has high levels of niacin (73.3 mg/100 g) and ascorbic acid (144 mg/100 g) (Raman, 2020). However, studies conducted on *P. pulmonarius* (Fr.) Quél. grown on fruit tree bark indicate a lower concentration of B vitamins, suggesting a marked influence of the substrate on nutritional composition (Okwulehie et al., 2015).

Table 1.1. Approximate composition of *Pleurotus* species. Values are expressed as g/100 g of dried mushrooms (from Raman et al., 2020).

Species	Carbohydrates	Proteins	Lipids	Fiber	References
<i>Pleurotus eryngii</i>	39.85	11.95	7.50	28.29	Mehmet & Sevda 2010
<i>Pleurotus ostreatus</i>	50.90	32.00	3.10	6.20	Yehia 2012
	45.59	24.10	4.73	15.91	Raman 2020
<i>Pleurotus flabellatus</i>	57.40	21.60	1.80	11.90	Raman 2020
<i>Pleurotus florida</i>	42.83	20.56	2.31	11.50	Ahmed et al. 2009
<i>Pleurotus sajor-caju</i>	39.82	24.63	2.29	10.90	Alan et al. 2008
<i>Pleurotus pulmonarius</i>	34.00	20.30	2.62	9.00	Silva et al. 2002
<i>Pleurotus citrinopileatus</i>	42.50	30.00	3.90	20.78	Singh 2011
<i>Pleurotus djamor</i> var. <i>roseus</i>	44.75	35.50	1.72	14.60	Jegadeesh et al. 2018
<i>Pleurotus tuber-regium</i>	63.03	22.10	1.06	10.86	Ijeh et al. 2009

About the mineral content, *Pleurotus* spp. are distinguished by their high potassium and phosphorus content, compared to low sodium concentrations, thus contributing to a favourable electrolyte balance and the prevention of hypertension (Raman, 2020). They are also a significant source of iron and zinc: in particular, *P. eryngii* has iron concentrations ranging from 5.5 to 13.4 µg/g (Mehmet and Sevda, 2010). Potassium and sodium levels vary between species, as evidenced by: *P. djamor* var. *roseus* (2218.33 and 67.12 mg/kg), *P. sajor-caju* (2146 and 220 mg/kg), *P. ostreatus* (1950 and 270

mg/kg), and *P. flabellatus* (1537 and 686 mg/kg) (Alan et al., 2008; Yehia, 2012; Jegadeesh et al., 2018; Raman, 2020). Another important element is selenium, the bioavailability of which in *P. sajor-caju*, *P. ostreatus*, *P. flabellatus*, and *P. eryngii* varies between 0.011 and 0.512 mg/100 g (Table 1.2). Regular intake of adequate amounts of selenium has been associated with a potential protective effect against some types of cancer (Raman, 2020).

The broad spectrum of therapeutic activities exhibited by *Pleurotus* extracts highlights the need for the detailed characterization of their bioactive compounds, both for pharmacological and clinical applications. Moreover, understanding the possible synergistic interactions among these metabolites could strengthen their therapeutic potential, developing innovative bioproducts useful in medicine and pharmacy.

Table 1.2. Micronutrient content in selected *Pleurotus* species (mg/kg). Values refer to dry weight. Missing values are indicated with a dash (–). Adapted from Raman (2020).

Species	Se	Zn	Fe	Mg	K	Ca	Na	References
<i>Pleurotus eryngii</i>	4.50	33.5	620	14.8	15.3	0.51	100	Mehmet & Sevda 2010
<i>Pleurotus ostreatus</i>	0.11	38	15.8	900	1950	260	270	Patil et al. 2010
<i>Pleurotus flabellatus</i>	–	141	248	40	1537	120	686	Mshandete et al. 2008
<i>Pleurotus florida</i>	0.013	2.3	12.3	92	920	305	90	Ahmed et al. 2009
<i>Pleurotus sajor-caju</i>	0.025	20.9	33.4	20.2	2146	22.5	220	Alan et al. 2008
<i>Pleurotus citrinopileatus</i>	–	–	560	102	162.	80.0	90	Raman 2020
<i>Pleurotus pulmonarius</i>	–	–	75	2593	2709	38.0	–	Silva et al. 2002
<i>Pleurotus eous</i> (Berk.) Sacc.	–	1.9	2.3	7.6	121	3.7	60	Kortei et al. 2015
<i>Pleurotus djamor</i> var. <i>roseus</i>	–	1.21	17.3	167	2218	49.6	67.10	Jegadeesh et al. 2018
<i>Pleurotus tuber-regium</i>	0.0025	0.14	0.5	0.04	0.02	0.08	0.08	Ijeh et al. 2009

Biotechnological potential

Pleurotus species, beyond their economic value as edible mushrooms, are increasingly recognized for their biotechnological potential in bioremediation. Compared to bacterial consortia, white rot fungi, particularly those of the *Pleurotus* genus, demonstrate a unique capacity to degrade complex aromatic pollutants due to their broad-spectrum oxidative enzymes linked to their nature of ligninolytic fungi (Salmones, 2017; El-Ramady et al., 2022). Moreover, several studies have demonstrated that *Pleurotus* species exhibit a remarkable capacity to bioaccumulate heavy metals from the substrate, particularly cadmium and zinc, making them promising candidates for bioremediation strategies. Recent studies have reinforced the role of *Pleurotus* spp., particularly *P.*

ostreatus, as effective biosorbents for environmental contaminants. Murtic et al. (2024) reported high bioaccumulation factors for Zn and Cd, suggesting strong metal-binding affinity. However, variations in substrate composition, as evidenced by arsenic overaccumulation in sugarcane bagasse substrates (Saha et al., 2024) underline the need for rigorous monitoring of growth media. Atila et al. (2024) demonstrated that the use of sugar mill wastewater not only enhanced biological efficiency in *P. ostreatus* cultivation but maintained metal content within safe consumption limits. Notably, selenium supplementation has shown promise in mitigating Cd and Pb accumulation (De Oliveira et al., 2022), pointing to emerging strategies for enhancing food safety. *Pleurotus* spp. have been extensively studied not only for their capacity to bioaccumulate heavy metals but also for their enzymatic degradation of a wide range of organic pollutants, including synthetic dyes and xenobiotics. For example, *P. ostreatus* has been effectively employed in the decolorization of various industrial dyes, such as Congo red and Brilliant green, achieving removal efficiencies of up to 98% under optimized conditions (Chaurasia & Bharati, 2019). Similarly, *P. pulmonarius* has demonstrated the ability to degrade soils heavily contaminated with pentachlorophenol (C₆HCl₅O) and dioxins (C₄H₄O₂) via solid-state fermentation, highlighting its potential for the breakdown of persistent organic pollutants (Kaewlaoyoon et al., 2020). Another notable application relates to control of plant and animal parasitic nematodes. For instance, *Pleurotus* spp. have shown nematocidal activity attributed to specific compounds such as trans-2-decenedioic acid (Castañeda-Ramírez et al., 2020). Considering the wide range of biotechnological applications discussed, *Pleurotus* mushrooms could become a cornerstone of the circular bioeconomy, bridging environmental restoration, human health, and sustainable food production. As highlighted by Mastranzo-Pérez et al. (2025), the future of *Pleurotus* employment lies not only in its nutritional and pharmacological potential but also in its contribution to waste reduction, bioenergy generation, bioplastic production, and agro-industrial valorisation.

Pleurotus eryngii

Key features

Among the species of the *Pleurotus* genus, *P. eryngii* occupies a distinctive ecological niche due to its association with plants of the Apiaceae family, from which it derives both its name and its unique ecological behavior. Unlike other *Pleurotus* species that primarily colonize wood, *P. eryngii* acts as a weak root parasite or saprotroph, growing on the decaying roots and stems of plants in the genera *Eryngium*, *Ferula*, *Ferulago*, *Cachrys*, *Laserpitium*, *Diplotaenia*, and *Elaeoselinum* (Gyorfi, 2007). Fruiting typically occurs between autumn and late spring (Zervakis et al., 2001), particularly on the dead roots of *Eryngium campestre* L. and other Apiaceous plants in dry meadows and pastures (Györfi et al., 2007). Reports from Central Europe describe basidiomata appearing from October to

December, occasionally extending into early spring (Gyorfi et al., 2007). *Pleurotus eryngii* forms fleshy fruiting bodies with an eccentric stipe and a pileus from convex- to funnel-shaped as it matures. The cap surface ranges from whitish to pale brown, becoming slightly sticky when wet; the gills are decurrent, thin, and turn ochraceous with age, while the stipe is cylindrical, compact, and white, with a mild aroma and firm texture (Saitta, 2021). The first attempts at intensive cultivation were carried out in Hungary during the 1950s (Kószó, 1997), subsequently spreading across southern Europe and Asia. Today, *P. eryngii*, the king oyster mushroom, has become one of the most economically and agronomically significant *Pleurotus* species worldwide. Its combination of high-quality protein, delicate taste, and long shelf life has contributed to its status as both a gourmet and functional food (Li et al., 2024). *Pleurotus eryngii* alone accounts for about 5% of total global mushroom output (Pathak et al., 2009; Singh et al., 2020). Chinese production continues to rise, reaching about 2 million tons in 2021 (Zhou et al., 2023; China Edible Fungi Association, <https://www.dongfangqib.com/article/1693>). *Pleurotus eryngii* has also attracted research attention for its metabolic versatility and environmental adaptability. Comparative proteomic analyses revealed that this species modulates its extracellular enzymatic profile according to the lignocellulosic substrate used for cultivation such as sawdust, bagasse, or peanut shells by differentially expressing lignin-degrading enzymes like laccase, manganese peroxidase, and aryl-alcohol oxidase (Li et al., 2024). This enzymatic plasticity makes it suitable for biotechnological valorisation of agricultural residues and sustainable waste recycling. For example, the use of corncobs, a byproduct of corn processing, provides a renewable and cost-effective substrate, reducing environmental impact while supporting circular bioeconomy principles (Leong et al., 2022). In Italy, the local strain C-142-c, commercially known as “cardoncello”, has been described for its excellent productivity, nutritional composition, and adaptability to open-field cultivation (Venturella et al., 2015). Field trials yielded an average of 0.91 kg of mushrooms per 4 kg cultivation bag, with 70% of production achieved in the first flush. The fruiting bodies exhibit low caloric value (29.7 kcal/100 g), high water content (93.5%), and low lipid content (0.56 g/100 g), along with relevant concentrations of proteins, carbohydrates, and B-group vitamins, notably niacin (5.9 mg/100 g) and vitamin B₁₂ (1.7 µg/100 g).

Taxonomy

Pleurotus eryngii and sister species have been subjected to extensive revisions over time. Early analyses based on rDNA genes led to the description of multiple varieties, such as *P. eryngii* var. *nebrodensis* (Inzenga) Sacc. and *P. eryngii* var. *tuoliensis* C.J. Mou and, later, to the recognition of *P. nebrodensis* (Inzenga) Quél. as a separate species. The *P. eryngii* complex currently includes several cultivated varieties of economic relevance, notably *P. eryngii* var. *eryngii* (DC.) Quél. (Xingbaogu) and *P. eryngii* var. *ferulae* (Lanzi) Sacc. (Aweigu), both widely cultivated in Asia

(Zhang et al., 2018). The advent of molecular techniques such as DNA barcoding and genomic fingerprinting has refined the taxonomic resolution of the *P. eryngii* group, enabling precise species delimitation (Zhao et al., 2021). Phylogenetic analyses targeting loci such as *efl**a*, *rpb1*, and *rpb2* have confirmed *Pleurotus tuoliensis* (C.J. Mou) M.R. Zhao & Jin X. Zhang (the Chinese variety Bailinggu) as a distinct evolutionary lineage, supported by strong bootstrap and posterior probability values (He et al., 2016; Zhao et al., 2016). Six well-supported clades have been identified across the group *P. eryngii* var. *ferulae*, *P. eryngii* var. *eryngii*, *P. nebrodensis*, *P. tuoliensis*, *P. ostreatus*, and *P. pulmonarius*, with *P. tuoliensis* forming a well-supported monophyletic group. Integrative approaches combining phylogenetic, molecular, and chemical data, GC-content analysis, and secondary metabolite profiling, have been proposed to improve classification consistency (Lin et al., 2022).

Chapter 2

Diversity and ecology of nivicolous *Peziza* from the glacial basin of the Gran Sasso massif (Central Apennines, Italy)

This chapter is based on the manuscript by Martina De Mattheis et al., published in Fungal Ecology and scheduled for release in February 2026 (volume 79: 101481). The full text of the manuscript is included here. Only a subset of the original figures and tables is presented, while an additional photographic image of the sampling site and *Peziza* specimens has been added to complement the study.

Introduction

The family Pezizaceae (Pezizales, Pezizomycetes) harbours epigeous and hypogeous, mycorrhizal and saprobic fungi. These fungi form fleshy, often fragile ascomata, ranging in diameter from a few millimetres to several centimetres. The asci are operculate, opening by a lid allowing the forcible discharge of ascospores into the turbulent air currents. Asci of species in the Pezizaceae generally become blue in iodine solutions. In the genus *Peziza*, the bluing reaction is located at the tip of the ascus where a ring zone is generally observed staining deep blue. Building on previous studies of lineages within the family, Van Vooren (2020) proposed several genera, and recent work has added genera based on genetic data (Pfister and Healy, 2021). Previously, the name *Peziza* was applied to a

wide range of species both in the Pezizaceae and Pezizales. In the early 2000s, thorough molecular work showed divergence among the species and suggested a narrow circumscription of *Peziza* (Hansen et al., 2001, 2002, 2005). The distribution of the species of *Peziza sensu stricto* is wide, with a total of 20–30 species mainly described from the northern hemisphere but several have been found in Southern America, New Zealand and Australia (Pfister et al., 2022). *Peziza* in its narrow sense encompasses saprobic species (Pfister et al., 2024) that grow in a wide range of habitats, including forests, urban areas and coastal sandy environments (Donadini, 1981; Hansen et al., 2002; Vizzini et al., 2016), and colonize diverse substrates such as wood and plant debris, dung, burnt and waterlogged soils (Hansen et al., 2002; Pfister et al., 2022). In stark contrast, several *Peziza* species were observed at the snowmelt margins (nivicolous species), including *P. flos-nivium*, *P. heimii*, *P. ninguis*, *P. fortoulia*, *P. phaeotheca* (McKnight and Dublin, 1979; Donadini, 1980; Breitenbach and Kränzlin, 1984; Pfister, 1992; Fedorenko, 2020) and the cosmopolitan species *P. nivalis* which was firstly described from the French Alps (Heim and Rémy, 1932). The latter was subsequently noted in other regions of Europe, as well as in North America, Australia and New Zealand (Voitk et al., 2021; Pfister et al., 2024). Recent surveys have found nivicolous species of *Peziza* at Campo Pericoli, a glacial basin located on the eastern side of the highest peak of the Apennines chain (Gran Sasso, Abruzzi, Italy). We hypothesized this site could represent a notable hot-spot for nivicolous *Peziza*, suitable for studying their diversity and ecology. In this study, we examined the diversity of *Peziza* species associated with melting snowbanks by using a multigene sequencing approach and we correlated the genetic diversity with spatial distributions of the specimens and four topographical variables obtained by remote sensing. Finally, we inferred the climate preferences of *P. nivalis* and *P. heimii* based on the distribution data available from literature.

Materials and Methods

Study area, sample collection and processing

A total of 100 *Peziza* ascomata were collected in May/June 2020 and May 2021 from Campo Pericoli. It is a typical snow valley around 2200-2300 m asl. It has been profoundly shaped by glaciations and influenced in the past by pastoral activities (Calandra 1999, De Simone et al. 2021). The soil is snow free for only a few months (June to September) and is characterized by cryoturbation which led to the formation of "sorted stripes structures" (Giraudi 2001). This area encompasses a large and unique region of biodiversity (De Simone et al. 2021). It has been designated a Special Protection Area (SPA) and a Site of Community Interest (SCI) and includes various habitats such as Alpine heaths, calcareous grasslands, and important orchid sites of the Natura 2000 habitats (Biondi et al. 1999, Conti and Bartolucci 2016). The climate of Campo Pericoli is mediterranean montane with an average

precipitation of 1170 mm per year and a mean annual temperature of 3.7 °C (max 17.4 °C and min - 8.9 °C) (Petriccione and Bricca 2019). Daily precipitation, air moisture, temperature (mean, max and min) and wind (direction and speed) spanning a period of 28 days (from May 10 to June 6, 2020, and 2021) were obtained from the meteorological station at Campo Imperatore (2152 m asl) located about 1 km from the sampling area. (Fig. 2.1).

Sixty-two fruiting points (20 in 2020 and 42 in 2021) were selected for sampling along the melting snowbanks. Each fruiting point covered an area no larger than half a square meter. One or two ascomata were collected from each fruiting point. GPS coordinates of each fruiting point were registered by a Garmin GPSMAP 66sr. Ascomata were stored at 4 °C during transport to the laboratory and processed within 24 h.

Each ascoma was cleaned of organic residues, cut into small pieces and placed in a 2 mL tube containing a sterile Tween 20 water solution (1:1000, v:v). The tubes were vortexed for 30 s and spun for 1 min at maximum speed. Samples were then rinsed three times in sterile water following the same procedure. After washing, the samples were freeze-dried at -50 °C in a BenchTop 2K (Virtis) for three days and stored at 4 °C pending molecular analyses. Three additional specimen vouchers of the *Peziza nivalis* group (IBF 19480079, IBF 19650025, IBF 19790026) from the Innsbruck herbarium (IB) were included in the analysis, as they were deposited by M.M. Moser, who proposed the new combination transferring *Galactinia nivalis* to *Peziza* (Moser, 1963).

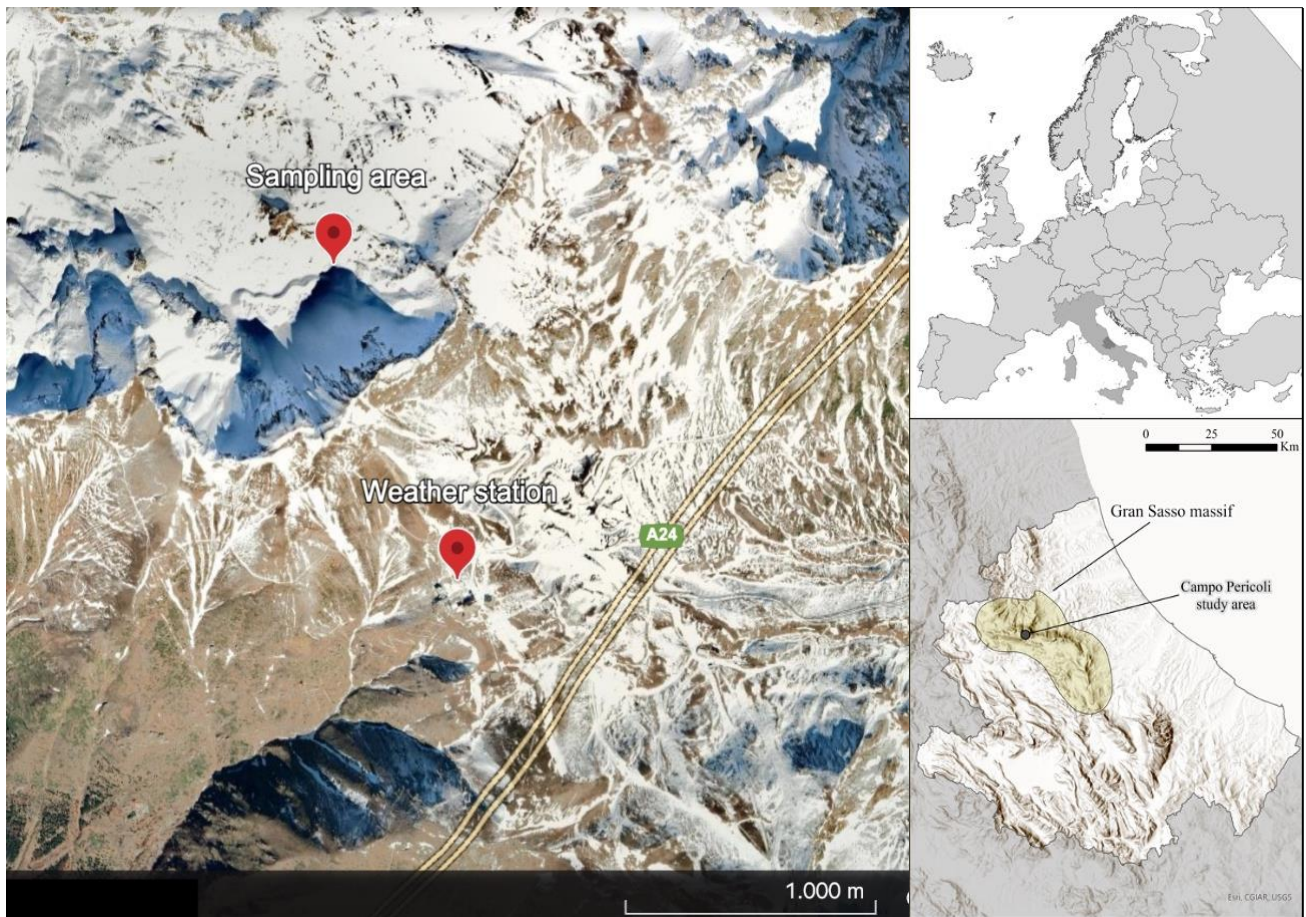


Figure 2.1. Location of the Campo Pericoli study area (Gran Sasso Massif, Central Apennines, Italy). The main panel shows the position of the sampling area and the meteorological station. Insets indicate the geographic context within Italy and Europe.

Morphological analysis

Portions of ascomata collected in Campo Pericoli were air-dried at room temperature before the washing procedure. Among these, 25 were selected based on genotyping results and used in morphological analyses along with the three *P. nivalis* specimens from the IB. Spores were analysed microscopically using free-hand sections mounted in a solution of 5% KOH. Examination and measurements were carried out on a Zeiss Axiomat compound microscope (Germany). Due to the low standard deviation previously detected, spore dimensions were calculated measuring 10 mature spores for each analysed ascoma at 1000× magnification. Statistical differences in spore dimensions, length-to-width ratio of the spores were analysed using one-way ANOVA, and pairwise differences were assessed using Tukey post hoc test ($p < 0.05$).

Molecular analyses

DNA extraction was performed on about 20 mg of lyophilized sample (excipulum and hymenium) of all collected ascomata, using the NucleoSpin II kit (Macherey-Nagel), according to the modified protocol for fungal material proposed by the manufacturer. Isolated DNA was eluted in 100 µL of

ultrapure water and quantified by a Qubit fluorometer (Invitrogen) with dsDNA high-sensitivity assay kit. All PCRs were performed in 30 µL volume in a SimpliAmp thermal cycler (ThermoFischer). Four target genes were amplified with the following primer pairs: ITS1F-ITS4 (White et al., 1990; Gardes and Bruns, 1993) for the ITS region, Bt2a-Bt2b (White et al. 1990) for the β -tubulin (β -tub), Rpb26f-Rpb27cR (Liu et al. 1999) for the subunit II of RNA polymerase (RPB2), and LR0R-LR7 (Vilgalys and Hester, 1990) for the LSU region. Each PCR reaction was run with 5-10 ng of DNA, 1x Takara Ex Taq DNA polymerase, 6 µg of BSA and 350 nM of each primer. PCR products were sequenced in both directions using the following primers: ITS5 (White et al. 1990) and ITSi (Redecker et al., 2003) for the ITS region; Bt2a-Bt2b for the β -tub; LR0R and LR5 (Hopple and Vilgalys, 1994) for the LSU. The RPB2 gene was amplified and sequenced for a few samples using the Rpb26f and Rpb27cR primers, but the amplification of nonspecific amplicons led us to design new internal primers (RPB2_pez_for 5'-CGAAACACCAGAAGGTCAAG-3', RPB2_pez_rev 5'-AGTGAAGCCAAAGACAGACC-3'), also used for sequencing reactions. The thermal cycling conditions adopted for this new primer pair were: 95 °C for 6 min, 35 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 7 min. All PCR products were purified and sequenced at Microsynth (Balgach, Switzerland). Sequences were edited by Chromas Pro 1.34 (Technelysium) and aligned by ClustalW (Thompson 1994) implemented in MEGA11 (Tamura et al. 2021).

Genetic analyses

Clustering and phylogenetic analyses were conducted using the neighbor-joining (NJ) method, Maximum Likelihood (ML), and Bayesian (MB) estimations. First, single gene and concatenated datasets were prepared for the ITS, LSU, RPB2 and β -tub sequences generated in this work to identify the main groups. NJ trees were generated with the Kimura's 2-parameter model implemented in MEGA11 software (Tamura et al. 2021). Mean genetic distances among and within clades for all molecular markers were calculated in MEGA11 by applying p-distance and Kimura's 2-parameter models. Next, a concatenated dataset was prepared with ITS, LSU and/or RPB2 sequences to infer the phylogenetic relationships among our samples and the species belonging to *Peziza sensu stricto*. Our sequences were compared with those obtained by Hansen et al. (2001, 2002, 2005), Medardi et al. (2012), Pfister et al. (2016, 2024), Van Vooren (2020). *Peziza vesiculosa* Bolton (ITS: AF491626; LSU: AY500552; RPB2: AY500489) was selected as outgroup. ML and MB analyses were conducted by RAxML GUI (Stamatakis 2014) and MrBayes (Ronquist et al. 2012), respectively. ML analyses were carried out by applying the GTR model, 100 runs and 1000 rapid bootstrap replicates. For each gene (ITS1, ITS2, LSU, RPB2), the best model of nucleotide substitution was evaluated using jModelTest (Darriba et al. 2012) under the Akaike information criterion: TIM2+I for ITS1,

TPM2uf+G for ITS2, TrN+I+G for LSU and TPM2+G for RPB2. Then, MB analysis was carried out by two independent chains run for 5000000 generations each, sampling every 5000 generations and a majority-rule consensus tree was generated after the exclusion of the first 250 trees and posterior probabilities were computed for each node. Genetic divergences between and within clades were calculated by MEGA11 using both p-distance and Kimura's 2-parameters models.

Landscape profiling

The features of the sampling area were characterized through four topographical variables, namely the elevation, the slope, the aspect, and the Topographic Wetness Index (TWI, a numerical indicator used to quantify the spatial distribution of soil moisture and the potential for water accumulation in a landscape, often used to assess areas that are likely to be wet or prone to saturation). These were generated starting from a Digital Elevation Model (DEM) obtained through a dedicated UAV (Unmanned Aerial Vehicle, using a DJI Phantom 4 Pro) flight, to obtain a high spatial resolution of 40 cm/pixel. Once produced, the DEM through photogrammetry was processed in ArcGIS Pro 3.3.0 (ESRI 2024) using the 'Surface parameters' tool, obtaining the slope and aspect maps, with the former used in turn (together with other maps obtained by using the 'Flow Direction' and 'Flow Accumulation' tools) to generate the TWI data.

Statistical and geostatistical analysis

Once the topographic predictors were generated (as described above), those were checked for possible multicollinearity by measuring Pearson's correlation ('Band collection statistics' tool), and the corresponding values were extracted for each sampling point by reading the predictor values (elevation, aspect, slope, and TWI) precisely at the GPS coordinates of each fruiting point. This process was carried out for all collected specimens. After extracting the topographic data, the values were categorized based on the clades inferred for the *Peziza* specimens. A Kolmogorov-Smirnov test for normality ($p = 0.05$) was applied to the distributions of each topographic parameter within these groups. Given the non-normality of the distributions, a Kruskal-Wallis test ($p = 0.05$) was subsequently performed to compare the topographic parameters across the different clades.

To further analyze the spatial distribution of the gathered ascomata occurrences, with particular attention to the clade they belong to, we used a Kernel Density Estimation (KDE) in GIS environment. This technique is employed to estimate the density of features over a defined area (Bernabò et al. 2022), creating a smooth surface that shows the clustering of spatial features, in our case occurrence points. It works by placing a kernel function (usually, a Gaussian one) over each point and summing the contributions from all kernels at each location to generate a continuous density surface; outliers are thus lowered in their contribution to the overall trend, distinguishing the KDE from a simple point

density (ESRI, 2024). This approach enabled visualizing the spatial distribution of fruiting points based on different clades, highlighting areas of the territory with a higher clustering of each group. In this way, a visual representation of the relationship between the distribution of clades and the environmental characteristics of the territory was provided.

We further assessed the spatial patterns by measuring the raster correlation (Band Collection Statistics tool, $p = 0.05$) among the KDEs obtained for each clade inferred. Quantifying this parameter helps to support or reject the significance of a geographic overlap among the spatial arrangement (and, thus, spatial preferences) of the different clade/subclades found.

Finally, to better characterize the ecological profiles of the target species identified through phylogenetic analysis, we extracted mean annual temperature (Fick and Hijmans, 2017) and elevation values at a global scale from the known localities of 33 specimens identified as *P. nivalis* (17), *P. ninguis* (3), *P. fortoullii* (1) and *P. heimii* (12). The localities of each specimen were retrieved by the literature, the GBIF portal (<https://doi.org/10.15468/dl.c9vg58>; accessed on June 4, 2025), GenBank or herbarium metadata. Then, we calculated the correlation between these two variables for *P. nivalis* group and *P. heimii*, to assess how tightly their distributions are associated with elevation-dependent temperature gradients.

Results

Sampling and phylogenetic analyses

All 100 ascomata collected in 2020 and 2021 were found up to a maximum distance of 40 cm from the melting snowbanks regardless of the date or fruiting point. The fruiting points were characterized by frost-burned vegetation and wet soil due to snowmelt (Fig. 2.2).



Figure 2.2. Overview of Campo pericoli during a sampling survey and image of *Peziza* ascomata in different fruiting points.

No pezizalean ascomata were found in the surrounding grassland. One to five ascomata were found in each fruiting point. The mean distance among fruiting points was 156 m (min 3 m, max 384 m) in 2020 and 158 m (min 3 m, max 412 m) in 2021 within an area of 3.65 Ha in 2020 and 8.49 Ha in 2021 (total 9.23 Ha – intersect 2.91 Ha). The climate parameters registered by the meteorological station placed 200 m downstream (1 km from the sampling area) over the period of fruiting (roughly between the second week of May and the first week of June) were the followings: mean daily temperature of about 6 °C (max ~ 10 °C and min ~2.5 °C), relative humidity ~70% and a cumulative rainfall of 194 mm in 2020 and 28 mm in 2021 (Fig. 2.3a,b).

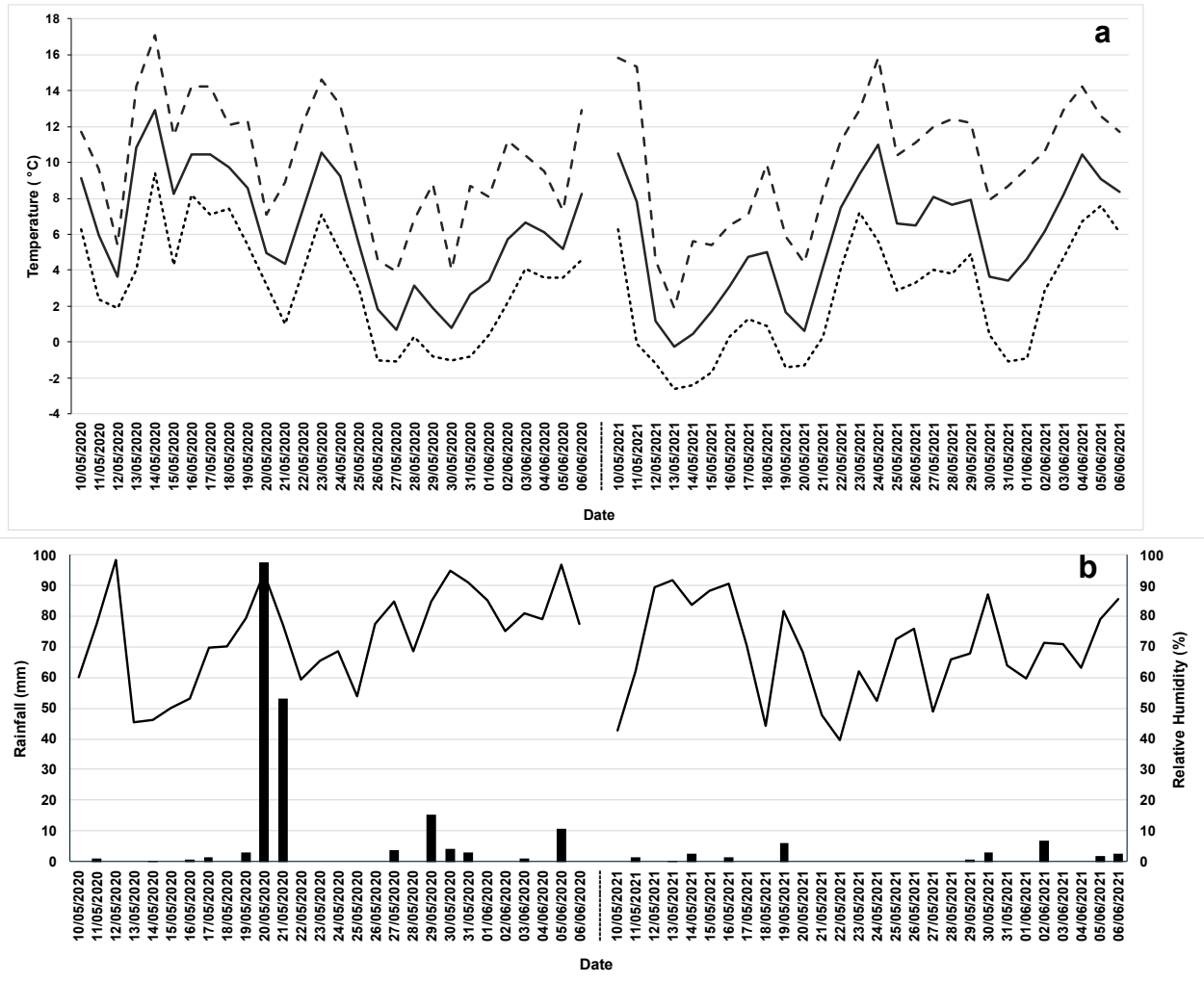


Figure 2.3. Temperature, relative air humidity and rainfall recorded during the fruiting period of *Peziza* spp. in Campo Pericoli from May 10 to June 6, 2020, and 2021. a) minimum (dotted line), maximum (dashed line), and mean (solid line) daily temperatures (°C); b) daily rainfall (mm) and relative humidity (%). Climate data were recorded by a station located at Campo Imperatore about 1 Km from and 200m below Campo Pericoli.

Ascomata occurred in elevations between 2251 and 2335 m asl (2287 m asl on average), in sites with a mainly western aspect and an average slope of 14°. Moreover, the TWI of fruiting sites ranged from 5.1 to 11.6 (9.3 on average). We generated and analyzed 103 new sequences for each of the four genetic markers included in this study (412 sequences in total). We did not find evidence of polymorphic sites in any Sanger chromatograms except one position in ITS sequences of the samples P12b and P13a. ITS, LSU, β -tub and RPB2 alignments were 567 bp, 905 bp, 397 bp and 716 bp, respectively. The parsimony informative sites were 25 for ITS (8 in ITS1 and 17 in ITS2), 4 for LSU, 18 for β -tub and 20 for RPB2.

Phylogenetic analysis conducted on the single and concatenated datasets grouped the 100 ascomata collected in Campo Pericoli in two main clades with 95 (clade I) and 5 (clade II) specimens (Fig. 2.4, 2.5).

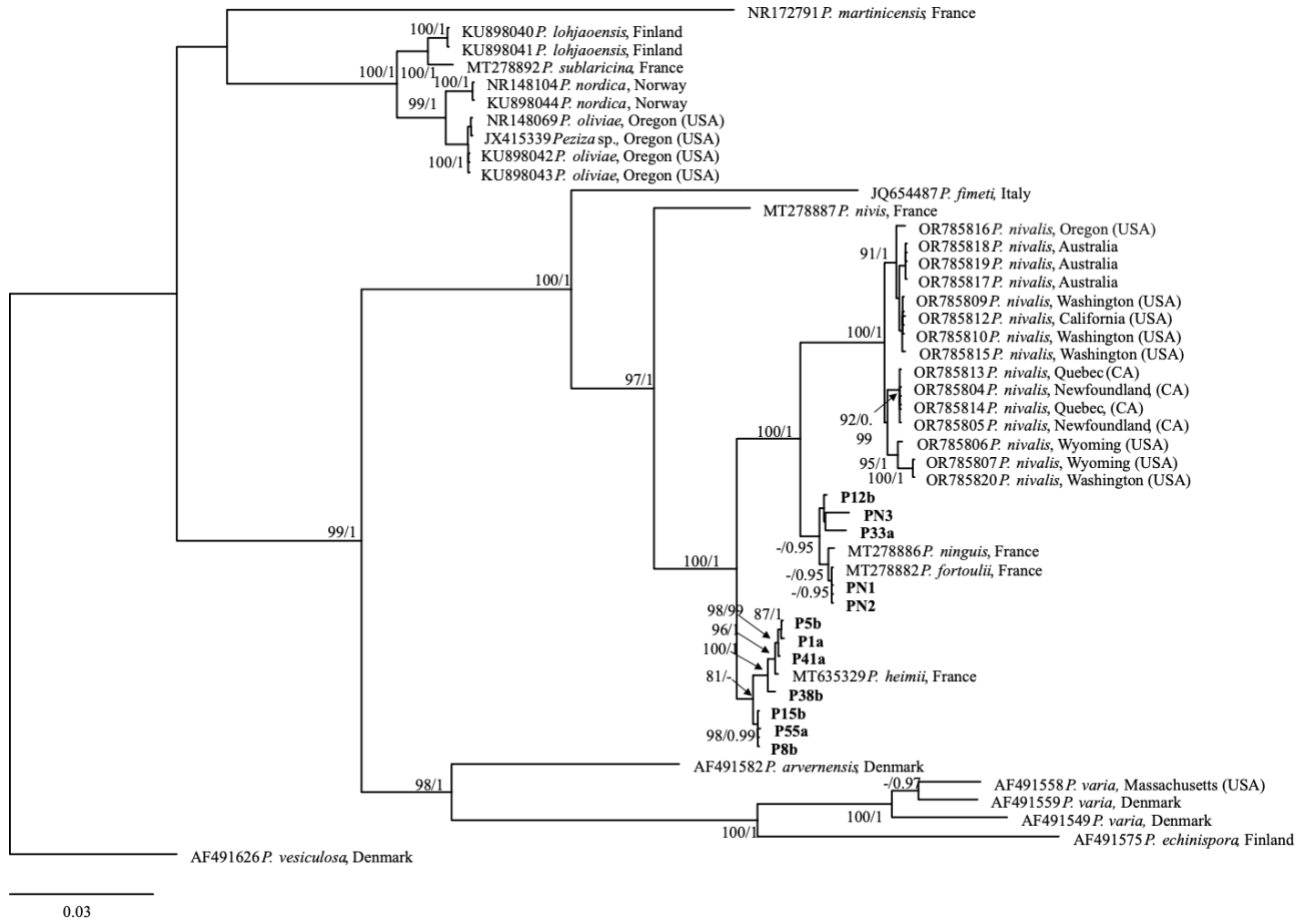


Figure 2.4. Phylogenetic tree inferred from Maximum Likelihood (ML) based on ITS1, ITS2, LSU, and RpbII regions. *Peziza vesiculosa* was used as the outgroup. Bootstrap support values > 70 and posterior probabilities > 0.95 are indicated at the nodes. The specimens from this study are in bold and those from GenBank are identified by ITS accession number, species name and country of origin.

