University of Zululand

Genome data mining, annotation, and phylogenetic analysis of cytochrome P450 monooxygenases in the fungal class *Pezizomycetes*

Nomfundo Ntombizinhle Nsele 201721035

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Faculty of Science and Agriculture

Department of Biochemistry and Microbiology

University of Zululand

Private Bag X 1001 KwaDlangezwa

3886

Supervisor: Prof Khajamohiddin Syed

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DECLARATION

I, Nomfundo Ntombizinhle Nsele, declare that this dissertation is completely my original work, with the exception of quotations from other sources that have been properly recognized and referenced. This dissertation has never been submitted to a university for review toward any degree. Considering that quality is more important than quantity (Gould, 2016), care has been taken to present the dissertation in a publication format to enable the presentation of data concisely and for easy understanding of the work. As stated in the research outcomes section, I certify that the work provided in this dissertation has been published (see Research output section).

Signed on the day of August 2023

Reference: Gould, J., 2016. Future of the thesis. Nature, 535(7610), pp.26-29.

ABSTRACT

Cytochrome P450 monooxygenases (CYPs/P450s) are heme proteins that play a role in organisms' primary and secondary metabolism. P450s play an important role in organism adaptation since lifestyle influences P450 composition in their genome. This phenomenon is well-documented in bacteria but less so in fungi. This study observed this phenomenon where diverse P450 complements were identified in saprophytic and ectomycorrhizal Pezizomycetes. Genome-wide data mining, annotation, and phylogenetic analysis of P450s in 19 Pezizomycetes revealed 668 P450s that can be grouped into 153 P450 families and 245 P450 subfamilies. Only four P450 families, namely, CYP51, CYP61, CYP5093, and CYP6001, are conserved across 19 Pezizomycetes, indicating their important role in these species. A total of 5 saprophyte Pezizomycetes have 103 P450 families, whereas 14 ectomycorrhizal Pezizomycetes have 89 P450 families. Only 39 P450 families were common, and 50 and 64 P450 families, respectively, were unique to ectomycorrhizal and saprophytic Pezizomycetes. These findings suggest that the switch from a saprophytic to an ectomycorrhizal lifestyle led to both the development of diverse P450 families as well as the loss of P450s, which led to the lowest P450 family diversity, despite the emergence of novel P450 families in ectomycorrhizal Pezizomycetes.

DEDICATION

I would like to specially dedicate this work to my family my mother Bonisiwe Ngubane, my sister Celokuhle Nsele, my brother Lungani Manqele, my sister Simangele Mthembu and my very special friend and boyfriend Kwanele Mthethwa.

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Thirdly I would like to thank Tiara Padayachee for her continued support and assistance in completing this work.

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RESEARCH OUTPUT

- **Article**

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ABBREVIATIONS

Anti-SMASH	Antibiotics & Secondary Metabolite Analysis Shell
BGC	Biosynthetic gene cluster
BLAST	Basic Local Alignment Search Tool
CYPs/P450s	Cytochrome P450 monooxygenase
ECM	Ectomycorrhizal
iTOL	Interactive Tree of Life
MAFFT	Multiple alignment using fast Fourier transform
MeV	Multi-experiment viewer
NAD	Nicotinaminde adenine dinucleotide
NCBI	National Center for Biotechnology Information
NRPS	Non-ribosomal peptide synthases
SAP	Saprotrophic
T-REX	Tree and Reticulogram Reconstruction

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Chapter 1: INTRODUCTION

1.1. Background and problem statement

Cytochrome P450 monooxygenases (CYPs/P450s) are one-of-a-kind enzymes due to their catalytic versatility and widespread presence in organisms, including viruses (Nelson, 2018, Lamb *et al*., 2019). P450s carry out stereo- and regio-specific enzyme activities in primary and secondary metabolism. Due to this capability, the use of P450s in all areas of biology, mainly their function in drug metabolism, has been investigated.

Fungi are the largest kingdom of microbes and contain the most incredible variety of ecologically niche-adapted species. P450s were found in fungi when genome sequencing was done. The generation of secondary metabolites and the adaptability of fungi to various ecological niches are both facilitated by the catalytic versatility of fungal P450s (Črešnar and Petrič, 2011, Syed *et al*., 2014, Durairaj et al., 2016, Keller, 2019, Zhang et al., 2021). Furthermore, azole medicines have fungal P450s as a therapeutic target (Jawallapersand *et al*., 2014, Lepesheva *et al*., 2018, Lamb *et al.,* 2021).

Due to the enormous applications of fungal P450s, researchers worldwide are looking for more P450s to explore their biotechnological potential. In this direction, many fungal species' genomes have been sequenced (Grigoriev *et al*., 2011, Grigoriev *et al*., 2014). However, very few P450s were annotated (assigning the family and subfamily) as per the International P450 Nomenclature Committee rules, i.e., >40% identity for a family and >55% identity for a subfamily (Nelson, 2018).

In this study, for the first time, analysis of P450s was carried out in the fungal class *Pezizomycetes*. A total of 25 species genomes were mined for P450s to enable their annotation, phylogenetic analysis, and unraveling of their role in the biosynthesis of secondary metabolites.

1.2. Value to the body of knowledge

1.2.1. Novelty and Scientific contribution to new knowledge

This is the first study to look at genome data mining, annotation, and phylogenetic analysis of cytochrome P450 monooxygenases in the fungus class *Pezizomycetes*.

1.2.2. Socio-economic impact

- The study could identify novel secondary metabolite biosynthetic gene clusters (BGCs). These gene clusters can be used to synthesize antibiotics useful for humans.
- Human capacity development – A black female candidate training in Bioinformatics, which is a very important and scarce skilled research area.

1.2.3. Scientific outcomes

- MSc dissertation
- One publication in an internationally reputed and accredited journal

1.3. Aim and Objectives

1.3.1 Aim of the Study

Genome data mining, annotation, and phylogenetic analysis of cytochrome P450 monooxygenases in the fungal class *Pezizomycetes*

1.3.2 Objectives of the Study

- To perform genome data mining of P450s in 25 Pezizomycetes species
- To assign family and subfamilies to the identified P450s
- To carry out a phylogenetic analysis of P450s
- To identify natural metabolite biosynthetic gene clusters in Pezizomycetes species
- To identify P450s part of natural metabolite biosynthetic gene clusters in Pezizomycetes species

1.4. Dissertation overview

This dissertation is divided into five chapters. Chapter 1 is an introduction that provides an overview of the background and problem statement, value to the body of knowledge, and study aim and objectives. Chapter 2 consists of a literature review. Chapters 3-5 consist of methodology, results & discussion, and conclusion & future perspectives respectively. Chapters 3-5 are presented in research article format, the same as the published article in the journal of Fungi (https://doi.org/10.3390/jof9080830). Furthermore, the information provided under sections abstract, literature review and background & problem statement is the same as the published article.

Chapter 2 : LITERATURE REVIEW

2.1 Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases (CYPs/P450s) are a heme-containing enzyme superfamily that was identified nearly six decades ago (Garfinkel, 1958, Klingenberg, 1958, Omura and Sato, 1962, White and Coon, 1980, Omura, 2011). In the name, "cytochrome" refers to a protein with heme, "450" indicates that these proteins absorb light at 450 nm wavelength, and "monooxygenases" means they incorporate one oxygen atom in a variety of substrates (Figure 2.1) (Garfinkel, 1958, Klingenberg, 1958, Omura and Sato, 1962, White and Coon, 1980, Omura, 2011).