The LSU gene showed the lowest clustering resolution whereas ITS, β -tub, RPB2 and the four concatenated genes supported the two clades by high bootstrap values. In turn, clade I was formed by two well supported subclades, containing 61 (subclade Ia) and 32 (subclade Ib) specimens, regardless of the dataset considered for analyses. Only two specimens from the same fruiting point, P38a and P38b, were clustered differently depending on the genetic marker (e.g., within clade II for LSU and clade I for ITS/RPB2 or diverged from both for β -tub) but never fell into clade II. The three *P. nivalis* specimens from Innsbruck herbarium grouped in clade II with samples P12b, P13a, P13b, P33a and P33b, regardless of genetic marker sequenced in this study. The lowest genetic divergence within and between clades I and II was obtained for LSU, regardless of the model of substitution used for computation. The other three markers (ITS, β -tub, RPB2) gave a genetic divergence always ≤ 0.005 within the two clades and greater than 0.018 (RPB2) or 0.032 (ITS and β -tub) between them.

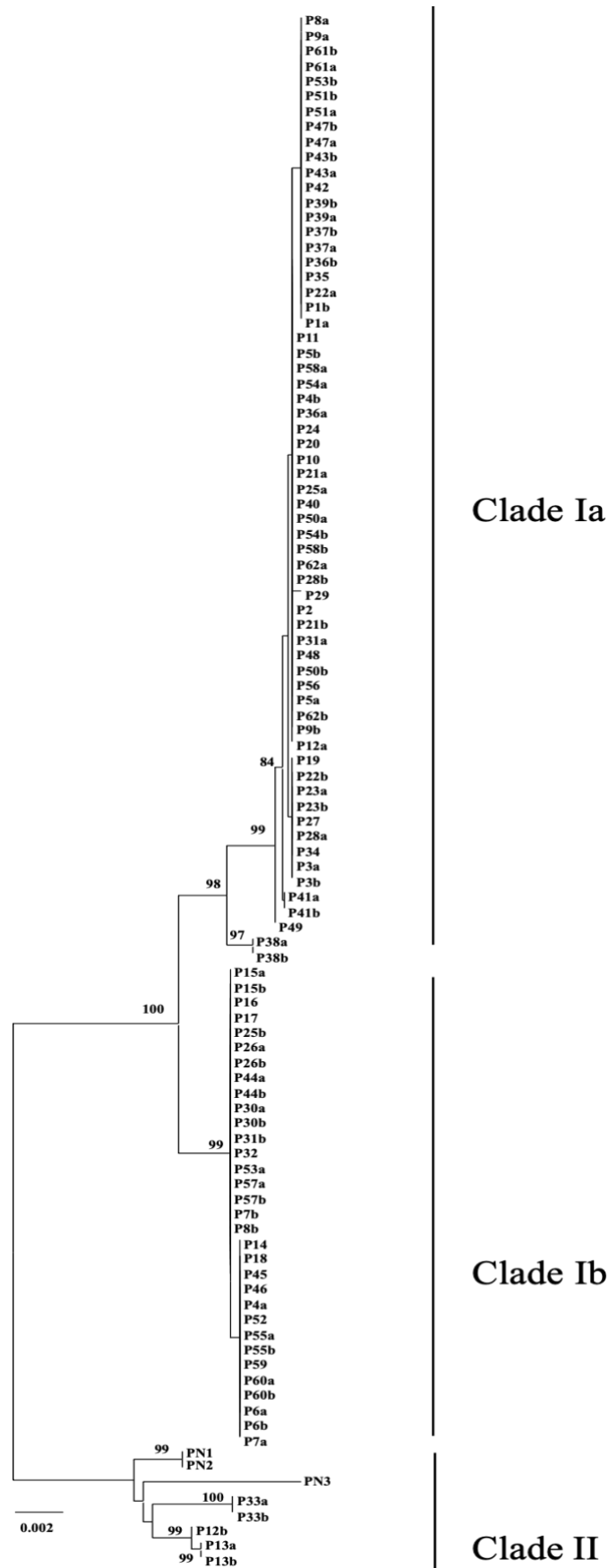


Fig. 2.5. NJ tree based on concatenated ITS, LSU, RPB2, and β -tub sequences from this study, generated using the Kimura 2-parameter model in Mega11. Specimens from IB herbarium are indicated by the code PN. Bootstrap values > 70 are reported at each node.

When compared to the available published sequences of *Peziza* species, our sequences were all clustered as *P. heimii* or *P. nivalis*, which were supported by high bootstrap values at the respective nodes. In particular, the specimens of clades Ia and Ib grouped with the French collection MPU:JCD174-78 identified as *P. heimii* by several authors (Van Vooren 2020, Pfister et al. 2024) while the specimens of clade II and IB herbarium samples formed a larger monophyletic group together with many collections of *P. nivalis* from North America and Oceania (Pfister et al. 2024) as well as *P. fortoulia* (MPU:JCD77-78) and *P. ninguis* (MPU:JCD398-74) from France.

Ascomatal morphology

The apothecial collected in Campo Pericoli did not exceed 4 cm in diameter, were sessile and flattened in age. The hymenium was brown or dark brown with the outer surface of the ascomata lighter (cream to brown). In addition to the three *P. nivalis* IB specimens, micromorphological analysis were carried out on 13, 8 and 4 specimens from Campo Pericoli belonging to the subclade Ia, subclade, Ib and clade II, respectively. Paraphyses are septate with a large swelling at the apex. The spores were ellipsoidal, smooth, without oil droplets and characterized by a mean Q-value of 1.61 ± 0.14 with no significant differences between members of the different clades and/or subclades (Table 2.1, Fig. 2.6). Specimens of the clade I produced significantly larger spores than those of clade II and IB herbarium samples specimens ($p < 0.001$).

Table 2.1. Dimensions (mean and standard deviation) of ascospores from ascomata of different clade/subclades and IB specimens. Different letters in the same column indicate significant differences for $p < 0.05$ by Tukey post hoc test.

Clade	n. ascomata	Spore length		Spore width		Q-value	
		mean	SD	mean	SD	mean	SD
Ia	13	25.7a	1.6	15.7a	1.1	1.64	0.12
Ib	8	25.1a	2.0	16.3a	2.0	1.55	0.15
II	4	18.0b	2.0	11.2b	1.1	1.61	0.17
IB specimen	3	19.9b	1.1	11.2b	1.7	1.80	0.20

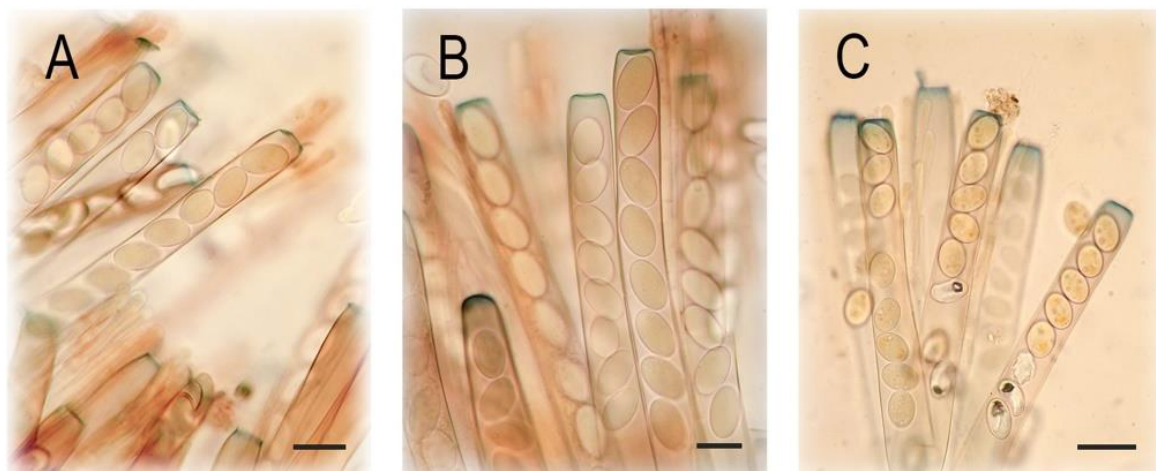


Figure 2.6. Morphology of asci and ascospores from specimens belonging to clades Ia (A), Ib (B), and II (C), stained with Melzer's reagent. Scale bars = 30 μ m.

Statistical and geostatistical analyses

The Pearson's correlation calculated for all the possible predictors' combination pairs resulted in low values: elevation - aspect (0.47), elevation - slope (0.32), elevation - TWI (−0.17), aspect - slope (0.18), aspect - TWI (−0.022), and slope - TWI (−0.30). The Kolmogorov-Smirnov test revealed that most of the topographic predictor distributions were non-normal. Also, none of the Kolmogorov-Smirnov tests showed statistically significant differences among the clades. Specifically, the p -values obtained for each predictor were: altitude $p = 0.134$, aspect $p = 0.059$, slope $p = 0.056$, and TWI $p = 0.059$ (Fig. 2.7).

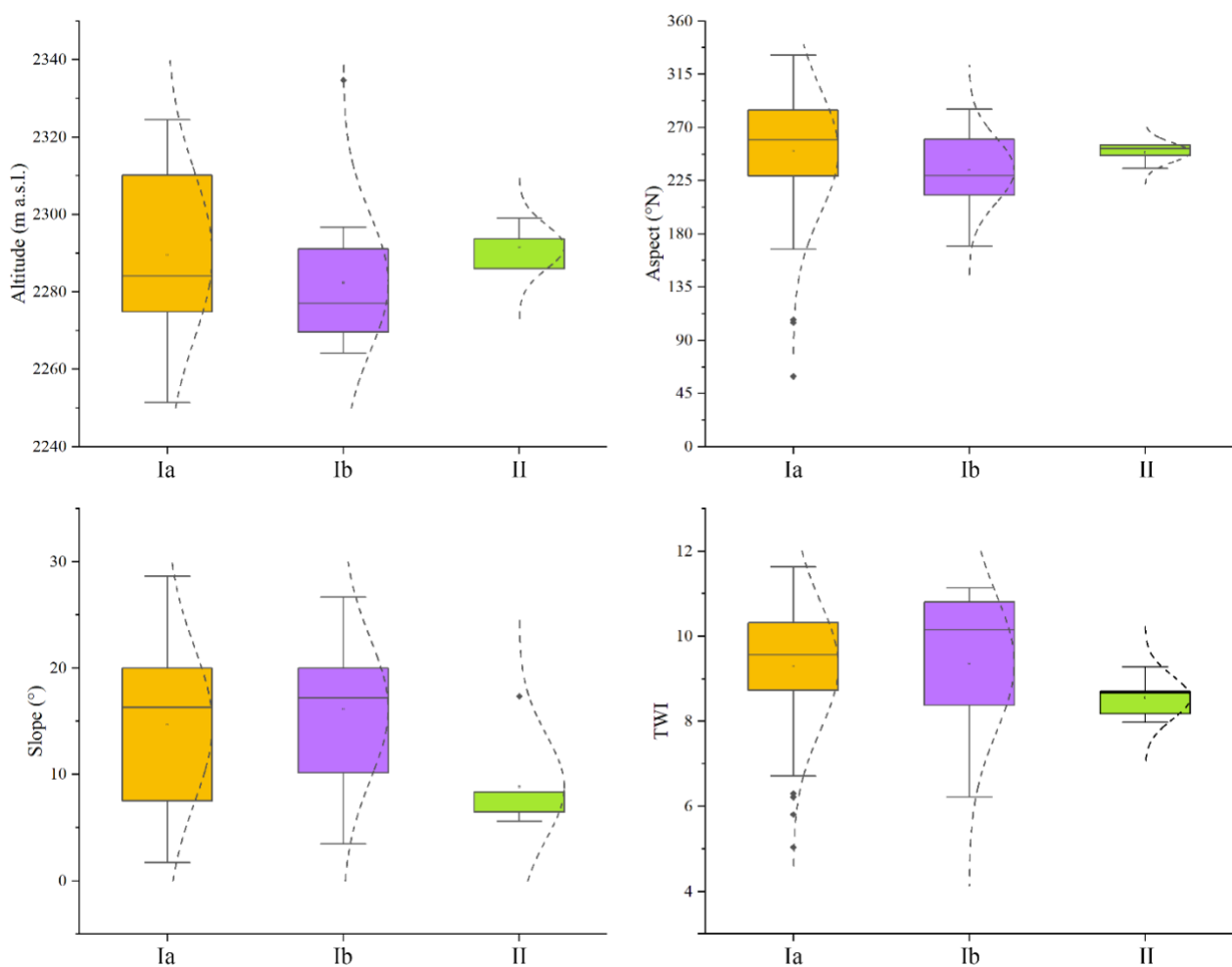


Figure 2.7. Boxplots resulting from the Kolmogorov-Smirnov test assessing the normality of the distributions of topographic predictor variables among clades/subclades. a) altitude; b) aspect; c) slope; d) TWI. The test did not detect statistically significant differences between groups.

Kernel Density Estimation (KDE) analysis cartographically indicated that clade Ia was the most widespread, occupying the largest spatial extent. Clade Ib was found to be largely nested within clade Ia, with the two main KDE density peaks of Ib falling well within the distribution of the clade Ia. In contrast, specimens of clade II exhibited a distinct spatial distribution, independent of Ia and Ib. This

pattern was further supported by the KDE raster correlation matrix (Fig. 2.8), which showed a strong correlation between specimens of clades Ia and Ib ($r = 0.489$) but a much weaker correlation between them and the clade II ($1a$ vs 2 : $r = 0.168$; $1b$ vs 2 : $r = 0.299$), suggesting a clear spatial differentiation of specimens belonging to clade II.

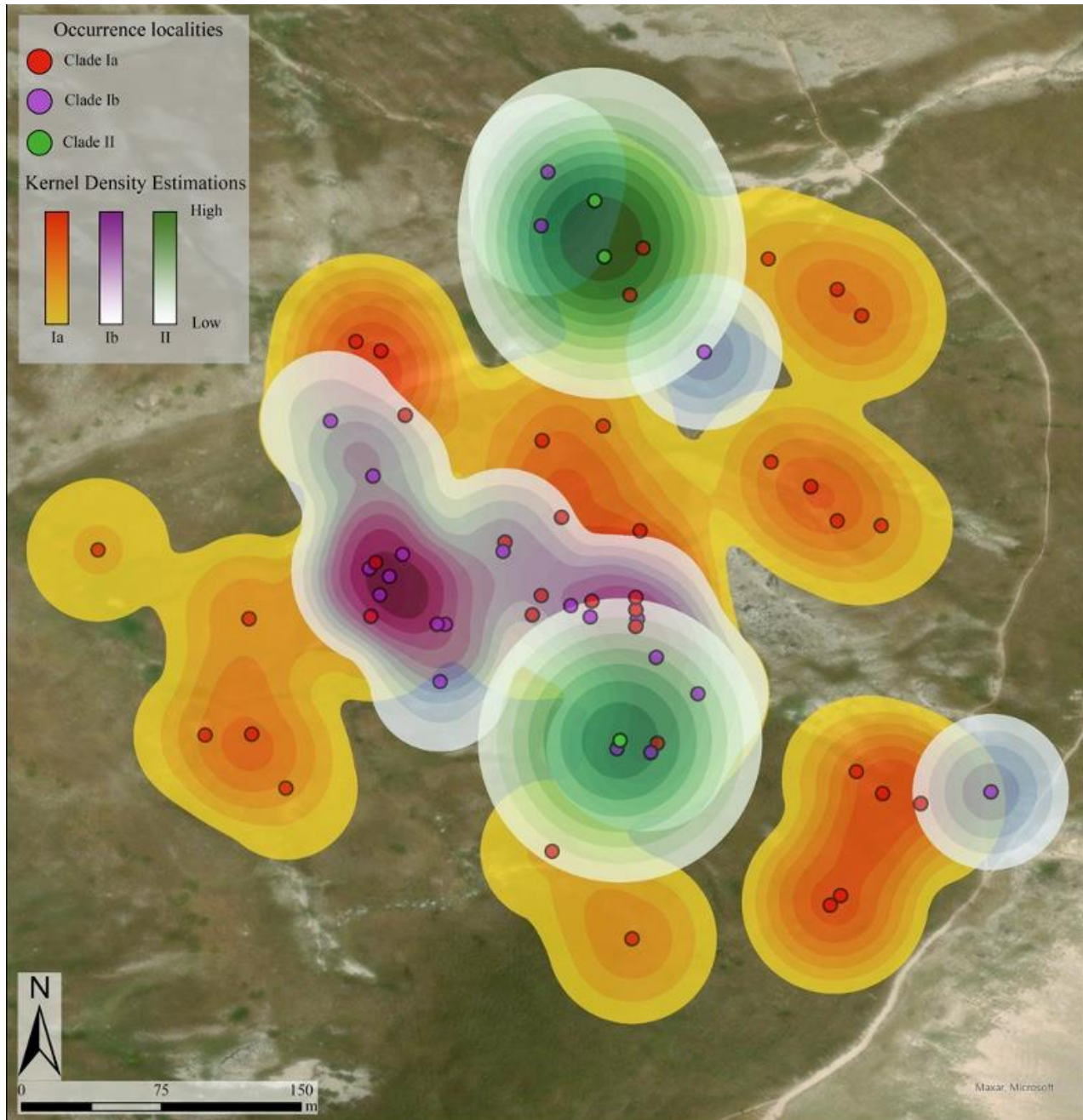


Figure 2.8. Kernel Density Estimation (KDE) map of clade/subclade distribution. The map illustrates the overlapping spatial patterns of fruiting points of the subclades Ia (red) and Ib (purple) contrasted by the discrete distribution of clade II (green).

Finally, the correlation between mean annual temperature and elevation across the global occurrence localities of the two clades reported a strong negative correlation ($r = -0.97$) for specimens in clade I

(*P. heimii*), while those in clade II (*P. nivalis*, *P. fortoului*, *P. ninguis*) exhibited a weaker negative correlation ($r = -0.34$).

Discussion

Many of the studies carried out up to now on *Peziza* species have been focused on taxonomy and phylogeny. The distribution range and ecological requirements of many *Peziza* species remain largely unknown or assumed. In this study, we investigated for the first time the diversity and the ecology of a population that develops at the edge of melting snowbanks, members of the *P. nivalis*-*P. heimii* group. Moreover, the study was carried out in a peculiar and ecologically peculiar location, which is characterized by an alpine climate in a Mediterranean context. Campo Pericoli could be the highest site in the Apennines and southern Europe where nivicolous *Peziza* species are found. The potential fruiting period in Campo Pericoli is short (maximum three weeks): it starts as soon as the ground becomes free from snow, and the mean temperature exceeds 0 °C and lasts until the snowbanks completely disappear. Ascomata appear to have a short life span, typically lasting no more than 1–2 days, and are found along a narrow strip of ground bordering the shrinking snowbanks where grasses have yet to resume growth. Consequently, the fruiting sites move quickly following the snow edge. Most probably this fruiting habit allows efficient aerial spore dispersion from cup-shaped ascomata that are small, sessile and at ground level. Considering the fruiting season of nivicolous *Peziza* species in Campo Pericoli, spore release occurs under harsh climate conditions, generally unsuitable for most fungal species. It is known that temperature, precipitation, and relative humidity are the main factors affecting the concentration of airborne fungal spores (Jones & Harrison 2004, Wyka et al. 2018). In our case, in Campo Pericoli *Peziza* species are thought to release spores when temperatures are below 10 °C and the relative humidity exceeds 50–60%. The amount of precipitation in the period under consideration is low. Specifically, rainfall during the studied period accounted for 25.71% of the total annual precipitation in 2020 and only 2.80% in 2021. It is likely that the amount of soil water allowing the asci to attain turgidity and explosively release spores is guaranteed by the melting snow. As the snow recedes, the ascomata dry up.

Our phylogenetic analyses showed that two different species of nivicolous *Peziza* co-occur in Campo Pericoli. *Peziza heimii* was the most common with 95% of the collected ascomata were from *Peziza heimii*, followed by *P. nivalis* with only 5%. This difference in abundance did not seem affected by the sampling year. Surprisingly, the genetic diversity within the two species was similar despite the difference in the number of ascomata collected for each of them. *Peziza nivalis* is a cosmopolitan species found so far in Europe, North America and Oceania, but it could be also present in Asia or South America. Pfister et al. (2024) revealed the presence of 4 genetically distinct lineages in *P.*

nivalis without any geographic correlation among them and our phylogeny confirmed these results, with the specimens from Campo Pericoli and IB distributed among all these lineages. *Peziza nivalis* clade also included *P. ninguis* and *P. fortoulia* from French alps already noted as synonyms of *P. nivalis* by different authors (Pfister 1992, Perić and Perić 2009, Van Vooren and Mauruc 2020, Pfister et al. 2024). *Peziza heimii* seems to be genetically structured into two strongly supported subclades, but the current genetic data available for this species are too scarce to make further conclusions.

Peziza heimii was proposed by Pfister (1992) based on ascospores that are larger than those of *P. nivalis*. Our investigation statistically confirmed the difference in spore size after comparison of several specimens of *P. nivalis* and *P. heimii* from Campo Pericoli. *Peziza heimii* seems to have less extent of occurrence than *P. nivalis* but its real distribution range is largely unknown but could be regarded as a European endemic. In addition to the type specimen collected in the French Alps at 2260 m asl, the other two findings of *P. heimii* were made by Ribes et al. (2016) and Urben (2015) in high elevation grassland located in the Ordeza-Monte Perdido national park (2050–2350 m asl, Torla municipality, Pyrenees, Spain) and at the Gemmi pass (2300–2500 m asl, Bernese Alps, Switzerland) but their identity was not confirmed by the DNA data. One ITS sequence deposited in GenBank from a soil samples collected at the Rotmoosferner glacier foreland in the Austrian Alps (Kuhnert et al. 2012) has a similarity over 99% to our *P. heimii* specimens. There are many other ITS accessions in Genbank with an identity between 97% and 99% for which it is impossible to infer well-supported phylogenetic relationships by only using the ITS region (data not shown). These species falling within this range of identity have been associated with different ecological niches: e.g., *P. domiciliana* typically found on damp walls, plaster, and mortar; *P. alcis* on elk (*Alces alces*) dung in mesic acid coniferous heath forests and *P. ampliata* on various woody substrates (Hansen et al. 2002).

In Campo Pericoli, *P. heimii* and *P. nivalis* share the same territory although they occur in slightly different microhabitats. Weak differences in slope, aspect and TWI were found between *P. heimii* and *P. nivalis*; the *p*-values were marginally significant ($p < 0.06$). On the contrary, elevation profiles were similar between the two species. No difference was found in any topographical parameters between specimens of the subclades Ia and Ib of *P. heimii*. This aspect is also reported from the KDE analyses since subclade Ib is spatially nested within subclade Ia, with clade II being rather completely outside the spatial ranges of these two. Indeed, the raster correlation supported the difference in spatial structure.

The current literature and metadata associated with *P. heimii* and *P. nivalis* suggest that these species could be the only obligately psychrophilic species in the genus. They are confined to elevations above 2000 m asl in temperate and Mediterranean regions and below this elevation in boreal climates. *Peziza*

nivalis has been found below 2000 m asl in more temperate climates, including low elevation subalpine areas such as Washington (Hurricane Ridge, 168 m) and Canada (Newfoundland and Quebec), suggesting a broader ecological tolerance than previously thought (Pfister et al. 2024, Voitek et al. 2021). It is the case that few collectors undertake field work in the late winter as snow is melting. There is the possibility that other species might be found and that these fungi occur more commonly than our specimen records indicate.

Climate warming, which is particularly pronounced in the Mediterranean basin (Lionello and Scarascia, 2018), could have consequences for the competition between these two species. *Peziza heimii* is putatively more vulnerable than *P. nivalis* to increasing temperatures and its migration to higher elevations is limited by the grassland extension. This has been documented in the last 18 years for many psychrophilic plant species in calcareous grassland of the central Apennines (Varricchione et al. 2022). Similarly, *P. heimii* could also disappear from Campo Pericoli due to climate changes. Indeed, although both species currently occur at similar elevations in our study area, this alone does not necessarily indicate similar thermal tolerances. At a global scale, *P. heimii* shows a clear association with high elevations in warmer regions, as evidenced by the strong negative correlation between annual mean temperature and elevation. In contrast, *P. nivalis* appears to exhibit broader ecological adaptability, showing a much weaker correlation.

The life cycle and the ecological role of the nivicolous *Peziza* species are largely unknown, particularly those, such as *P. heimii* and *P. nivalis*. Current knowledge does not allow speculation on the requirements for spore germination or subsequent growth and development of *P. heimii* and *P. nivalis* or the survival strategies they adopted to overwinter. They are most likely saprotrophs, but specific studies on the enzymatic capabilities and nutritional habit of these species do not exist. Several of the *P. nivalis* collections are burn associated (Perić and Perić 2009, Voitek et al. 2021) but in Campo Pericoli no fire has been ever reported. Significant insight into their biology could result from the analysis of genomes and specific *in vitro* tests after isolating pure cultures.

Chapter 3

Population genomics of *Tuber borchii*: comparative diversity between Sardinian and continental isolates

Introduction

Population genetics investigates how allele frequencies are distributed and change within and among populations, providing a framework to infer key evolutionary processes such as gene flow, genetic drift, selection and mating systems (Hartl & Clark, 2007; Hedrick, 2011). In fungi, these approaches are particularly powerful because morphological traits often fail to distinguish recently diverged or cryptic lineages, and because dispersal, reproductive modes and population boundaries differ substantially from those of plants and animals (Taylor et al., 2006). This means that genetic isolation frequently precedes morphological divergence, making population genetics essential for identifying true evolutionary units. Population-level approaches have been widely applied to mushrooms to investigate patterns of gene flow, isolation by distance, reproductive biology and ecological specialization. For example, multilocus sequence analyses revealed multiple cryptic species within *Russula griseocarnosa* X.H. Wang, Zhu L. Yang & Knudsen, showing that accurate species delimitation is a prerequisite for any meaningful population genetic inference (Li et al., 2010). Likewise, studies on ectomycorrhizal *Russula* spp. have demonstrated how genetic structure can be shaped by mating systems, host association and dispersal limitation (Wang et al., 2015). Within this framework, ectomycorrhizal fungi represent one of the most intensively studied models in fungal population genetics, due to their ecological importance and their tight dependence on host plants. Ectomycorrhizal fungi establish mutualistic associations with the roots of trees and shrubs, and population genetic studies have shown that the diversity and spatial structure of ectomycorrhizal communities are shaped by the interaction between ecological factors and limitations in spore dispersal (Peay et al., 2007; Murat et al., 2011). One of the most studied mechanism of spore dispersal in ectomycorrhizal fungi is typical of species that form hypogeous fruiting bodies for which it is necessary intentional consumption by animals followed by passive transport and defecation, during which spores may pass through the gut undamaged (Urban, 2016; Vernes et al., 2016; Elliott et al., 2019; Caiafa et al., 2021; Cooper & Vernes, 2011). In true truffles (genus *Tuber*), ascospore deposition in faecal material near the root systems of potential host plants represents a selective advantage for successful mycorrhizal establishment. Moreover, several recent studies demonstrated that spores moved by mycofagous animals often act as a paternal partner in fertilization of *Tuber* species, allowing ascoma production (Taschen et al., 2016; De La varga et al., 2017; Leonardi et al.,

2020). The evolution of hypogeous fruiting in *Tuber* spp. is thought to have arisen as an adaptation to increasingly dry environments (Trappe & Claridge, 2005), and many species have evolved specific traits that enhance animal-mediated dispersal, most notably the production of volatile organic compounds acting as chemical attractants to stimulate mycophagy (Splivallo et al., 2011; Pacioni et al., 2015). This aspect is therefore particularly critical for hypogeous fruiting taxa such as *Tuber* spp., which rely primarily on animal-mediated dispersal through mycophagy (Trappe, 2009). Because this dispersal strategy is tightly linked to animal movement and feeding behavior, it inherently constrains long-distance natural gene flow and often results in pronounced isolation-by-distance and fine-scale genetic structuring within populations. This pattern has been clearly documented in *Tuber melanosporum* Vittad., where strong genetic isolation by distance at the metric scale was observed, likely driven by animal-mediated dispersal, soil spore persistence, and vegetative incompatibility (Taschen et al., 2016). Experimental evidence further confirms the effectiveness of mycophagy as the dominant natural dispersal mechanism in this species: feeding experiments showed that ascospores can survive gut passage and remain viable after deposition in faeces, highlighting the ecological role of both vertebrates and invertebrates as dispersal agents (Thomas & Thomas, 2022). At the same time, recent genomic studies have revealed that *Tuber* species can maintain unexpectedly high levels of standing genetic variation even in the absence of deep geographic isolation. This pattern reflects a combination of biological features, including heterothallism, extensive recombination, large effective population sizes, and broad ecological amplitude, which together promote the maintenance of high allelic diversity across landscapes despite limited dispersal (Murat et al., 2011; Taschen et al., 2016).

The increasing sequencing of fungal genomes stimulated the application of population genomics which represent a powerful approach that can address several issues pertaining to fungal evolution, adaptation and diversity more efficiently than population genetics (Grünwald et al., 2016). In truffles, genome-wide population genomics was first applied to *T. melanosporum* by Payen et al. (2015), who compared seven genomes from geographically distinct populations across the northern and southern range of the species. This study revealed high levels of nucleotide diversity (more than 440,000 single nucleotide polymorphisms) and identified genomic regions potentially subjected to positive or negative selection. These results provided the first genome-scale insight into adaptive processes in a truffle species and opened the way to marker-assisted selection for truffle cultivation and management. Despite this pioneering contribution, population genomic approaches have not yet been systematically applied to other ecologically and economically relevant species of the genus *Tuber*. *Tuber borchii*, known as the “bianchetto” truffle, is a European endemic ectomycorrhizal fungus of ecological and economic relevance. It has a broad latitudinal and ecological range, extending from the Iberian Peninsula to Iran and from Mediterranean islands to Northern Europe (Lancellotti et al.,

2016). To date, it has not yet been reported in North Africa as the other European widespread truffle *T. aestivum* Vittad. (Henkrar et al., 2023). *Tuber borchii* associates with a variety of host plants including members of the genera *Quercus*, *Pinus*, *Corylus* and *Tilia* and it can be found from coastal to mountain forests (Lancellotti et al., 2016). Ecology and systematic of *T. borchii* have been studied by many authors, but the population genetic studies focused on this species remain scarce compared to other valuable truffle species (Molinier et al., 2016; Taschen et al., 2016; Belfiori et al., 2020). Genetic characterization of *T. borchii* has begun since the mid-1990s with amplification and analysis of ITS rDNA regions, application of RAPD approaches and design of species-specific primers (Stocchi et al., 1995; Amicucci et al., 1998, 2000; Bertini et al., 1998). Later, a multilocus phylogenetic study conducted on 61 specimens from Italy identified two cryptic species within *T. borchii* of which haplotype H1 dominates across most of the Italian peninsula (Bonuso et al., 2010). After the release of the genome in 2018 (Murat et al., 2018), SSRs and mating type genes were used to assess the genetic structure of a *T. borchii* population in a truffle plantation and the reproductive biology of this truffle (Leonardi et al., 2020). However, the extent of genetic diversity of *T. borchii* populations at regional and continental scale has never been investigated so far.

Sardinia represents a biodiversity hotspot for fungi and truffles (Comandini et al., 2018) and offers an exceptional natural setting for investigating the population genomics of *T. borchii*, given its pronounced environmental heterogeneity and its location in the center of Mediterranean. To address this aspect, whole-genome resequencing of 24 Sardinian and 5 mainland Italian ascomata was carried out. By jointly analyzing Sardinian and continental genomes, this study provides the first high-resolution genomic assessment of *T. borchii* population structure at a regional scale. This study is part of a broader regional initiative coordinated by the “Associazione Tartufai Sardi Paolo Fantini” and funded by the “Regione Sardegna” focused on the valorization and sustainable management of *T. borchii* in Sardinia. Moreover, it implements the data obtained from the ongoing JGI project entitled “Investigating the *Tuber borchii* pan-genome for insights into local-scale adaptive evolution,” which aims to integrate more than twenty new European genomes and advance our understanding of phylogeographic structure and local adaptation in this species.

Materials and Methods

Sampling and collection of Tuber borchii ascocarps

A total of 29 ascomata of *Tuber borchii* were collected in natural truffle grounds of Sardinia (n = 24) and mainland Italy (n = 5) between 2021 and 2024 (Fig 3.1).

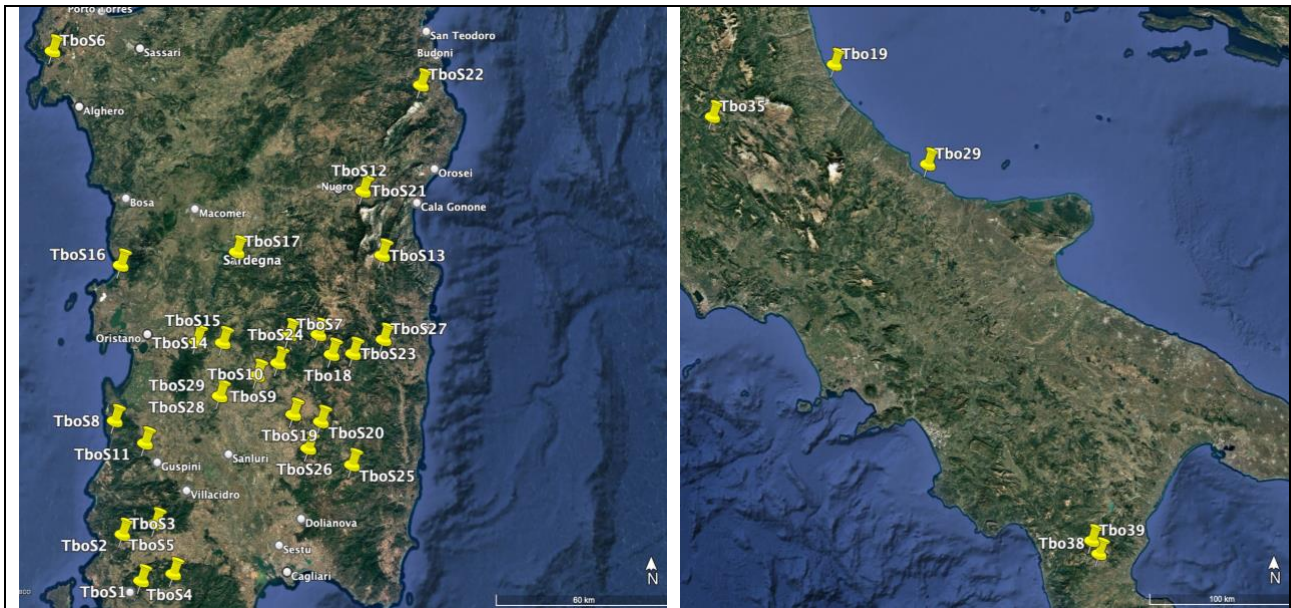


Figure 3.1. Geographic distribution of *Tuber borchii* sampling sites in Sardinia (left) and mainland Italy (right). Yellow pins indicate the locations of collected ascomata (TboS = Sardinian isolates; Tbo = continental isolates). Base map from Google Earth.

All Sardinian specimens (TboS samples) were harvested between January and March 2024 across 17 municipalities spanning southern, central and northern Sardinia, covering an altitudinal range from 18 to 1043 m a.s.l. (mean = 394 m). Sampling sites encompassed a broad spectrum of Mediterranean forest types and plantation systems, including *macchia evoluta* (e.g. Serbariu, Monte Arci), coastal pine plantations (*Pinus pinea*, *P. halepensis*), montane *Quercus ilex* stands (Laconi, Ussassai, Seulo), *Q. suber* woodlands (Villa Sant'Antonio), and mixed plantations with conifers and sclerophyllous shrubs (e.g. Urzulei: *Cedrus atlantica*, *Q. ilex*, *Cistus* spp.). Geographic coordinates and elevation were recorded for each location, and the dominant host tree(s) are reported in Table 3.1.