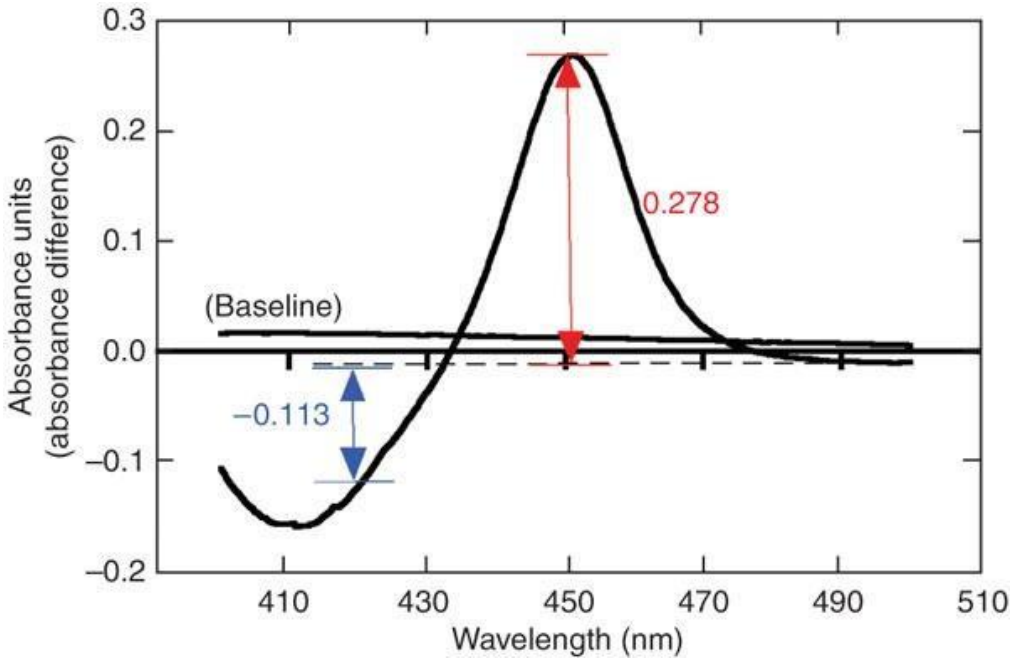

Figure 2.1 P450 enzyme spectrum, where absorbance at 450 nm is shown (Guengerich *et al*., 2009).

P450s are found in archaea, bacteria, and eukarya because they play an important role in an organism's primary and secondary metabolism (Table 2.1) (Woese and Fox, 1977, Nelson, 2018, Ngcobo *et al*., 2023). P450s have been discovered in viruses, which are thought to be non-living (Table 2.1) (Lamb *et al*., 2009, Lamb *et al*., 2019).

Table 2.1 P450 analysis across the domains of life (Ngcobo *et al*., 2023).

Species category	**Species subcategory**	**Species subcategory P450 count**	**Species category P450 count**	**P450 families**
Animals			37,149	1,948*
	Mammals	4,558		18
	Other vertebrates	3,268		19
	Insects	22,173		1,031
	Non-insect invertebrates	7150		880
Plants			42,102	819
Fungi			28,260	3,204
Protozoa			5,807	1,374
Bacteria			17,236	1,910
Archaea			1,204	34
Viruses			37	13
Total			131,795	9,302

Note: The data provided in the table for all categories except for archaea is from the previous report (Nelson, 2018). However, the information has been updated since 2018 with the latest numbers from the P450 library as of 28 November 2022 (Nelson, 2009). The symbol * indicates families found in multiple groups are counted once, i.e., the total of families in all animals is less than the sum of the numbers for the individual groups of animals listed.

2.2. Catalytic diversity of P450s

Even though these enzymes are called monooxygenases, research has demonstrated that they perform a variety of enzymatic processes with regio- and stereo-selectivity (Figure 2.2) (Sono *et al*., 1996, Bernhardt, 2006, Yan *et al*., 2022). Their remarkable catalytic capabilities prompted researchers to investigate these enzymes' applicability in all areas of biology (Kelly and Kelly, 2013, Girvan and Munro, 2016, Lepesheva *et al*., 2018, Urlacher and Girhard, 2019, Li *et al*., 2020).

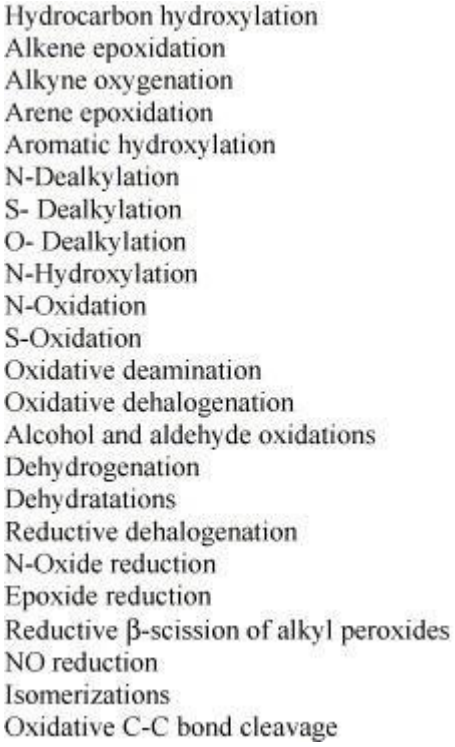

Figure 2.2 Reactions catalyzed by cytochrome P450 monooxygenases (Sono *et al*., 1996, Bernhardt, 2006)

2.3. Nomenclature and classification of P450s

P450s have a distinct nomenclature and categorization system (Nelson *et al*., 1993, Nelson, 1998, Nelson, 2006, Nelson, 2009). The nomenclature scheme starts with the prefix "CYP" for cytochrome P450 monooxygenase, followed by an Arabic numeral indicating the family, a capital letter indicating the subfamily, and an Arabic digit indicating the individual P450 in a family. The annotation/classification criteria include assigning a family and subfamily with more than 40% identity to the same family and all P450s with more than 55% identity to the same subfamily (Nelson *et al*., 1993, Nelson, 1998, Nelson, 2006, Nelson, 2009).

2.4. Applications of P450s

Initially, the P450s gained popularity due to their role in testing the toxicity of the drugs. Researchers used these enzymes to assess how the human metabolism reacts to the drugs and xenobiotics (Figure 2.3) (Urlacher and Girhard, 2019, Guengerich, 2021). Because these enzymes catalyze a wide range of reactions in a regio- and stereo-selective manner, their properties have been studied for a variety of pharmaceutical, biotechnological, and environmental applications such as drug discovery and development, fine chemical, fragrance, pharmaceutical compounds, and biofuels production, biosensing, and bioremediation (Zhang *et al*., 2011, Girvan and Munro, 2016, Urlacher and Girhard, 2019)

For example, in drug development research, one well-established commercial application of P450 monooxygenases is the biotransformation of steroids to medicines, such as 11-hydroxylation of Reichstein S to hydrocortisone (van Beilen *et al*., 2003) and conversion of progesterone to cortisol (Hogg, 1992). Another example is the production of the macrolide antibiotic erythromycin and glycopeptide antibiotics, which involves hydroxylation steps mediated by P450eryF (CYP107A1) from *Saccharopolyspora erythraea* (Andersen *et al*., 1993) and P450 OxyA, OxyB, and OxyC from *Amycolatopsis orientalis* (Bischoff *et al*., 2005). CYP725A1 from yew (*Taxus cuspidate*) and CYP153 from *Mycobacterium* sp. (van Beilen *et al*., 2005) have recently been employed in the manufacture of the anticancer medicines taxol and perillyl alcohol. Biosensors based on mammalian P450s CYP1A2, CYP2B4, and CYP11A have been developed to detect pharmaceuticals (clozapine), xenobiotic chemicals (styrene), and fatty acids (cholesterol), respectively (Paternolli *et al*., 2004). Biofuel production from alkanes or fatty acids has been investigated utilizing modified bacterial P450s CYP153A6 (Koch *et al*., 2009) and OleTje (a CYP152 family P450) (Rude *et al*., 2011). CYP153A6 mutants converted butane to 1-butanol, whereas OleTje oxidized fatty acids to 1-alkenes (terminal olefins). Attempts have been made to design model bacterial P450s CYP101 and CYP102 to broaden their substrate range to environmental contaminants for possible bioremediation applications (Kumar and Kapur, 2016, Bhattacharya and Yadav, 2018).