Mainland Italian specimens (Tbo samples) were collected in 2021 from Abruzzo, and Molise (central Italy) and Calabria (southern Italy), at different elevations (3 to 1120 m a.s.l.). Sites included coastal *P. pinea* stands (Pineto, Petacciato Marina), montane *Q. pubescens* woodlands (Petrella Salto), and mixed *Pinus nigra* – *Q. ilex* forests in the Pollino massif (Castrovillari, Morano Calabro). All ascomata were collected by trained dogs and sent to the laboratory at 4 °C, within 48 h from harvesting. Specimens were cleaned from soil debris under tap water and then the peridium was flame sterilized. Each ascoma was divided in half by a scalpel under sterile conditions and 100-200 mg of the internal gleba excised and preserved at -80 °C pending lyophilization. Ascomata with evident signs of rots or/and larval galleries were excluded from the analysis (not included in table 3.1). The remaining parts of the selected ascomata were dried at 40 °C for 24 h and deposited in the AQUI herbarium as a reference. Before DNA extraction the gleba fragments were lyophilized at 50 °C in a

VirTis Benchtop 2-K freeze dryer (SP industries) for 48 h. Taxonomic identity as *T. borchii* was confirmed through PCR amplification by species-specific primers (Amicucci et al., 1998).

Table 3.1. Host plant associations and habitat types of *Tuber borchii* isolates collected in Sardinia and mainland Italy.

Host plant	Sample IDs	Habitat type
<i>Quercus ilex</i>	TboS5 (plantation), TboS7, TboS10, TboS11, TboS12, TboS18, TboS21, TboS23, TboS24, TboS27, Tbo39	Mediterranean evergreen oak woodland (montane or mesic)
<i>Pinus pinea</i>	TboS2, TboS3 (plantation), TboS6 (plantation), Tbo19	Coastal pine plantation
<i>Pinus pinaster</i>	TboS19 (mixed plantation with oaks and pines), TboS22	Montane <i>P. pinaster</i> plantation
<i>Pinus halepensis</i>	TboS4 (plantation), TboS17 (mixed plantation with oaks and pines)	Mixed conifer plantation
<i>Quercus pubescens</i>	TboS20, Tbo35	Deciduous oak woodland
<i>Quercus ilex</i> + <i>Cistus</i> spp. + <i>Arbutus unedo</i>	TboS1	Mature Mediterranean macchia (shrubland)
<i>Pinus pinea</i> + <i>Cistus monspeliensis</i>	TboS8	Coastal pine forest with Mediterranean shrubs
<i>Quercus ilex</i> + <i>Pinus pinaster</i>	TboS9 (plantation)	Mixed Mediterranean woodland
<i>Pinus</i> sp.	Tbo29	Coastal pine stand
<i>Pinus nigra</i>	Tbo38	Mountain <i>P. nigra</i> forest
<i>Cedrus atlantica</i> + <i>Quercus ilex</i> + <i>Cistus</i> spp.	TboS13 (plantation)	mixed conifer–oak plantation

DNA extraction, PCR, and sequencing

Genomic DNAs were isolated from 20–25 mg of lyophilized gleba by using the DNeasy Plant Mini Kit (Qiagen), following the manufacturer’s protocol optimized for fungi. DNA was eluted in sterile TE buffer and stored at –20 °C. DNA quality was evaluated by measuring 260/280 and 260/230 OD ratios with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and by electrophoresis on a 1.5% agarose gel. DNAs were quantified with a Qubit Fluorometer (Invitrogen) using the High Sensitivity dsDNA quantification Kit. DNA extracts with concentrations < 20 mg mL⁻¹, OD ratios < 1.4 and/or showing smears on agarose gel were discharged and DNA extraction repeated. Libraries were prepared sequenced at BIODIVERSA company (Treviso) using standard Illumina-compatible workflows and sequencing was carried out on an Illumina NovaSeq 6000 platform, using paired end configuration (2 × 150 bp). The average output was approximately 4 Gb per sample with an average depth of 30×. Raw reads were provided in FASTQ format and subjected to downstream quality control and bioinformatic analyses.

Read quality control, alignment, and variant calling

Raw paired-end Illumina reads (R1 and R2) generated from *T. borchii* were processed for quality assessment and variant calling to obtain a high-confidence genome-wide SNP dataset suitable for population genomic analyses. Initial read quality was evaluated using FastQC v0.12.1 (Andrews,

2010), assessing Phred quality distribution, adapter contamination, and GC content. Low-quality bases and adapters were removed using fastp v0.23.2 (Chen et al., 2018; Chen, 2023) with default parameters, including automatic adapter detection, sliding-window trimming for Phred < 20, and exclusion of reads shorter than 15 bp. Cleaned reads were aligned to the *T. borchii* reference genome (strain Tbo3840, 98 Mb; Murat et al., 2018), available from the MycoCosm database, using BWA-MEM v0.7.17 (Li, 2013) with default parameters. The resulting SAM files were converted to BAM format, sorted, and indexed using SAMtools v1.6 (Li et al., 2009). Variant discovery was conducted using the HaplotypeCaller module of the Genome Analysis Toolkit (GATK) v4.6.1.0 (McKenna et al., 2010; Van der Auwera & O'Connor, 2020) in single-sample mode. Each BAM file was processed individually to generate sample-specific VCF files aligned to the *T. borchii* Tbo3840 reference genome. Variant quality control was performed with the VariantFiltration tool of GATK v4.6.1.0, applying the following thresholds to exclude low-confidence sites: QUAL < 30.0, DP < 10, MQ < 40.0, QD < 2.0, FS > 60.0, and SOR > 3.0. Only variants labeled as “PASS” were retained for downstream analyses.

Variant filtering and dataset preparation

Population genomic analyses were conducted starting from a multi-sample VCF file including Sardinian and continental *T. borchii* isolates, with the aim of generating a high-confidence and statistically robust SNP dataset for downstream analyses of population structure. Only single nucleotide polymorphisms (SNPs) were retained by extracting biallelic sites ($-m2 - M2 - v$ snps) using bcftools v1.3 (Danecek et al., 2021), to avoid biases introduced by multiallelic variants in distance- and variance-based analyses. Variants were filtered according to quality (QUAL ≥ 35) and missingness ($\leq 20\%$ missing data per site), generating a high-quality dataset suitable for population genomic inference. Locus-level missingness was estimated with VCFtools v0.1.17 (Danecek et al., 2011), and sites exceeding the 20% threshold were excluded. Samples with a call rate below 0.90 were removed prior to downstream analyses to ensure consistency and reliability of genotype-based distance and variance estimates. Two SNP datasets were derived from the initial filtering procedure. The first dataset, including all high-quality biallelic SNPs (QUAL ≥ 35 ; $\leq 20\%$ missing data), was used for population genetic statistics such as F_{ST} estimation. The second dataset was further pruned for linkage disequilibrium and used for multivariate analyses (PCA) and genotype-based distance calculations (IBS), to minimize redundancy among linked markers and better capture genome-wide patterns of variation.

Hierarchical clustering

A hierarchical clustering analysis was performed to assess relationships among isolates based on genotype similarity. The biallelic SNP matrix (0 = homozygous reference, 1 = heterozygous, 2 = homozygous alternate) was converted into a pairwise Manhattan distance matrix normalized by SNP count. Clustering was computed using the average linkage method [`hclust(method = "average")`] in R v4.5.0 (R Core Team, 2025). Dendrograms were visualized with custom color annotations to distinguish Sardinian (blue) and Continental (red) isolates. Bootstrap resampling of loci (1,000 replicates) was applied to evaluate the robustness of major clades.

Principal Component Analysis (PCA)

Genetic structure among isolates was explored by Principal Component Analysis (PCA), a multivariate, model-free approach used to investigate patterns of genetic differentiation and potential clustering between Sardinian and continental samples without imposing a priori population assignments. PCA was implemented in SNPRelate v1.36.0 (Zheng et al., 2012) within R. To ensure that the analysis reflected genome-wide structure rather than local linkage effects or technical artifacts, the final filtered VCF was converted into Genomic Data Structure (GDS) format and pruned for linkage disequilibrium (LD) using a sliding window of 50 kb, step = 5, and a pairwise LD threshold of $r^2 \leq 0.2$, retaining only statistically independent SNPs with minor allele frequency ≥ 0.05 . This filtering strategy was adopted to avoid the overrepresentation of tightly linked genomic regions and to guarantee that each variant contributed independently to the multivariate signal. PCA was performed using the `snpgdsPCA` function with the option `autosome.only = FALSE`, due to the scaffold-based structure of the reference genome. The first three principal components (PC1–PC3) were visualized using `ggplot2` v3.5.1 (Wickham, 2016) and `ggrepel` v0.9.6. Population labels (Sardinian vs Continental) were used exclusively for visualization purposes and were not imposed during the ordination.

Identity-By-State (IBS), PERMANOVA and AMOVA analyses

To explore genome-wide patterns of genetic similarity and population structure, pairwise genetic distances were estimated using the Identity-By-State (IBS) approach implemented in SNPRelate v1.36.0 (Zheng et al., 2012). IBS coefficients were calculated from LD-pruned genotypes and converted into genetic distances as $(1 - \text{IBS})$, generating a symmetric pairwise distance matrix. This matrix was visualized as a clustered heatmap using `pheatmap` v1.0.12 (Kolde et al., 2019), applying Euclidean distance and average linkage for both rows and columns. Sample annotations were color-coded according to population origin (Sardinian = blue; Continental = red). This approach allows for

a direct visualization of overall genomic similarity among individuals and provides an intuitive representation of potential clustering patterns between island and mainland samples. To statistically test whether the observed genomic patterns reflected significant population differentiation, a permutational multivariate analysis of variance (PERMANOVA) was applied to the same IBS-derived distance matrix. The IBS matrix was computed in SNPRelate from the filtered dataset of high-quality biallelic SNPs ($QUAL \geq 35$; $\leq 20\%$ missing data per site). Pairwise distances were calculated as $(1 - IBS)$ and imported into R for analysis using the `adonis2` function implemented in `vegan` v2.6-10 (Oksanen et al., 2025). The analysis was performed using 999 unrestricted permutations under a reduced model, with population origin (Sardinian vs Continental) specified as the grouping factor. The resulting F-statistics, R^2 and p-values were used to quantify the proportion of total genetic variance explained by population structure and to assess its statistical significance. PERMANOVA thus provides a formal multivariate test of whether genome-wide genetic distances differ between geographic groups. In parallel, an Analysis of Molecular Variance (AMOVA) was conducted to further partition genetic variability across hierarchical levels. AMOVA was performed using the `amova` function implemented in the `pegas` package v1.3-1 (Paradis, 2010) on the IBS distance matrix derived from SNPRelate. The analysis included 27 isolates and a dataset of high-quality biallelic SNPs. Population structure was assessed using 999 random permutations to test the significance of variance components, expressed as Φ_{ST} . Molecular variance was partitioned between populations and within populations, allowing estimation of the fraction of genome-wide diversity attributable to geographic origin. While PERMANOVA evaluates overall multivariate separation between groups, AMOVA provides a complementary hierarchical quantification of genetic structure directly comparable to traditional F-statistics.

Population differentiation

Genetic differentiation between Sardinian and continental populations was quantified using Weir and Cockerham's F_{ST} estimator (Weir & Cockerham, 1984) implemented in `VCFTools` (v0.1.17), to assess the magnitude of genome-wide divergence at a regional scale. Because F_{ST} is a relative measure of differentiation whose values can increase when within-population diversity is locally reduced (Nei, 1973; Charlesworth et al., 1997; Charlesworth, 1998; Jakobsson et al., 2013), window-based F_{ST} estimates were interpreted with caution. Elevated F_{ST} peaks may arise from reduced local polymorphism rather than true barriers to gene flow (Cruickshank & Hahn, 2014). Analyses were conducted on the filtered biallelic SNP dataset. Both genome-wide and window-based estimates were calculated using non-overlapping 50 kb windows to capture broad-scale differentiation as well as potential localized signals of divergence. Plots were generated in R using `ggplot2`.

Results

Read mapping and alignment quality

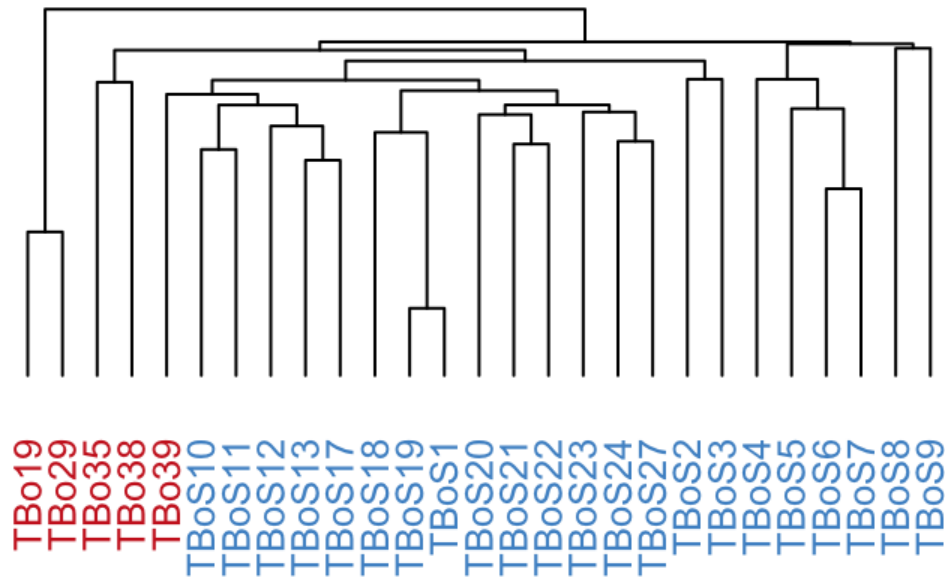
Molecular barcoding confirmed that all 29 sequenced isolates belonged to haplotype H1. After quality filtering and adapter trimming, all samples yielded high-quality Illumina reads suitable for downstream analyses. For the Sardinian isolates, the number of clean paired-end reads ranged from approximately 3.8×10^6 to 111.8×10^6 per sample. The proportion of successfully mapped reads to the *T. borchii* Tbo3840 reference genome (Murat et al., 2018) varied between 67 % and 88 %, with an average mapping efficiency of ≈ 79 % across the 24 isolates. Two samples (TBoS24 and TBoS25) showed markedly lower mapping rates (< 50 %) and were therefore excluded from subsequent variant calling. The final Sardinian dataset comprised 22 isolates. Among the continental isolates, total read counts ranged from 14.8×10^6 (TBo39) to 502×10^6 (TBo29), with mapping percentages between 65 % and 92 %. The percentage of successfully mapped reads was generally comparable to that observed for the Sardinian group. Overall, these mapping results confirmed the suitability of the sequencing data for high-confidence variant discovery. The relatively high mapping rates observed (> 75 % in most samples) indicate good coverage of the *T. borchii* Tbo3840 reference genome, consistent with previous genomic studies (Murat et al., 2018). Minor variations in mapping efficiency likely reflect biological divergence between Sardinian and continental lineages or local genomic polymorphisms not represented in the reference assembly (e.g., different mating type idiomorph).

Variant filtering and dataset quality

Starting from 85,250 raw SNPs in the multi-sample VCF, filtering for quality ($QUAL \geq 35$) and missing data ($\leq 20\%$) yielded 8,359 high-quality biallelic variants (sc.mm.filtered.clean.vcf.gz). Variant filtering produced a high-quality dataset comprising 27 isolates and 8359 biallelic SNPs across the nuclear genome scaffolds of *T. borchii*. All variants passed a minimum quality score of 35 and had $\leq 20\%$ missing data per site. The average call rate across individuals exceeded 97%, indicating good sequencing and genotyping consistency.

Hierarchical clustering and genetic relationships

Hierarchical clustering based on the normalized Manhattan distance produced a topology consistent with PCA results (Figure 3.1).



`hclust (*, "average")`

Figure 3.1. Hierarchical clustering of 27 *T. borchii* isolates based on Manhattan genetic distance.

Four out of five continental isolates (TBo19, TBo29, TBo35, TBo38) grouped together in a single major clade, while TBo39 from Calabria showed affinity with Sardinian samples, without forming a clearly distinct or isolated lineage. Hierarchical clustering was performed using Manhattan distance. Cluster robustness was assessed using pvclust (Suzuki & Shimodaira, 2006) with multiscale bootstrap resampling (average linkage, $n = 1,000$). Cluster support was evaluated using AU (Approximately Unbiased) p-values, with values $\geq 95\%$ indicating strong support. The continental cluster showed very high support (AU = 97–100%), confirming its robustness despite the limited sample size (Fig. 3.2).

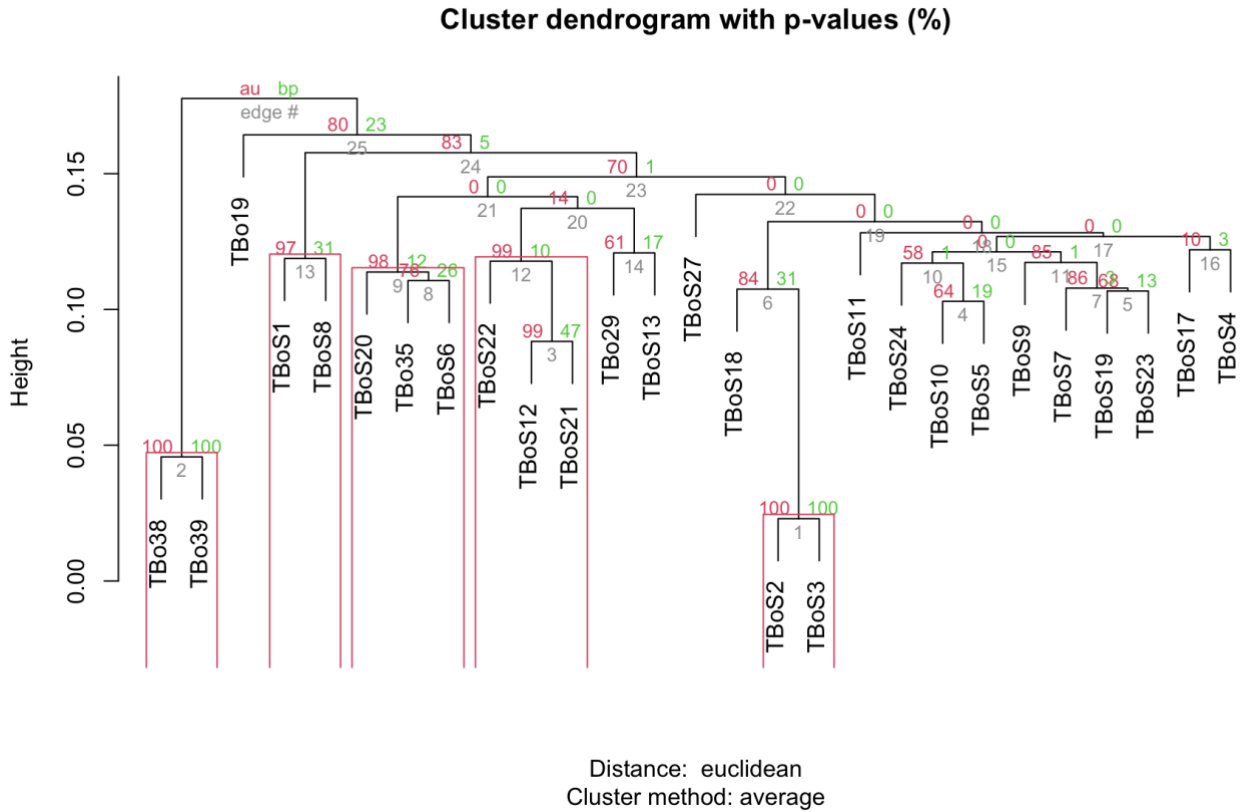


Figure 3.2. Bootstrap validation of the hierarchical clustering using pvclust (Euclidean distance, average linkage, $n = 1,000$). AU p-values (%) are indicated in red. The analysis confirms strong support for the continental clade (AU = 97–100%).

Sardinian isolates displayed short internal branch lengths, indicative of high genetic similarity within the island population, whereas the continental cluster showed slightly greater internal divergence.

Principal Component Analysis (PCA)

The PCA based on 1,361 LD-pruned SNPs revealed a weak but consistent separation between Sardinian and continental isolates (Fig. 3.3). The first two principal components explained 17.26% of total variance (PC1 = 9.12%; PC2 = 8.14%). Continental isolates clustered loosely on the left of the plot, while most Sardinian isolates formed a diffuse group toward the right. This pattern was further confirmed by the third principal component (PC3 = 7.20%), which captured subtle within-group variation but did not identify strong substructure within Sardinia (table 3.1).

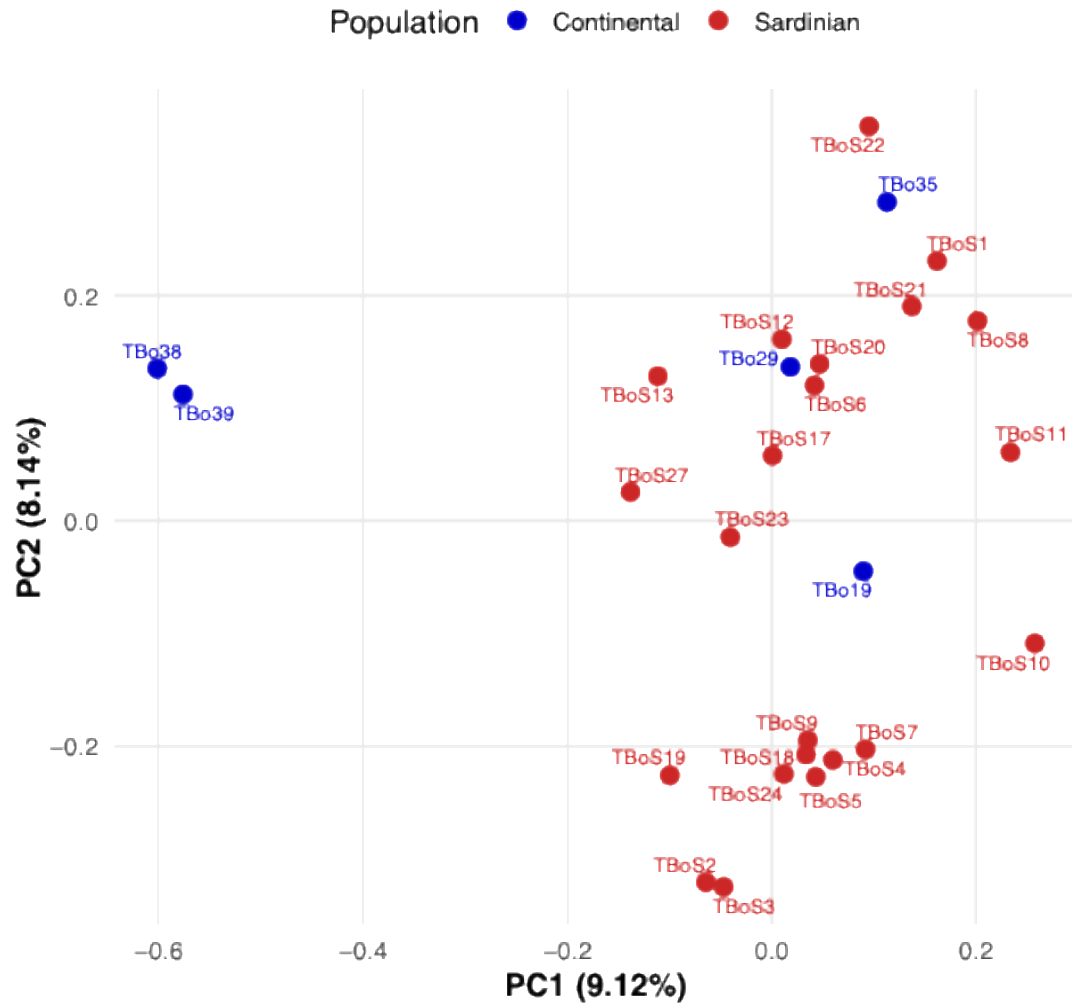


Figura 3.3. Principal Component Analysis (PC1 × PC2) showing the genetic structure of *T. borchii* isolates.

Table 3.1. Variance explained by the first five principal components.

Principal Component	Variance explained (%)
PC1	9.12
PC2	8.14
PC3	7.20
PC4	6.57
PC5	5.73

Identity-By-State (IBS) analysis

IBS-based pairwise distances provided a quantitative assessment of within- and between-population genomic divergence (Figure 3.4b). Average within-group distances were 0.0781 for continental isolates and 0.0832 for Sardinian isolates, while the mean between-group distance was higher

(0.0964), indicating a moderate level of differentiation between island and mainland populations (Table 3.3).

Table 3.3. Mean pairwise IBS distances within and between *Tuber borchii* populations

Population comparison		Mean IBS distance
Continental–Continental		0.0781
Sardinian–Sardinian		0.0832
Continental–Sardinian		0.0964

The IBS heatmap (Fig. 3.4) revealed a clear block-like structure separating continental and Sardinian isolates, in agreement with the patterns observed in the PCA and F_{ST} analyses. Continental samples formed a compact cluster with low internal variability, whereas Sardinian isolates showed slightly higher intra-group distances, possibly reflecting microgeographic structuring within the island.

Overall, intragroup distances were consistently lower than intergroup distances, indicating higher genomic similarity within populations than between populations. To statistically assess the robustness of this pattern, a PERMANOVA performed on the IBS distance matrix revealed a weak but statistically significant effect of population origin ($R^2 = 0.063$; $F = 1.69$; $p = 0.028$), indicating that approximately 6% of the total genetic variance was attributable to the Sardinian versus continental grouping. Consistently, AMOVA based on the same distance matrix detected a significant partitioning of molecular variance between populations ($\Phi_{ST} = 0.078$; $p = 0.019$), with 7.8% of the total variance explained by population structure and 92.2% residing within populations. Together, IBS, PERMANOVA and AMOVA converge in indicating a weak but statistically supported genetic differentiation between Sardinian and continental *T. borchii* populations. This pattern is consistent with the very low genome-wide F_{ST} (mean $F_{ST} = 0.008$) and supports a scenario of limited but non-negligible divergence between island and mainland lineages maintained under substantial gene flow.

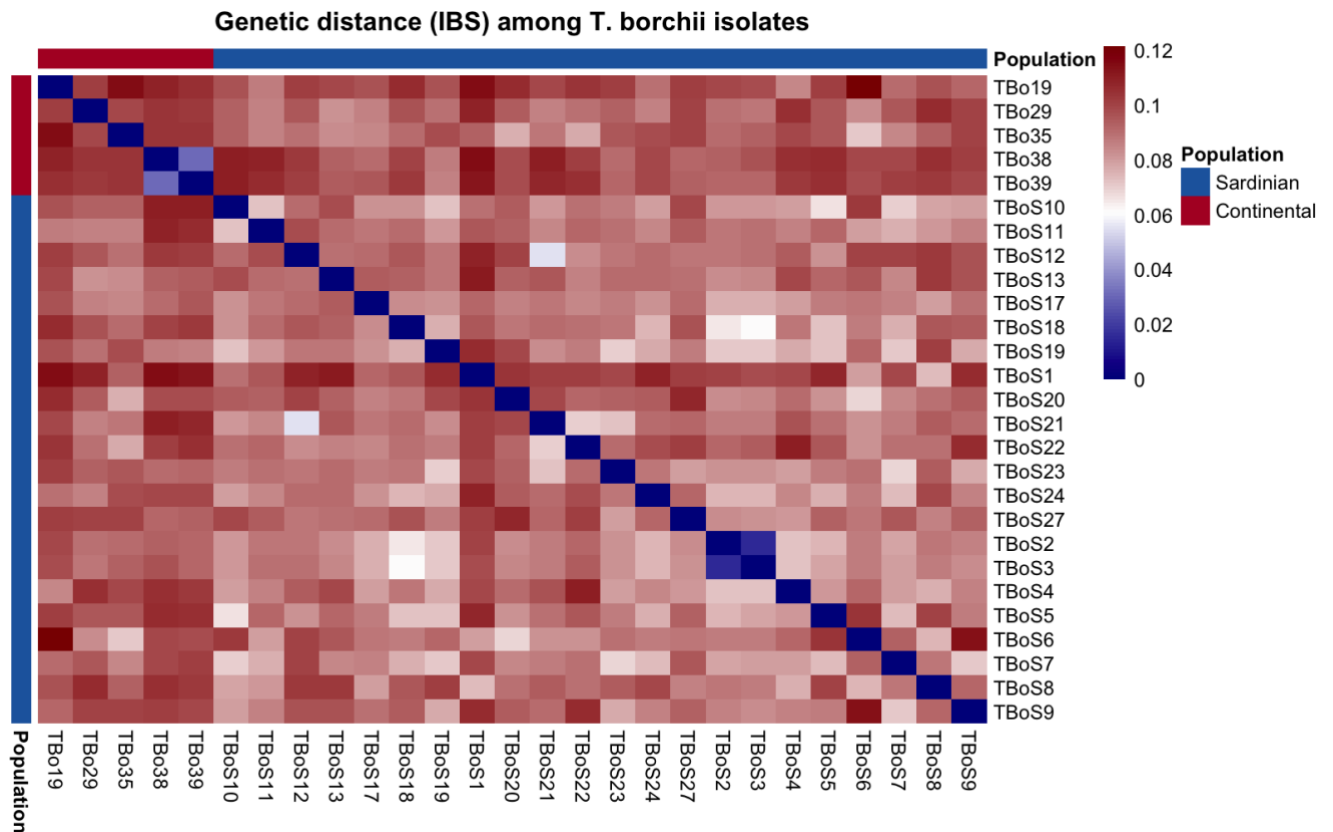


Figure 3.4. Heatmap of pairwise Identity-By-State (IBS) genetic distances among 27 *T. borchii* isolates. Blue and red bars indicate Sardinian and continental populations, respectively. Darker blue regions correspond to higher genetic similarity, while lighter or red areas indicate greater divergence.

Population differentiation

Weir and Cockerham's fixation index (F_{ST}) revealed very low genetic differentiation between Sardinian and continental populations of *T. borchii* (Table 3.4).

Table 3.4. Summary of global and windowed Weir & Cockerham F_{ST} estimates between Sardinian and continental* *T. borchii* populations. Values are based on 8,359 high-quality biallelic SNPs; windowed F_{ST} was computed using non-overlapping 50 kb windows.

Statistic	Value	Description
Mean F_{ST}	0.0047	Average across all 8359 SNPs
Weighted F_{ST}	0.0059	Weir & Cockerham weighted estimate
Mean weighted (windowed) F_{ST}	~0.0058	Average across 50 kb non-overlapping genomic windows
95% Confidence Interval (bootstrap)	0.0021 0.0103	Genome-wide interval from windowed analysis
SNPs analyzed	8359	After quality and LD filtering
Isolates	27	22 Sardinian, 5 Continental

These values indicate minimal genome-wide differentiation between populations. The slightly higher window-based estimates (~ 0.0058) and narrow confidence interval (0.0021–0.0103) confirm that population structure is weak but consistent across loci. This pattern aligns with previous evidence of broad gene flow and shared allelic variation in *T. borchii* across its distribution range. In addition to genome-wide averages, a window-based analysis identified 41 genomic windows ($\approx$ top 5%) with F_{ST} values above the 95th percentile threshold ($F_{ST} > 0.075$). The top 10 windows, summarized in Table 3.5, reached weighted F_{ST} values up to 0.53, located mainly on scaffolds 30, 88, 194, 196, and 365. These isolated peaks likely reflect localised divergence hotspots, rather than genome-wide selection or strong population subdivision.

Table 3.5. High- F_{ST} genomic windows (95th percentile) between Sardinian and continental *T. borchii* populations. Only the top 10 windows exceeding the 95th percentile ($F_{ST} > 0.075$) are shown. Values correspond to 50 kb non-overlapping bins computed with VCFtools.

SCAFFOLD ID	WINDOW START (BP)	WINDOW END (BP)	N. VARIANTS	WEIGHTED F_{ST}	MEAN F_{ST}
SCAFFOLD_365	50,001	100,000	1	0.528	0.528
SCAFFOLD_88	200,001	250,000	1	0.511	0.511
SCAFFOLD_30	1	50,000	1	0.332	0.332
SCAFFOLD_194	50,001	100,000	1	0.332	0.332
SCAFFOLD_196	1	50,000	8	0.298	0.269
SCAFFOLD_317	50,001	100,000	11	0.198	0.245
SCAFFOLD_97	200,001	250,000	4	0.180	0.173
SCAFFOLD_30	100,001	150,000	1	0.178	0.178
SCAFFOLD_77	200,001	250,000	1	0.177	0.177
SCAFFOLD_82	150,001	200,000	3	0.177	0.177

Such patterns are consistent with stochastic drift or region-specific barriers to gene flow rather than genome-wide selection. Across all 8,359 high-quality SNPs, the mean F_{ST} was 0.0047, and the weighted F_{ST} was 0.0059, confirming the weak overall population structure inferred from PCA and IBS analyses (Figure 3.5).

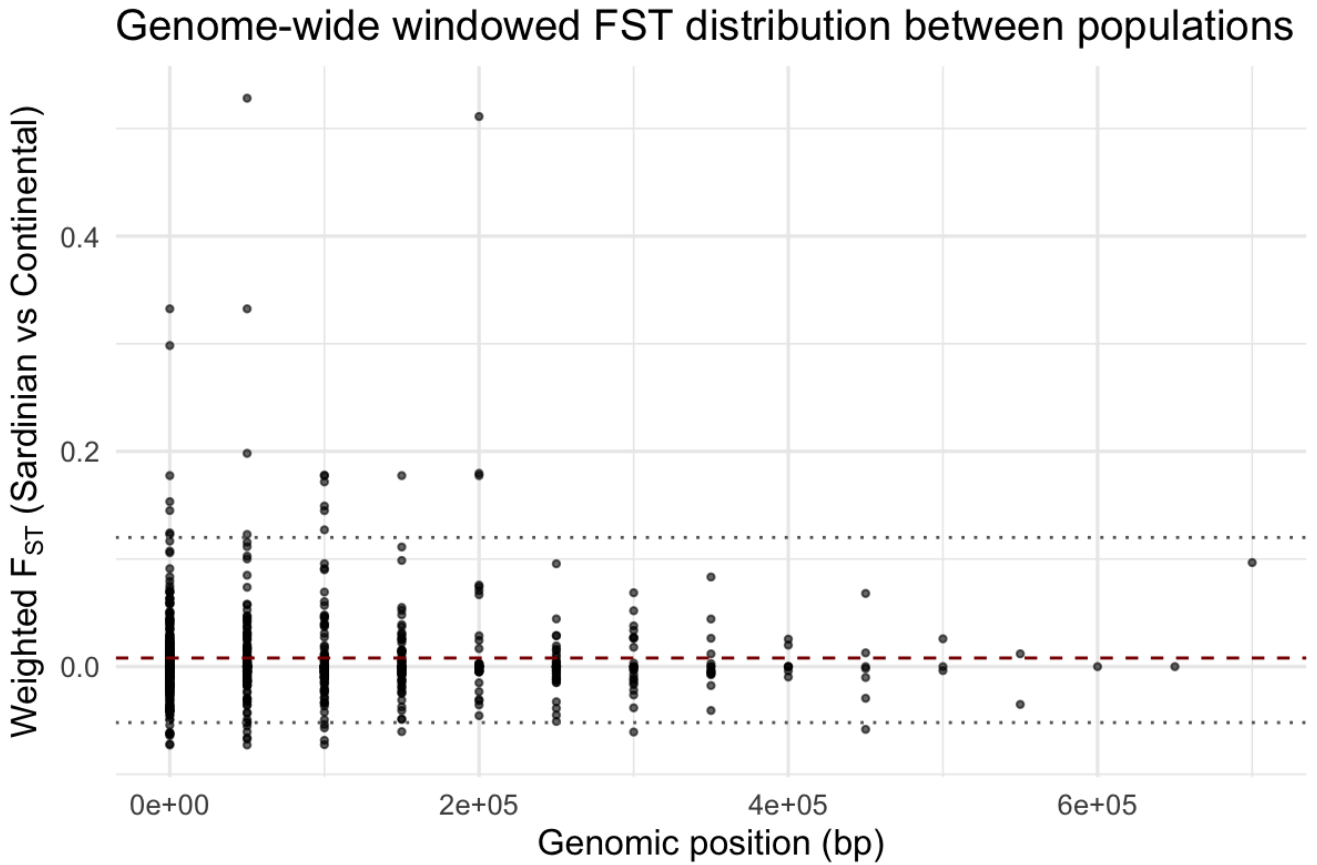


Figure 3.5. Genome-wide distribution of windowed weighted F_{ST} (50 kb non-overlapping windows) between Sardinian and continental *T. borchii* populations. The red dashed line indicates the genome-wide mean, and dotted lines represent the 95% confidence interval. A few elevated peaks (> 0.25) suggest potential localized divergence hotspots, but overall differentiation remains low.

Genome-wide windowed estimates (50 kb non-overlapping windows) ranged from -0.07 to 0.53 , with a mean of 0.0080 and a 95 % confidence interval of -0.052 to 0.120 . Only a small number of genomic windows displayed elevated F_{ST} values (> 0.25), which may correspond to localized regions of restricted gene flow or early divergence, but none reached the levels typically associated with strong adaptive differentiation. Combined with the IBS and PCA results, the F_{ST} analysis supports a scenario of incipient population structure, where geographic isolation has only begun to shape allele-frequency patterns.