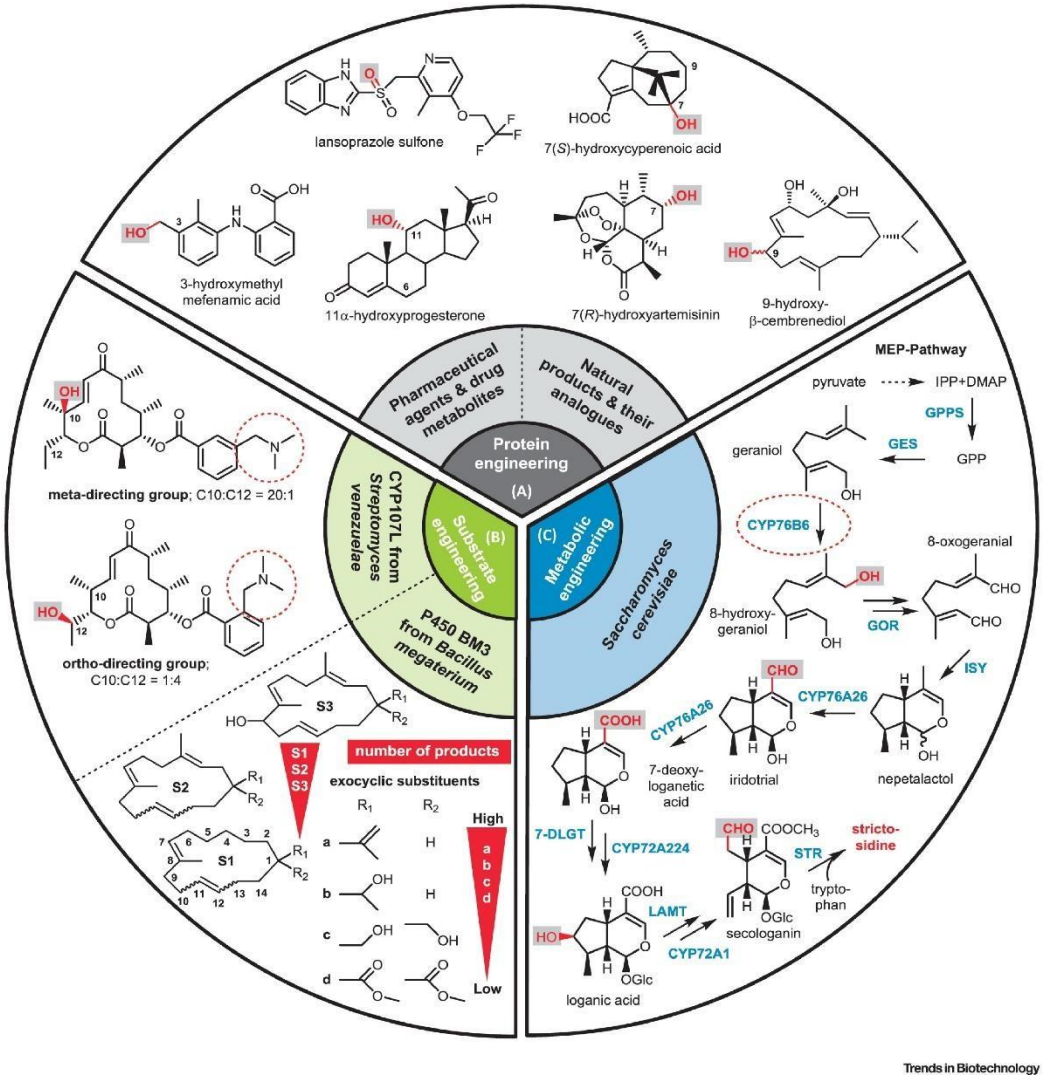

Figure 2.3. Applications of Cytochrome P450 monooxygenases (Urlacher and Girhard, 2019).

2.5. Fungal P450s

Fungi are a vast and complex kingdom of lower eukaryotic organisms that are classed morphologically as yeasts, filamentous fungi, or dimorphic fungi. Many live as saprophytes, digesting dead matter, while others have evolved to be obligatory or opportunistic pathogens, obtaining nourishment from animal and plant hosts (Stajich *et al*., 2009, Carris *et al*., 2012). The filamentous fungus has evolved an amazing ability to adapt to changing environments, owing mainly to enzymatic defense systems that protect them from poisonous foreign substances known as xenobiotics. As a result, filamentous fungi play an essential role in the breakdown and mineralization of a wide range of environmental contaminants, as well as in

catalyzing key processes in the biotechnological manufacture of plant and human hormones (Gadd *et al*., 2007, Carris *et al*., 2012).

Like other organisms, fungi have P450s in their genomes (Table 2.1). In fungi, P450s are known to play a role in their primary and secondary metabolism and detoxification or degradation of xenobiotics (Črešnar and Petrič, 2011).

2.5.1. Fungal P450 classes

P450 systems are categorized into ten groups based on the architecture of the protein components involved in electron transfer, with fungal P450s falling into classes II, VIII, and IX (Figure 2.4) (Hanukoglu, 1996, Hannemann *et al*., 2007, Črešnar and Petrič, 2011).

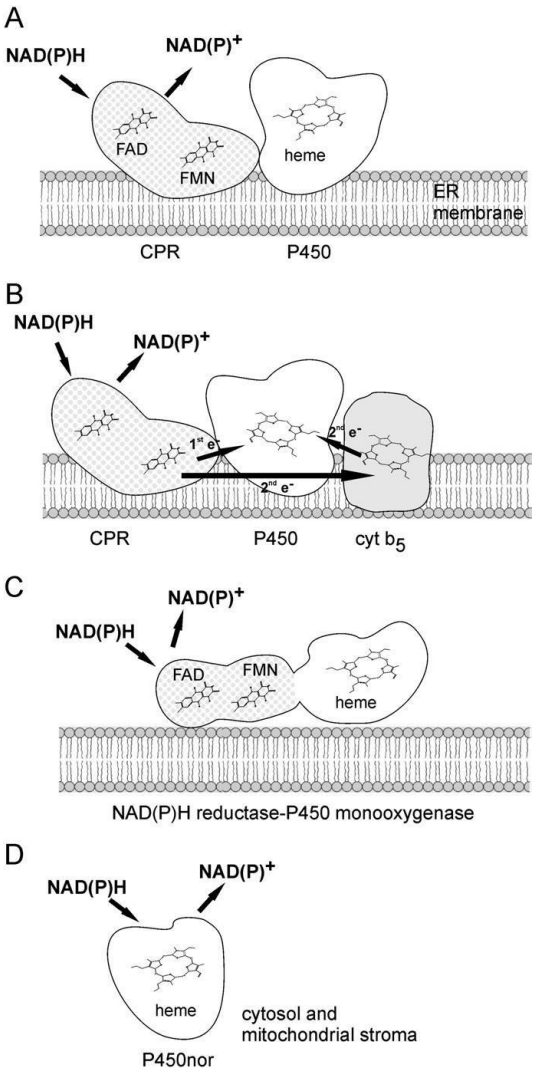

Figure 2.4. Cytochrome P450 systems in fungi (Črešnar and Petrič, 2011). (A) and (B) class II microsomal systems; (C) class VIII fungal and bacterial fusion systems; (D) class IX fungal soluble system.

Class II proteins are P450 and cytochrome P450 reductase (CPR), which include the prosthetic cofactors FAD and FMN, which carry two electrons from NAD(P)H to the heme moiety (Figure 2.4A). It could include a third protein component, cytochrome b5 (Cyt b5), which transmits a second electron to the oxyferrous P450 (Figure 2.4B). In the absence of CPR, certain P450s (CYP5150A2 and CYP63A2) can be directly activated by Cyt b5 and NADHdependent Cyt b5 reductase (CB5R) (Syed *et al*., 2011, Ichinose and Wariishi, 2012). Class VIII proteins are fused proteins in which a short protein linker links the N-terminal heme domain to a C-terminal diflavin reductase partner (CPR) (Figure 2.4C). Electrons are transported from NADPH to the active site of CYP via the reductase domain (Figure 2.4C). Class VIII CYPs are self-sufficient in catalysis and do not require electron transport proteins such as CPR or Cyt b5. Class IX is composed of a single protein, whereby electrons are directly transferred from NAD(P)H to CYP without the need for any additional redox partners (Figure 2.4D) (Shoun and Takaya, 2002). Class IX differs functionally from the rest of the CYPs because they catalyze the reduction of two molecules of NO to N_2O (Shoun and Takaya, 2002).

2.5.2. Catalytic versatility

Most fungal P450s, in particular, belong to class II and perform a wide range of catalytic processes (Figure 2.5).

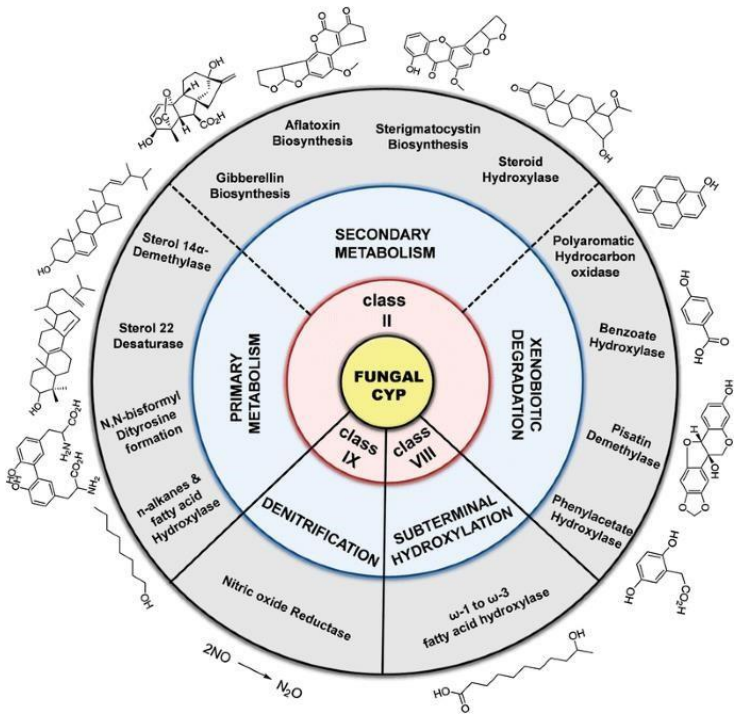

Figure 2.5. Catalytic versatility of fungal P450s as per their P450 classes (Durairaj *et al*., 2016).