Discussion

Studies on population genomics conducted until now on a *Tuber* species are very scarce. Payen et al. (2015) analyzed SNPs from six genomes of *T. melanosporum* collected in different areas of the distribution range of the species. Truffle genome resources have been most exploited to identify SSR markers for population studies in *T. melanosporum* (Murat et al., 2011), *T. aestivum* (Molinier et al., 2016a,b), *T. magnatum* Vittad. (Uncu and Uncu, 2019), *T. indicum* Cooke & Masee (Qiao et al.,

2018) and *T. borchii* (Leonardi et al., 2020). However, population genomic studies have been carried out on other ectomycorrhizal fungi like the widely distributed *Cenococcum geophilum* Fr. (Dauphin et al., 2021) and the more valuable *Boletus edulis* Bull. (Tremble et al., 2022). In this work we re-sequenced and analysed 27 genomes of *T. borchii* to study its population structure on a regional scale in Sardinia in comparison with a limited set of specimens from central and southern Italy. Molecular barcoding confirmed that all analysed isolates belonged to haplotype H1. Consequently, the dataset does not include representatives of the haplotype H2 described by Bonuso et al. (2010). This likely reflects the dominance of haplotype H1 across most of the Italian peninsula, as previously reported. It should be noted that variant calling was performed in single-sample mode followed by merging into a multi-sample VCF, rather than using a joint genotyping approach. While this strategy is commonly adopted in non-model organisms, it may affect the consistency of genotype calls across samples, particularly for low-frequency variants and missing data, and should therefore be considered a potential limitation when interpreting fine-scale patterns of genetic variation. We observed a weak but significant separation by distance of *T. borchii* samples from the different geographic areas although these differences stay within the intraspecific diversity. Sardinian samples taken together seem to be genetically divergent from continental provenances, although partial overlap is observed. The two southern samples (Calabria region; TBo38 and TBo39) form a closely related pair but do not appear genetically isolated from the rest of the dataset, instead showing connectivity with other samples. These patterns should also be interpreted considering the unbalanced sampling design, with a higher number of Sardinian isolates compared to continental ones. This asymmetry may influence estimates of genetic structure and reduce the power to detect subtle patterns within the continental group. A geographical separation after analysis of genome SNPs was found also for *T. melanosporum* by Payen et al. (2015). They analyzed only 7 specimens but were able to identify 3 distinct clusters: one from northern France, the second from south-eastern France and Italy, and last including Spanish samples. Also, our results reinforce the hypothesis that SNPs can represent a useful diagnostic array to reveal geographical provenance of natural population of the *Tuber* genus. Considering only Sardinian samples we found several clades highly supported by approximately unbiased P values (> 90) but this clustering does not seem correlated to the host plant or the habitat type. The few genomic windows with elevated F_{ST} values greater than 0.25 observed in our dataset are unlikely to represent true reproductive barriers. They more plausibly reflect locally reduced diversity or weak selective pressures, a phenomenon widely documented in genome-wide divergence landscapes where heterogeneous differentiation emerges from processes occurring after speciation rather than from strict isolation (Cruickshank and Hahn, 2014). Interpreting low F_{ST} in *T. borchii* requires acknowledging that these metric decreases when within-population diversity is high. As a result, it

may underestimate differentiation even when ecological or demographic structure is present (Nei, 1973; Gregorius and Roberds, 1986; Charlesworth et al., 1997; Meirmans and Hedrick, 2011; Jakobsson et al., 2013). Low F_{ST} therefore does not imply biological homogeneity. It is instead consistent with a species characterized by strong internal polymorphism, broad ecological amplitude, and a shallow but detectable structure between island and continental lineages, which may reflect historical connectivity rather than direct evidence of ongoing gene flow. The slightly higher between-group IBS distance (0.0964) compared to within-population values (0.078–0.083) supports the presence of early geographic divergence between Sardinian and continental samples, although it does not indicate reproductive isolation. Comparable patterns have been reported in other ectomycorrhizal fungi. Studies on *T. melanosporum* documented pronounced fine-scale structure at the level of individual truffle grounds but only modest differentiation between spontaneous and cultivated populations across regions (Taschen et al., 2016). Similarly, populations of *Suillus luteus* (L.) Roussel introduced into Patagonian pine plantations show moderate local diversity but extremely weak differentiation between sites separated by hundreds of kilometres. This pattern has been attributed to anthropogenic movement of inoculum, nursery material, and plantation practices (Pildain et al., 2021). These results illustrate how extensive human-assisted dispersal can maintain high connectivity across landscapes, effectively reducing large-scale genetic structure even in organisms with limited natural dispersal capabilities. In hypogeous fungi such as *Tuber*, natural dispersal constraints are particularly strong. Ascospores are dispersed exclusively through animal mycophagy, which restricts their movement to local scales and makes long-distance transport across sea barriers highly improbable. Under purely natural dynamics, one would therefore expect a more pronounced genomic separation between Sardinian and mainland populations. The differentiation observed in our data is unlikely to reflect natural gene flow that could be affected by human forestry activity. Historical reforestation campaigns, the movement of *Pinus* seedlings from continental nurseries, translocation of soils and inoculated roots, and the circulation of truffles for cultivation have likely facilitated sporadic gene flow between regions that would otherwise remain genetically isolated. These processes can homogenize genomic backgrounds across distant localities, preventing deeper divergence. However, it should be also considered that cultivation of *T. borchii* has been limited so far with respect to *T. melanosporum* or other edible ectomycorrhizal fungi. These results have important implications for conservation, traceability, and the sustainable economy of the bianchetto truffle. For traceability and fraud prevention, even shallow genomic structure, when supported by high-resolution SNP datasets, can provide a foundation for developing diagnostic markers capable of distinguishing truffles from different regions, host associations, or soil environments. Establishing such markers is crucial for valorising the truffle supply chain, improving the cultivation and

conservation strategies and supporting local economies. The genomic characterization of Sardinian isolates, historically underrepresented in the literature, provides essential baseline information for future studies linking genetic variation to functional traits. In this context, phylogenomics represents a powerful and promising approach to address key questions in fungal research, including evolutionary history, ecological resilience and genetic diversity.

Chapter 4

Genomics of cultivated *Pleurotus eryngii* strains

Introduction

Genomic resources for *Pleurotus* spp. represent a fundamental starting point to improve cultivation strategies and for understanding the genetic basis of metabolic performance, stress responses, and environmental adaptation. Genome sequencing and comparative genomics have increasingly been applied to *Pleurotus* species to investigate the evolution of lignocellulolytic systems, ecological strategies, and strain-level functional divergence. Recent studies have revealed substantial variation in gene content, regulatory architectures, and epigenomic features among strains, contributing to phenotypic and metabolic diversity within this commercially and ecologically important genus (Dai et al., 2019). *Pleurotus eryngii* is widely recognized as part of a species complex, characterized by pronounced intraspecific variability and a broad geographical distribution (Stajić et al., 2009). The biological complexity of this mushroom group has long complicated species delimitation and the genome-scale approaches of investigation will resolve taxonomic ambiguities and explore the whole intraspecific diversity. A notable contribution to the genomic characterization of *P. eryngii* was provided by et Zhang et al. (2019), who performed whole-genome sequencing of *P. eryngii* and *P. toluensis* using PacBio RS II long-read technology. The resulting genome assemblies sized approximately 49.9 Mb for *P. eryngii* and 48.2 Mb for *P. nebrodensis*. Phylogenetic and epigenomic analyses, including comparisons with *P. ostreatus*, demonstrated that *P. nebrodensis* represents an independent evolutionary lineage within the *P. eryngii* species complex. Despite these advances, genomic coverage within the genus *Pleurotus* remains uneven. To date, only 43 genome assemblies representing 14 *Pleurotus* species have been deposited in GenBank, with highly variable levels of assembly completeness and annotation quality (Table 4.1, accessed December 2025).

Table 4.1. List of *Pleurotus* genome assemblies currently available in GenBank (accessed December 2025), with information on accession number, species, strain, assembly level, release date, and number of scaffolds/contigs.

Accession	Species	Strain	Assembly Level	Release Date	Scaffold n.
GCA_017312325.1	<i>P. citrinopileatus</i>	HfpriPC05-1-Y1	Contig	02/03/2021	88
GCA_003314595.1	<i>P. citrinopileatus</i>	MG63	Scaffold	11/07/2018	10689
GCA_019677325.2	<i>P. cornucopiae</i>	CCMSSC00406	Chromosome	25/08/2021	11
GCA_029747585.1	<i>P. djamor</i>	MPG-05	Scaffold	14/04/2023	2603
GCA_049864135.1	<i>P. djamor</i>	PL01-White	Contig	23/04/2025	38658
GCA_003243765.1	<i>P. eryngii</i>	JKXB130DA	Contig	18/06/2018	153
GCA_001717165.1	<i>P. eryngii</i>	183	Scaffold	30/08/2016	166
GCA_015484515.1	<i>P. eryngii</i>	ATCC 90797	Scaffold	15/11/2020	609
GCA_003314755.1	<i>P. eryngii</i>	MG61	Scaffold	11/07/2018	104407
GCA_029874175.1	<i>P. eryngii</i> var. <i>elaeoselini</i>	CCMJ2131	Contig	26/04/2023	42
GCA_029467805.1	<i>P. eryngii</i> var. <i>eryngii</i>	CCMJ2132	Contig	29/03/2023	47
GCA_029467795.1	<i>P. eryngii</i> var. <i>ferulae</i>	CCMJ970	Contig	29/03/2023	51
GCA_019395345.1	<i>P. floridanus</i>	CCMSSC00406	Scaffold	29/07/2021	425
GCA_032158425.1	<i>P. giganteus</i>	PG46	Contig	28/09/2023	16
GCA_036873075.1	<i>P. giganteus</i>	zhudugu2	Contig	26/02/2024	27
GCA_005298045.1	<i>P. ostreatoroseus</i>	DPUA 1720	Contig	13/05/2019	619
GCA_014466165.1	<i>P. ostreatus</i>	PC9	Contig	03/09/2020	16
GCF_014466165.1	<i>P. ostreatus</i>	PC9	Contig	03/09/2020	16
GCA_947034855.1	<i>P. ostreatus</i>		Chromosome	07/10/2022	14
GCA_051307915.1	<i>P. ostreatus</i>	PC80	Chromosome	14/07/2025	14
GCA_029852705.2	<i>P. ostreatus</i>	PC9.15	Chromosome	15/05/2023	13
GCA_003313235.2	<i>P. ostreatus</i>	CCMSSC03989	Chromosome	07/06/2019	89
GCA_001956935.2	<i>P. ostreatus</i>	CCMSSC00389	Chromosome	07/06/2019	136
GCA_947034875.1	<i>P. ostreatus</i>		Contig	08/10/2022	53
GCA_000697685.1	<i>P. ostreatus</i>	PC15	Scaffold	05/06/2014	12
GCA_024195665.1	<i>P. ostreatus</i>	595	Contig	08/07/2022	329
GCA_003313735.1	<i>P. platypus</i>	MG11	Scaffold	11/07/2018	6484
GCA_012980535.1	<i>P. pulmonarius</i>	PM_ss5	Contig	07/05/2020	23
GCA_012979565.1	<i>P. pulmonarius</i>	PM_ss13	Contig	07/05/2020	17
GCA_012980525.1	<i>P. pulmonarius</i>	PM_ss2	Contig	07/05/2020	23
GCA_032158505.1	<i>P. pulmonarius</i>	CCMSSC 04423	Contig	28/09/2023	111
GCA_047301645.1	<i>P. pulmonarius</i>	LGAM 28684	Scaffold	29/01/2025	2651
GCA_002583695.1	<i>P. salmoneostramineus</i>	N60	Scaffold	04/10/2017	1372
GCA_002933715.1	<i>P. salmoneostramineus</i>	N4	Scaffold	25/01/2018	1597
GCA_001742905.1	<i>P. salmoneostramineus</i>	NBRC 31859	Scaffold	09/08/2016	26934
GCA_026743855.1	<i>P. sapidus</i>	DSM 8266	Contig	08/12/2022	63
GCA_014058305.1	<i>P. tuber-regium</i>	ACCC 50657-18	Contig	04/08/2020	65
GCA_019693315.1	<i>P. tuber-regium</i>	JN18	Contig	18/08/2021	123
GCA_003314355.1	<i>P. tuber-regium</i>	KL18	Scaffold	11/07/2018	1283
GCA_036872985.1	<i>P. tuoliensis</i>	Ptuo-s1	Contig	26/02/2024	14
GCA_029874165.1	<i>P. tuoliensis</i>	CCMJ1077	Contig	26/04/2023	34
GCA_003243755.1	<i>P. tuoliensis</i>	JKBL130LB	Contig	18/06/2018	106
GCA_002847145.1	<i>P. tuoliensis</i>	CCMSSC00489P1	Scaffold	22/12/2017	1409
GCA_003313115.1	<i>P. tuoliensis</i>	MG79	Scaffold	11/07/2018	7458

Pleurotus ostreatus is the most extensively represented species, with genomes available for ten strains, followed by *P. eryngii*, for which seven genomes have been published, including three formally recognized varieties (*P. eryngii* var. *elaeoselini*, var. *eryngii*, and var. *ferulae*). For most other *Pleurotus* species, only one or two genome assemblies are currently available, severely limiting comparative and population-level genomic analyses (Villani et al., 2025). This fragmented genomic scenario highlights the need for integrative studies combining experimental data and genomic resources. In this context, the present work leverages selected genomic datasets to support downstream comparative and functional analyses within a broader framework of physiological and biotechnological investigation. A key genomic resource for *P. eryngii* is the standard draft genome of strain ATCC 90797, generated within the framework of the DOE Joint Genome Institute (JGI) and made publicly available through the MycoCosm portal. This genome was generated within the JGI proposal “Study of the lignocellulolytic machinery in saprobic wood and leaf litter degrading Agaricales” and has been extensively used to investigate the evolution of fungal lifestyles and ligninolytic enzyme systems. Comparative genomic analyses based on this assembly have provided key insights into the diversification of class II peroxidases and other oxidative enzymes involved in lignin degradation (Ruiz-Dueñas et al., 2021). The genome of the ATCC 90797 isolate was sequenced using Illumina technology and assembled with the AllPathsLG pipeline, resulting in a standard draft assembly released in 2015. The assembly has a total size of 44.61 Mb, with an average sequencing depth of 96.7×, and is composed of 1,684 contigs organized into 609 scaffolds, of which 487 exceed 2 kb in length. The scaffold N50 is 54 kb, with the three largest scaffolds measuring 1.22, 1.02, and 0.83 Mb, respectively. Gaps account for approximately 3.0% of the total scaffold length. Gene prediction and annotation identified 15,954 protein-coding genes, supported by extensive transcriptomic evidence, as indicated by the high proportion of expressed sequence tags (ESTs) mapping to the genome (97.8%). The availability of a well-annotated gene set makes this genome a valuable resource for functional and comparative analyses. A second high-quality genomic resource considered in the present study is the reference genome assembly of *Pleurotus eryngii* var. *eryngii*, deposited under accession GCA_029467805.1 in the NCBI database and associated with BioProject PRJNA529267. This assembly corresponds to strain CCMJ2132, sequenced and assembled by Jilin Agricultural University and released in March 2023. The assembled genome comprises 47 scaffolds with a total size of approximately 53.46 Mb and a GC content of 49.5%, reflecting a relatively contiguous and complete representation of the genomic landscape for this varietal taxon. Although gene counts are not explicitly reported in the current NCBI summary, the scaffold number and assembly size indicate a substantial improvement in continuity compared to earlier draft genomes. Owing to its higher assembly continuity and reduced fragmentation compared to earlier draft

genomes, the GCA_029467805.1 assembly was selected as the primary reference framework for the genomic analyses conducted in the present work. In addition, its taxonomic assignment to *Pleurotus eryngii* var. *eryngii* makes it particularly suitable for comparative analyses of closely related strains. The adoption of this reference genome complements the draft assembly of strain ATCC 90797, strengthening the genomic backbone for integrative analyses presented in this thesis. Within this context, the present study was developed in the framework of a PNRR PhD project aimed at the genomic characterization of cultivated strains of *P. eryngii* (cardoncello) to support the optimization of production processes and the development of high-added-value products. The analyzed cultivated strains were provided by the industrial partner NaturAgri S.r.l. (Matera) and represent genotypes currently employed in commercial production systems. To place these strains within a broader evolutionary and genomic context, a wild-type strain was included as a comparative reference, allowing the investigation of genomic variation and strain-specific features in a comparative framework, with particular attention to differences among cultivated and wild-type isolates. By integrating industrially relevant genotypes with a wild reference, this work aims to elucidate the genomic basis underlying phenotypic variability and functional traits of interest for both production efficiency and biotechnological applications.

Material and Methods

Fungal strain collection

Five strains of *Pleurotus eryngii* were included in this study. Strain P1, representing the wild-type isolate, was obtained from a basidioma collected on a wild *Ferula communis* plant in Avezzano (Abruzzo, Italy) in November 2023. The other strains (P2, P3, P4, and P6) were provided by NaturAgri S.r.l. (Basilicata, Italy). Among them, P2, P3, and P4 were classified as slow-growing industrial strains, whereas P6 was classified as a fast-growing strain. This classification was based on phenotypic observations provided by the industrial partner, referring to mycelial growth rate and production cycle length under standardized cultivation conditions, with fast-growing strains typically producing two flushes and slow-growing strains up to four flushes. All isolates were analyzed in their dikaryotic state, corresponding to the vegetative mycelium used in commercial cultivation, and were not derived from monokaryotic isolates obtained from basidiospores. All strains were maintained on potato dextrose agar (PDA) in the dark at 22°C.

Molecular analyses

To confirm species identity of the pure cultures, the internal transcribed spacer (ITS) region of the ribosomal DNA was amplified by direct PCR using the universal primers ITS1F and ITS4 (White et al., 1990; Gardes & Bruns, 1993). PCR reactions were performed in a final volume of 50 μ L, containing a small portion of fungal tissue directly as template, 1 $\times$ Takara Ex Taq DNA polymerase buffer, 40 μ g of BSA, and 350 nM of each primer. Amplifications were carried out in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific). PCR products were verified by agarose gel electrophoresis and subjected to Sanger sequencing in both directions using primers ITS5 (White et al., 1990) and ITSi (Redecker et al., 2003) at Microsyth (Balgach, Switzerland). The resulting sequences were compared against the NCBI GenBank database using BLASTn to confirm the taxonomic identity of each specimen. Genomic DNA was extracted from lyophilized mycelium using the DNeasy Plant Mini Kit (Qiagen), following the manufacturer's protocol optimized for fungal material. DNA quality and concentration were assessed by measuring 260/280 and 260/230 optical density (OD) ratios using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and by electrophoresis on a 1.5% agarose gel. DNA concentration was further quantified using a Qubit Fluorometer (Invitrogen) with the High Sensitivity dsDNA Assay Kit. High-quality genomic DNA (concentration > 20 ng mL⁻¹, OD ratios > 1.4, no smear on agarose gel) from all *P. eryngii* strains was sent to BIODIVERSA S.r.l. (Treviso, Italy) for Illumina paired-end sequencing (2 $\times$ 150 bp) on a NovaSeq 6000 platform. Library preparation was performed using standard Illumina-compatible workflows according to the manufacturer's instructions. Sequencing generated an average of approximately 2 Gb of data per sample, corresponding to an estimated genome-wide coverage of $\sim$ 40 $\times$ (Q30 > 90%).

Genomic analyses

All computational analyses were performed in a Linux environment using the High-Performance Computing (HPC) facilities of the Department of Plant, Soil and Microbial Sciences, Michigan State University, and the Department of Information Engineering, Computer Science and Mathematics (DISIM), University of L'Aquila. Activities were executed on the *Caliban* cluster and managed through the SLURM workload manager, employing multicore CPU parallel processing. Software environments were configured via *Anaconda* (v23.9.0; Anaconda, Inc., Austin, TX, USA) to ensure reproducibility and dependency control. All statistical, genomic, and graphical analyses were performed in *R* (v4.5.1; R Core Team, 2025) within the *RStudio* environment (v2025.05.1+513; Posit Software, PBC, Boston, MA, USA).

Read quality control, alignment, and variant calling

Raw paired-end Illumina reads (R1 and R2) generated from NovaSeq 6000 platform (2×150 bp) were first subjected to quality assessment using FastQC v0.12.1 (Andrews, 2010). Trimming and quality filtering were performed using fastp v0.23.2 (Chen et al., 2018; Chen, 2023) with default parameters, including automatic adapter detection, sliding-window trimming for Phred quality < 20 , and removal of reads shorter than 15 bp. Cleaned reads were aligned to the *P. eryngii* var. *eryngii* reference genome (assembly GCA_029467805.1; strain CCMJ2132; assembly name ASM2946780v1, Jilin Agricultural University, 2023) using BWA-MEM v0.7.17 (Li, 2013) with default parameters. The choice of the GCA_029467805.1 reference genome was supported by its higher assembly continuity and reduced fragmentation compared to earlier draft genomes. In addition, preliminary alignment tests indicated a consistently high mapping rate of sequencing reads from the analyzed strains to this reference, confirming its suitability for variant calling and downstream genomic analyses. Alignment files in SAM format were converted to BAM, sorted, and indexed using SAMtools v1.6 (Li et al., 2009).

Variant calling was performed using the HaplotypeCaller module of the Genome Analysis Toolkit (GATK) v4.6.1.0 (McKenna et al., 2010; Van der Auwera & O'Connor, 2020) in single-sample mode, independently processing each BAM file to generate individual VCF outputs. Variants were called against the *P. eryngii* reference genome (GCA_029467805.1). Variant quality control was carried out using the VariantFiltration tool of GATK v4.6.1.0 to retain only high-confidence variants. Filtering thresholds ($QUAL < 20.0$, $DP < 15.0$, $MQ < 40.0$, $QD < 5.0$, $FS > 60.0$) were applied to remove low-quality, low-depth, or poorly supported sites, following GATK best-practice recommendations for non-model eukaryotic genomes. Only variants marked as PASS were retained for downstream analyses. PASS-filtered VCF files were merged into a multi-sample dataset using bcftools (v1.3; Li et al., 2009; Danecek et al., 2021). Per-sample variant statistics were computed by considering only PASS sites with non-missing genotypes ($GT \neq \text{"./."}$). Variant types were assigned based on the reference allele and the first alternate allele, classifying sites as SNPs, INDELs, or OTHER (e.g., complex or multi-nucleotide substitutions). The transition/transversion (Ts/Tv) ratio was calculated exclusively on canonical single nucleotide substitutions ($A \leftrightarrow G$ and $C \leftrightarrow T$) among SNP sites. Mean sequencing depth was computed as the average per-sample FORMAT/DP across PASS variant sites, while mean variant quality was derived from the QUAL field of the VCF. The resulting high-confidence variant dataset was used for all downstream population genomic and comparative analyses.

SNP filtering and distance-based clustering

Single nucleotide polymorphisms (SNPs) were extracted from the merged PASS VCF and restricted to biallelic sites to generate a dataset suitable for downstream population-level analyses. Additional filtering and pruning steps were subsequently applied to obtain a clean, informative, and non-redundant SNP dataset for multivariate and distance-based analyses. Biallelic SNPs were retained using `bcftools view -m2 -M2 -v snps`. All variant filtering and dataset preparation steps were performed using `bcftools v1.3`. The filtering strategy included:

1. **Minor Allele Frequency (MAF > 0.10)** Rare variants (e.g., singletons present in only one sample) were excluded as they are often uninformative or error-prone, especially with small sample sizes ($n = 5$). A MAF threshold of 0.10 ensured that retained SNPs were present in at least two individuals.
2. **Missingness filter ($\leq 20\%$)** Variants with excessive missing genotypes (i.e., not called in ≥ 2 out of 5 samples) were discarded to avoid unreliable sites and maximize interpretability.
3. **Linkage Disequilibrium (LD) pruning ($r^2 \leq 0.2$)** To eliminate redundancy, highly correlated SNPs were pruned based on pairwise LD (r^2), retaining only quasi-independent loci suitable for population structure analyses.

Following filtering, the VCF was normalized to split multi-allelic variants. The cleaned VCF was converted into GDS format using the `snpgdsVCF2GDS` function from the *SNPRelate* R package v1.42.0 (Zheng et al., 2012).

Both Principal Component Analysis (PCA) and Identity-by-State (IBS) distance calculations were conducted on the LD-pruned SNP set within the RStudio environment, using the packages *SNPRelate*, *vcfR* (v1.15.0; Knaus & Grünwald, 2017), *ape* (v5.8-1; Paradis & Schliep, 2019), *phangorn* (v2.12.1; Schliep, 2011), *ggplot2* (v3.5.2; Wickham, 2016), *tidyverse* (v2.0.0; Wickham et al., 2019), and *dplyr* (v1.1.4; Wickham et al., 2025). IBS-based hierarchical clustering was performed using the `ward.D2` method, which minimizes intra-cluster variance and maximizes inter-cluster separation, providing a robust genetic stratification among isolates.

Variant annotation and functional impact analysis

Input dataset

Single-nucleotide polymorphism (SNPs) and short insertions/deletions (indels) were analyzed across five *Pleurotus eryngii* strains (wild-type P1; industrial low-growth strains P2, P3, P4, and fast-growth P6) using a multi-sample VCF obtained after variant calling, hard filtering, and merging of per-sample callsets. Only variants flagged as PASS were retained for downstream annotation and

comparative analyses. Variant calling and primary filtering were performed as described in Section Read quality control, alignment, and variant calling.

Reference genome and projected gene annotation

All downstream analyses were performed using the *Pleurotus eryngii* var. *eryngii* reference assembly (GCA_029467805.1; ASM2946780v1; strain CCMJ2132) as the coordinate system for variant calling and functional interpretation. Because an official curated GFF3 annotation was not available for this assembly at the time of analysis, gene models derived from the JGI *Pleurotus eryngii* annotation (Pleery1; ATCC90797) were used as a coordinate-consistent projected annotation on the CCMJ2132 reference (genes.gff). This annotation was adopted as the common gene model set for variant-effect prediction and downstream gene-level analyses.

Construction of a custom SnpEff database

Functional annotation of variants was performed using SnpEff v5.2f (build 2025-02-07) executed under OpenJDK 21. A custom SnpEff database (dataset label: pleurotus_CNv4) was built using (i) the reference genome FASTA and (ii) the projected gene annotation (genes.gff) described above. The resulting SnpEff predictor was then used to annotate variant effects across all strains.

Variant effect prediction (SnpEff annotation)

The merged multi-sample PASS VCF file was annotated with SnpEff using the pleurotus_CNv4 database, generating functional consequence terms for each variant. (e.g., missense_variant, synonymous_variant), predicted impact classes (MODIFIER, LOW, MODERATE, HIGH), and gene-level identifiers consistent with the projected annotation (e.g., gene_XXXXX). SnpEff produced an annotation-rich VCF containing the ANN field and an HTML report summarizing overall annotation metrics (e.g., number of annotations, warnings, and processed variants).

Filtering of coding and splice-related variants and VCF normalization

To focus on variants with potential functional relevance, the SnpEff-annotated VCF was filtered to retain only variants carrying coding or splice-proximal consequences in the ANN field, including missense_variant, synonymous_variant, stop_gained, stop_lost, start_lost, frameshift_variant, inframe insertions and deletions, as well as splice_acceptor_variant, splice_donor_variant, and splice_region_variant. This step generated a curated subset enriched for variants potentially affecting gene structure or protein sequence. To simplify downstream genotype-aware analyses and avoid complex multi-allelic genotype configurations, variants in which at least one sample carried a third or additional alternative allele (i.e., genotypes encoded with allele index “2” or higher in the GT field) were removed. The remaining multi-allelic variant records were then normalized by splitting them

into independent biallelic entries, ensuring a consistent site-based representation across all samples. Finally, symbolic placeholder alleles introduced by VCF representation (such as <NON_REF>) and spanning deletion alleles (*) were excluded, producing a fully cleaned dataset of coding and splice-related variants in a standardized biallelic format. The resulting file constituted the analysis-ready variant set used for all downstream parsing, genotype-aware comparisons, and gene-level aggregation. All compressed files were indexed with Tabix to enable efficient querying.

Extraction of genotype-aware variant tables

Variant-level information was exported from the cleaned coding/splice VCF using bcftools query into tab-delimited tables, including chromosome, position, reference and alternative alleles, the full SnpEff annotation field (ANN), and per-sample genotypes. These tables provided the input for custom parsing of functional consequences, classification of variant effect categories, and subsequent aggregation of variant counts at the gene and functional levels.

Parsing of SnpEff annotations and classification of functional classes

The ANN field was parsed using custom shell and awk scripts to extract, for each variant record, the associated gene identifier, functional consequence term, and predicted impact category. When multiple consequences were reported for a single variant (e.g., due to overlapping transcript models or composite annotations such as splice_region_variant&intron_variant), composite labels were retained for descriptive summaries. For gene-level counts analyses, “nonsynonymous coding” variants were conservatively defined as the union of missense_variant (MODERATE impact) and high-impact coding consequences, including frameshift_variant, stop_gained, stop_lost, and start_lost. Splice-proximal variants were retained in the curated dataset for completeness but were excluded from the nonsynonymous count metric due to their heterogeneous and often uncertain functional impact.

Gene-level aggregation of synonymous and nonsynonymous variants

Synonymous and nonsynonymous variants were analyzed separately at the gene and functional-category levels to characterize the distribution of coding variation across *Pleurotus eryngii* strains. From the curated SnpEff-annotated coding and splice-related dataset, variants were classified as synonymous (synonymous_variant) or nonsynonymous, conservatively defined as missense_variant and high-impact coding consequences, including stop_gained, stop_lost, start_lost, and frameshift_variant. For each gene, synonymous and nonsynonymous variant counts were quantified by counting distinct biallelic variant sites associated with that gene and showing at least one non-

reference genotype in a given strain. Thus, each polymorphic site was counted once per gene regardless of zygosity, and counts reflect the presence of variant positions rather than allele copy number. To explore functional patterns, gene-level variant counts were further summarized at the Gene Ontology (GO) level by aggregating synonymous and nonsynonymous sites across all genes annotated with a given GO term. To ensure robustness and comparability across functional categories, only GO terms represented by at least five annotated genes ($\text{minG} = 5$) and shared by at least three strains were retained for downstream descriptive analyses. For each retained GO category, the total number of synonymous and nonsynonymous variant sites was calculated and normalized by the number of annotated genes, yielding mean variant counts per gene for each functional class. These GO-level summaries were used exclusively as descriptive representations of the functional landscape of coding variation and were not intended as formal tests of selection or enrichment.

Gene product mapping for candidate gene tables

To facilitate the biological interpretation of genes showing the strongest differences in nonsynonymous variant counts in the pairwise comparisons, gene product descriptions were extracted from the projected GFF3 annotation (genes.gff) using the `product_name` attribute, generating a two-column mapping table linking gene identifiers to functional product labels. This mapping was joined to candidate gene tables using standard UNIX-based operations, producing final outputs reporting gene IDs, nonsynonymous variant metrics, and associated product descriptions. Gene Ontology (GO) terms were assigned to projected gene models based on Blast2GO functional annotation and mapped to gene identifiers using transcript- and protein-to-gene relationships defined in the projected GFF3 annotation.

*Functional annotation of the *Pleurotus* genome*

To obtain an updated functional annotation framework for the *Pleurotus eryngii* CCMJ2132 reference genome, predicted coding sequences (CDS) were annotated through sequence similarity searches against the NCBI RefSeq protein database using DIAMOND v2.0.14.152 in blastx mode. Up to five top hits were retained per query, applying an e-value threshold of $<1 \times 10^{-4}$. DIAMOND outputs were processed in Blast2GO v5.1 to perform Gene Ontology (GO) mapping and functional assignment following the standard annotation workflow. GO annotations were subsequently mapped back to projected gene identifiers using the relationships defined in the projected GFF3 annotation. Consolidated gene-to-GO and GO ID-to-name lookup tables were generated and used for all downstream functional summaries and descriptive analyses. Gene product descriptions were additionally extracted to support interpretation of GO-associated gene sets.

Identification of shared GO categories across strains

To characterize the functional distribution of coding variation across *Pleurotus eryngii* strains, a Gene Ontology (GO)-based analysis was performed explicitly distinguishing between synonymous and nonsynonymous variants. Variant sites were classified according to their predicted amino acid effect as synonymous or nonsynonymous. For each strain and each GO category, the following metrics were calculated: (i) the number of genes annotated to the category (n_genes); (ii) the number of distinct sites carrying synonymous variants (syn_sites); and (iii) the number of distinct sites carrying nonsynonymous variants (nonsyn_sites). To allow direct comparison among GO categories with different gene counts, site counts were normalized by the number of annotated genes, yielding the average number of synonymous and nonsynonymous variant sites per gene (syn_per_gene and nonsyn_per_gene, respectively). The analysis focused on functional categories shared by at least three strains, including GO terms conserved across all strains as well as those shared by specific subsets of four or three strains. To prioritize the most variant-rich functional classes while maintaining robustness, GO terms were retained if they ranked among the top 30 categories based on total coding variant load per gene (synonymous + nonsynonymous), with a minimum of five annotated genes per GO category (minG = 5), in at least three strains (“sharedAtLeast3”). For each retained GO term, mean synonymous and nonsynonymous variant counts per gene were calculated by averaging syn_per_gene and nonsyn_per_gene values across only the strains in which the category was present. The set of strains contributing to each GO category defined the corresponding “Shared_in” group reported in summary tables.

Per-gene nonsynonymous variant counts between strains

To identify genes displaying differential accumulation of nonsynonymous coding variants among strains, pairwise comparisons were conducted using per-gene nonsynonymous variant counts. For each gene and each strain, the number of nonsynonymous variants was quantified by counting distinct biallelic variant sites exhibiting at least one non-reference genotype ($GT \neq 0/0$), thus representing the presence of variant positions rather than genotype dosage. Sites with missing genotype calls (./.) were treated as missing data and did not contribute to the count calculation; accordingly, genes for which no genotype information was available in a given strain were reported as NA in the corresponding tables, while zero values indicate the absence of observed variants at positions with available genotype information. Pairwise contrasts were computed as per-gene differences in nonsynonymous variant counts (Δ), defined as the count in one strain minus that in the comparison strain. Positive Δ values indicate enrichment of nonsynonymous variants in the first strain, whereas negative values indicate higher counts in the second strain. For each contrast, genes were ranked by the absolute value

of Δ , and top-ranked genes were retained for descriptive and functional interpretation. To avoid pivot-related artifacts when summarizing top-ranked genes across multiple contrasts, the final wide-format tables were rebuilt directly from the gene-by-strain count matrix prior to annotation joins. Pairwise analyses were performed both between the wild-type strain (P1) and each industrial strain (P2, P3, P4, and P6), and among industrial strains with contrasting growth phenotypes, comparing the fast-growing strain (P6) with the slow-growing strains (P2, P3, and P4). This strategy allowed strain-specific divergence patterns to be assessed without aggregation across groups. Exploratory group-based summaries were initially generated during pipeline development to evaluate robustness; however, all final interpretations were based exclusively on strain-level pairwise contrasts due to the limited number of available strains.

Results

Read quality control, alignment, and variant calling

Comparison between raw variant calls obtained from GATK HaplotypeCaller and the final filtered PASS dataset showed a reduction of approximately 15–25% in the total number of variants, accompanied by a marked increase in mean QUAL values (approximately 50–100%) and sequencing depth (approximately 15–30%). Transition/transversion ratios remained consistently high after filtering, indicating that variant quality was substantially improved without introducing systematic bias. After variant calling and quality filtering, between 956,762 and 1,236,167 high-confidence (PASS) variant sites with non-missing genotypes were retained per *Pleurotus eryngii* isolate (Table 4.2). Single nucleotide polymorphisms (SNPs) represented the majority of detected variants (approximately 89–90%), while insertion–deletion variants (INDELs) accounted for 8–10% of sites. A minor fraction (1–2%) consisted of other variant types, including complex or multi-nucleotide substitutions. The transition/transversion (Ts/Tv) ratio ranged from 3.79 to 5.01 across isolates, indicating a low false-positive rate. Mean sequencing depth at PASS variant sites ranged from 30.7× to 54.6×, and mean QUAL scores were consistently high across samples. The resulting high-confidence variant dataset was used as the basis for all downstream population genomic and comparative analyses.

Table 4.2. Summary statistics of high-confidence (PASS) variants across *P. eryngii* isolates

Sample	Total variants	SNPs	indels	other	TS	TV	TS/TV	MEAN_DP	MEAN_QUAL
P1	1,101,084	986,905	98,911	15,268	822,588	164,317	5.006104	30.87	1100.87
P2	1,196,678	1,061,711	118,739	16,228	849,935	211,776	4.013368	45.15	1578.99
P3	1,140,015	1,012,782	112,044	15,189	808,359	204,423	3.954345	37.10	1615.54
P4	956,762	850,641	93,875	12,246	673,079	177,562	3.790670	30.67	1709.48
P6	1,236,167	1,102,046	117,695	16,426	890,157	211,889	4.201053	54.57	1527.46

Table note: Variant counts and summary statistics were computed on variants with FILTER = PASS only. Per-sample statistics were calculated considering PASS sites with non-missing genotypes (GT $\neq$ “./.”). Variant types (SNP, INDEL, OTHER) were assigned based on the reference allele and the first alternate allele. Mean depth refers to per-sample FORMAT/DP.

SNP filtering and distance-based clustering

After successive filtering steps, the number of retained SNPs was progressively reduced from over 2 million biallelic variants to approximately 465,000 quality-filtered SNPs, and finally to 32,092 LD-pruned SNPs suitable for population structure analyses (Table 4.3).

Table 4.3. Reduction of SNP datasets across successive quality-control and LD-pruning steps.

Filtering Stage	SNP Count
Biallelic VCF	2,145,602
QC SNP (MAF > 0.10, missing $\leq$ 0.20)	465,401
LD-pruned SNP ($r^2 \leq 0.2$)	32,092

The IBS-based hierarchical clustering (Fig. 4.1) distinguished the wild-type isolate (P1, collected from *Ferula* sp. in Avezzano) from most of the commercial strains (P2–P6) provided by NaturAgri S.r.l., revealing both intra- and inter-group genetic differentiation. Notably, the fast-growing strain P6 showed a closer affinity to P1 compared to the other commercial strains.