P450s play a role in producing a variety of primary and secondary metabolites in the fungal kingdom, with stereo and regio-specificity. Housekeeping tasks such as ergosterol biosynthesis, meiotic spore-wall biogenesis, and n-alkane hydroxylation are examples of primary fungal metabolism. In contrast, fungal secondary metabolism involves the biosynthesis of hormones, mycotoxins, and like compounds (Figure 2.5). Fungal P450s may also detoxify and degrade a wide range of xenobiotic chemicals found in their surroundings, including polycyclic aromatic hydrocarbons (PAHs), phenolic compounds, and other hazardous environmental contaminants (Figure 2.5).

2.5.3. Natural product biosynthesis

Natural products are metabolites, either primary or secondary, produced by organisms. Primary metabolites play a role in the physiology of organisms. On the other hand, secondary metabolites, while not directly involved in organism physiology, indirectly influence organism survival. Fungi are well-known producers of natural metabolites (Table 2.2) (Črešnar and Petrič, 2011, Keller, 2019, Zhang *et al*., 2021). The involvement of several fungal P450s in the biosynthesis of natural products, such as secondary metabolites, polyketides, non-ribosomal peptides, terpenes, and other substances, has been well-reviewed (Črešnar and Petrič, 2011, Keller, 2019, Zhang *et al*., 2021).

Table 2.2 Information on a few fungal P450s involved in the biosynthesis of natural metabolites (Črešnar and Petrič, 2011).

P450 family	Function	Species name
CYP58	Aflatoxin biosynthesis	*Aspergillus flavus*, A. parasiticus
CYP65	Trichothecene biosynthesis	*Fusarium graminearum, F. sporotrichoides*
CYP505	Fumonisin biosynthesis	*F. verticilliodes*
CYP68	Gibberellin biosynthesis	*F. fujikori*
CYP57	Pisatin detoxification	*Nectria haematococca*

2.5.4. Drug target

2.5.4.1. CYP51

CYP51, also known as sterol 14-demethylase, is the most conserved P450 across biological kingdoms and is the primary target of conventional antifungal azole drugs (Figure 2.6)

(Lepesheva *et al*., 2018, Lamb *et al*., 2021). CYP51 demethylates lanosterol, a critical step in forming cell membrane ergosterol (Figure 2.6) (Lepesheva *et al*., 2018).

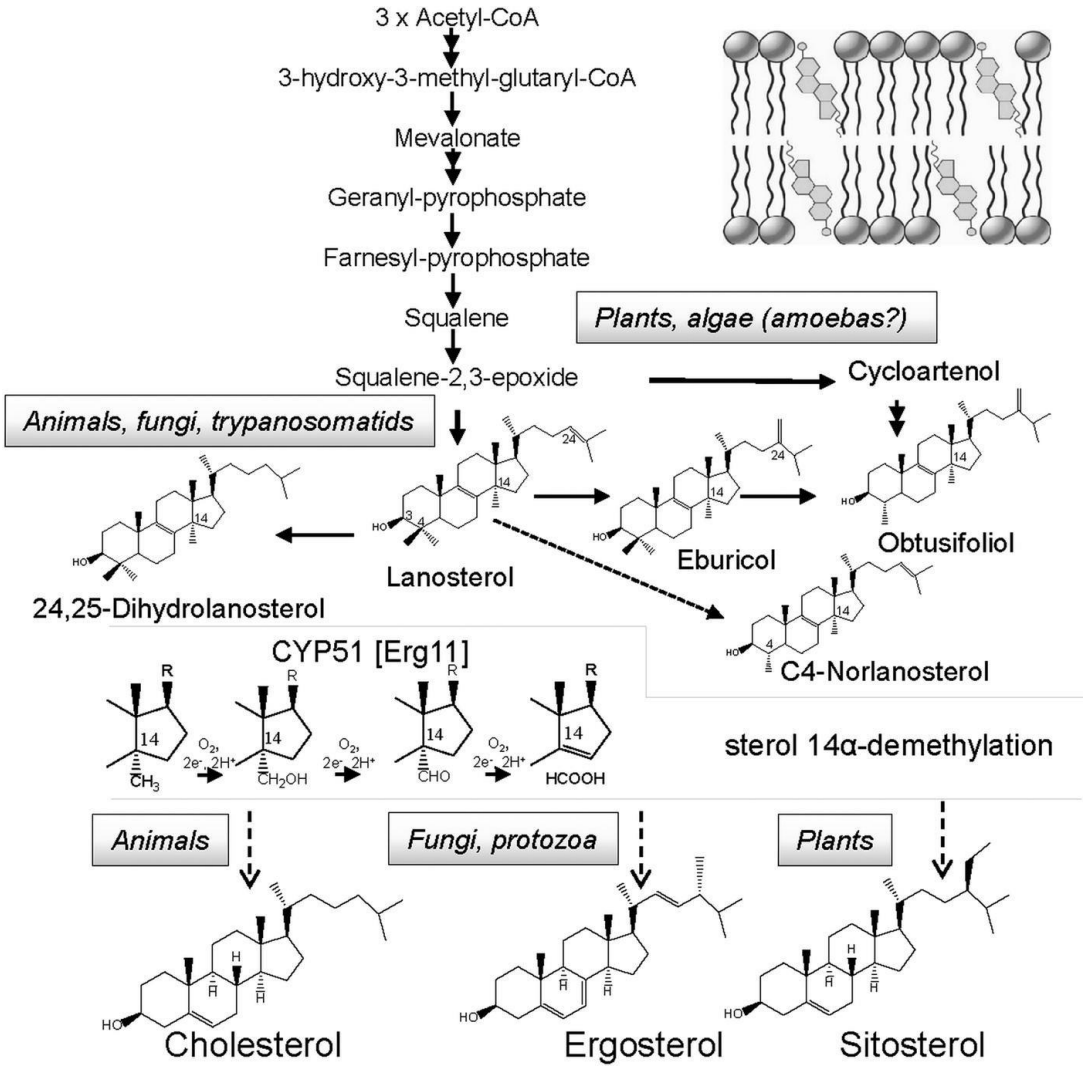

Figure 2.6 The role of CYP51 in the biosynthesis of sterols in organisms (Lepesheva *et al.,* 2018).

CYP51s catalyze the oxidative removal of the 14-methyl group from one or more of the five naturally occurring cyclized sterol precursors, lanosterol, 24,25-dihydrolanosterol, eburicol, obtusifoliol, and C4-lanosterol, in three steps (Figure 2.6). The reaction occurs in the endoplasmic reticulum of eukaryotes and is required for the biosynthesis of sterols, which serve as essential components of plasma membranes (bulky sterols, cholesterol in humans, ergosterol in fungi) as well as precursors for regulatory molecules that modulate growth, division,

differentiation, and development processes (sparking sterols) (Lepesheva and Waterman, 2007, Nes, 2011). For rapidly multiplying cells, fast sterol production is critical. It is also worth noting that while humans can get cholesterol from their diets, inhibiting ergosterol formation in unicellular human pathogens is deadly.

Even though sterol biosynthesis involves several stages, only two have become key targets for systemic therapeutic medicines (Figure 2.6). Statins (cholesterol-lowering agents), which act upstream of the pathway at the step of mevalonate production, are currently the most commonly prescribed medications (Superko *et al*., 2012), whereas azoles, inhibitors of sterol 14-demethylase, are the most widely used antifungals (Lass-Flörl, 2011, Denning and Bromley, 2015), and are being researched for use in the treatment of human infections with protozoa (Buckner and Urbina, 2012).