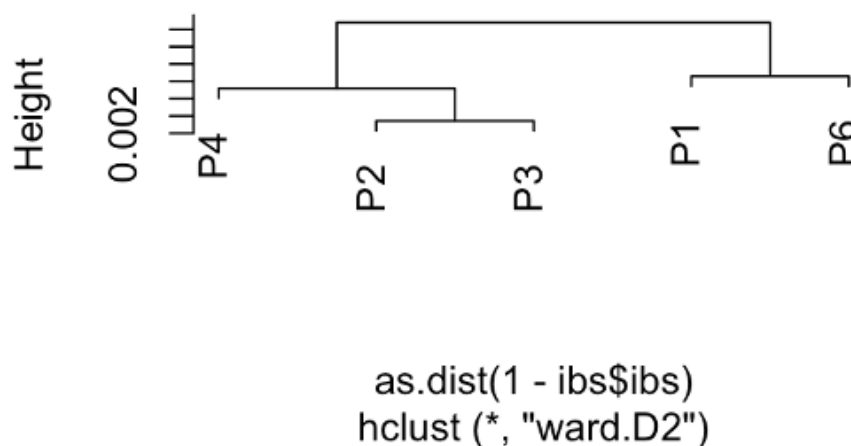


Fig. 4.1 Hierarchical clustering based on Identity-by-State (IBS) distances is calculated on 32 092 SNPs (QC+LD). Filters MAF > 0.10 and missing < 0.20.

The PCA (Fig. 4.2), computed on 32,092 LD-pruned SNPs ($MAF > 0.10$, $missing \leq 0.20$), confirmed this pattern. The three slow-growing commercial strains (*P2*, *P3*, *P4*) clustered tightly together, while the wild-type isolate (*P1*) and the fast-growing commercial strain (*P6*, *MIP*) were positioned on opposite sides along the first principal component (PC1).

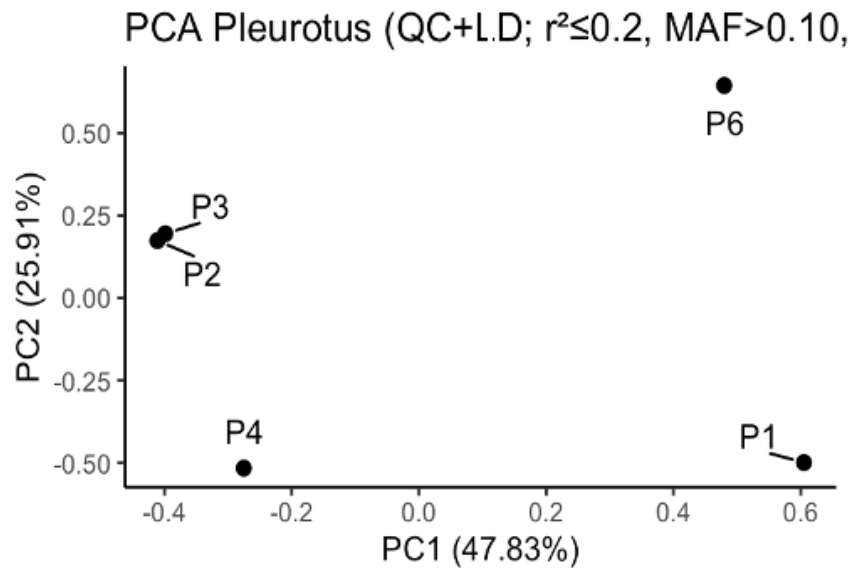


Fig. 4.2. PCA of *Pleurotus eryngii* isolates based on 32,092 LD-pruned SNPs ($MAF > 0.10$; $missing \leq 0.20$; $r^2 \leq 0.2$). The first two principal components reveal clear genetic differentiation between the wild-type isolate and commercial strains.

The explained variance was 47.8% for PC1 and 25.9% for PC2, indicating strong differentiation along the primary axis (Table 4.4).

Table 4.4. PCA coordinates of *Pleurotus eryngii* isolates along PC1–PC3

Sample	PC1	PC2	PC3
P1 (wild type)	0.605	−0.499	0.429
P2 (slow-growing)	−0.411	0.174	0.261
P3 (slow-growing)	−0.399	0.195	0.376
P4 (slow-growing)	−0.275	−0.516	−0.674
P6 (fast-growing)	0.480	0.645	−0.391

Both analyses support a clear genetic separation between slow-growing commercial strains and the two divergent isolates (*P1* and *P6*), reflecting intra-lineage homogeneity among slow growers and marked differentiation of the wild and fast-growing genotypes.

Total and coding variants

Variant annotation using SnpEff was first performed on the complete multi-sample PASS VCF, identifying a total of 2,419,913 high-quality variant sites across the analyzed strains, predominantly SNPs (~89%), followed by small insertions and deletions. Most variants were in non-coding regions, including intergenic and upstream/downstream regulatory contexts, whereas approximately 6.8% mapped to coding sequences. Functional consequence terms and predicted impact classes (MODIFIER, LOW, MODERATE, HIGH) were assigned to all variants through the ANN field. Because a single variant may generate multiple predicted effects due to alternative transcripts or composite functional consequences, impact-class distributions were summarized at the annotation level. Within the complete annotated dataset, the majority of predicted effects were classified as MODIFIER (9,464,920 annotations; 92.5%), reflecting the predominance of variants in non-coding regions. LOW-, MODERATE-, and HIGH-impact annotations accounted for 385,050 (3.8%), 331,883 (3.2%), and 53,286 (0.5%) entries, respectively. To focus on variants with potential functional relevance, the SnpEff-annotated VCF was subsequently filtered to retain only coding and splice-proximal variants, generating a curated and normalized dataset used for downstream analyses. Within this coding/splice subset, impact-class distributions shifted markedly, with MODIFIER effects accounting for 2,843,977 annotations (78.1%), while LOW-, MODERATE-, and HIGH-impact categories increased proportionally to 388,163 (10.7%), 346,858 (9.5%), and 62,027 (1.7%), respectively. This shift reflects the exclusion of most non-coding variants and the enrichment of functionally relevant consequences within gene-associated regions. Overall, the complete annotated dataset provides a genome-wide overview dominated by non-coding variation, whereas the curated coding/splice subset represents the analysis-ready variant collection used for all genotype-aware comparisons, gene-level aggregation, and functional interpretation.

Distribution of synonymous and nonsynonymous coding variants across functional categories

Functions involved in core cellular maintenance and fundamental cellular processes, including ATP hydrolysis activity, DNA repair, chromatin remodeling, and transcription-related processes, displayed the highest coding-variant loads per gene. Broad compartment-level categories such as plasma membrane, nucleus, and cytosol also accounted for large fractions of total coding variation, reflecting their extensive gene content (Table 4.5). At the strain level, commercial isolates account for a higher total number of coding and splice-related variant sites than the wild-type strain. The fast-growing industrial strain P6 exhibited the highest coding-variant count (421,267 sites), followed by P2 (399,558), P3 (389,591), and P4 (363,968), whereas the wild-type strain P1 showed the lowest number of coding variants (348,328 sites). Across all strains, synonymous variants outnumbered

nonsynonymous variants, a pattern commonly observed in coding regions and consistent with expectations under functional constraint (Fig. 4.3).

GO categories shared across all strains

These GO categories shared across all analyzed *P. eryngii* strains were primarily related to membrane transport and solute trafficking, including ABC-type transporter activity, ATPase-coupled transmembrane transporter activity, metal ion transport, and monoatomic cation transmembrane transport. Within these core functional classes, synonymous variants predominated over nonsynonymous variants, with mean nonsynonymous counts per gene generally ranging between approximately 15 and 25 sites (table 4.6). In addition to transport-related processes, GO categories shared across all strains encompassed essential cellular and structural functions such as ATP hydrolysis activity, ligase activity, serine-type peptidase activity, intracellular signal transduction, cytoskeleton organization, and microtubule binding (Figure 4.4). Notably, GO categories associated with genome maintenance and RNA metabolism, including helicase activity, single-stranded DNA binding, and double-strand break repair via homologous recombination, exhibited among the highest mean nonsynonymous variant counts per gene within the shared functional set. This indicates that these functional groups harbor relatively high coding variation across both wild-type and industrial lineages. A complete summary of shared GO categories and corresponding mean synonymous and nonsynonymous variant counts is reported in Table 4.6.

Table 4.5. GO terms with the highest number of variants in coding regions per gene (synonymous + nonsynonymous), considering only GO categories represented by at least 20 annotated genes. VS = mean variant sites per gene; NVS = non-synonymous variant sites per gene.

GO_ID	Gene number	Syn.	Nonsyn.	Total	VS	NVS	GO_name
GO:0016887	53	2528	1119	3647	68.8	21.1	ATP hydrolysis activity
GO:0006281	28	876	680	1556	55.6	24.3	DNA repair
GO:0009987	24	795	464	1259	52.5	19.3	cellular process
GO:0004553	24	730	483	1213	50.5	20.1	hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0006355	40	1301	720	2021	50.5	18.0	regulation of DNA- templated transcription
GO:0005524	188	6211	3198	9409	50.0	17.0	ATP binding
GO:0005886	69	2227	1223	3450	50.0	17.7	plasma membrane
GO:0006338	31	986	550	1536	49.5	17.7	chromatin remodeling
GO:0055085	96	2985	1564	4549	47.4	16.3	transmembrane transport
GO:0016705	29	804	565	1369	47.2	19.5	oxidoreductase activity
GO:0006508	48	1387	861	2248	46.8	17.9	proteolysis
GO:0004497	33	885	642	1527	46.3	19.5	monooxygenase activity

GO:0008168	21	520	445	965	4650	21.2	methyltransferase activity
GO:0005730	29	689	639	1328	45.8	22.0	nucleolus
GO:0016798	22	598	393	991	45.0	17.9	hydrolase activity, acting on glycosyl bonds
GO:0005829	108	3248	1594	4842	44.8	14.8	cytosol
GO:0050660	20	542	354	896	44.8	17.7	flavin adenine dinucleotide binding
GO:0004674	37	1139	504	1643	44.4	13.6	protein serine/threonine kinase activity
GO:0005506	33	849	614	1463	44.3	18.6	iron ion binding
GO:0016787	97	2705	1591	4296	44.3	16.4	hydrolase activity

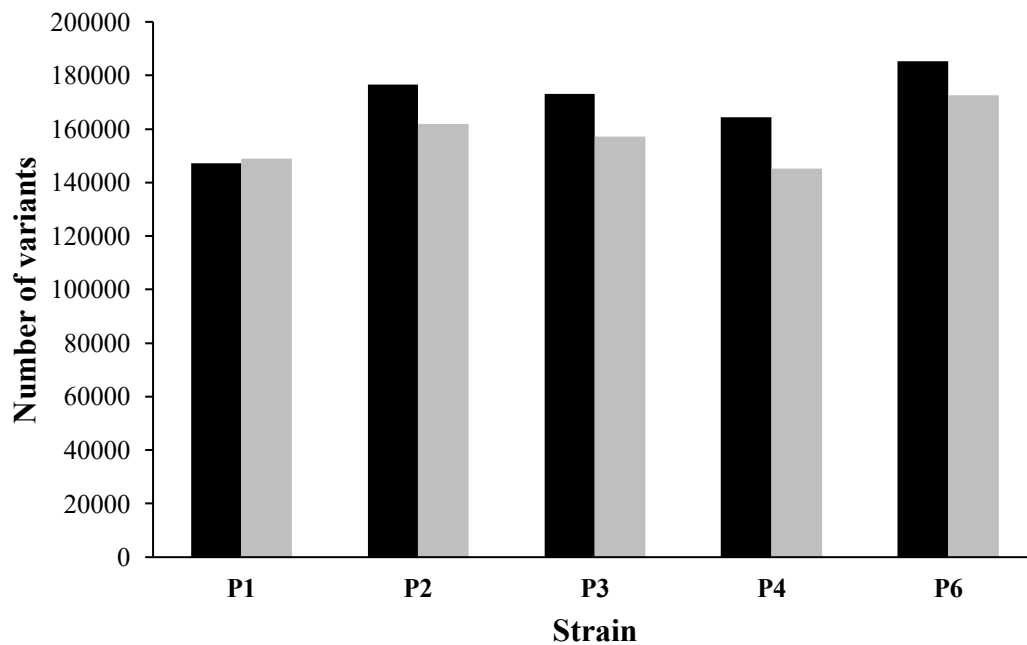


Figure 4.3. Global distribution of coding variants by strain. Histogram showing the total number of coding and splice-related variant sites identified in each strain (P1–P6), partitioned into synonymous (black columns) and nonsynonymous (gray columns) functional classes.

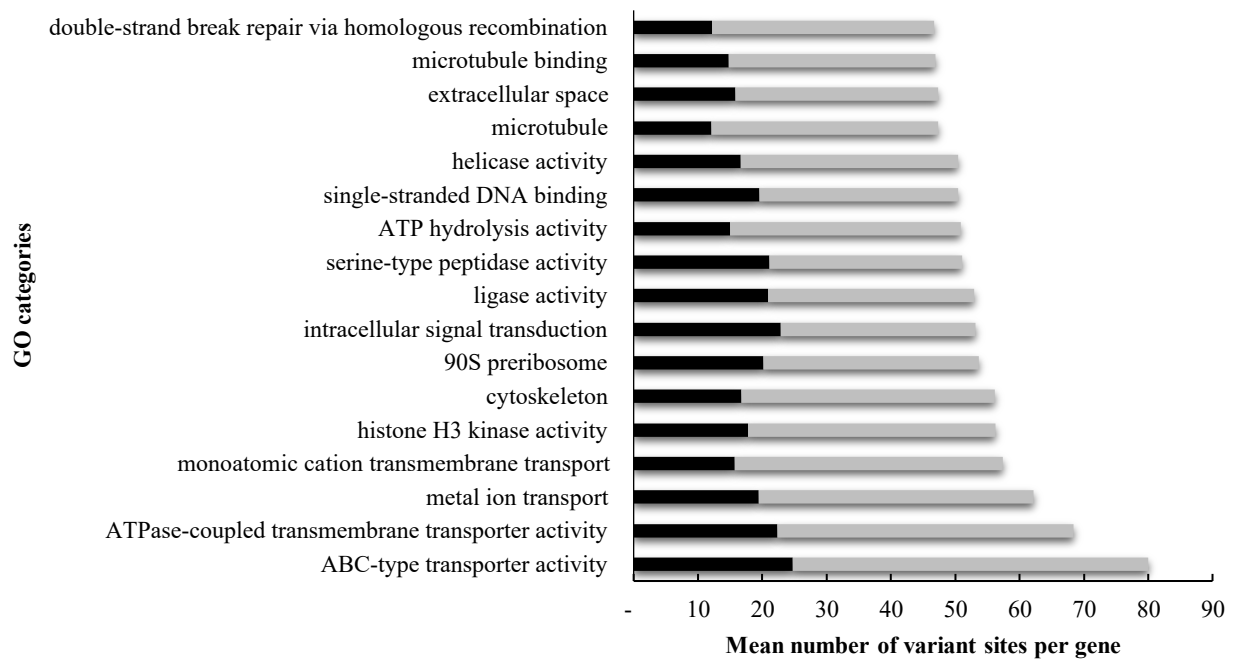


Figure 4.4. Mean nonsynonymous (black columns) and synonymous (gray columns) coding variant counts per gene across GO categories shared by all strains (P1–P2–P3–P4–P6).

Table 4.6. GO categories shared across all strains and specific strain subsets, with corresponding mean nonsynonymous (MNG) and synonymous (MSG) variants per gene. Terms recurrent across strains were selected by the following strategy: top-30 per strain, minG=5, shared in ≥ 3 strains.

GO	GO name	Shared in	MNG	MSG
GO:0140359	ABC-type transporter activity	P1-P2-P3-P4-P6	25	55
GO:0042626	ATPase-coupled transmembrane transporter activity	P1-P2-P3-P4-P6	22	46
GO:0030001	metal ion transport	P1-P2-P3-P4-P6	19	43
GO:0098655	monoatomic cation transmembrane transport	P1-P2-P3-P4-P6	16	42
GO:0140996	histone H3 kinase activity	P1-P2-P3-P4-P6	18	38
GO:0005856	cytoskeleton	P1-P2-P3-P4-P6	17	39
GO:0030686	90S preribosome	P1-P2-P3-P4-P6	20	33
GO:0035556	intracellular signal transduction	P1-P2-P3-P4-P6	23	30
GO:0016874	ligase activity	P1-P2-P3-P4-P6	21	32
GO:0008236	serine-type peptidase activity	P1-P2-P3-P4-P6	21	30
GO:0016887	ATP hydrolysis activity	P1-P2-P3-P4-P6	15	36
GO:0003697	single-stranded DNA binding	P1-P2-P3-P4-P6	20	31
GO:0004386	helicase activity	P1-P2-P3-P4-P6	17	34

GO:0005874	microtubule	P1-P2-P3-P4-P6	12	35
GO:0005615	extracellular space	P1-P2-P3-P4-P6	16	31
GO:0008017	microtubule binding	P1-P2-P3-P4-P6	15	32
GO:0000724	double-strand break repair via homologous recombination	P1-P2-P3-P4-P6	12	34
GO:0005096	GTPase activator activity	P2-P3-P4-P6	23	34
GO:0044550	secondary metabolite biosynthetic process	P2-P3-P4-P6	27	30
GO:0006874	intracellular calcium ion homeostasis	P2-P3-P4-P6	13	41
GO:0044238	primary metabolic process	P2-P3-P4-P6	18	35
GO:0007018	microtubule-based movement	P2-P3-P4-P6	12	40
GO:0006635	fatty acid beta-oxidation	P2-P3-P4-P6	16	34
GO:0006351	DNA-templated transcription	P2-P3-P4-P6	21	30
GO:0036503	ERAD pathway	P1-P2-P3-P4	17	30
GO:0035672	oligopeptide transmembrane transport	P1-P2-P3-P4	10	35
GO:0003777	microtubule motor activity	P2-P3-P6	15	34
GO:0005085	guanyl-nucleotide exchange factor activity	P2-P3-P4	17	29
GO:0035673	oligopeptide transmembrane transporter activity	P1-P2-P3	10	35

GO categories shared exclusively among commercial strains (P2–P3–P4–P6)

Several functional categories were shared exclusively among the commercial strains, highlighting a common functional profile shaped by cultivated lineages. These included metabolic and biosynthetic processes such as secondary metabolite biosynthetic process, fatty acid beta-oxidation, primary metabolic process, and intracellular calcium ion homeostasis. Among these industrial-specific shared categories, secondary metabolite biosynthetic process displayed one of the highest mean nonsynonymous variant counts per gene, indicating elevated coding variability within genes involved in specialized metabolic functions. In contrast, functions such as DNA-templated transcription and GTPase activator activity showed a high number of synonymous variant, suggesting substantial accumulation of neutral coding variation in these regulatory functional compartments (Figure 4.5).

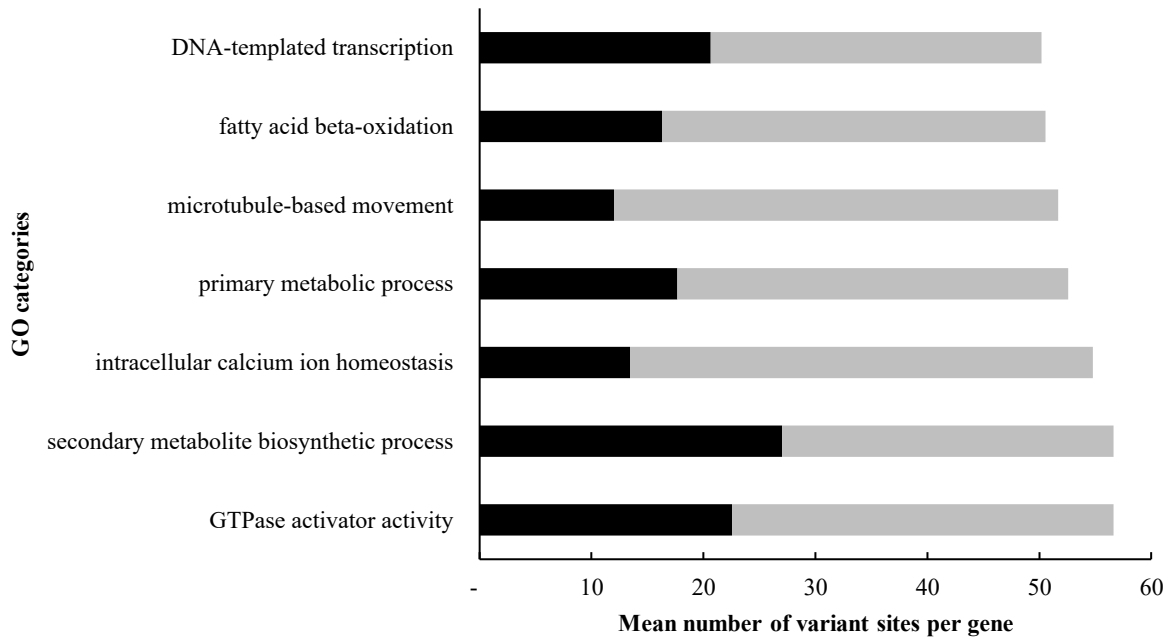


Figure 4.5. Mean nonsynonymous (black columns) and synonymous (gray columns) coding variant counts per gene across GO categories shared exclusively by industrial strains (P2–P3–P4–P6).

GO categories shared by strain subsets

Additional functional categories were shared by specific subsets of strains (e.g., P1–P2–P3–P4; P2–P3–P6; P1–P2–P3), encompassing biological processes related to proteostasis (ERAD pathway), peptide transport (oligopeptide transmembrane transport and transporter activity), and intracellular signaling (guanyl-nucleotide exchange factor activity). Across these subset-shared categories, coding variation was frequently dominated by synonymous mutations, consistent with the broader genome-wide pattern observed in coding regions.

Pairwise comparison between the fast-growing and slow-growing commercial strains

Pairwise contrasts of per-gene nonsynonymous variant counts revealed a set of genes displaying marked divergence between the fast-growing commercial strain (P6) and the slow-growing commercial strains (P2, P3, and P4), highlighting strain-specific patterns of coding variation. Several genes showed a pronounced enrichment of nonsynonymous variants in P6 relative to the slow-growing strains, as indicated by high positive Δ values. Among these, genes associated with mitochondrial function and energy metabolism were particularly prominent. These included gene_11085, encoding a component of the TIM23 mitochondrial inner membrane translocase complex, and gene_14478, annotated with fatty acid synthase activity and fatty acid metabolism-

related processes. Additional P6-enriched genes comprised gene_11679, linked to ligase activity and phosphopantetheine binding, and gene_17791, associated with oxidoreductase activity, collectively suggesting an increased accumulation of coding variation in metabolic and redox-related pathways in the fast-growing strain. Genes involved in RNA processing and nuclear regulation also exhibited elevated nonsynonymous counts in P6, including gene_18483 and gene_13662, associated with mRNA splicing and rRNA processing, respectively, indicating potential divergence in gene expression regulatory machinery. For a subset of loci, such as gene_17791 and gene_15890, nonsynonymous variants were detected exclusively in P6, reflecting strain-specific accumulation of coding variation rather than true biological absence. Conversely, multiple genes displayed higher nonsynonymous variant counts in the slow-growing strains compared to P6 (negative Δ values). These included genes related to enzymatic regulation and methylation processes, such as gene_10371 involved in glutamate metabolism and gene_10580 and gene_5108 encoding methyltransferase-related activities. In addition, genes associated with kinase signaling pathways (e.g., gene_3764) and with translation and cellular structural organization (e.g., gene_2035, gene_2977, gene_257, and gene_12834) were enriched for nonsynonymous variants in the slow-growing strains. Overall, the FAST versus SLOW pairwise comparison revealed a clear asymmetry in nonsynonymous variant distribution, with P6 enriched in genes related to mitochondrial function, energy metabolism, and gene expression regulation, whereas the slow-growing strains exhibited higher nonsynonymous counts in genes associated with enzymatic control, translation, and cytoskeletal organization. These strain-specific patterns of coding divergence suggest that growth rate differences among commercial isolates may be associated with functional differentiation in central metabolic pathways and regulatory mechanisms (Supplementary Table 1).

Pairwise comparison between wild-type (P1) and commercial strains (P2–P3–P4–P6)

The pairwise analysis highlighted genes displaying marked differences in the per-gene nonsynonymous variant counts between the wild-type strain (P1) and the commercial slow- and fast-growing strains (P2, P3, P4, P6). Counts represent the number of distinct nonsynonymous polymorphic sites per gene showing non-reference genotypes. Delta values ($\Delta P1 - P_x$) allowed the identification of loci characterized by higher variant loads in the wild-type (positive deltas) or in industrial strains (negative deltas). Several of the strongest contrasts involved genes associated with nucleolar functions, RNA binding, and ribosome biogenesis. For instance, gene_4581, involved in RNA binding, nucleolar localization, and SSU rRNA maturation, showed substantially higher variant

counts in the wild-type ($P1 = 90$) compared with industrial strains ($P2 = 0$; $P3 = 15$; $P4 = 15$; $P6 = 16$), resulting in large positive delta values (up to +75). This suggests an increased density of coding variation in P1 for genes related to rRNA processing. Conversely, gene_10480, associated with ribosome biogenesis and nuclear lumen organization, exhibited much higher variant loads in industrial strains ($P2$ – $P4 \approx 108$; $P6 = 104$) than in the wild-type ($P1 = 36$), with strongly negative deltas (up to –72). Together, these results indicate that genes involved in ribosomal function and RNA metabolism represent major targets of divergence in coding variant load between WT and industrial strains, although with opposite trends across specific loci. Multiple genes involved in transcriptional control and chromatin-associated complexes displayed higher variant loads in industrial strains. Notably, gene_5060, linked to the TFIIC complex and RNA polymerase III-dependent transcription (including 5S rRNA transcription), showed much higher values in commercial strains (≈ 68 – 76) compared to the wild-type (17), with consistently negative delta values (up to –59). Similarly, gene_4421, associated with the Mediator complex and transcriptional coactivator activity, and gene_13089, related to the Sin3-type complex, both exhibited elevated variant loads in industrial strains relative to P1. Genes associated with chromatin binding and epigenetic regulation also followed this trend. For example, gene_6869, encoding a DNA cytosine-5 methyltransferase involved in chromatin modification and methylation, showed a substantially higher variant load in industrial strains (≈ 38) compared to the wild-type (5). Overall, these patterns suggest increased coding variability in industrial strains affecting transcriptional regulation and chromatin-related processes. A consistent enrichment of higher variant loads in industrial strains was observed for genes involved in lipid metabolism and oxidoreductase activity. Key examples include gene_3717 (acyl-CoA dehydrogenase, fatty acid beta-oxidation), gene_13025 (acyl-CoA oxidase), and gene_12725 (cytochrome P450). In all cases, variant counts were markedly higher in commercial strains than in the wild-type, producing strongly negative delta values (up to –42). Contrasting trends were observed for genes involved in DNA processing and helicase activity. Gene_10102, annotated with helicase activity, showed higher variant counts in the wild-type ($P1 = 88$) compared with industrial strains (≈ 52 – 54), yielding positive delta values ($\sim +34$ to $+36$), suggesting increased coding variability in the WT for specific helicase-related loci. In contrast, gene_17151, associated with kinase activity, DNA repair, and transcriptional regulation complexes (including SAGA and NuA4), exhibited higher variant loads in industrial strains than in P1, with deltas reaching –39 (Supplementary Table 2).

Discussion

Although several genome assemblies and functional genomics resources have been generated for *P. eryngii* and related species, much of the available work has focused on single-strain genome

characterization or on specific functional aspects, including lignocellulolytic enzyme repertoires, phylogenomic relationships, and epigenomic features (Dai et al., 2019; Ruiz-Dueñas et al., 2021; Zhang et al., 2019). Comparative analyses within *Pleurotus* remain constrained by uneven assembly quality and by the limited number of studies integrating genome-wide variant discovery with population-level patterns and gene-level interpretation in a cultivation- and ecological-focused context (Villani et al., 2025). Comparison between wild and cultivated *P. eryngii* strains using systematic variant profiling, population structure analyses, and per-gene nonsynonymous variant counts remain scarce. In this context, the present study provides an exploratory comparative framework across a small set of *P. eryngii* genomes, including one wild-derived strain and four cultivated strains. By integrating whole-genome variant calling, genome clustering, and gene-level nonsynonymous variant counts, this work offers an initial descriptive view of genomic divergence patterns potentially associated with breeding of commercial strains within this species complex.

Genome clustering and genomic differentiation

Analyses based on IBS distances and PCA revealed a separation between the wild-type strain (P1) and most of the commercial strains (P2–P6), indicating that industrial genotypes generally share a distinct genetic background relative to the wild pool. However, the distinction between P1 and the fast-growing strain P6 was less pronounced. This pattern is consistent with what has been reported for other domesticated edible fungi, in which breeding and selection activities followed by clonal or semi-clonal propagation have strongly affected the genetic diversity of cultivated lineages and led to a marked differentiation from natural populations. (Ling et al., 2026; McTaggart et al., 2023; Zhang et al., 2023). Similar dynamics have also been described in systems characterized by extremely narrow founder events, such as *Pholiota microspora*, where cultivated lines originate from a single domestication event associated with a strong loss of genetic variability (Hirao et al., 2022). Within the pool of commercial *Pleurotus eryngii* strains, the slow-growing cluster (P2–P4) suggests a high degree of genomic homogeneity among these strains, consistent with a shared origin or with strong selection acting on closely related genetic backgrounds, as expected in production systems based on the large-scale propagation of a few improved genotypes. In contrast, the distinct position of the fast-growing strain P6 with respect to the slow cluster indicates that P6 represents a differentiated lineage or a distinct breeding strategy. This intra-commercial strain divergence is plausibly associated with phenotypically relevant traits such as mycelial growth rate and fruiting body yield. This is consistent with observations in other cultivated fungi, where selection for specific production traits has resulted in genetically differentiated cultivated lines from the same domesticated genotypes (Ling et al., 2026).

*Genomic differentiation between wild-type and commercial strains in *Pleurotus eryngii**

The comparative genomic analyses revealed a clear differentiation between the wild-type strain (P1) and the commercial strains (P2, P3, P4, and P6), consistent across global variant distributions and gene-level nonsynonymous count differences. Commercial strains exhibited a higher total number of coding variants than the wild-type strain, with all variants defined relative to the reference genome. This pattern may reflect both the closer similarity of the wild-type isolate to the reference sequence and underlying biological differences among strains. In particular, divergence among industrial strains could be influenced by selection associated with domestication and cultivation; however, these effects cannot be disentangled from reference bias or directly inferred from the present dataset. Across all strains, synonymous variants consistently exceeded nonsynonymous variants, a pattern commonly observed in coding regions and potentially consistent with expectations under functional constraint, although no formal test of selection (e.g., dN/dS) was performed. Although this trend was consistent across most loci, a subset of genes showed the opposite pattern, with higher nonsynonymous counts in the wild-type strain. This general pattern is typical of population-scale genomic datasets and indicates that most mutations are either neutral or mildly deleterious and maintained at low functional impact. The overall higher variant number observed in commercial strains compared to the wild-type may reflect the combined effects of domestication history, artificial selection, and repeated propagation under controlled cultivation systems. Similar patterns of genomic restructuring associated with domestication have been reported in other cultivated mushrooms, including *Agaricus bisporus*, where cultivated lineages show clear divergence from wild populations at both nuclear and mitochondrial levels (Ling et al., 2026; Zhang et al., 2023). Severe genetic bottlenecks linked to the establishment of cultivated stocks from a limited number of initial strains have also been documented in *Pholiota microspora* and clandestinely cultivated *Psilocybe* species, leading to constrained but structured genomic diversity compared to natural populations (Hirao et al., 2022; McTaggart et al., 2023). In this context, the wild-type strain P1 likely represents a genomic background closer to natural *Pleurotus eryngii* populations, whereas commercial strains reflect lineages shaped by human-mediated selection and propagation. Moreover, P1 showed overall lower coding variant counts. Since variants are defined relative to the reference genome (CCMJ2132), this pattern likely reflects a closer similarity of P1 to the reference sequence rather than an intrinsic lower level of genetic variation. This close relationship between P1 and the wild-type-derived reference gene model may partially contribute to the lower apparent divergence observed in P1; however, the consistent differences across multiple functional categories and gene-level contrasts suggest that domestication-driven divergence represents a major biological signal rather than a purely technical artifact.

Functional profile of coding variation across shared GO categories

The descriptive aggregation of synonymous and nonsynonymous variants across Gene Ontology categories shared among strains provided a neutral functional context for interpreting directional contrasts. Across all strains, GO categories associated with core cellular maintenance and information processing, including ATP hydrolysis activity, DNA repair, transcription-related functions, chromatin remodeling, and ribonucleoprotein-associated processes displayed high average coding-variant loads per gene. Broad compartment-level categories such as membrane, nucleus, and cytoplasm also accounted for a substantial proportion of total coding variation, reflecting their large gene content. Among the GO categories shared by all strains, transport-related functions were consistently represented, including ABC-type transporter activity, ATPase-coupled transmembrane transport, metal ion transport, and monoatomic cation transmembrane transport. In this core functional group, synonymous variants predominated over nonsynonymous variants, with mean nonsynonymous counts per gene typically ranging between approximately 15 and 25. This pattern suggests long-term accumulation of neutral or weakly constrained coding variation in highly conserved functional systems that are essential across both wild-type and cultivated lineages.

Shared GO categories with relatively high mean nonsynonymous counts included transport-related functions (e.g., ABC-type and ATPase-coupled transmembrane transport), intracellular signal transduction, and ligase or peptidase activities; genome-maintenance functions such as helicase activity and single-stranded DNA binding also showed substantial nonsynonymous loads, consistent with large and domain-rich proteins providing multiple mutational targets. High coding variability in these functional compartments has been reported across genomes and may reflect both large gene sizes and complex domain architectures that provide numerous mutational targets (Spence et al., 2024). Importantly, this descriptive GO distribution does not imply functional enrichment or selection but instead highlights functional traits that harbor substantial coding variation across *Pleurotus* strains. GO categories shared exclusively among commercial strains further emphasized metabolic and biosynthetic functions, including secondary metabolite biosynthetic processes, fatty acid beta-oxidation, primary metabolic processes, and calcium ion homeostasis. These industrial-specific shared categories suggest that cultivated lineages retain a common functional traits particularly related to energy metabolism and biosynthesis, potentially reflecting adaptation to standardized growth substrates and controlled environmental conditions.

Pairwise comparison between fast-growing and slow-growing commercial strains (GO categories)

The pairwise comparison between the fast-growing industrial strain (P6) and the slow-growing strains (P2, P3, and P4) revealed marked asymmetries in nonsynonymous variant counts across specific functional gene groups. Genes showing higher nonsynonymous variant count in P6 were predominantly associated with mitochondrial function, fatty acid metabolism, ligase activity, and RNA processing. Genes involved in mitochondrial protein import (TIM23 complex), fatty acid synthase activity, and metabolic redox-related functions showed consistently higher counts in the fast-growing strain. These patterns suggest that divergence in energy metabolism and mitochondrial-associated processes may contribute to growth-rate differences among commercial strains. In filamentous fungi, metabolic fluxes and mitochondrial efficiency are tightly linked to biomass accumulation and hyphal extension, supporting the functional relevance of such associations (Navarro-Espíndola et al., 2020; Li et al., 2024). Conversely, slow-growing strains exhibited higher nonsynonymous counts in several genes related to kinase signaling pathways, methyltransferase activity, translation-associated factors, and cytoskeletal or microtubule-related components. These functional classes are involved in cellular regulation, protein turnover, and structural organization, processes that are fundamental to hyphal growth dynamics and cellular homeostasis (Fischer et al., 2008). Together, the FAST vs SLOW comparison indicates that growth-rate divergence among cultivated strains may involve coordinated variation in metabolic capacity, regulatory networks, and cellular architecture rather than single-gene effects. Such multilayered functional divergence aligns with the concept that quantitative traits like growth performance in fungi arise from polygenic restructuring of central cellular systems rather than from isolated major-effect mutations.

Pairwise comparison between wild-type and industrial strains (GO categories)

The pairwise contrasts between the wild-type strain and industrial strains further highlighted extensive divergence affecting genes involved in RNA metabolism, ribosome biogenesis, transcriptional regulation, chromatin organization, and lipid metabolism. Several of the strongest differences involved nucleolar and ribosome-associated genes, including loci linked to SSU rRNA maturation and ribosome biogenesis, showing opposite trends of nonsynonymous count between wild type and cultivated strains. This indicates that translational machinery components represent major targets of coding divergence under cultivation-associated evolutionary trajectories. Commercial strains also consistently displayed higher nonsynonymous counts in genes associated with transcriptional control complexes (e.g., TFIIC, Mediator, Sin3-type complexes) and chromatin modification processes, including DNA methyltransferase activity. These results evidence a

substantial reorganization of gene regulatory frameworks during domestication and industrial propagation. In parallel, multiple genes involved in fatty acid metabolism, oxidoreductase activity, and cytochrome P450-mediated processes exhibited a higher variant rate in commercial strains. Metabolic remodeling is a common feature of domesticated organisms, reflecting adaptation to artificial environments and optimized resource utilization. Similar metabolic shifts have been observed in cultivated *Agaricus bisporus* and other domesticated fungi (Ling et al., 2026; Hirao et al., 2022). Together, these findings suggest that domestication in *Pleurotus eryngii* has involved coordinated genomic restructuring affecting transcriptional regulation, chromatin organization, translational machinery, and metabolic pathways, consistent with broader patterns of domestication-driven evolution reported across fungal species (McTaggart et al., 2023; Ling et al., 2026).

Functional implications of transporter-rich and metabolic divergence in basidiomycetes

Transport-related functions emerged as a recurrent functional group in the descriptive GO summaries shared across strains, including ABC-type transporter activity and ATPase-coupled transmembrane transport, together with metal ion and cation transmembrane transport. This is consistent with the basidiomycete lifestyle, where transporter systems contribute to nutrient acquisition, metabolite trafficking, detoxification, and environmental adaptation (Perlin et al., 2014; Coleman & Mylonakis, 2009). In lignocellulose-associated fungi, diversified transporter repertoires have been linked to substrate colonization and chemical stress tolerance (Mäkinen et al., 2019; Bai et al., 2025). In our strain-level comparisons, transporter-related loci were also represented among top divergent candidates (e.g., an ABC-type oligopeptide transporter), supporting the idea that variation in membrane transport and associated metabolic throughput may contribute to differentiation among cultivated lineages. Importantly, these observations provide functional relevance rather than evidence of selection and should be interpreted as functional hypotheses to be tested in future multi-isolate datasets and expression/phenotype-linked analyses (Guerillot et al., 2023).

Methodological considerations and interpretation limits

While the gene-level nonsynonymous count approach provides a powerful descriptive framework for focusing on divergent loci, it represents a ranking strategy rather than direct evidence of functional causality. Variant accumulation is influenced by gene length, background mutation rates, and genomic architecture, and longer genes are more likely to harbor higher variant counts purely by chance (Guo et al., 2016; Spence et al., 2024). The predominance of hypothetical or poorly characterized genes among top candidates further reflects the incomplete functional annotation typical of fungal genomes (Haas et al., 2011; Mohanta & Al-Harrasi, 2021). Accordingly, the functional interpretations proposed here should be viewed as biologically plausible hypotheses that

require future validation through transcriptomic profiling, functional assays, and phenotypic correlation analyses.

Chapter 5

Bioactive properties of *Pleurotus eryngii* extracts in hepatic cell models

Introduction

In recent years, *Pleurotus eryngii* has gained increasing attention for its potential applications in nutraceutical and pharmaceutical fields. Several preclinical studies have reported that the consumption of fruiting bodies or their extracts exerts beneficial effects on lipid metabolism. In particular, *P. eryngii* has been shown to reduce obesity and LDL cholesterol levels in obese mouse models (Nakahara et al., 2020). Other investigations have attributed to this species a wide range of biological activities, including antidiabetic, antihypercholesterolemic, anti-aging, antimicrobial, and antioxidant effects, confirming its value as a functional food (Madhanraj et al., 2019). Clinical observations, although still limited, are promising consumption of *P. eryngii* by obese individuals modulated postprandial glucose absorption and improved glycemic control (Kleftaki et al., 2022). Efforts to enhance the nutritional value of *P. eryngii* have included changes in the growth substrate; for instance, supplementation with wood distillate increased soluble proteins, phenolic compounds, and vitamin C without affecting yield (Fedeli et al., 2024). Among its bioactive constituents, triterpenoid compounds isolated from ethanolic extracts have shown significant inhibitory activity against human breast cancer (MCF-7) cell lines. Two triterpene acids with dose-dependent cytotoxicity were identified for the first time in *P. eryngii* (Xue et al., 2015). Cold-water extracts of *P. eryngii* var. *ferulae* (CWE-Pef) exhibited potent antitumor activity against human colon carcinoma (HCT116) cells, reducing cell viability and migration, promoting apoptosis via increased Bax/Bcl-2 ratios, and downregulating intracellular signalling pathways such as tyrosine phosphorylation and ERK1/2 activation (Fontana et al., 2014). Similarly, ethanol extracts of *P. ferulae* (*Agaricus ferulae* Lanzi, syn. of *P. eryngii*) enriched in terpenoids inhibited the proliferation of melanoma and gastric cancer cell lines while sparing normal cells (Wang et al., 2014). The same work also demonstrated that in vivo treatment delayed and reduced melanoma growth in a murine melanoma model, suggesting that. In addition, to antitumor potential, *P. eryngii* is recognized as a rich source of antioxidant compounds. Two polysaccharides, identified as α - and β -glucans, displayed strong radical-scavenging activity and cytotoxic effects against colorectal adenocarcinoma cells,

demonstrating both antioxidant and antitumor potential (Cateni et al., 2018). Other studies confirmed the antioxidant performance in polysaccharide fractions (Zhang et al., 2014) from fruiting bodies of *P. eryngii* var. *eryngii*. Further advancements have focused on peptide-based antioxidant systems: selenium-enriched peptides (SePEPs) extracted from *P. eryngii* cultivated under selenium-enriched conditions showed significantly stronger antioxidant activity than selenium-free analogues, especially within the 0–3500 Da molecular range. Spectroscopic and computational analyses revealed that selenium substitution enhanced peptide compactness and increased hydrogen-bonding and hydrophobic interactions with DPPH radicals, achieving a scavenging rate of 68.5 ± 2.2 % at 10 mg/mL (Chen et al., 2025). These findings reveal an advanced mechanism of redox modulation, reinforcing the role of *P. eryngii* as a source of high-efficiency antioxidant biomolecules. Beyond its antioxidant and anticancer effects, *P. eryngii* fruiting body extracts have demonstrated cytotoxicity against leukemia cells (Ngai & Ng, 2006) and antimicrobial activity against human pathogenic bacteria (Schillaci et al., 2013). Two types of β -glucans and proteoglycan complexes isolated from *P. eryngii* exhibited synergistic potential when combined with probiotic strains for intestinal health (Synytsya et al., 2009). The fruiting bodies themselves contain high levels of both soluble and insoluble β -glucans, key components of functional dietary fiber that contribute to its hypocholesterolemic and immunomodulatory effects (Synytsya et al., 2008). Several investigations have also demonstrated the anti-inflammatory and hepatoprotective properties of *P. eryngii*. An ethanol extract suppressed delayed-type (type IV) hypersensitivity responses in murine models (Sano et al., 2002), while water-soluble polysaccharides exhibited hepatoprotective and antioxidant effects by scavenging free radicals and increasing hepatic antioxidant enzyme activity (Chen et al., 2012). In addition, a glycosphingolipid isolated from *P. eryngii* induced interferon- γ (IFN- γ) and interleukin-4 (IL-4) secretion from murine T cells, supporting its immunomodulatory role (Nozaki et al., 2008). Complementary studies confirmed anti-inflammatory activity in models of acute lung injury, where *P. eryngii* extracts mitigated lipopolysaccharide-induced inflammation and oxidative damage (Kawai et al., 2014). Emerging research has also highlighted the neuroprotective potential of *P. eryngii*. Polysaccharides have been shown to exert protective effects against β -amyloid-induced neurotoxicity in PC12 neuronal cells and aging rats, reducing apoptosis and downregulating APP, iNOS, and COX-2 expression levels (Zhang et al., 2020). Moreover, extracts have been found to activate the transcription factor Nrf2, a master regulator of antioxidant defense. Phytochemical profiling identified N-acetyltryptamine (NAT), a serotonin and melatonin precursor, as a competitive inhibitor of acetylcholinesterase, while transcriptomic data from HepG2 cells suggested modulation of gene expression associated with neurodegenerative disorders, oxidative stress, and lipid metabolism (Teniou et al., 2022). Finally, post-harvest by-products from *P. eryngii* cultivation have been reported

as a promising source of antioxidants agents. Polysaccharides extracted from the spent mushroom substrate were characterized as a novel acid heteropolysaccharide with high molecular weight ($\approx 8.3 \times 10^6 \text{ g mol}^{-1}$) and rigid-rod conformation (He et al., 2016). Both crude and refined fractions of the substrate displayed strong ABTS, superoxide, hydroxyl, and DPPH radical-scavenging activity. In 2017, Soilem et al. reported some differences in antioxidant, anti-inflammatory and cytotoxic properties between mycelium and fruiting body extracts of a wild *P. eryngii* specimen collected in Portugal. Considering the longtime activities of mushroom growers for obtaining isolates with improved traits, genetics of cultivated *Pleurotus* species may have been modified by repeated breeding (Pánek et al., 2019). This work aimed to evaluate anti-proliferative, cytotoxicity, antioxidant, anti-inflammatory and anti-steatotic effects on liver cancer cell lines of both mycelium and fruiting body ethanolic extracts obtained from a commercial strain of *P. eryngii* marketed in Italy.

Material and Methods

Mycelial isolation and sample preparation

The basidioma of *P. eryngii* used for analyses was obtained from the industrial strain P3 (slow-growing), as described in Chapter 4. Pure culture was isolated by excising a fragment of inner tissue of the intersection zone between stipe and pileus and placing it onto a potato dextrose agar (PDA, 25 g L⁻¹) plate. The obtained pure culture was subcultured every 20 days on PDA at 20 °C, in the dark. The remaining portion of inner tissues of the basidioma was excised and lyophilized at -50 °C in a VirTis benchtop 2K freeze dryer (SP Industries) for two days. The lyophilized sample was stored at -20 °C pending metabolite extraction. The identity of the pure culture was verified by sequencing ITS region following the procedure described by De Mattheis et al. (2025).

Preparation of liquid cultures and ethanol extracts

Liquid cultures were prepared by inoculating two mycelial plugs (0.7 cm in diameter), taken from the edge of 1-week-old colonies, into fifteen 150 mL Erlenmeyer flasks containing 150 mL of the following medium: glucose 50 g L⁻¹, peptone 2 g L⁻¹, yeast extract 2 g L⁻¹, KH₂PO₄ 2.5 g L⁻¹, MgSO₄ 2.5 g L⁻¹, and maltose 10 g L⁻¹, adjusted to pH 5.7. The medium was autoclaved at 120 °C for 15 min prior to inoculation. Cultures were incubated for 14 days at 20 °C in the dark, then the mycelium was harvested, washed with sterile distilled water, squeezed out and freeze-dried for three days. Dried mycelia and fruiting body were then ground to a fine powder in a mortar with liquid nitrogen. A total of 1.5 g of both mycelial and fruiting body powder were mixed with 15 mL of 70% ethanol (70:30, v/v, ethanol: water) and stirred at room temperature for 24 h on a rotary shaker (30 rpm). The mixture was centrifuged at 1500 rpm for 15 min in a ROTANTA 460R centrifuge (Hettich), and the resulting

supernatant was collected. The remaining pellet was subjected to a second extraction round with other 4 mL of 70% ethanol for 4 h under the same conditions. After centrifugation, the supernatants were pooled together, distributed in several 2 mL centrifuge tubes and dried overnight at 45 °C by using a vacuum concentrator (concentrator plus, Eppendorf). The final dried extracts were resuspended in dimethyl sulfoxide (DMSO) to a concentration of 100 µg mL⁻¹ and preserved at -20 °C pending later processing.

Cell cultures

HepG2 and Huh7 cells were obtained from the American Type Culture Collection (ATCC) and the JCRB Cell Bank, respectively. Both cell lines were propagated in clarified polystyrene flasks or multiwell plates using low-glucose DMEM (Euroclone) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 µg mL⁻¹ streptomycin and 100 µg mL⁻¹ penicillin). Cultures were maintained at 37 °C in a humidified incubator with 5% CO₂. Dried ethanol extracts obtained from both mycelium (Myc) and fruiting bodies (FB) of *Pleurotus eryngii* were resuspended in dimethyl sulfoxide (DMSO) to obtain concentrated stock solutions (200 mg mL⁻¹) and stored at -20 °C until use. For cell treatments, the extracts were diluted in culture medium at appropriate ratios depending on the experimental design, including a 1:2000 dilution to obtain a final concentration of 100 µg mL⁻¹. For experimental procedures, HepG2 and Huh7 cells were plated under exponential growth conditions at densities of 1 × 10⁴ cells cm⁻² and 8 × 10³ cells cm⁻², respectively. After a 24 h stabilization period, cells were exposed to different concentrations of Myc and FB extracts, as specified for each assay. Control groups received equivalent volumes of DMSO, ensuring that the final solvent concentration never exceeded 0.01%.

Assessment of cell viability and cytotoxicity

Trypan blue exclusion assay

Trypan Blue exclusion assay was first applied to evaluate the dose-dependent effects of Myc and FB extracts *P. eryngii* on HepG2 and Huh7 cell viability and to determine the concentrations that reduced cell numbers by 50% (IC₅₀). In addition to the control (no ethanol extract added), four increasing extract concentrations (50, 100, 150, and 200 µg mL⁻¹) were selected based on previous studies where *P. eryngii* extracts were shown to inhibit proliferation of various tumour cell lines in a dose-dependent manner (Xue et al., 2014; Önder et al., 2024). HepG2 and Huh7 cells were treated for 48 h and then harvested from the plates to check their viability. Cell suspensions were mixed 1:1 (v:v) with 0.5% Trypan Blue (Euroclone), incubated for 5 min at room temperature and then loaded onto a Bürker counting chamber. Unlike viable cells, dead cells displayed blue-stained nuclei following the

absorption of the dye. Viable and dead cells were counted and viability at different extract concentrations were expressed as percentage of viable cell with respect to the controls. Mean values and standard deviations were calculated for each group (cell type $\times$ fungal material $\times$ extract concentration) from independent experiments and three technical replicates for each experiment. IC₅₀ values were derived by using the AAT Bioquest online IC₅₀ calculator.

Lactate Dehydrogenase (LDH) release assay

In support of Trypan Blue exclusion assay data, necrosis induced by Myc and FB extracts was assessed using the LDH-Glo™ Cytotoxicity Assay (Promega). HepG2 and Huh7 cells were plated at the abovementioned concentrations in clear-bottom 96-well plates and incubated for 48 h with 0, 50, or 100 $\mu\text{g mL}^{-1}$ of *P. eryngii* extracts. Following treatment, 50 μL of culture supernatant were transferred to a separate plate, mixed with an equal volume of LDH-Glo™ reagent prepared according to the manufacturer's protocol, and incubated for 30 min at room temperature in the dark. Luminescence was recorded using a plate reader configured for luminometric detection, and values were expressed as relative luminescence units (RLU). Each experiment included a lysis-induced positive control to determine maximal LDH release and untreated cells (no ethanol extract added) as negative control. Necrotic cells were counted and cytotoxicity were expressed as percentage of necrotic cell with respect to the positive controls for the negative control and two extract concentrations (50, 100 $\mu\text{g mL}^{-1}$). Mean values and standard deviations were calculated for each group (cell type $\times$ fungal material $\times$ extract concentration) from three technical replicates for three independent experiments. Cytotoxicity was calculated as:

$$\text{Cytotoxicity (\%)} = \frac{\text{RLU sample} - \text{RLU negative control}}{\text{RLU positive control} - \text{RLU negative control}} \times 100$$

Antioxidant activity

The HepG2 cell line was selected for antioxidant assays due to its active hepatic metabolic profile and stable oxidative response, making it a suitable model for evaluating the antioxidant potential of *P. eryngii* extracts (Liu et al., 2018). The intracellular accumulation of reactive oxygen species (ROS) was quantified with (basal conditions) and without (oxidative stress) H₂O₂ exposure using a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorometric assay kit (Immunological Sciences). HepG2 cells were seeded at 3×10^4 cells/cm² in 96-well culture plates and allowed to stabilize for 24 h. Subsequently, cells were exposed for 1 h at 37 °C to *P. eryngii* ethanol extracts (0, 50, 100 $\mu\text{g mL}^{-1}$

¹) under two experimental conditions: (i) treatment with the extract alone and (ii) co-treatment with the extract and 100 μM hydrogen peroxide (H_2O_2), used as an exogenous oxidative challenge. Untreated cultures and cells treated exclusively with 100 μM H_2O_2 served as negative and positive controls, respectively.

At the end of the exposure period, wells were aspirated and gently rinsed with Hank's Balanced Salt Solution (HBSS). DCFH-DA was freshly prepared in HBSS at a final concentration of 25 μM and added to each well. Probe loading was performed for 30 min at 37 $^\circ\text{C}$ in the absence of light to prevent premature oxidation. Following incubation, excess dye was eliminated by an additional wash with HBSS, and fluorescence was measured right after by using a Tecan multiwell fluorometer (ditta) set to an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Data were expressed as relative fluorescence units (RFU) and normalized to control values to exclude background fluorescence and plate-to-plate variability.

Intracellular lipid accumulation

Since HepG2 cells display an inadequate lipid content which does not reflect a pathological steatotic phenotype, lipid accumulation was experimentally induced using palmitic acid (PA) to investigate the anti-steatotic effects of *P. eryngii* extracts. Intracellular neutral lipid deposition was evaluated by Oil Red O (Sigma) staining. HepG2 cells were seeded at a density of 1.5×10^4 cells/cm² in 96-well plates and allowed to adhere for 24 h. To induce steatosis, cells were exposed to 0.2 mM PA for 24 h and treated with *P. eryngii* ethanol extracts at 0, 50, or 100 $\mu\text{g mL}^{-1}$. Experimental controls included a basal negative control (untreated cells) and a positive control (steatotic control) consisting of cells treated with PA alone. At the end of the induction period, the culture medium was removed, and the cells were washed twice with PBS. Fixation was performed using 4% paraformaldehyde for 30 min at room temperature, followed by two additional PBS washes. Prior to staining, cells were briefly conditioned with 60% isopropanol for 5 min and allowed to air-dry.

Oil Red O working solution was freshly prepared by diluting the stock solution (0.5% w/v dye in 100% isopropanol) 1:1 (v:v) in distilled water, equilibrated for 10 min, and filtered. Fixed cells were incubated with the working solution for 15–30 min at room temperature. Excess dye was removed by repeated washing with distilled water until background staining was eliminated. Stained lipid droplets were visualized using an optical microscope in bright-field mode. For quantitative analysis, the retained dye was extracted with 100% isopropanol for 10 min under gentle agitation, and the absorbance of the resulting eluate was measured at 500–520 nm using a Tecan microplate reader. Results were expressed as relative lipid content normalized to positive control.

Anti-inflammatory potential

The anti-inflammatory potential of *P. eryngii* extracts was evaluated by measuring the extracellular active form of caspase-1, a marker of inflammasome activation, in the culture medium of HepG2 cells, both in the presence and absence of lipopolysaccharide (LP) stimulation. Extracellular caspase-1 activity was quantified using the Caspase-1 Glo® Inflammasome Assay (Promega), following the manufacturer's instructions with minor adaptations for HepG2 cells. Cells were seeded in 96-well plates at a density of 1×10^4 cells/cm² and allowed to adhere for 24 h under standard culture conditions. After stabilization, cells were treated for 24 h with ethanol extracts of *P. eryngii* at 0, 50, or 100 µg mL⁻¹ under two experimental conditions: (i) basal condition (extract alone, no inflammatory stimulus) (ii) Inflammatory condition (extract + inflammasome activation). For inflammasome priming, cells were exposed to LPS (1 µg/mL) for 4 h. Following LPS priming, inflammasome activation was induced by adding ATP (3 mM) for 30 min at 37 °C. Experimental controls included: (i) untreated cells (negative control), (ii): LPS (1 µg/mL) + ATP (3 mM) (positive inflammasome control), (iii) assay reagent without biological sample (reagent blank).

At the end of the stimulation protocol, 50 µL of culture supernatant were collected from each well and transferred into a white, clear-bottom 96-well plate. An equal volume (50 µL) of Caspase-1 Glo® reagent was added to each sample, and the plate was incubated for 1 h at room temperature in the dark. Luminescence was quantified using a Tecan microplate reader configured for luminometric detection. Caspase-1 activity was expressed as relative luminescence units (RLU) and normalized to control values to account for background and plate-to-plate variability.

Statistics

Statistics were performed separately for Huh7 and HepG2 cells by applying one-way ANOVA and considering either extract concentrations or fungal material (Myc vs FB) as independent variables. Statistical analyses were conducted on the following metrics: percentage of viable cells for trypan blue exclusion assay, percentage of necrotic cells for LDH release assay, relative fluorescence units for antioxidant activity, optical density values for anti-steatotic potential and RLU for inflammatory assay. A post hoc Tukey test was applied to identify statistical significances between means ($p < 0.05$). Statistics were performed using Past 4.03 (Hammer et al., 2001).

Results

Assessment of cell viability and cytotoxicity

Treatment with both Myc and FB extracts significantly reduced the viability of Huh7 cells, in particular between 50 µg mL⁻¹ and 100 µg mL⁻¹ (Fig. 5.1a, Table 5.1). No significant differences were found between Myc and FB extracts at any concentrations tested. The IC₅₀ values for Huh7 cells were

105 $\mu\text{g mL}^{-1}$ for Myc extract and 100 $\mu\text{g mL}^{-1}$ for FB extract. HepG2 cells exhibited lower sensitivity (Fig. 5.1b) and they did not reach 50% inhibition even at concentrations of 200 $\mu\text{g mL}^{-1}$ for both types of extract. Significant differences were only found for FB extracts between 50 and 200 $\mu\text{g mL}^{-1}$ (Table 1). Percentage of viable HepG2 cells were always lower when treated with FB extracts at any concentrations tested but significant difference was only obtained at 200 $\mu\text{g mL}^{-1}$.

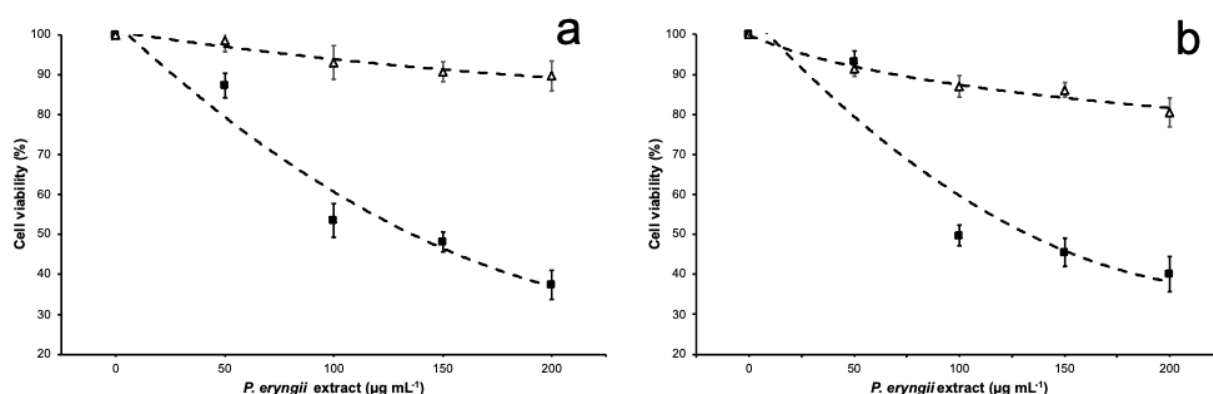


Fig. 5.1: Effect of *P. eryngii* ethanolic extracts on the viability of hepatocarcinoma cells by trypan blue exclusion assay. Dose-response effect on HepG2 (white triangles) or Huh7 (black squares) cell viability at 48 h of treatment with mycelium (a) or fruiting body (b) extracts. Percentages were calculated based on the values of the negative control (0 $\mu\text{g mL}^{-1}$ extract). Data are shown as the mean $\pm$ SD of at least three independent experiments and three replicates for each experiment.

Table 5.1: mean percentages and standard deviations of viable Huh7 and HepG2 cells at different concentrations of mycelium and fruiting body ethanolic extracts. Percentages were calculated based on the values of the negative control (0 $\mu\text{g mL}^{-1}$ extract). Different letters on the same row and asterisk in the same column indicate significant differences between means ($p < 0.05$). ns = not significant.

	Ethanolic extract concentration ($\mu\text{g mL}^{-1}$)			
	50	100	150	200
Huh7				
Mycelium	87.3 $\pm$ 3.0a	53.5 $\pm$ 4.1b	48.2 $\pm$ 2.5b	37.4 $\pm$ 3.7c
Basidioma	93.2 $\pm$ 2.7a	49.7 $\pm$ 2.6b	45.5 $\pm$ 3.6bc	40.0 $\pm$ 4.4c
	ns	ns	ns	ns
HepG2				
Mycelium	98.7 $\pm$ 5.1	93.0 $\pm$ 7.2	90.7 $\pm$ 4.1	89.7 $\pm$ 4.0
Basidioma	91.4 $\pm$ 1.9a	87.0 $\pm$ 2.6ab	86.1 $\pm$ 1.8ab	80.5 $\pm$ 3.6b
	ns	ns	ns	*

Lactate released from necrotic cells remained low across all tested concentrations, fungal materials and cell types ($\leq 2\%$) (Fig. 2a). Treatments with 50 and 100 $\mu\text{g mL}^{-1}$ Myc extracts significantly increased cytotoxicity in Huh7 cells with respect to the negative control, while significant differences were only found at 100 $\mu\text{g mL}^{-1}$ by using FB extracts (Table 2). Moreover, FB extracts showed a

significantly lower cytotoxicity than Myc extracts both at 50 and 100 $\mu\text{g mL}^{-1}$. HepG2 cells exhibited a significant higher number of necrotic cells with respect to the negative control only at 100 $\mu\text{g mL}^{-1}$ of both Myc and FB extracts (Fig. 2b). Also in these cells, FB extracts showed a significantly lower cytotoxicity than Myc extracts but only at 100 $\mu\text{g mL}^{-1}$ (Table 2).

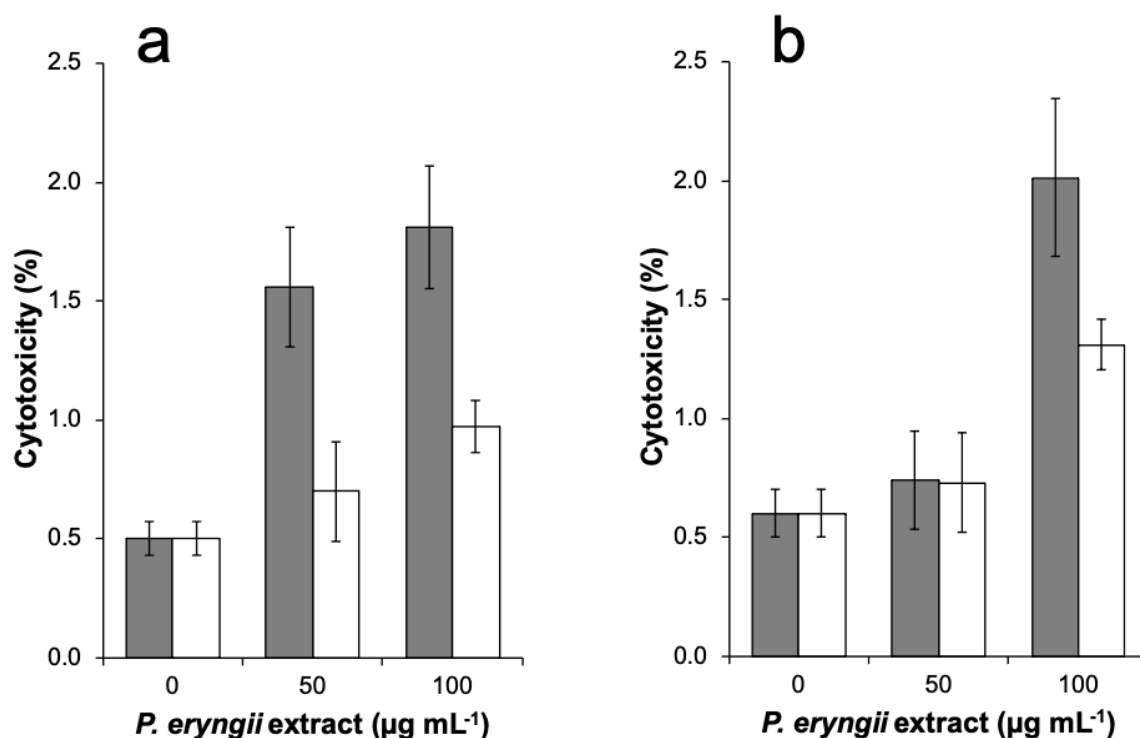


Fig. 5.2: Effect of *P. eryngii* ethanolic extracts on the cytotoxicity (%) of hepatocarcinoma cells by LDH cytotoxicity test. Dose-response effect on Huh7 (a) and HepG2 (b) cells at 48 h of treatment with mycelium (grey columns) or fruiting body (white columns) extracts. Percentages were calculated based on the values of the lysis-induced positive control. Data are shown as the mean $\pm$ SD of three independent experiments and three replicates for each experiment.

Table 5.2: mean percentages and standard deviations of necrotic hepatocarcinoma cells at different concentrations of mycelium and fruiting body ethanolic extracts. Different letters on the same row and asterisk in the same column indicate significant differences between means ($p < 0.05$). ns = not significant.

		Ethanolic extract concentration (µg mL ⁻¹)	
		50	100
Huh7			
Mycelium	0.50 ± 0.07a	1.56 ± 0.25b	1.81 ± 0.26b
Basidioma	0.50 ± 0.07a	0.70 ± 0.21a	0.97 ± 0.11b
		*	*
HepG2			
Mycelium	0.60 ± 0.10a	0.74 ± 0.21a	2.01 ± 0.33b
Basidioma	0.60 ± 0.10a	0.73 ± 0.21a	1.31 ± 0.11b

Antioxidant activity

Under basal conditions, ROS levels measured in HepG2 cells treated with Myc and FB extracts increased moving from 50 to 100 $\mu\text{g mL}^{-1}$ regardless the fungal material (Fig. 5.3a). However, under these conditions, Myc and FB extracts did not differ statistically from those of the negative control with the exception of 50 $\mu\text{g mL}^{-1}$ Myc extract which induced a significant lower level of ROS than either the negative control (approximately 77% less) or 100 $\mu\text{g mL}^{-1}$ Myc extract (Table 5.3). Significant differences in ROS production were also obtained between Myc and FB extracts at 50 $\mu\text{g mL}^{-1}$.

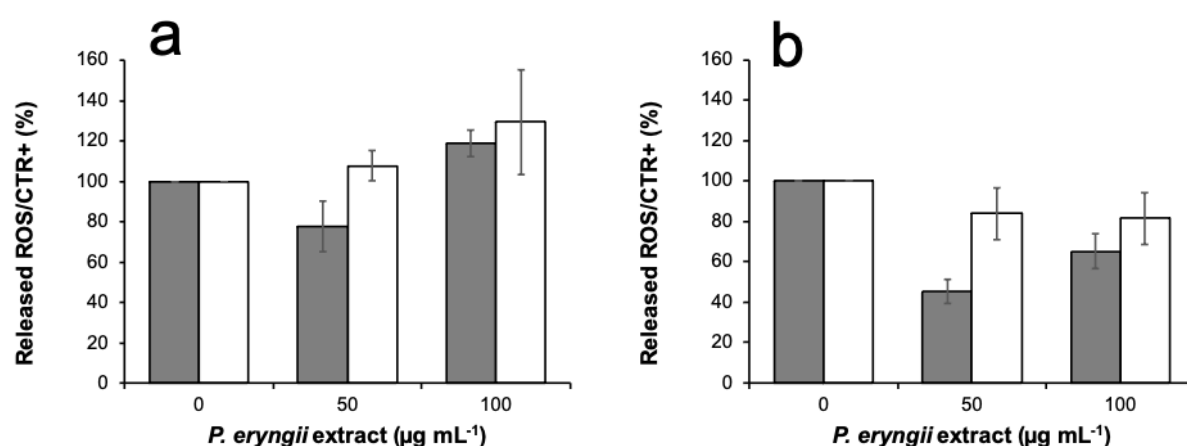


Fig. 5.3: Effect of *P. eryngii* ethanolic extracts on ROS release (%) of HepG2 cells without (a) or with (b) H_2O_2 addition. Percentages were calculated based on the values of the negative (a) and positive (b) control. Grey and white columns are referred to mycelium and fruiting body extracts, respectively. Data are shown as the mean $\pm$ SD of three independent experiments and three replicates for each experiment.

Table 5.3: mean and standard deviations of relative fluorescence units (RFU) from HepG2 cells at different concentrations of mycelium and fruiting body ethanolic extracts. Different letters on the same row and asterisk in the same column indicate significant differences between means ($p < 0.05$). ns = not significant.

	- H_2O_2			+ H_2O_2		
	Negative control	Ethanolic extract concentration ($\mu\text{g mL}^{-1}$)		Positive control	Ethanolic extract concentration ($\mu\text{g mL}^{-1}$)	
		50	100		50	100
Mycelium	30864 $\pm$ 2391a	23860 $\pm$ 3218b	36623 $\pm$ 1278a	46193 $\pm$ 4864a	20924 $\pm$ 3478b	29815 $\pm$ 829b
Basidioma	30864 $\pm$ 2391	33264 $\pm$ 3291	39635 $\pm$ 6078	46193 $\pm$ 4864	38346 $\pm$ 3849	37480 $\pm$ 6844
		*	ns		*	ns

Following oxidative stress induction with H₂O₂, both Myc and FB extracts reduced ROS levels in a dose-dependent manner (Fig. 3b). Myc extracts were significantly effective with respect to negative control by lowering ROS to 45% and 65% at 50 and 100 µg mL⁻¹, respectively. In contrast, FB extracts did not significantly reduced ROS level, which only decreased to 83% (50 µg mL⁻¹) and 81% (100 µg mL⁻¹). Significant differences were also found between Myc and FB 50 µg mL⁻¹ extracts.

Intracellular lipid accumulation

Treatment with the extracts reduced intracellular lipid content regardless fungal material in a concentration-dependent manner (Fig. 5.4). In particular, the Myc extract induced a significant reduction of PA accumulation only at 100 µg mL⁻¹ with respect to the control (85%) (Table 4). The FB extract had a stronger effect, lowering significantly lipid accumulation to 79% and 77% of control values both at 50 and 100 µg mL⁻¹, respectively (Table 5.4). Significant differences were also found between Myc and FB extracts at both extract concentrations, with FB extract proved more effective anti-steatotic agent (Table 5.4).

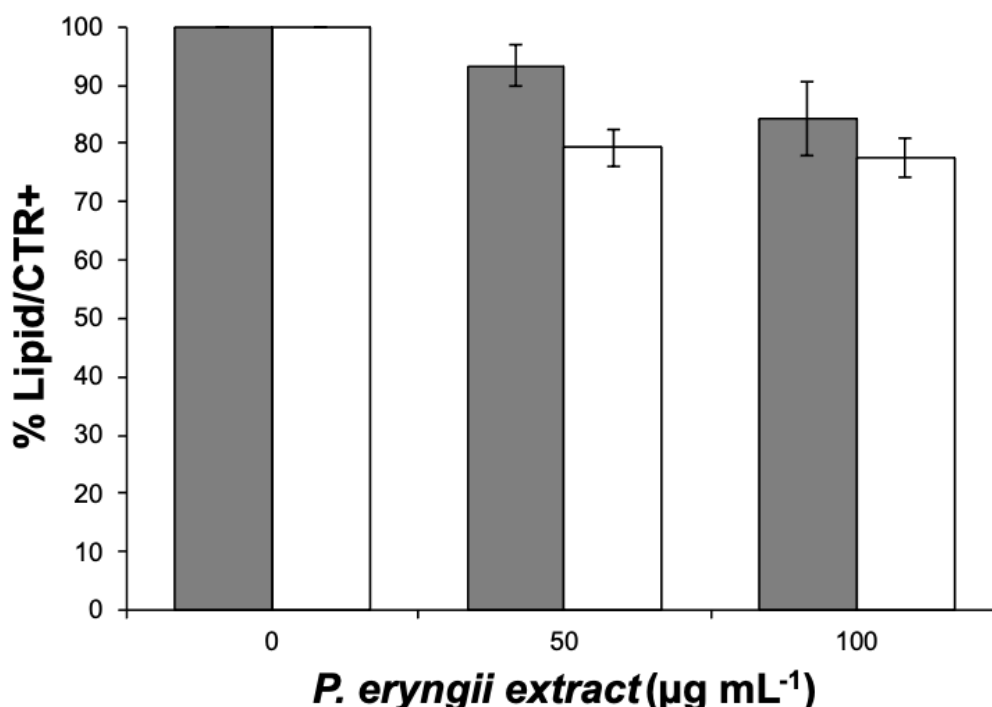


Fig. 5.4. Effect of *P. eryngii* ethanolic extracts on HepG2 cell lipid accumulation at 48 h of treatment with mycelium or fruiting body extracts. Percentages were calculated based on the values of the positive control (with PA). Grey and white columns are referred to mycelium and fruiting body extracts, respectively. Data are shown as the mean ± SD of three replicates.

Table 5.4. mean and standard deviations of absorbance values from HepG2 cells at different concentrations of mycelium and fruiting body ethanolic extracts. Different letters on the same row and asterisk in the same column indicate significant differences between means ($p < 0.05$).

	Negative control	Ethanolic extract concentration ($\mu\text{g mL}^{-1}$)	
		50	100
Mycelium	$0.231 \pm 0.010\text{a}$	$0.216 \pm 0.007\text{a}$	$0.195 \pm 0.006\text{b}$
Basidioma	$0.231 \pm 0.010\text{a}$	$0.183 \pm 0.002\text{b}$	$0.179 \pm 0.0004\text{b}$
		*	*

Anti-inflammatory potential

Under basal conditions (no LPS), 3 out of 4 extract treatments significantly reduced caspase-1 release compared to the negative control, with the most pronounced effect observed for FB extract at $100 \mu\text{g mL}^{-1}$ (~76% of control) (Fig. 5.5a, Table 5.5). A significant drop of caspase-1 was also found for FB extract with respect to Myc extract at $100 \mu\text{g mL}^{-1}$. Following LPS stimulation, only $50 \mu\text{g mL}^{-1}$ *P. eryngii* extracts reduced caspase-1 activity with respect to the positive control (Fig. 5.5b, Table 5.5). However, significant differences were only found between FB and Myc extracts at $50 \mu\text{g mL}^{-1}$, with FB extracts that reduced caspase-1 activity to ~78% of control.