2.5.4.2. CYP53

According to research on fungal P450s, the P450 family CYP53 potentially serves as a unique alternative antifungal therapeutic target (Podobnik *et al*., 2008, Jawallapersand *et al*., 2014). CYP53 family members are well-known as benzoate para-hydroxylases, which are involved in benzoate molecule detoxification (Faber *et al*., 2001). Benzoate is a naturally occurring antifungal plant material (Amborabé *et al*., 2002) as well as an intermediary in the breakdown of aromatic compounds in fungi (Durham *et al*., 1984, Jensen Jr *et al*., 1994, Lapadatescu *et al*., 2000).

In fungi, benzoate causes toxicity by disrupting the membrane, blocking critical cellular functions, affecting pH balance, and producing a stress response (Brul and Coote, 1999, Amborabé *et al*., 2002). The only known mechanism in fungi that finally directs this hazardous chemical into the -ketoadipate pathway is CYP53 P450-mediated para-hydroxylation of benzoate (Harwood and Parales, 1996).

Furthermore, the CYP53 gene was necessary for fungal species' survival (Fraser *et al*., 2002). The accumulation of hazardous intermediate benzoate impeded the growth of the CYP53 gene-knock-out fungus strain (Fraser *et al*., 2002). This indicates that this P450 plays an essential function in the survival of fungal species by detoxifying benzoate.

A genome-wide investigation of fungal species indicated the presence of CYP53 members in ascomycetes and basidiomycetes (Jawallapersand *et al*., 2014). According to phylogenetic and gene-structure analyses, basidiomycetes have an enrichment of CYP53 P450s

due to widespread duplication of CYP53 P450s in their genomes. A total of 103 amino acids were found to be conserved in ascomycetes CYP53 P450s, compared to only seven in basidiomycetes CYP53 P450s (Jawallapersand *et al*., 2014). Data from protein modeling and active-site cavity mapping revealed that the ascomycetes' CYP53 P450s have a highly conserved protein structure, with 78% of the amino acids in the active-site cavity being conserved. Because of the rigidity of the active site cavity of ascomycetes CYP53 P450s, any inhibitor directed against this P450 family can serve as a common antifungal therapeutic target, particularly against pathogenic ascomycetes (Jawallapersand *et al*., 2014).

2.5.5. Role in adaptation to diverse ecological niches

It is well known that ecological niches, including the host (a parasite or a symbiont or a commensal), play a key role in shaping the genome content of an organism (Qhanya *et al*., 2015). This phenomenon has been observed for P450s in fungi, where P450s have been discovered to play an important part in fungi adaptation regarding lifestyle impacts on P450 content in their genomes. This has been demonstrated in numerous fungal species where distinct P450 families and subfamilies within P450 families bloomed (present in many copies within the same species) to help fungi adapt to ecological niches (Jawallapersand *et al*., 2014).

- The CYP53 family bloomed in basidiomycetes due to its involvement in forming the wooddegrading oxidant veratryl alcohol and the breakdown of wood-derived chemicals (Jawallapersand *et al.*, 2014). CYP53's different role appears to have enriched this P450 family by significantly duplicating its members in basidiomycetes genomes (paralogous evolution) (Jawallapersand *et al*., 2014).
- Analysis of potential P450s in basidiomycete biotrophic plant diseases indicated the presence of distinct P450 families, which may reflect the order's features (Qhanya *et al*., 2015). A comparison of P450 families at the order level amongst biotrophic plant pathogens indicated the presence of distinct P450 family patterns in these species, which may reflect the properties of their order. Comparing P450 families with non-pathogenic basidiomycetes indicated that biotrophic plant pathogens have unique P450 families in their genomes. Compared to other Agaricomycotina saprophytes, the CYP63, CYP5037, CYP5136, CYP5137, and CYP5341 P450 families were expanded in *Armillaria mellea*, as were the CYP5221 and CYP5233 P450 families in *Puccinia graminis* and *Melampsora laricispopulina* (Qhanya *et al*., 2015). The presence of distinct P450 families in these biotrophic plant pathogens demonstrates how a host can shape an organism's P450

composition. These distinct P450 family members may be necessary for the host's successful infection.

2.6. Fungi- *Pezizomycetes*

The fungal class *Pezizomycetes* forms a monophyletic group, yet Pezizomycetes have diverse lifestyles, including saprophytic, mycorrhizal, and parasitic (Ekanayaka *et al*., 2018). Species in this class have significant scientific, ecological, and economic importance (Table 2.3). Some species have been used as model organisms to understand the development of multicellular structures, rehabilitation of post-fire soils, and as an income source, as many species in this class are truffles with great economic importance (Table 2.3). General information on some of the Pezizomycetes species focusing on their importance is listed in Table 2.3.

Some Pezizomycetes genomes were sequenced to understand the molecular mechanisms underlying the transition from saprophytic to mycorrhizal lifestyle (Kuo *et al*., 2013). The authors discovered that transitions from saprotrophic to symbiosis involve (i) loss of lignin and cellulose-degrading genes, (ii) the ancestral genes gaining novel functions, (iii) new, lineage-specific symbiotic gene diversity, (iii) multiplication of transposable elements, and (v) diverse genetic innovations behind the ectomycorrhizal guild’s convergent origins (Miyauchi *et al*., 2020, Murat *et al.*, 2018).

Table 2.3 Information about various Pezizomycetes and their well-known characteristics (Nsele *et al*., 2023).

Species Name	Family	Lifestyle (Well-Known/Common Name)	General Information	Reference
Ascobolus immersus RN42	*Ascobolaceae*	Saprotroph (coprophilous fungus)	This fungus lives on herbivore dung and is used as a model fungus for epigenetic research.	(Murat *et al*., 2018)
Ascodesmis nigricans CBS 389.68	*Ascodesmidaceae*	Saprotroph (coprophilous fungus)	This fungus lives on both omnivorous and herbivore dung and is ideal for studying the complex multicellular structure in ascomycetes.	(Lütkenhaus *et al*., 2019)
Choiromyces venosus 120613-1	*Tuberaceae*	Ectomycorrhizal truffle (pig truffle)	This symbiotic species coexists with coniferous and deciduous plants on clayey soils. Because of the potent and distinctive order of the fruiting body, different European regions place different values on the gourmet attributes of this white truffle.	(Murat *et al*., 2018)
Kalaharituber pfeilii F3	*Pezizaceae*	Ectomycorrhizal truffle (Kalahari or desert truffle)	This desert truffle is a food and economical source for the people who live in the dry regions of Southern Africa, which range from South Africa's Northern Cape Province through Botswana, Namibia, and Angola. The truffle is remarkably resistant to harsh desert conditions. This truffle is the only one to create ectomycorrhizal relationships with dicot and monocot plants. It demonstrates extraordinary adaptability to harsh desert conditions.	(Miyauchi *et al*., 2020)
Morchella importuna CCBAS932	*Morchellaceae*	A saprotrophic morel	This fungus belongs to the true morel fungi and lives in pre- and post-fire environments. This fungus maintains the fertility of the site and stabilizes the soil after a fire. Despite being widely	(Murat *et al*., 2018)

			prized as edible species, cultivation has proven difficult.	
Morchella importuna SCYDJ1-A1	*Morchellaceae*	A saprotrophic morel	This species can be artificially grown in typical agricultural soil; thus, a popular variety of gourmet mushrooms contributes significantly to the global economy.	(Tan *et al*., 2019)
Peziza echinospora CBS 144458	*Pezizaceae*	Saprotroph (pyrophilous fungus)	This fungus is a moderate-size cup fungus with a contrast in color between its upper and lower surfaces. It strictly grows in post-fire environments and is thus an ideal candidate to study its enzymatic abilities.	(Steindorff *et al*., 2022)
Pyronema confluens CBS100304	*Pyronemataceae*	Saprotroph	It is a saprobe that lives in the soil and is found in temperate forests. After a forest fire, its fruiting bodies typically appear on the ground. This fungus serves as a model for investigating cell biology and forming fruiting bodies in filamentous ascomycetes.	(Traeger *et al.,* 2013)
Pyronema domesticum CBS 144463	*Pyronemataceae*	Saprotroph (pyrophilous fungus)	This fungus grows rapidly on post-fire soils and also on sterilized materials.	(Steindorff *et al.,* 2022)
Pyronema omphalodes CBS 144459	*Pyronemataceae*	Saprotroph (pyrophilous fungus)	This fungus grows rapidly on post-fire soils.	(Steindorff *et al*., 2022)
Terfezia boudieri ATCC MYA-4762	*Terfeziaceae*	Ectomycorrhizal truffle (desert truffle)	This desert truffle has been an important food since dating back to 4000 years in the arid areas of the Middle East.	(Murat *et al*., 2018)
Terfezia claveryi T7	*Terfeziaceae*	Ectomycorrhizal truffle (desert truffle)	This desert truffle has been an important food in the Mediterranean Basin, Near East, and Middle East. It has a pleasant flavor, an unusual texture, significant antioxidant activity, and antibacterial properties.	(Miyauchi *et al*., 2020)

Tirmania nivea G3	*Pezizaceae*	Ectomycorrhizal truffle (desert truffle)	It is one of the most appreciated desert truffles in the north of Africa, the Near East, and the Middle East. It grows to a diameter of more than 10 cm,	(Miyauchi *et al*., 2020)
			has a mild flavor and a fungal odor, and is highly prized in the market. The heat and water stress this species can withstand in deserts is exceedingly unfavorable for other fungus.	
Tricharina praecox CBS 144465	*Pezizaceae*	Saprotroph (pyrophilous fungus)	This fungus grows only on post-fire soils.	(Steindorff *et al*., 2022)
Tuber aestivum var. *urcinatum*	*Tuberaceae*	Ectomycorrhizal truffle (Burgundy truffle)	Burgundy truffle, summer truffle, and scorzone are all names for the edible fruiting bodies that are produced by *Tuber aestivum*. This truffle is widely distributed from Morocco to Sweden in the north and from Ireland to Kazakhstan.	(Murat *et al*., 2018)
Tuber borchii Tbo3840	*Tuberaceae*	Ectomycorrhizal truffle (the white truffle or bianchetto)	Due to its highly prized gourmet qualities, this ectomycorrhizal ascomycete is regarded as the tuber species with the broadest biological distribution in Europe. It is growing in popularity as an Italian delicacy. *T. borchii* is one of the most extensively researched truffle species because it is amenable to laboratory manipulations.	(Murat *et al*., 2018)
Tuber brumale	*Tuberaceae*	Ectomycorrhizal truffle (the winter truffle)	This species is widespread in Europe, and its edible fruiting body (truffle) is harvested during the winter.	(Morin *et al*., 2021)
Tuber indicum	*Tuberaceae*	Ectomycorrhizal truffle (the Chinese black truffle)	At an elevation of 2.000 to 2.500 m in a temperate climate, this ectomycorrhizal Ascomycota forms a mutualistic association with oak and mountain pines in the Chinese provinces of Yunnan and Sichuan, and it has unintentionally spread to North America and Italy.	(Morin *et al*., 2021)

Tuber magnatum	*Tuberaceae*	Ectomycorrhizal truffle (the white truffle—the icon of European gastronomy)	This white truffle, a "cult food," is a well-known symbol of European cuisine and culture. *T. magnatum*'s fruiting body is an edible truffle (also known as a hypogeous ascocarp) prized for its exquisite organoleptic qualities (i.e., taste and perfumes). In Italian and Balkan soils, it is generally found as mycelia. It forms a mutualistic mycorrhizal connection with the roots of deciduous trees such as poplars, oaks, and willows.	(Murat *et al*., 2018)
Wilcoxina mikolae CBS 423.85	*Pyronemataceae*	Ectomycorrhizal fungus	This fungus is a significant ectomycorrhizal symbiont of Pinaceae and numerous hardwood species. *Wilcoxina* species are among the most frequent colonizers of young pine, spruce, and larch trees and are found in nurseries and in forests that have experienced a fire or other disturbance.	(Miyauchi *et al*., 2020)
Sphaerosporella brunnea Sb_GMNB300	*Pyronemataceae*	Ectomycorrhizal	This fungus is considered a vital pioneer ectomycorrhizal symbiont due to its ability to associate with diverse trees and shrub species.	(Benucci *et al*., 2019)
Trichophaea hybrida UTF0779	*Pyronemataceae*	Ectomycorrhizal	This species is distributed throughout Northern and Central Europe and predominantly inhabits old forests, contrary to the *Wilcoxina* species.	(Miyauchi *et al.,* 2020)
Tuber melanosporum Mel28	*Pyronemataceae*	Ectomycorrhizal (Périgord black truffle)	This species is native to Southern Europe, and its fruiting body (truffle) is one of the most expensive edible mushrooms in the world.	(Martin *et al*., 2010, Murat *et al*., 2018)

Chapter 3 : METHODOLOGY

3.1. Species and database information

Nineteen Pezizomycetes were used in the study (Table 3.1). All the species' genomes used in the study have been published and are available for public use at the Joint Genome Institute MycoCosm portal (Grigoriev *et al*., 2014).

Table 3.1 Pezizomycetes used in the study and their genome database links, along with reference articles, are listed in the table. The genome database of Pezizomycetes was accessed on 31 May, 2023.

Species Name	Genome Version	Genome Database Link	Reference
Ascobolus immersus RN42	v1.0	https://mycocosm.jgi.doe.gov/Ascim1/Ascim1.home.html	(Murat *et al*., 2018)
Ascodesmis nigricans CBS 389.68	v1.0	https://mycocosm.jgi.doe.gov/Ascni1/Ascni1.home.html	(Lütkenhaus *et al*., 2019)
Choiromyces venosus 120613-1	v1.0	https://mycocosm.jgi.doe.gov/Chove1/Chove1.home.html	(Murat *et al*., 2018)
Kalaharituber pfeilii F3	v1.0	https://mycocosm.jgi.doe.gov/Kalpfe1/Kalpfe1.home.html	(Miyauchi *et al*., 2020)
Morchella importuna CCBAS932	v1.0	https://mycocosm.jgi.doe.gov/Morco1/Morco1.home.html	(Murat *et al*., 2018)
Morchella importuna SCYDJ1-A1	v1.0	https://mycocosm.jgi.doe.gov/Morimp1/Morimp1.home.html	(Tan *et al*., 2019)
Pyronema confluens CBS100304		https://mycocosm.jgi.doe.gov/Pyrco1/Pyrco1.home.html	(Traeger *et al*., 2013)

Sphaerosporella brunnea Sb_GMNB300	v2.0	https://mycocosm.jgi.doe.gov/Sphbr2/Sphbr2.home.html31	(Benucci *et al*., 2019)
Terfezia boudieri ATCC MYA-4762	v1.1	https://mycocosm.jgi.doe.gov/Terbo2/Terbo2.home.html	(Murat *et al*., 2018)
Terfezia claveryi T7	v1.0	https://mycocosm.jgi.doe.gov/Tercla1/Tercla1.home.html	(Miyauchi *et al*., 2020)
Tirmania nivea G3	v1.0	https://mycocosm.jgi.doe.gov/Tirniv1/Tirniv1.home.html	(Miyauchi *et al*., 2020)
Trichophaea hybrida UTF0779	v1.0	https://mycocosm.jgi.doe.gov/Trihyb1/Trihyb1.home.html	(Miyauchi *et al*., 2020)
Tuber aestivum var. *urcinatum*	v1.0	https://mycocosm.jgi.doe.gov/Tubae1/Tubae1.home.html	(Murat *et al.,* 2018)
Tuber borchii Tbo3840	v1.0	https://mycocosm.jgi.doe.gov/Tubbor1/Tubbor1.home.html	(Murat *et al*., 2018)
Tuber brumale	v1.0	https://mycocosm.jgi.doe.gov/Tubbr1_1/Tubbr1_1.home.html	(Morin *et al*., 2021)
Tuber indicum	v1.0	https://mycocosm.jgi.doe.gov/Tubin1_1/Tubin1_1.home.html	(Morin *et al*., 2021)
Tuber magnatum	v1.0	https://mycocosm.jgi.doe.gov/Tubma1/Tubma1.home.html	(Murat *et al*., 2018)
Tuber melanosporum Mel28	v1.2	https://mycocosm.jgi.doe.gov/Tubme1v2/Tubme1v2.home.html	(Martin *et al*., 2010)

Wilcoxina mikolae CBS 423.85	v1.0	https://mycocosm.jgi.doe.gov/Wilmi1/Wilmi1.home.html	(Miyauchi *et al*., 2020)

3.2. Genome data mining and identification of P450s

Genome data mining and identification of P450s in Pezizomycetes was carried out following the protocol previously published by our laboratory (Mthethwa *et al*., 2018, Malinga *et al*., 2022). Each Pezizomycetes genome was searched for P450s using the InterPro code "IPR001128". The hit protein sequences were downloaded and searched for P450 characteristic motifs, including the EXXR and CXG motifs (Gotoh, 1992, Syed and Mashele, 2014). Proteins with all P450 characteristic motifs were considered P450s, and proteins with one of these motifs or short in amino acid length (less than 350 amino acids) were considered P450 fragments. Proteins considered P450s were subjected to P450 family and subfamily analysis.