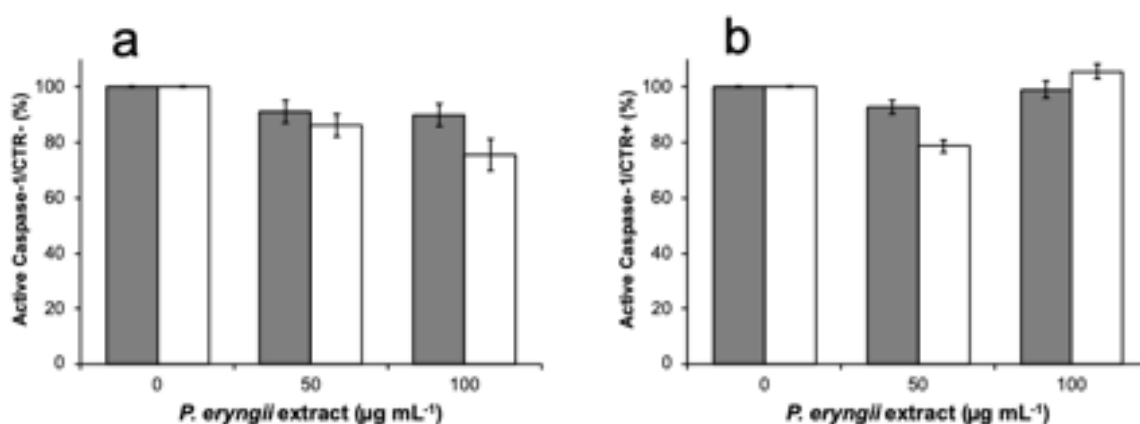


Fig. 5.5: Effect of *P. eryngii* ethanolic extracts on HepG2 cell Caspase-1 activity released into the culture medium at 24 h of treatment with mycelium or fruiting body extracts. Caspase-1 activity was measured under basal conditions (a) and after stimulation by LPS (b) and percentages were calculated based on the values of negative (a) or positive (b) controls. Grey and white columns are referred to mycelium and fruiting body extracts, respectively. Data are shown as the mean $\pm$ SD of three replicates.

Table 5.5. mean and standard deviations of relative fluorescence units (RFU) from HepG2 cells at different concentrations of mycelium and fruiting body ethanolic extracts. Different letters on the same row and asterisk in the same column indicate significant differences between means ($p < 0.05$). ns = not significant.

- LPS		+ LPS	
Negative control	Ethanolic extract concentration ($\mu\text{g mL}^{-1}$)	Positive control	Ethanolic extract concentration ($\mu\text{g mL}^{-1}$)

		50	100		50	100
Mycelium	631 ± 33a	575 ± 23b	566 ± 6b	784 ± 57	729 ± 68	777 ± 76
Basidioma	631 ± 33a	543 ± 8b	476 ± 14c	784 ± 57ab	617 ± 51a	829 ± 69b
		ns	*		ns	ns

Discussion

Investigations on medicinal and nutraceutical properties of *Pleurotus* spp. have increased considerable in recent years (Masri et al., 2017; Venturella and Gargano 2017; Kumar 2020; Wal et al., 2023; Medihi et al., 2024). Antioxidant, antiproliferative and anti-inflammatory activities have been examined in different species, using a variety of tumour cell lines (Elhusseiny et al., 2021; Bassi et al., 2024; Pandey et al., 2024). However, almost all these works did not compare the biological activities of fruiting body and mycelium extracts obtained from the same *Pleurotus* strain (Souilem et al., 2017). Overall, the results obtained in this study further confirm several medicinal properties attributed to *P. eryngii* and reinforce the perspectives for its application in nutraceutical and therapeutic contexts either as mycelium or fruiting body. The antiproliferative activity observed in this work on hepatocellular carcinoma (HCC) cells is consistent with the extensive evidence documenting the antitumor potential of *P. eryngii* extracts across various human cancer cell lines of lung, liver, stomach, breast, colon, prostate and cervix (Fontana et al., 2014; Mariga et al., 2014; Souilem et al., 2017; Acay et al., 2021; Contreras-Ochoa et al., 2022; Önder et al., 2024). In our experiments, both Myc and FB ethanol extracts significantly reduced the viability of Huh7 cells, particularly when concentration gets closer to 100 µg mL⁻¹. Antiproliferative activity of *P. eryngii* extracts have never been tested on Huh7 cells but similar results were obtained on these cells with water extracts of *P. pulmonarius* exceeding 50 µg mL⁻¹ (Xu et al., 2012). In contrast, HepG2 cells showed a low sensitivity to both Myc and FB extracts and did not reach 50% inhibition at any tested concentrations. The same anti-proliferative behaviour of HepG2 cell was observed by Souilem et al. (2017) which obtained IC₅₀ values only at concentrations of methanol extracts higher than 200 µg mL⁻¹ regardless the fungal material (mycelium or basidioma). In literature, lower IC₅₀ values have been reported only when HepG2 cells have been treated with some specific protein fractions of *P. eryngii* extracts (Li et al., 2013; Mariga et al., 2014) but no with other compounds as polysaccharides (Al-Saffar et al., 2020; Choi et al., 2017; Li & Shah, 2016). The differential susceptibility between Huh7 and HepG2 aligns with previous studies showing that hepatocellular carcinoma cell lines often display heterogeneous responses to mushroom-derived extracts and purified fungal bioactives (Xu et al., 2012; El-Maradny et al., 2025). Regarding cell viability, Myc and FB extracts displayed a broadly comparable biological behaviour. This pattern is consistent with previous reports in *Pleurotus*

species. In *P. pulmonarius*, polysaccharides derived from mycelium and fruiting bodies exerted similar inhibitory effects on the proliferation of several colon cancer cell lines (Lavi et al., 2010). Nevertheless, this behaviour cannot be considered universal. For instance, Souilem et al. (2017) reported a clear divergence between vegetative and reproductive stages of *Suillus bellinii*, with fruiting body extracts showing a substantially stronger anti-proliferative effect than mycelial extracts. These observations highlight that stage-dependent bioactivity is species-specific. The low cytotoxicity detected in our work by the LDH assay indicates that the observed decrease in viability is primarily due to proliferation inhibition rather than necrotic cell death due to cell membrane damage, supporting a mechanism of action compatible with controlled antiproliferative effects rather than nonspecific toxicity. Cytotoxicity increased significantly as the concentration of ethanolic extract increased regardless the cell type or fungal material. However, the percentage of necrotic cells never exceeded 2% with respect to the control and FB extract proved to be significantly less cytotoxic than Myc extract. In this respect, different authors reported that specific protein and polysaccharide purified fractions from *P. eryngii* fruiting bodies considerably increase LDH release when applied to HepG2 cells at high concentrations (Mariga et al., 2014b; Ma et al., 2016; Ren et al., 2016). The antioxidant activity measured in HepG2 cells provides additional support for *P. eryngii* as a valuable source of redox-active biomolecules. Myc extracts were the most effective in attenuating oxidative stress, especially at the lowest concentration of 50 $\mu\text{g mL}^{-1}$, both under basal conditions and following H_2O_2 -induced oxidative stress. In contrast, FB extracts showed only minimal interference with physiological redox homeostasis. The higher activity of the mycelium extract observed in our system may reflect differences in chemical composition between the two fungal materials, which are known to display distinct profiles of proteins, polysaccharides, and secondary metabolites (Berger et al., 2022). For example, α - and β -glucans display strong antioxidant capacity (Cateni et al., 2018; Zhang et al., 2014; Liang et al., 2013) and their composition very greatly between mycelium and fruiting body of the same *Pleurotus* strain (Devi et al., 2015). Although direct comparisons are constrained by methodological differences in evaluating antioxidant activity, our results did not align with previous reports conducted on *P. eryngii*, underscoring that the relative contribution of each fungal material strongly depends on the strain, growth conditions, and analytical approach. Souilem et al. (2017) found that mycelial and fruiting body extracts display a comparable antioxidant activity in multiple chemical assays (DPPH, reducing power, β -carotene bleaching, and TBARS) whereas Liang et al (2013) reported higher antioxidant effectiveness for fruiting bodies compared to mycelia. It worth noting that, the chemical assays applied by the latter authors mainly reflect chemical scavenging capacity of extracts or metabolites, whereas our DCFH-DA-based model evaluates intracellular redox machinery, which is controlled by complex biochemical pathways. To our

knowledge, no previous studies have directly investigated the intracellular antioxidant activity of *P. eryngii* extracts in HepG2 cells using a DCFH-DA-based approach. Our results also indicated that *P. eryngii* ethanolic extracts can mitigate palmitate-induced steatosis, probably through modulation of lipid metabolism or enhancement of antioxidant defences. Both Myc and FB extracts reduced intracellular lipid accumulation in a dose-dependent manner, with a more pronounced effect observed for FB extract, which was already effective at concentration of 50 $\mu\text{g mL}^{-1}$ and reduced lipid accumulation over 20% with respect to the negative control at 100 $\mu\text{g mL}^{-1}$. Although there are no investigations comparing the effects of Myc and FB extracts of *Pleurotus* spp. on lipid accumulation in liver cell lines, it is known that both crude extracts and polysaccharides of *P. eryngii* mycelia and fruiting bodies improve non-alcoholic fatty liver disease and obesity in mice (Chen et al., 2012; Ren et al., 2014; Xu et al., 2017; Gao et al., 2018; Jo et al., 2019; Nakahara et al., 2020). No evidence of detection of anti-inflammatory activity on HepG2 cells by *P. eryngii* metabolites is present in literature. The evaluation of this biological property is usually conducted on macrophage models but, in our study, we focused specifically on evaluating the bioactive properties of our *P. eryngii* strain on HCC cells. Under basal conditions, all extracts significantly reduced caspase-1 release, with FB extracts showing the best performances as its concentration increased. On the contrary, no anti-inflammatory effects relative to the control were observed when cells were stimulated by LPS, except for 50 $\mu\text{g mL}^{-1}$ FB extract. Anti-inflammatory effect of *P. eryngii* methanolic extracts on RAW264.7 macrophages determined by Soilem et al. (2017) varied greatly depending on the growth conditions of mycelium, with liquid cultures proving to be less efficient than solid cultures. In the same work, fruiting body extracts obtained intermediated results, with an IC_{50} slightly over 200 $\mu\text{g mL}^{-1}$, a result that is probably similar to that obtained by our HepG2 cells.

Conclusion

The functional activities characterized in this study integrate seamlessly with the studies on bioactive properties of *P. eryngii*, which arise as a multifunctional edible organism with antitumor, antioxidant, anti-steatotic, and immunomodulatory properties. In particular, the high pronounced antiproliferative activity revealed for Huh7 cells, which have a high tumorigenic and metastatic potential, further strengthens the knowledge on the bioactivity profile of *P. eryngii*.

Both Myc and FB extracts have proven to be effective as sources of bioactive molecules against HHC cells. However, several differences between the two fungal materials have been significantly disclosed depending on the extract concentration but especially on the type of biological processes assayed. While the effects on cell viability and inflammation of the two extract types are almost similar, Myc extract appears to be more effective as antioxidant and FB extract is more helpful to protect liver cells from lipid accumulation. The comparison of metabolite profiles differentially

expressed by Myc and FB extract might help to better understand how each molecule and its concentration rate can affect the different biological activities.

Chapter 6

Cadmium bioaccumulation in *Pleurotus eryngii* mycelia

Introduction

Because heavy metals persist in contaminated matrices, biological traits of organisms such as biosorption capacity, tolerance abilities, and enzymatic responses are increasingly explored as complementary remediation options. In this respect, *P. eryngii* mycelium expresses effective metal-binding, tolerance, and enzymatic response abilities that can be exploited for mycoremediation processes (Joo et al., 2011; Wu et al., 2016; Tang et al., 2016). In vitro experiments have shown that *P. eryngii* mycelium can efficiently accumulate metallic contaminants in liquid culture systems, including nanoparticles and radionuclides (Jakubiak et al., 2014; Asztemborska et al., 2016). Moreover, exposure to cadmium induces pronounced antioxidative and metabolic responses in the mycelium, reflecting a high degree of metal tolerance (Huang et al., 2022). At the molecular level, transcriptomic evidence indicates that cadmium stress triggers an oxidoreductive/heat-shock response in *P. eryngii* mycelium, and that nitric oxide can enhance biomass and tolerance by activating metabolic pathways connected to cellular redox and cofactor biosynthesis (Li et al., 2018). Beyond investigation conducted under in vitro conditions, pot experiments also support the remediation potential of *P. eryngii* in complex contaminated environments (Wang et al., 2018); in soil co-contaminated with cadmium and the organochlorine pesticide endosulfan, co-cultivation of *P. eryngii* and *Coprinus comatus* increase the cadmium removal and almost completely degraded the organic pollutant in the substrate. Complementarily, experiments carried out on industrial wastewater confirms that *P. eryngii* mycelium tolerate and accumulate high concentration of lead and cadmium, reinforcing the idea that mycelial biomass of this mushroom can be efficiently used in mycoremediation-oriented systems (Goligar et al., 2023).

Given the dual relevance of cadmium as a food contaminant and a target in bioremediation processes and the importance of *P. eryngii* as edible mushrooms, it is essential to further investigate this specific fungus/metal combination, both in terms of consumer safety and the potential use in bioremediation strategies. *P. eryngii* can be considered a suitable fungal model for bioremediation studies because it is easy to isolate and maintain in pure culture, its mycelium is characterized by a high growth rate and can be cultivated on a vast range of substrates. This work aims to evaluate the cadmium

bioaccumulation capacity and the growth rate of a wild and a cultivated strain of *P. eryngii* mycelium, under invitro conditions.

Materials and Methods

Isolation and maintenance of fungal strains

Experimental activities were conducted using two out the four strains of *P. eryngii* involved in genome analysis in Chapter 4: the wild-type strain Per1 and the cultivated strain Per3. Strain isolation and maintenance procedures were performed according to the protocol described in Chapter 4.

Experimental design of mycelial growth under cadmium exposure

Two complementary experimental approaches were adopted to investigate cadmium (Cd^{2+}) bioaccumulation in Per1 and Per3 mycelia growth in liquid culture. These two approaches differed by the growth rate of mycelial strains cultured in Cd-enriched media. In total, 36 samples were examined (2 strains $\times$ 3 treatments $\times$ 6 repetitions).

Direct exposure to cadmium during mycelial growth (method I):

Liquid cultures were prepared using Potato Dextrose Broth (PDB; 25 g L^{-1}) distributed into 36 flasks (125 mL of PDB per flask) and sterilized by autoclaving at 121°C for 30 min. A cadmium chloride (CdCl_2) stock solution (10 g L^{-1}), prepared in deionized water and sterilized by autoclaving (121°C , 15 min), was added to the culture medium to obtain the following experimental treatments:

- control (no Cd addition);
- Cd-50 concentration (50 μL stock solution; $2.45 \text{ mg L}^{-1} \text{ Cd}^{2+}$);
- Cd-100 concentration (100 μL stock solution; $4.9 \text{ mg L}^{-1} \text{ Cd}^{2+}$).

Mycelial plugs (0.6 cm diameter) were excised from the edge of 20-day-old PDA cultures and inoculated into the liquid medium (one plug per flask). For each strain (Per1 and Per3) and treatment (control, Cd-50 and Cd-100), six biological replicates were prepared. Cultures were incubated at 22°C in the dark on a rotary shaker (60 rpm) for approximately 20 days to promote mycelial development.

Two-step cultivation with delayed cadmium exposure (method II):

In addition to direct inoculation of the strains on cadmium supplemented medium, a two-step cultivation approach was applied to evaluate cadmium bioaccumulation following preliminary biomass production under non-stress conditions. In the first stage, mycelia were grown in Cd-free PDB (25 g L^{-1}) distributed into 36 flasks (40 mL per flask) and incubated at 22°C in the dark for

approximately 10 days. Subsequently, mycelial colonies were rinsed in sterile water and transferred into 36 flasks containing Cd-enriched PDB (2.45 and 4.9 mg L⁻¹ Cd²⁺) or free-Cd PDB (controls). Preparation of culture medium and inoculation procedure were the same as described above. Cultures were incubated at 22 °C in the dark on a rotary shaker (60 rpm) for an additional 10 days.

Mycelial harvesting and sample preparation

At the end of the incubation period, mycelial biomass was recovered by filtration and thoroughly washed with sterile autoclaved water to remove any residue of culture medium components. During all handling steps, contact with metallic tools was avoided, and only plastic materials were used to minimize contamination. Excess water was removed by gentle manual pressure using a fine-mesh sieve. Washed mycelia were transferred into polypropylene tubes and stored at -20 °C pending lyophilization. Samples were subsequently freeze-dried at -50 °C for 72 h in a Virtis Benchtop lyophilizer (SP industries), ground to a fine powder under liquid nitrogen, and stored at -20 °C pending chemical analysis.

Acid digestion of mycelial samples

Acid digestion of mycelial samples was performed using an oxidizing mixture of nitric acid (HNO₃, 67–69%) and hydrogen peroxide (H₂O₂, 30%). Ten mg of freeze-dried and homogenized mycelium were weighed into Teflon digestion tubes and treated with 2 mL HNO₃ and 2 mL H₂O₂. Samples were pre-digested for 24 h under a fume hood and subsequently heated in a thermostated water bath at 190 °C until complete mineralization. After cooling, digests were quantitatively transferred into 50 mL polypropylene volumetric flasks, spiked with rhodium (Rh) as internal standard (final concentration 10 ppb), and brought to volume with Milli-Q ultrapure water. Procedural blanks were prepared following the same protocol without addition of the dried mycelium.

ICP-MS analysis

Metal quantification was performed by Inductively Coupled Plasma mass spectrometer (Mass Scan – Single Quadrupole – Kinetic Energy Discrimination), which allows precise and interference-free determination of trace elements. The triple-quadrupole configuration (Q1–Q3) enables efficient removal of spectral interferences through a collision/reaction cell operated in KED (Kinetic Energy Discrimination) mode, ensuring high mass resolution and accuracy (Balcaen et al., 2015; Thermo Fisher Scientific, 2020). Helium was used as the collision gas in KED mode to minimize polyatomic interferences. Samples are nebulized into an argon plasma, where the elements are atomized and ionized. The resulting ions are separated according to their mass-to-charge ratio (*m/z*) and detected by a ICP–MS/MS, Sample introduction was performed via a peristaltic pump connected to a

nebulization system and a cyclonic spray chamber, ensuring the formation of a fine aerosol directed into the plasma torch. Instrumental conditions (plasma power, gas flow rates, dwell time) followed manufacturer specifications. Cadmium concentrations were determined by monitoring the ^{111}Cd isotope, with ^{107}Rh used as internal standard. All samples were analyzed in triplicate (technical rereplicates), and a mean value was calculated.

Statistical analysis

Statistical analyses were conducted separately for the two experimental approaches. Data were analyzed using two-way analysis of variance (ANOVA) with strain (Per1 and Per3) and cadmium concentration (0, 2.45, and 4.9 mg L⁻¹ Cd²⁺) as independent variables. Statistical significance was evaluated by post-hoc comparisons using Tukey's HSD test ($p < 0.05$). In the presence of significant interaction effects, one-way ANOVA followed by Tukey's HSD test or t-student test were applied separately to each strain or cadmium concentration, respectively. Student's *t*-test was used for direct comparisons between strains under identical cadmium treatments. All statistical analyses were performed using PAST software v. 4.03 (Hammer et al., 2001).

Results

Mycelial biomass production

In the first experimental approach (method I), lyophilized mycelial biomass showed a marked reduction in cadmium-treated samples compared to controls for both *P. eryngii* strains (Table 6.1). For the wild-type strain Per1, the control sample displayed a mean dry weight of 0.33 g, whereas samples Cd-50 and Cd-100 showed substantially lower weights of 0.03 g and 0.02 g, respectively. Similarly, for the industrial strain Per3, the control samples weighed 0.34 g on average, while lyophilized mycelial biomass decreased to 0.04 g in both cadmium treatments. In the second experimental approach (method II), higher mycelial biomass was generally observed. For Per1, the control sample showed a dry weight of 0.39 g, while samples Cd-50 and Cd-100 had a dry weight of 0.03 and 0.30 g. For strain Per3, the control and Cd-50 samples weighed 0.46 g on average, while exposure to 4.9 mg L⁻¹ of Cd led to a reduction of mycelial dry weight to 0.22 g.

Table 6.1. Dry weight (g) of lyophilized mycelial samples obtained under the two experimental methods.

Strain	Control	Cd-enriched medium	
	0-Cd	50-Cd	100-Cd
Method I			
Per1	0.331 ± 0.050	0.030 ± 0.006	0.020 ± 0.004
Per3	0.338 ± 0.055	0.040 ± 0.008	0.040 ± 0.007
Method II			
Per1	0.391 ± 0.070	0.337 ± 0.060	0.300 ± 0.055
Per3	0.456 ± 0.080	0.462 ± 0.075	0.430 ± 0.070

Cadmium accumulation in mycelial biomass

Control samples exhibited consistently lower cadmium concentrations compared to treated samples, regardless of strain and experimental approach (Table 6.2). Mean Cd^{2+} concentrations in controls ranged from 0.2 to 3.2 $\mu\text{g g}^{-1}$ dry weight. For the wild-type strain Per1, direct exposure to cadmium from the beginning of cultivation (method I) resulted in mean cadmium concentrations of 214.7 $\mu\text{g g}^{-1}$ dry weight for 50-Cd treatment and 160.2 $\mu\text{g g}^{-1}$ for 100-Cd. When cadmium was added after 10 days of mycelial growth (method II), higher mean concentrations were detected, reaching 404.5 $\mu\text{g g}^{-1}$ for 50-Cd treatment and 447.8 $\mu\text{g g}^{-1}$ for 100-Cd. A similar trend was observed for the industrial strain Per3. Under method I, mean cadmium concentrations were 221.0 $\mu\text{g g}^{-1}$ and 249.5 $\mu\text{g g}^{-1}$ dry weight for 50-Cd and 100-Cd treatments, respectively. Under method II, mean concentrations increased to 235.7 $\mu\text{g g}^{-1}$ for 50-Cd treatment and 362.8 $\mu\text{g g}^{-1}$ for 100-Cd.

Table 6.2. Cadmium concentrations ($\mu\text{g g}^{-1}$ mycelium dry weight) measured in mycelial samples. Values are reported for three biological replicates (A–C), together with mean concentration and standard deviation.

Strain	Control	Cd-enriched medium	
	0 $\mu\text{L CdCl}_2$	50 $\mu\text{L CdCl}_2$	100 $\mu\text{L CdCl}_2$
Method I			
Per1	0.8 ± 0.1	214.7 ± 34.7	160.2 ± 5.9
Per3	3.2 ± 0.2	221.0 ± 9.3	249.5 ± 7.4
Method II			
Per1	0.2 ± 0.2	404.5 ± 66.2	447.8 ± 3.3
Per3	0.6 ± 0.1	235.7 ± 27.9	362.8 ± 44.5

For the first experimental approach (method I), a two-way ANOVA was initially performed considering strain and Cadmium concentration as independent variables. The analysis revealed a significant interaction between strain and concentration ($p = 0.02$), indicating that cadmium accumulation differed between strains depending on the applied concentration. For strain Per1, no statistically significant difference was detected by one-way ANOVA between 50-Cd and 100-Cd treatments ($p = 0.2$), while both treatments differed significantly from the control ($p < 0.004$) (Figure 6.1a).

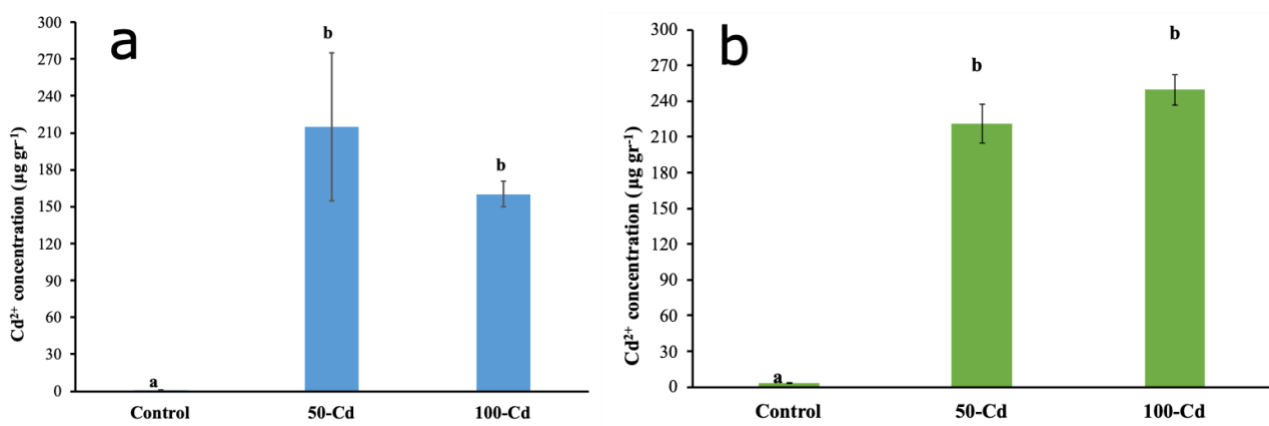


Figure 6.1. Mean Cd concentrations (µg g⁻¹ mycelium dry weight) measured in the three experimental conditions of method I for strain Per1 (a) and Per3 (b). Error bars represent the standard deviation. Different letters indicate significant differences between means ($p < 0.05$).

For strain Per3, a trend toward significance was instead observed between the two cadmium treatments ($p = 0.058$), whereas highly significant differences were found between the control and cadmium-exposed samples (Figure 6.1b). Direct comparison between strains for method I, performed using Student's *t*-test, did not reveal statistically significant differences in Cadmium accumulation between Per1 and Per3 at 50-Cd treatment but Cadmium accumulation was significantly higher in Per3 mycelium at 100-Cd ($p = 0.0007$) treatments (Figure 6.2).

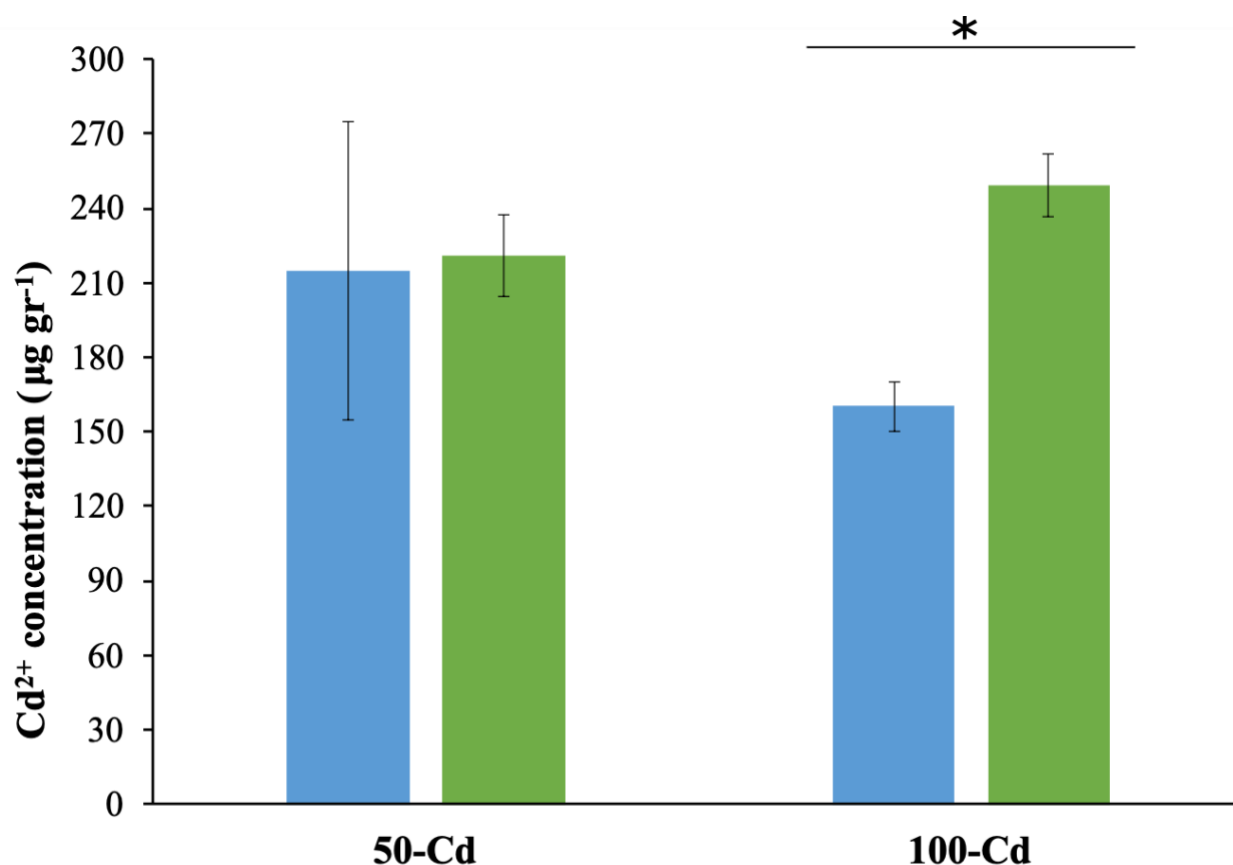


Figure 6.2. Comparison of mean cadmium concentrations ($\mu\text{g g}^{-1}$ dry weight) between strains Per1 and Per3 under method I. Error bars represent the standard deviation. Statistical analysis was performed using Student's *t*-test. * indicates significant differences between mean ($p < 0.001$).

For method II, two-way ANOVA again indicated a significant interaction between strain and cadmium concentration ($p = 0.004$), consequently, statistical analyses were conducted separately for each strain. For strain Per1, no significant differences were detected by one-way Anova between the 50 μL and 100 μL CdCl_2 treatments ($p = 0.40$), although both treatments showed also for this strain highly significant differences compared to the control condition ($p < 0.00004$) (Figure 6.3a). In contrast, strain Per3 exhibited a statistically significant difference between the two cadmium treatments ($p = 0.005$), with higher intracellular cadmium concentrations measured in 100-Cd treatment compared to the 50-Cd treatment (Figure 6.3b). As observed for method I, control samples differed significantly from cadmium-treated samples for both strains. Comparison between strains for method II using Student's *t*-test showed that strain Per3 accumulated significantly lower cadmium levels than strain Per1 at both cadmium concentrations. Specifically, significant differences were detected at 50 μL CdCl_2 ($p = 0.015$) and at 100 μL CdCl_2 ($p = 0.03$) (Figure 6.4).

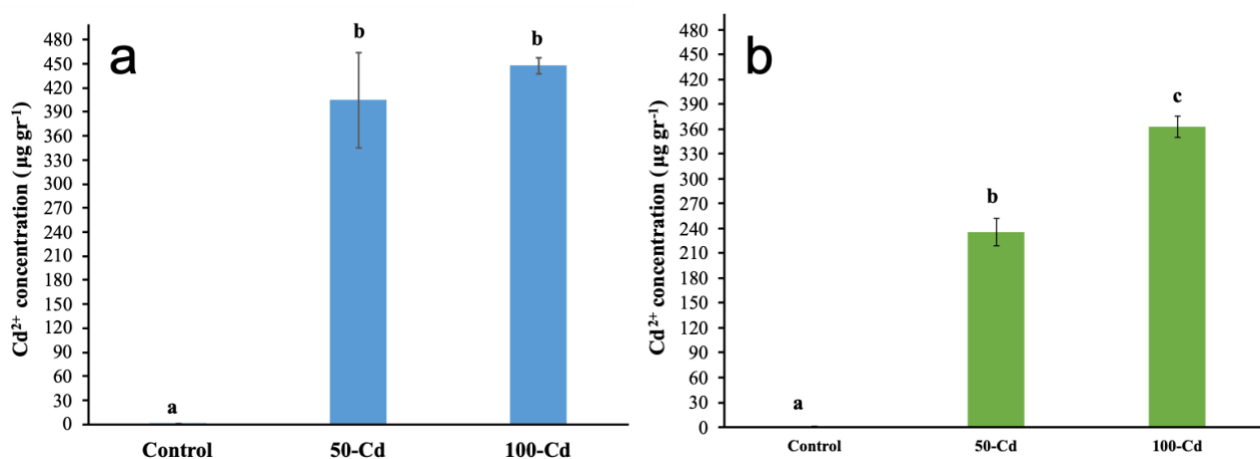


Figure 6.3. Mean Cadmium concentrations ($\mu\text{g g}^{-1}$ mycelium dry weight) measured in the three experimental conditions of method I for strain Per1 (a) and Per3 (b). Error bars represent the standard deviation. Different letters indicate significant differences between means ($p < 0.05$).

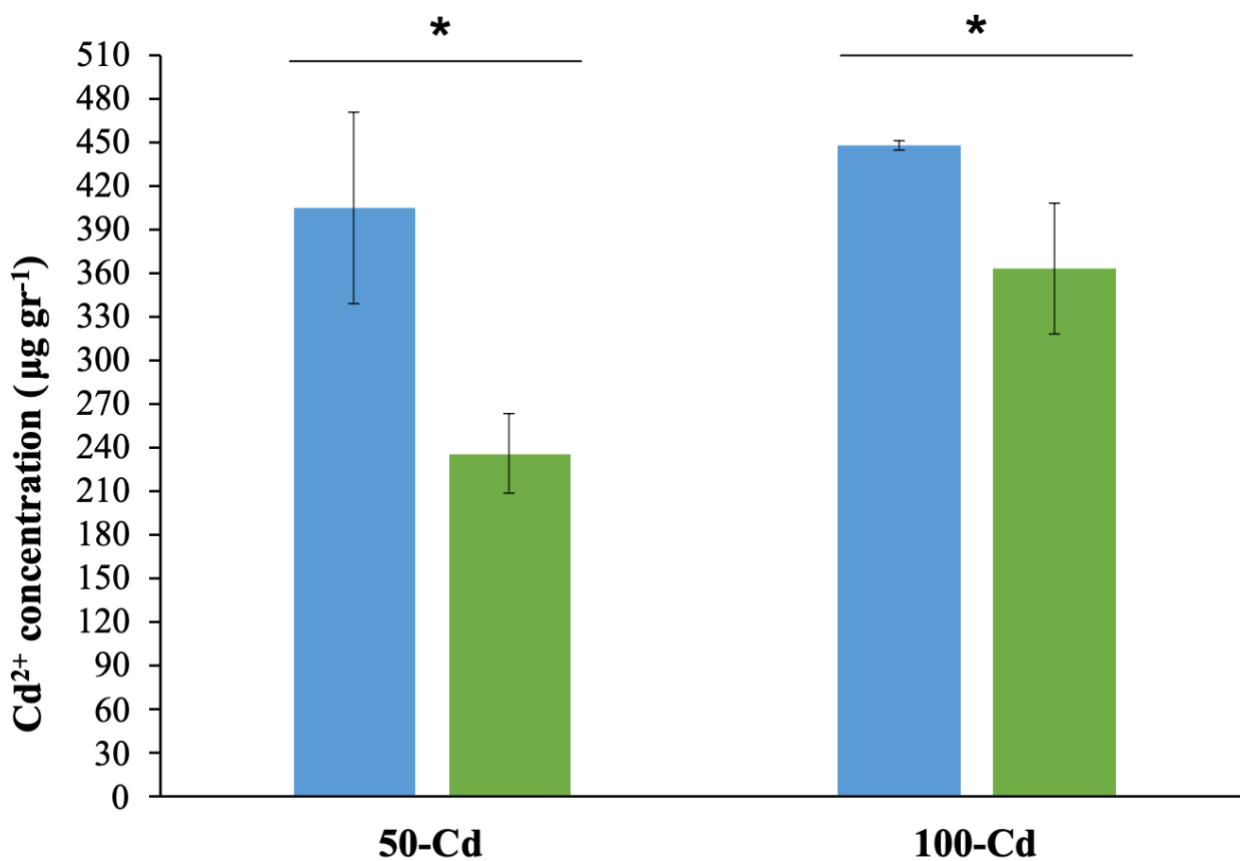


Figure 6.4. Comparison of mean cadmium concentrations ($\mu\text{g g}^{-1}$ dry weight) between strains Per1 and Per3 under method II. Error bars represent the standard deviation. Statistical analysis was performed using Student's *t*-test. * indicates significant differences between mean ($p < 0.05$).