3.3. Assigning P450 family and subfamily

To assign P450 families and subfamilies, we performed a Basic Local Alignment Search Tool (BLAST) analysis of Pezizomycetes P450s against all named fungal sequences on the Cytochrome P450 Homepage (Nelson, 2009) to identify the percentage identity with named homolog P450s. The proteins were then grouped into different P450 families and subfamilies following the International P450 Nomenclature criteria (Nelson, 1998, Nelson, 2006), i.e., proteins with >40% and >55% amino acid identity were grouped under the same P450 family and subfamily. Proteins with less than 40% identity to the named homologs were assigned to the new P450 family. The P450s along with their assigned names and P450 fragment sequences are presented in Supplementary Dataset 1.

3.4. Phylogenetic analysis of P450s

Phylogenetic analysis of P450s was carried out following the procedure previously published by our laboratory (Akapo et al., 2019, Ngcobo *et al*., 2023). Briefly, the P450 protein sequences were aligned by the MAFFT v6.864 (Katoh *et al*., 2005) program available at the T-REX web server (Boc *et al*., 2012). The alignments were then automatically subjected to interpret the best tree using the Trex web server (Boc *et al*., 2012). Finally, the best-inferred tree was visualized, colored, and generated by the Interactive Tree Of Life (iTOL) (Letunic and Bork, 2019).

3.5. P450 family conservation analysis

The presence or absence of P450s belonging to different families in Pezizomycetes was shown with heat maps generated using P450 family data following the method described by our laboratory (Akapo *et al*., 2019, Malinga et al., 2022). The data were represented as −3 for gene absence (green) and 3 for gene presence (red). A tab-delimited file was imported into a multiexperiment viewer (MeV) (Saeed et al., 2003). Hierarchical clustering using a Euclidean

distance metric was used to cluster the data. A total of 19 Pezizomycetes form the vertical axis, and 153 P450 families form the horizontal axis.

3.6. Identification of P450s that are part of natural metabolite biosynthetic gene clusters

P450s, part of natural metabolite biosynthetic gene clusters (BGCs), were identified following the procedure published by our laboratory (Msweli *et al*., 2022) with slight modification. Each of the fungal genome National Center for Biotechnology Information (NCBI) genome accession numbers (Table 3.2) was submitted for BGCs analysis at the Antibiotics and Secondary Metabolite Analysis Shell (anti-SMASH) program (Blin *et al*., 2021). Anti-SMASH results were downloaded in gene cluster sequences and Excel spreadsheets representing species-wise cluster information. P450s that formed part of a specific gene cluster were identified by manual data mining of gene cluster sequences. Standard gene cluster abbreviation terminology available at the anti-SMASH database (Blin *et al*., 2021) was maintained in this study. Among the 19, only 12 Pezizomycetes NCBI genome accession numbers were successful at the anti-SMASH program, and the remaining seven species accession numbers have yet to provide any results. Thus, we presented information on BGCs for twelve species in this study.

Table 3.2 Information on Pezizomycetes and their NCBI genome accession numbers used to analyze natural metabolite biosynthetic gene clusters at the anti-SMASH database (Blin *et al*., 2021).

Species name	NCBI genome accession number
Ascobolus immersus	PZQT00000000.1
Tuber borchii	NESQ00000000
Terfezia claveryi	WHUX00000000
Wilcoxina mikolae CBS 423.85	WITH00000000
Tirmania nivea	WHUY00000000
Tuber brumale	JACCEG00000000
Tuber melanosporum	CABJ00000000.1
Trichophaea hybrida	WHVE00000000
Tuber indicum	JACCEH00000000
Sphaerosporella brunnea	VXIS00000000
Morchella importuna SCYDJ1-A1	SSHS00000000.1
Tuber magnatum	DYWC00000000.1

Chapter 4 : RESULTS AND DISCUSSION

4.1. Saprotrophs have more P450s than Ectomycorrhizal Pezizomycetes

Genome-wide-data mining of P450s in nineteen Pezizomycetes resulted in 779 hit proteins (Table 4.1). Further analysis of hit proteins for characteristic P450 motifs (as indicated in section 3.2) revealed that not all hit proteins are P450s. Among hits, 668 hits have all the P450 characteristic motifs and are thus considered P450s; 88 were P450 fragments, 7 were false positives, and 16 were different proteins, thus noting them as no hits. The presence of false positives and no hits indicates that automated allocation of P450s is not always accurate, and manual curation of P450 is needed to assess an accurate number of P450s in an organism.

Table 4.1 Genome-wide analysis of P450s in 19 Pezizomycetes.

Species Name	**Lifestyle**	**Total hits**	**P450s**	**No hits**	**False Positive**	**Fragments**
Ascobolus immersus RN42	SAP	63	58	4	1	0
Ascodesmis nigricans CBS 389.68	SAP	31	28	2	0	1
Morchella importuna CCBAS932	SAP	40	37	3	0	0
Morchella importuna SCYDJ1-A1	SAP	41	37	3	0	1
Pyronema confluens CBS100304	SAP	55	44	1	2	8
Choiromyces venosus 120613-1	ECM	52	33	1	1	17
Kalaharituber pfeilii F3	ECM	35	32	0	0	3
Sphaerosporella brunnea Sb_GMNB300	ECM	49	47	0	0	2
Terfezia boudieri ATCC MYA-4762	ECM	24	19	1	2	2
Terfezia claveryi T7	ECM	19	17	0	0	2

Tirmania nivea G3	ECM	21	19	0	0	2
Trichophaea hybrida UTF0779	ECM	44	37	0	0	7
Tuber aestivum var. *urcinatum*	ECM	31	29	0	0	2
Tuber borchii Tbo3840	ECM	74	55	0	0	19
Tuber brumale	ECM	37	32	0	0	5
Tuber indicum	ECM	39	35	0	0	4
Tuber magnatum	ECM	32	27	0	1	4
Tuber melanosporum Mel28	ECM	35	30	0	0	5
Wilcoxina mikolae CBS 423.85	ECM	57	52	1	0	4

Note: The lifestyle of different Pezizomycetes is retrieved from the published articles (Murat *et al*., 2018, Miyauchi *et al*., 2020). Abbreviations: SAP: saprotrophic; ECM: ectomycorrhizal.

The number of P450s in 19 Pezizomycetes ranged from 17 to 58 P450s with an average of 35 P450s. Among Pezizomycetes, *Ascobolus immersus* RN42 has the highest number of P450s (58), and *Terfezia claveryi* T7 has the lowest number of P450s (17) in their genomes (Table 4.1). *Tuber borchii* Tbo3840, despite having the highest number of hit proteins due to 19 P450 fragments, the P450 count was confined to only 55 P450s, second only to *A. immersus* (Table 4.1).

A comparison of P450s revealed that saprotrophic Pezizomycetes have more P450s in their genome compared to ectomycorrhizal Pezizomycetes (Table 4.1). The average number of P450s was 41 in saprotrophs compared to 33 in ectomycorrhizal Pezizomycetes (Table 4.1). The difference observed concerning P450 numbers is not statistically significant due to one or two species as an outlier from saprophytes and ectomycorrhizal groups. However, the average P450s number difference indicates that most ectomycorrhizal have fewer P450s than saprophyte Pezizomycetes.

4.2. P450 Family and Subfamily analysis in Pezizomycetes

Following the International P450 Nomenclature Committee rules and the phylogenetic analysis (Figure 4.1), the 668 P450s found in 19 Pezizomycetes were grouped into 153 P450 families and 245 P450 subfamilies (Tables 4.2 and S1). Although P450s were assigned to different P450 families and subfamilies based on the percentage identity as indicated in section 3.3., phylogenetic analysis is critical in assigning the subfamilies to P450s that fall around 55% identity borderline with named homolog P450s. Based on the alignment on the phylogenetic tree, these borderline P450s were assigned to the correct subfamilies. Furthermore, phylogenetic analysis will also help find evolutionary relationships, such as the closeness of P450s from two different species.