Discussion

Cadmium represents a major contaminant of concern for fungal growth, and its accumulation significantly affects fungal physiology (Gao et al., 2020; Huang et al., 2022). The effects of cadmium accumulation on *P. eryngii* mycelia have been investigated by a few authors. Huang et al. (2022) evaluated the expression of antioxidant and sugar-metabolism enzymes in a strain cultured at different cadmium concentrations while Li et al. (2018) studied the mechanism related to enhancement of cadmium stress tolerance using NO-enriched substrates. In this study we evaluated cadmium bioaccumulation in two *P. eryngii* strains with different genomic traits (see chapter 4), using two experimental approaches differing in the timing of metal exposure. Statistical analyses showed that the two strains responded differently to cadmium exposure in both experimental approaches and significant interactions between strain and metal concentration variables were also proofed. In the method I, the mycelial dry weight of both strains obtained by cadmium treatments was much lower than those prepared with method II while biomass of controls was similar regardless of strain and timing cadmium exposure. Indeed, the ability of the mycelium to grow and accumulate cadmium is strongly compromised if mycelium is not actively growing. There is to be considered that with the method I the mycelium must go through a shorter or longer lag phase during which cadmium might interfere with the reactivation of the cell's metabolism. A significant decrease in mycelium biomass was also measured by Li et al. (2018) when *P. eryngii* inoculum was transferred to PDB broth containing 5.6 mg L⁻¹ of CdCl₂. With this experimental approach, the cadmium concentrations in the medium (2.45 and 4.9 mg L⁻¹ Cd²⁺) does not significantly affect the mycelium growth and cadmium biosorption of both strains. However, the commercial strain Per3 seemed to be more resilient as the concentration of the heavy metal double whereas the wild strain Per1 reduced both mycelial growth and cadmium uptake. The behaviour of the two strains changed when the mycelia were exposed to the cadmium during the exponential phase of the colony growth. The commercial strain Per3 showed a significant increase in cadmium accumulation at concentration of 4.9 mg L⁻¹ Cd²⁺ compared to 2.45 mg L⁻¹ Cd²⁺, but the mycelial growth was reduced. On the contrary the wild-type strain Per1 exhibited a more stable response of cadmium uptake across concentrations and limited reduction of the mycelial biomass. Direct strain comparisons indicated that Per3 significantly accumulated lower amounts of cadmium than Per1 under the same concentration although this phenomenon was more evident at the lowest cadmium concentration. These differences can be attributed to strain-specific genetic and physiological traits. Per3, an industrial strain derived from genetic crosses, likely differs from the wild-type strain Per1 in metabolic activity and growth dynamics, factors known to influence heavy-metal uptake. Similar intraspecific variability has been reported by Golian et al. (2022), who observed substantial differences in metal accumulation among 59 strains of *P. ostreatus* grown under the same

conditions, highlighting the importance of genetic background in determining bioaccumulation capacity. Previous studies support the role of genetic and metabolic factors in modulating fungal responses to cadmium. A comparative transcriptomic analysis of two *Agaricus brasiliensis* strains with contrasting cadmium sensitivity identified genes involved in metal uptake and detoxification, showing that mycelial growth rate, metabolic efficiency, and cell wall properties influence cadmium sequestration (Liu et al., 2020). Our work also highlighted that the growth rate is one of the most important factors affecting the ability of mycelia to uptake cadmium from the medium (method I vs method II), most likely due to the higher absorbing surface and vacuolar volume of the hyphae. Also, the differences among strains of the same species and among developmental stages can translate into marked variation in heavy-metal accumulation. At the cellular level, cadmium detoxification in fungi involves binding at the cell wall and subsequent transportation and sequestration into vacuoles, where the metal complexes with intracellular ligands, reducing cytotoxicity. Vacuole accumulation has been identified as the primary intracellular sink for cadmium in several mushroom species, including *Agrocybe aegerita* and *Lentinula edodes* (Li et al., 2019; Chen et al., 2025). However, the capacity of vacuolar transporters and ligands is limited, and saturation effects may occur, leading to a non-proportional increase in intracellular cadmium despite higher external concentrations (Gadd, 1993; Chen et al., 2018; Zhang et al., 2023). In our work, this mechanism is consistent with the lack of significant differences between the cadmium treatments observed in most of experimental conditions. Moreover, comparing the results of the two methods, the tenfold increase of the mycelial biomass occurring in method II has only corresponded to about two-fold increase of cadmium uptake although differences in the experimental procedures (mycelium washing and the substrate change in the method II) could have influenced the results. From an applied perspective, the lower cadmium accumulation observed in the industrial strain Per3 compared to the wild-type strain Per1 when mycelia were well developed suggests that strain selection might also consider the safety profile of cultivated *Pleurotus* mushrooms. Overall, these results highlight the combined influence of genetic background and mycelial development on cadmium bioaccumulation in *P. eryngii*. The findings support the need for accurate analytical monitoring of strains involved in cultivation systems to minimize potential risks associated with heavy-metal contamination in edible mushrooms. Conversely, with a view to environmental bioremediation, the choice of a more efficient strains in absorbing cadmium and other heavy metals is mandatory for the restoration of contaminated matrices.

Chapter 7

Heavy metal content in the cultivation system of *P. eryngii*

Introduction

In recent decades, *P. eryngii* has transitioned from a niche food to a large-scale cultivated mushroom. Unlike many other edible mushrooms that are still wild-harvested, *P. eryngii*, like some other *Pleurotus* species, is predominantly produced through controlled cultivation systems in greenhouses, using lignocellulosic substrates such as sawdust and agricultural residues, including rice straw, wheat straw, corn cobs, cottonseed hulls, and similar by-products (Zhang et al., 2014; Mehmet & Sevda, 2010). Cultivation practices, including substrate formulation, climate parameters (temperature, humidity, light, atmosphere features), and spawn type, strongly affect growth kinetics of mycelium, fruiting body yield, and biological efficiency, highlighting the high degree of technological setting that characterizes modern *P. eryngii* production (Zhang et al., 2014; Bellettini et al., 2019). In intensive cultivation systems, *P. eryngii* mycelium is commonly incubated in the dark at ~25 °C until the growth substrate is fully colonized, followed by a temperature downshift (e.g., ~15 °C for several days) to trigger primordia formation and support fruiting-body development (Yue et al., 2022). During this phase, environmental parameters such as relative humidity (~85%) and CO₂ concentration are managed through automated humidification and ventilation (Yue et al., 2022). In this context, product quality cannot be reduced to sensory or nutritional attributes alone, but must also include food safety parameters, especially in relation to potential biotic or abiotic contaminations. Among these, heavy metals such as Cd, Pb, Hg, and As represent a major concern, as they are non-biodegradable, persistent in the environment, and capable of entering the food chain through contaminated substrates used to cultivate mushrooms. Several studies have shown that mushrooms are efficient accumulators of potentially toxic elements, with accumulation patterns strongly depending on species-specific traits and substrate composition (Sakellari et al., 2019). In this scenario, ensuring the safety of this widely consumed food product becomes a critical issue, as heavy metals may represent a significant risk for human health. For this reason, the monitoring of metal concentrations in fruiting bodies is essential in order to assess contamination levels and the potential dietary exposure associated with mushroom consumption (Sithole et al., 2022). In Europe, food safety with respect to heavy metals is regulated by the Commission Regulation (EU) 2023/915, which establishes maximum levels for several contaminants, including cadmium, lead, mercury, and arsenic, in different food categories. For cultivated mushrooms, the currently applicable maximum levels

include, for example, 0.050 mg kg⁻¹ fresh weight for cadmium and 0.30 mg kg⁻¹ fresh weight for lead, while comparable thresholds are set for mercury (e.g., 0.050 mg kg⁻¹), with generally more permissive limits established for wild-growing species (European Commission, 2023). For *P. eryngii*, analyses performed on fruiting bodies grown on different agro-wastes revealed substantial variability in macro- and microelement contents, while heavy metals were not detected by using atomic absorption spectrophotometer (Akyüz & Kırbağ, 2010). More comprehensive investigations using ICP-MS have further demonstrated that *P. eryngii* exhibits specific bioaccumulation capacity depending on the growth matrix, although estimated dietary intakes of heavy metals generally remain below critical thresholds (Sakellari et al., 2019). Another work on marketed fruiting bodies also found that heavy metal concentrations were within the World Health Organization limits (Soceanu et al., 2024). Substrate composition has been shown to significantly modulate the concentrations of Pb, As, Cd, and Hg in *P. eryngii* fruiting bodies, even when values remain within safe ranges (Zhou et al., 2023). Experiments conducted on artificially enriched substrates demonstrated that the species could accumulate in the fruiting bodies selected metals (notably Zn and Co) while a weakest sorption was found for cadmium (Drzewiecka et al., 2012). However mycelial growth and fruiting body yield were negatively affected depending on the element type and concentration. Taken together, these observations indicate that *P. eryngii* represents a relevant case study for evaluating how cultivation practices influence the elemental profile of edible mushrooms, but the knowledge on heavy metal accumulation in *P. eryngii* fruiting body remain scarce. Therefore, the present study aims to compare the heavy metal content in fruiting bodies and growth substrates in a *P. eryngii* cultivation system, to assess their accumulation patterns.

Materials and Methods

Sampling and experimental design

Samples were collected in April from 6 different greenhouses during the last flush (the 4th) of the annual productive cycle. All samples derive from the commercial strain P3 (slow growing), as described in Chapter 4. Three different composite sample types were collected from each greenhouse: 1) fruiting bodies (FB), 2) covering soil (CS), and 3) inoculated spawn (IS). FB composite samples were obtained from the cap of 3 different fruiting bodies (10 g in total of fresh internal meat) grown in the same greenhouse. CS and IS samples were obtained by collecting a total of 30 g of covering soil and spawn collected under the 3 fruiting bodies previously sampled. Three further samples taken from the stock of covering soil (free-mycelium covering soil - FMCS) were included in the analysis

as an uninoculated reference to assess the background levels of heavy metals present in this matrix. Nineteen composite samples were analysed: 6 FB, 6 CS, 6 IS and 3 FMCS. All composite samples (herein only samples) were lyophilized for three days at – 50 °C in a Virtis benchtop freeze dryer (LB laboratories).

Sample preparation and digestion

All samples were finely ground with a ceramic mortar and pestle, sifted using a plastic sieve (1 mm mesh) to remove coarse debris, and then homogenized. Aliquots of 0.2 g of each sample type were weighed into Teflon beakers and digested with 12 mL of aqua regia (69% HNO₃: 25% HCl, 1:3 v/v) under a fume hood for organic matter mineralization. Digestion proceeded for 24 h in plates, followed by evaporation at 120 °C to near dryness. The residues were re-suspended in ultrapure water, sonicated for 10 min, and transferred into 50 mL polypropylene flasks. After acidification to 1% HNO₃ and addition of the Rhodium as internal standard for ICP-MS quantification (10 ppb final concentration), digests were brought to volume with ultrapure water and gently mixed. Rhodium (Rh) was employed as an internal standard to correct for signal drift and non-spectral interferences, improving analytical precision (Vanhaecke et al., 1992). Twelve milliliters of each digest were transferred to 13 mL tubes and centrifuged (1000 rpm, 5 min) to remove suspended particles. Supernatants were filtered through 0.22 µm nylon syringe filters and stored in polypropylene tubes for ICP-MS analysis. Each sample was analyzed in triplicate (technical replicates).

ICP-MS calibration and analysis

Quantitative analysis of Ni, Cu, As, Pb, and Cd was performed using a triple-quadrupole (ICP–MS) operated in MS–SQ–KED mode as described in the previous chapter. Calibration curves were prepared using a multi-element standard solution with increasing concentrations to evaluate the response of the analytes of interest. Calibration curves were generated from multi-element standards at concentrations ranging from 1 to 400 ppb (Table 7.1), yielding correlation coefficients (R^2) > 0.999. To prevent detector saturation, the two highest points (III and IV) were diluted 1:100 to match the expected concentration range in the analyzed samples. All standard and sample solutions were acidified to 1% HNO₃ prior to analysis.

Table 7.1. Calibration points used for ICP–MS standard curves. Values are reported as ppb.

Calibration point	Arsenic (As)	Cadmium (Cd)	Copper (Cu)	Lead (Pb)	Nickel (Ni)
Point I	3.80	4.00	4.00	4.00	1.00
Point II	7.60	8.00	8.00	8.00	2.00

Point III	38.0	4.00	4.00	40.0	10.0
Point IV	95.0	10.0	10.0	100	25.0

All samples were analyzed in triplicate, and results were expressed as $\mu\text{g g}^{-1}$ dry weight. Quality control included reagent blanks, certified reference materials (e.g., NIST 1573a – Tomato Leaves), and recovery tests (acceptable range = 85–115 %). The isotopes monitored for quantification were ^{60}Ni for Nickel, ^{63}Cu for Copper, ^{75}As for Arsenic, ^{111}Cd for Cadmium, and ^{208}Pb for Lead.

Statistical analysis

Statistics were carried out separately for each heavy metal analysed in this work. Quantitative data were analysed by applying one-way ANOVA considering the matrix type (CS, IS, FB and FMCS) as an independent variable. Statistical significance was evaluated by post-hoc comparisons using Tukey's HSD test ($p < 0.05$) and analysis was performed using PAST software v. 4.03 (Hammer et al 2001).

Results

The quantitative analysis of heavy metals in *P. eryngii* cultivation matrices revealed clear and consistent differences among elements and significant variations between materials (Table 7.2). Cadmium (Cd) was below the instrumental detection limit ($< 0.5 \mu\text{g g}^{-1}$) in almost all samples and was therefore excluded from statistics. For all the other analysed metals, a systematic decrease in concentration was observed from covering soils (CS and FMCS) to inoculated substrate and, finally, to fruiting bodies. Copper (Cu) exhibited the highest mean concentrations regardless the matrix type, while the other elements were detected at somewhat similar concentrations in the different materials. The concentration of the four heavy metals had not differed significantly between CS and FMCS although for Ni, Cu and As was slightly higher in FMCS than CS. An evident decrease in heavy metal concentration (from 15% for Cu to 25% for Pb) was observed between CS and IS samples for which significant differences were found for Ni, As and Pb but not for Cu. The same behaviour was observed between FMCS and IS samples although in this case the lack of significance was found for Pb. In fruiting bodies (FB samples), heavy metal concentrations decreased sharply, remaining consistently below $10 \mu\text{g g}^{-1}$ for Cu (decline $> 76\%$) and below $0.6 \mu\text{g g}^{-1}$ for the other elements (decline $> 96\%$). All FB samples showed highly significant decrease in heavy metal concentrations regardless of the substrate type (CS, FMCS and IS).

Table 7.2. Mean concentrations ($\mu\text{g g}^{-1}$) of Ni, Cu, As, Pb, and Cd in *P. eryngii* cultivation matrices (CS, covering soil; IS, inoculated spawn; FB, fruiting body; FMCS, free mycelium covering soil). Values represent the mean $\pm$ standard deviation of 6 biological replicates obtained from three technical replicates. Different letters in the same column indicate significant differences between means ($p < 0.05$)

Matrices	Ni	Cu	As	Pb
CS	21.35 $\pm$ 3.09a	47.88 $\pm$ 9.49ab	29.52 $\pm$ 3.37a	23.57 $\pm$ 2.63a
IS	17.12 $\pm$ 2.28b	40.86 $\pm$ 4.64b	22.34 $\pm$ 3.59b	17.74 $\pm$ 1.09b
FB	0.60 $\pm$ 0.27c	9.22 $\pm$ 3.85c	0.45 $\pm$ 0.57c	0.58 $\pm$ 0.42c
FMCS	22.30 $\pm$ 1.15a	55.60 $\pm$ 4.20a	31.00 $\pm$ 2.05a	20.03 $\pm$ 1.47ab

Dicussion

The present study provides an integrated assessment of heavy metal distribution across different matrices of a controlled *Pleurotus eryngii* cultivation system, including covering soil, inoculated substrate, and fruiting bodies. Unlike most previous investigations that primarily focused on metal concentrations in harvested mushrooms alone, this work simultaneously examined both growth substrates and fungal tissues, allowing a comprehensive evaluation of metal transfer and attenuation along the cultivation chain. This multi-matrix approach represents an original contribution to the understanding of heavy metal bioavailability and food safety in industrial *P. eryngii* production systems. The selection of nickel (Ni), copper (Cu), arsenic (As), lead (Pb), and cadmium (Cd) was based on their widespread occurrence in agricultural environments, their potential toxicity to fungi and humans, and their relevance in food safety regulations. These elements are commonly introduced into cultivation systems through contaminated soils, irrigation water, fertilizers, and agricultural residues, and display distinct mobility and bioaccumulation behaviours within biological matrices. Cadmium is a highly persistent and toxic heavy metal released through industrial activities such as metal refining, pigment production, and battery manufacturing, representing a major source of food-chain contamination (Jomova et al., 2025). Chronic exposure is associated with oxidative stress, renal damage, and carcinogenicity due to its strong bioaccumulation capacity (Genchi et al., 2020; Dukic et al., 2020; Jarup & Akesson, 2009). Arsenic, although formally classified as a metalloid, is widely regarded as a heavy metal pollutant due to its environmental persistence and multisystem toxicity, including oxidative stress, metabolic disruption, cardiovascular damage, and carcinogenic effects (Jomova et al., 2025; Medda et al., 2021; Muzaffar et al., 2023). Lead remains a major public health

concern due to its historical industrial use and long-term accumulation in mineralized tissues, inducing oxidative stress, neurotoxicity, and systemic damage (Tchounwou et al., 2012; Flora et al., 2006; Jomova et al., 2025). Copper, although essential, becomes toxic at elevated concentrations, disrupting enzymatic activity, respiration, photosynthesis, and soil microbial balance (Perić et al., 2020; Alengebawy et al., 2021). Nickel is widely used in industrial alloys and is ubiquitous in soils and water; excessive exposure induces oxidative stress, respiratory disorders, allergic reactions, and carcinogenic outcomes (Cempel et al., 2006; EFSA, 2020). All heavy metal concentrations detected in fruiting bodies were well below the maximum levels established by European regulations for cultivated mushrooms on a fresh-weight basis (European Commission, 2023). After conversion from dry-weight concentrations, values were further reduced due to the high water content of fresh tissues, confirming negligible dietary risk. Lead concentrations averaged $0.58 \pm 0.42 \mu\text{g g}^{-1}$ dry weight, corresponding to approximately $0.05\text{--}0.07 \text{ mg kg}^{-1}$ fresh weight, markedly below the regulatory limit of 0.30 mg kg^{-1} FW established for cultivated species such as *Agaricus bisporus*, *Pleurotus ostreatus*, and *Lentinula edodes*. Arsenic and nickel levels were extremely low (0.45 ± 0.57 and $0.60 \pm 0.27 \mu\text{g g}^{-1}$ DW, respectively), indicating minimal transfer into edible tissues. Copper showed higher concentrations ($9.22 \pm 3.85 \mu\text{g g}^{-1}$ DW) but remained within reported safe ranges. Cadmium was below the instrumental detection limit ($<0.5 \mu\text{g g}^{-1}$ DW) in almost all samples, well below the maximum level of 0.15 mg kg^{-1} FW permitted for cultivated mushrooms. The absence of detectable cadmium accumulation contrasts with reports on wild mushrooms frequently exceeding health-risk thresholds (Bucurica et al., 2024), highlighting the protective role of controlled cultivation conditions and substrate quality in mitigating cadmium bioavailability. A clear gradient in heavy metal concentrations was observed across cultivation matrices, with highest levels in covering soils, intermediate levels in inoculated substrates, and lowest levels in fruiting bodies. Arsenic, lead, and nickel concentrations in substrates were approximately 10–15 times higher than in fruiting bodies, a pattern consistently observed across all greenhouses. Elevated concentrations in covering soils likely reflect their natural forest origin, consistent with geochemically influenced environments in Southern Italy (Buccione et al., 2021), whereas lower concentrations in inoculated substrates may result from the quality of agricultural residues used. In industrial cultivation systems, inoculated substrates are obtained by mixing soil with straw and other lignocellulosic residues, leading to dilution of original metal content. Although straw generally represents the dominant component, substrate composition is often not fully standardized and may vary according to local availability and agricultural practices, resulting in variable elemental backgrounds (Bellettini et al., 2019; Miles & Chang, 2004). Despite measurable metal concentrations in substrates, fruiting bodies consistently exhibited markedly lower levels, suggesting the presence of physiological mechanisms limiting metal translocation. Similar

patterns have been observed in other edible basidiomycetes, such as *Agaricus spp.*, where metal concentrations decrease from substrate to fruiting bodies (Svoboda & Kalač, 2003). Species-specific traits appear to play a dominant role in accumulation behaviour (Campos et al., 2009). Metal homeostasis in fungi involves reduced cellular import, binding to metallothioneins, immobilization in cell walls, and sequestration in vacuoles (Robinson et al., 2021). Cell wall components such as chitin possess functional groups capable of binding metal ions, providing a mechanistic basis for retention within the mycelium and restricted translocation to reproductive structures (Campos et al., 2009). Although fungal genetics appears to be the primary determinant of accumulation patterns, substrate composition can modulate metal uptake, as demonstrated by supplementation studies in *Pleurotus* species (Golian et al., 2022; Ab Rahman et al., 2021). Overall, the marked decrease in metal concentrations from substrates to *P. eryngii* fruiting bodies reflects regulated, species-specific physiological controls rather than passive transfer. Beyond confirming compliance with food safety regulations, these findings contribute to the functional characterization of the industrial strains used by NaturAgri S.r.l. and provide a basis for future investigations into genomic determinants of selective metal uptake and detoxification.

Chapter 8

Conclusions

This PhD thesis provides an integrated, multi-scale assessment of fungal diversity and functional potential by combining multigene phylogenetics, population genomics, and experimental assays across three systems: nivicolous *Peziza* spp., the truffle *Tuber borchii*, and the edible basidiomycete *Pleurotus eryngii*, including wild-derived and commercial strains. Overall, the results show how fungal genomes and phenotypes are shaped by environmental constraints, geographic history, and human-mediated processes, while also highlighting fungi as valuable resources for sustainable biotechnology. In Campo Pericoli (Central Apennines), two nivicolous species, *Peziza heimii* and *P. nivalis*, were shown to co-occur within a spatially restricted snowbank habitat. Despite strong differences in abundance, both species exhibited comparable genetic diversity, supporting a complex micro-geographic structuring in psychrophilic fungi. *P. nivalis* confirmed a cosmopolitan, multi-lineage pattern, while *P. heimii* appears more geographically restricted and potentially more vulnerable to climate warming in Mediterranean alpine environments, where upward migration is limited by habitat availability. These findings support the need for continued monitoring and future genomic work to clarify adaptive mechanisms and resilience. The population genomic analysis of 27 *Tuber borchii* genomes revealed extremely low overall divergence between Sardinian and continental isolates (mean $F_{ST} \approx 0.008$), but a weak and consistent geographic signal emerged from PCA/IBS patterns and localized high- F_{ST} windows. This structure is compatible with high internal polymorphism and historical or recent connectivity rather than reproductive isolation. Given the dispersal limitations of hypogeous fungi, the observed pattern likely reflects, at least in part, human-assisted movement (e.g., forestry material, soils, and cultivation-related practices). Beyond evolutionary inference, these results provide a foundation for developing SNP-based traceability markers and region-specific conservation strategies. In *Pleurotus eryngii*, genome-wide analyses clearly separated the wild-derived strain (P1) from cultivated strains (P2–P6), consistent with domestication-driven divergence. Within commercial lines, slow-growing strains formed a homogeneous cluster, while the fast-growing strain (P6) was more distinct, suggesting differentiated breeding backgrounds. Gene-level nonsynonymous variant comparisons indicated coordinated divergence affecting regulatory machinery (transcription/chromatin), translation-related functions, metabolism, and transport, supporting a polygenic basis for traits associated with cultivation

performance. While descriptive, these findings provide testable hypotheses for future functional validation through multi-isolate datasets and multi-omics approaches. Functional assays further supported *P. eryngii* as a source of bioactive compounds. Ethanolic extracts from both mycelium and fruiting bodies showed antiproliferative effects on hepatocarcinoma cells (stronger in Huh7 than HepG2) with low cytotoxicity, and stage-dependent differences were observed across antioxidant, anti-steatotic, and immunomodulatory endpoints. These results reinforce the nutraceutical relevance of *P. eryngii* and indicate that vegetative and reproductive tissues may provide complementary bioactivities. Finally, heavy metal analyses in controlled *P. eryngii* production systems showed a clear attenuation gradient from covering soils to substrates and then to fruiting bodies, with all edible tissues remaining well below EU regulatory limits. Cadmium was largely undetectable in fruiting bodies, highlighting the importance of substrate quality and species-specific physiological barriers limiting translocation. In mycelial experiments, cadmium uptake varied by strain and depended strongly on growth phase, supporting the combined role of genetic background and developmental state in metal bioaccumulation. Together, these results support both food safety monitoring in industrial production and future strain selection strategies for cultivation and potential bioremediation applications. In conclusion, this thesis demonstrates that integrating genomic and experimental approaches can effectively connect fungal biodiversity research with applied outcomes, ranging from conservation and traceability to nutraceutical development and safe, sustainable production systems. Future work should expand genomic sampling, generate reference resources for cold-adapted taxa, and apply multi-omics and functional assays to directly link genotype, phenotype, and environmental responses.

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Supplementary materials

Supplementary Table 1. Pairwise comparison of nonsynonymous variant counts between fast-growing (P6) and slow-growing industrial strains (P2–P3–P4). Per-gene nonsynonymous variant counts for the fast-growing industrial strain (P6) and the slow-growing industrial strains (P2, P3, and P4). Delta values ($\Delta P6-Px$) represent the difference in nonsynonymous variant number between P6 and each slow-growing strain ($P6 - Px$), with positive values indicating higher variant counts in P6 and negative values indicating higher counts in the slow-growing strains. Functional annotations include gene product names and associated Gene Ontology (GO) terms.

gene	P6	P2	P3	P4	$\Delta P6-P2$	$\Delta P6-P3$	$\Delta P6-P4$	GO_IDs	GO_short
gene_11878	4	124	100	4	-120	-96	0	GO:0016020 GO:1990234	membrane transferase complex
gene_3614	131	92	85	55	39	46	76	GO:0016020	membrane
gene_10371	12	61	59	62	-49	-47	-50	GO:0004351 GO:0005829 GO:0006538 GO:0030170	glutamate decarboxylase activity cytosol L-glutamate catabolic process
gene_13480	40	8	8	8	32	32	32	GO:0005525	GTP binding
gene_3128	11	35	38	42	-24	-27	-31	GO:0000328 GO:0004180 GO:0046872 GO:0051603	fungus-type vacuole lumen carboxypeptidase activity metal ion binding
gene_14478	43	22	18	18	21	25	25	GO:0000287 GO:0004312 GO:0004313 GO:0004314 GO:0004315 GO:0004316 GO:0004318 GO:0004321 GO:0005835 GO:0006633 GO:0006635 GO:0008897 GO:0016297 GO:0019171	magnesium ion binding fatty acid synthase activity [acyl-carrier-protein] S-acetyltransferase activity
gene_18483	28	7	7	6	21	21	22	GO:0000244 GO:0000398	spliceosomal tri-snRNP complex assembly mRNA splicing, via spliceosome

								GO:0005634	nucleus
								GO:0008380	
								GO:0046540	
								GO:0071013	
								GO:0140513	
								GO:1990904	
gene_11085	24	3	4	3	21	20	21	GO:0005739	mitochondrion
								GO:0005743	mitochondrial inner membrane
								GO:0005744	TIM23 mitochondrial import inner membrane translocase complex
								GO:0015031	
								GO:0016020	
gene_11679	90	69	69	69	21	21	21	GO:0005737	cytoplasm
								GO:0016874	ligase activity
								GO:0031177	phosphopantetheine binding
								GO:0043041	
								GO:0044550	
gene_17791	19	NA	NA	NA	19	19	19	GO:0004033	obsolete aldo-keto reductase (NADPH) activity
								GO:0005737	cytoplasm
								GO:0016491	oxidoreductase activity
gene_10580	3	26	28	17	-23	-25	-14	GO:0008171	O-methyltransferase activity
								GO:0008173	RNA methyltransferase activity
								GO:0017069	snRNA binding
								GO:0032259	
								GO:0040031	
gene_5108	20	44	45	9	-24	-25	11	GO:0008168	methyltransferase activity
gene_3764	6	24	24	24	-18	-18	-18	GO:0004676	3-phosphoinositide-dependent protein kinase activity
								GO:0004677	DNA-dependent protein kinase activity
								GO:0004679	AMP-activated protein kinase activity
								GO:0004694	
								GO:0004711	
								GO:0005524	
								GO:0006338	
								GO:0035175	
								GO:0035402	
								GO:0035403	
								GO:0035979	
								GO:0044022	
								GO:0044023	
								GO:0044024	
								GO:0044025	
								GO:0072354	

								GO:0072518	
								GO:0140823	
								GO:0140855	
								GO:0140857	
								GO:1990244	
gene_13662	25	6	5	2	19	20	23	GO:0003723	RNA binding
								GO:0005634	nucleus
								GO:0006364	rRNA processing
								GO:1990904	
gene_18181	28	10	15	NA	18	13	28	GO:0005524	ATP binding
								GO:0008540	proteasome regulatory particle, base subcomplex
								GO:0016887	ATP hydrolysis activity
gene_18515	0	28	21	9	-28	-21	-9	GO:0003682	chromatin binding
gene_17958	31	13	13	13	18	18	18	GO:0016787	hydrolase activity
gene_174	43	25	25	25	18	18	18	GO:0005886	plasma membrane
								GO:0032153	cell division site
								GO:0035838	growing cell tip
gene_5684	19	42	13	44	-23	6	-25	GO:0003723	RNA binding
								GO:0005634	nucleus
								GO:0008033	tRNA processing
								GO:0009982	
								GO:0016853	
								GO:0031119	
								GO:0043231	
								GO:1990481	
gene_9875	46	28	28	27	18	18	19	GO:0000155	phosphorelay sensor kinase activity
								GO:0000160	phosphorelay signal transduction system
								GO:0005886	plasma membrane
								GO:0009927	
								GO:0016020	
								GO:0016301	
								GO:0016772	
gene_15890	16	NA	NA	NA	16	16	16	GO:0004843	cysteine-type deubiquitinase activity
								GO:0005634	nucleus
								GO:0005829	cytosol
								GO:0006508	
								GO:0008234	
								GO:0008270	
								GO:0016579	
								GO:0101005	

gene_283	21	39	44	24	-18	-23	-3	GO:0004222	metalloendopeptidase activity
								GO:0006508	proteolysis
								GO:0008237	metallopeptidase activity
gene_10024	26	38	47	34	-12	-21	-8	GO:0005524	ATP binding
								GO:0008540	proteasome regulatory particle, base subcomplex
								GO:0016887	ATP hydrolysis activity
gene_13822	37	29	26	15	8	11	22	GO:0003723	RNA binding
								GO:0005685	U1 snRNP
								GO:0045292	mRNA cis splicing, via spliceosome
								GO:0071004	
gene_2035	1	1	1	30	0	0	-29	GO:0003746	translation elongation factor activity
								GO:0003924	GTPase activity
								GO:0005525	GTP binding
								GO:0006414	
gene_2977	2	2	2	30	0	0	-28	GO:0005524	ATP binding
								GO:0006457	protein folding
								GO:0016887	ATP hydrolysis activity
								GO:0051082	
								GO:0140662	
gene_257	2	3	3	29	-1	-1	-27	GO:0003746	translation elongation factor activity
								GO:0003924	GTPase activity
								GO:0005525	GTP binding
								GO:0005829	
								GO:0006414	
								GO:0043022	
								GO:1990904	
gene_12834	NA	NA	NA	23	NA	NA	-23	GO:0005200	structural constituent of cytoskeleton
								GO:0005525	GTP binding
								GO:0005874	microtubule
								GO:0007010	
								GO:0007017	
								GO:0016787	

Supplementary table 2. Pairwise comparison of nonsynonymous variant counts between wild-type (P1) and industrial strains (P2–P3–P4–P6). Per-gene nonsynonymous variant counts for the wild-type strain (P1) and industrial strains (P2, P3, P4, and P6). Delta values ($\Delta P1-Px$) represent the difference in nonsynonymous variant load between P1 and each industrial strain (P1-Px), with positive values indicating higher variant counts in the wild-type and negative values indicating higher counts in industrial strains. Functional annotations include gene product names and associated Gene Ontology (GO) terms.

gene	P1	P2	P3	P4	P6	$\Delta P1-P2$	$\Delta P1-P3$	$\Delta P1-P4$	$\Delta P1-P6$	GO_IDs	GO term
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gene_1036	53	89	87	86	91	-36	-34	-33	-38	GO:0000329	funeral-type vacuole membrane
										GO:0016020	membrane
gene_10371	8	61	59	62	12	-53	-51	-54	-4	GO:0004351	glutamate decarboxylase activity
										GO:0005829	cytosol
										GO:0006538	L-glutamate catabolic process
gene_10480	36	107	107	107	103	-71	-71	-71	-67	GO:0030170	pyridoxal phosphate binding
										GO:0031981	nuclear lumen
gene_11348	31	67	67	67	68	-36	-36	-36	-37	GO:0042254	ribosome biogenesis
gene_11679	43	69	69	69	90	-26	-26	-26	-47	GO:0005634	nucleus
										GO:0005737	cytoplasm
										GO:0016874	ligase activity
										GO:0031177	phosphopantetheine binding
										GO:0043041	amino acid activation for nonribosomal peptide biosynthetic process
										GO:0044550	secondary metabolite biosynthetic process
gene_11878	72	124	100	4	4	-52	-28	68	68	GO:0016020	membrane
										GO:1990234	transferase complex
gene_12358	41	14	18	15	12	27	23	26	29	GO:0003677	DNA binding
										GO:0006310	DNA recombination
gene_12725	11	47	43	46	42	-36	-32	-35	-31	GO:0015074	DNA integration
										GO:0016491	oxidoreductase activity
gene_12762	9	44	44	44	41	-35	-35	-35	-32	GO:0046872	metal ion binding
										GO:0003729	mRNA binding
gene_13025	7	38	36	37	35	-31	-29	-30	-28	GO:0005730	nucleolus
										GO:0006635	fatty acid beta-oxidation
gene_13089	9	39	38	40	51	-30	-29	-31	-42	GO:0050660	flavin adenine dinucleotide binding
gene_1331	20	48	47	47	58	-28	-27	-27	-38	GO:0070822	Sin3-type complex
gene_1351	28	52	54	53	58	-24	-26	-25	-30	GO:0006113	fermentation
										GO:0016020	membrane
										GO:0000976	transcription cis-regulatory region binding
										GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific
										GO:0003677	DNA binding
										GO:0005634	nucleus
										GO:0006351	DNA-templated transcription
										GO:0006355	regulation of DNA-templated transcription
										GO:0006357	regulation of transcription by RNA polymerase II
										GO:0008270	zinc ion binding
gene_17006	28	NA	NA	NA	NA	NA	NA	NA	28	GO:0046872	metal ion binding
										GO:0003677	DNA binding
gene_17151	17	53	54	50	56	-36	-37	-33	-39	GO:0000124	SAGA complex
										GO:0004672	protein kinase activity

gene_2977	2	2	2	30	2	0	0	-28	0	GO:0005634	nucleus
										GO:0006281	DNA repair
										GO:0006355	regulation of DNA-templated transcription
										GO:0016301	kinase activity
										GO:0035267	NuA4 histone acetyltransferase complex
										GO:0005524	ATP binding
										GO:0006457	protein folding
										GO:0016887	ATP hydrolysis activity
gene_3128	9	35	38	42	11	-26	-29	-33	-2	GO:0051082	unfolded protein binding
										GO:0140662	ATP-dependent protein folding chaperone
										GO:0000328	fungal-type vacuole lumen
										GO:0004180	carboxypeptidase activity
										GO:0046872	metal ion binding
										GO:0051603	proteolysis involved in protein catabolic process
										GO:0016020	membrane
										GO:0003995	acyl-CoA dehydrogenase activity
gene_3614	71	92	85	55	131	-21	-14	16	-60	GO:0005737	cytoplasm
										GO:0033539	fatty acid beta-oxidation using acyl-CoA dehydrogenase
										GO:0050660	flavin adenine dinucleotide binding
										GO:0005524	ATP binding
										GO:0005743	mitochondrial inner membrane
										GO:0015421	ABC-type oligopeptide transporter activity
										GO:0016887	ATP hydrolysis activity
										GO:0090374	oligopeptide export from mitochondrion
gene_3717	2	41	42	40	39	-39	-40	-38	-37	GO:0003712	transcription coregulator activity
										GO:0003713	transcription coactivator activity
										GO:0005634	nucleus
										GO:0006357	regulation of transcription by RNA polymerase II
										GO:0016592	mediator complex
										GO:0045944	positive regulation of transcription by RNA polymerase II
										GO:0003723	RNA binding
										GO:0005730	nucleolus
gene_3974	16	45	51	61	46	-29	-35	-45	-30	GO:0030490	maturation of SSU-rRNA
										GO:0000127	transcription factor TFIIC complex
										GO:0003677	DNA binding
										GO:0005634	nucleus
										GO:0006384	transcription initiation at RNA polymerase III promoter
										GO:0042791	5S class rRNA transcription by RNA polymerase III
										GO:0016020	membrane
										GO:0003677	DNA binding
gene_4421	13	45	45	44	44	-32	-32	-31	-31	GO:0003682	chromatin binding
gene_4581	89	15	15	15	16	74	74	74	73		
gene_5060	16	73	73	74	66	-57	-57	-58	-50		
gene_5111	21	51	51	50	46	-30	-30	-29	-25		
gene_6869	5	38	38	38	38	-33	-33	-33	-33		

gene_7334	4	33	31	33	29	-29	-27	-29	-25	GO:0003886	DNA (cytosine-5-)-methyltransferase activity
										GO:0005634	nucleus
										GO:0008168	methyltransferase activity
										GO:0032259	methylation
										GO:0044027	negative regulation of gene expression via chromosomal CpG island methylation
										GO:0051719	DNA (cytosine-5-)-methyltransferase activity, acting on CpN substrates
										GO:0051720	DNA (cytosine-5-)-methyltransferase activity, acting on CpNpG substrates
gene_9875	16	28	28	27	46	-12	-12	-11	-30	GO:0005576	extracellular region
										GO:0030570	pectate lyase activity
										GO:0045490	pectin catabolic process
										GO:0000155	phosphorelay sensor kinase activity
										GO:0000160	phosphorelay signal transduction system
										GO:0005886	plasma membrane
										GO:0009927	histidine phosphotransfer kinase activity
										GO:0016020	membrane
										GO:0016301	kinase activity
GO:0016772	transferase activity, transferring phosphorus-containing groups										

Tesi redatta con il cofinanziamento dell'Unione europea-*NextGeneration EU*
Piano Nazionale di Ripresa e Resilienza Missione 4 – Componente 1 –
Investimento 4.1. del PNRR – “Ricerca PNRR” – CUP E11I22000170001



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