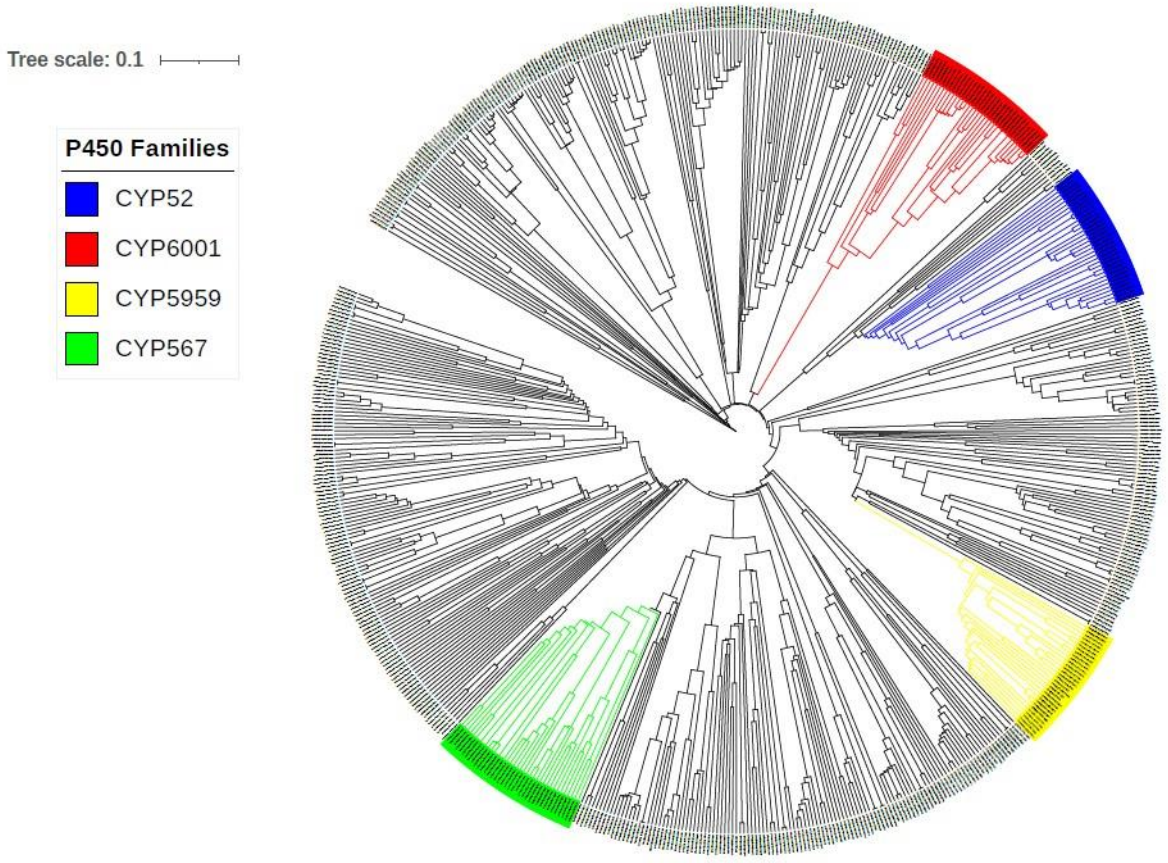

Figure 4.1 Phylogenetic analysis of Pezizomycetes P450s. P450 families that are populated in Pezizomycetes are highlighted in different colors. A high-quality figure is presented in Supplementary Figure S1.

Table 4.2 Analysis of P450 family and subfamily count in Pezizomycetes. The name of P450 families (F), their count (C), and the number of subfamilies (NSF) within a P450 family are presented in the table. A detailed analysis of the P450 families and subfamilies is presented in Table S1.

F	C	NSF	F	C	NSF	F	C	NSF	F	C	NSF
CYP567	38	12	CYP6608	4	1	CYP51075	2	2	CYP51066	1	1
CYP6001	35	3	CYP6637	4	2	CYP51079	2	2	CYP51068	1	1
CYP52	34	13	CYP6648	4	2	CYP51085	2	2	CYP51071	1	1
CYP5959	31	1	CYP6761	4	3	CYP51093	2	1	CYP51072	1	1
CYP548	24	2	CYP6855	4	4	CYP5142	2	1	CYP51076	1	1
CYP51	20	1	CYP50115	3	2	CYP5242	2	1	CYP51077	1	1
CYP5093	19	3	CYP50335	3	2	CYP540	2	1	CYP51078	1	1
CYP61	19	1	CYP5078	3	2	CYP578	2	1	CYP5108	1	1
CYP539	18	1	CYP51041	3	2	CYP6002	2	1	CYP51080	1	1
CYP6135	18	1	CYP51048	3	3	CYP6480	2	1	CYP51081	1	1
CYP6136	15	4	CYP51069	3	3	CYP6535	2	1	CYP51082	1	1
CYP617	15	4	CYP51083	3	3	CYP666	2	1	CYP51084	1	1
CYP663	14	2	CYP51089	3	2	CYP6683	2	1	CYP51086	1	1
CYP512	13	4	CYP51092	3	2	CYP6775	2	1	CYP51087	1	1
CYP5945	12	6	CYP5192	3	2	CYP6818	2	1	CYP51088	1	1
CYP6220	11	1	CYP5268	3	1	CYP6900	2	1	CYP5109	1	1
CYP51070	10	1	CYP532	3	2	CYP6958	2	1	CYP51090	1	1

CYP573	9	2	CYP584	3	2	CYP5004	1	1	CYP51091	1	1
CYP6271	9	1	CYP6470	3	1	CYP50147	1	1	CYP52486	1	1
CYP6713	8	6	CYP6497	3	2	CYP50183	1	1	CYP52487	1	1
CYP50043	7	1	CYP6521	3	2	CYP503	1	1	CYP55	1	1
CYP504	7	2	CYP6643	3	1	CYP5095	1	1	CYP566	1	1
CYP51062	6	3	CYP6685	3	1	CYP5104	1	1	CYP594	1	1
CYP530	6	1	CYP671	3	1	CYP51040	1	1	CYP596	1	1
CYP6188	6	1	CYP6742	3	2	CYP51043	1	1	CYP6006	1	1
CYP6498	6	1	CYP6902	3	2	CYP51047	1	1	CYP613	1	1
CYP6592	6	1	CYP50030	2	1	CYP51049	1	1	CYP65	1	1
CYP50194	5	2	CYP50042	2	2	CYP51050	1	1	CYP654	1	1
CYP505	5	1	CYP50241	2	1	CYP51053	1	1	CYP665	1	1
CYP675	5	2	CYP50308	2	1	CYP51054	1	1	CYP66608	1	1
CYP50026	4	2	CYP50320	2	1	CYP51055	1	1	CYP667	1	1
CYP50127	4	3	CYP50357	2	1	CYP51056	1	1	CYP676	1	1
CYP50251	4	2	CYP51042	2	2	CYP51058	1	1	CYP677	1	1
CYP51074	4	3	CYP51044	2	1	CYP51059	1	1	CYP6793	1	1
CYP53	4	1	CYP51045	2	1	CYP51060	1	1	CYP682	1	1
CYP6433	4	1	CYP51046	2	1	CYP51061	1	1	CYP6836	1	1

CYP6453	4	1	CYP51052	2	2	CYP51063	1	1			
CYP6501	4	1	CYP51057	2	1	CYP51064	1	1			
CYP6529	4	1	CYP51073	2	1	CYP51065	1	1			

Among 153 P450 families found in 19 Pezizomycetes, only four P450 families, namely, CYP567, CYP6001, CYP52, and CYP5959, have ≥30 members. Thus, one can safely say that P450 families in Pezizomycetes are not bloomed (a few P450 families with many genes) (Table 4.2). This is unlike some fungal species where P450 family blooming is common (Syed *et al*., 2014, Jawallapersand *et al*., 2014, Qhanya *et al*., 2015, Ngwenya *et al*., 2018). This indicates that the Pezizomycetes species have a high P450 diversity concerning P450 families in their genome. Analysis of P450 subfamilies revealed the blooming of two P450 subfamilies, A and C, in CYP5959 and CYP6001 families (Table 4.2 and S1).

The number of P450 families ranged from 14-40, with an average of 26 P450 families in 19 Pezizomycetes, where *Wilcoxina mikolae* CBS 423.85, and *T. claveryi* T7 had the highest and lowest number of P450 families in their genome (Tables 4.3). The number of P450 subfamilies ranged from 16-46, with an average of 31 P450 families *A. immersus RN42* and *T. claveryi* T7 had the highest and lowest number of P450 families in their genomes, respectively (Tables 4.3 and S2). Detailed analysis of P450 families and subfamilies in 19 Pezizomycetes is presented in Table S2.

Table 4.3 Comparative analysis of P450 families and subfamilies in Pezizomycetes. Detailed analysis on P450 families and subfamilies for each Pezizomycetes is presented in Table S2.

Species Name	**No of P450 families**	**No of P450 subfamilies**
Ascobolus immersus RN42	36	49
Ascodesmis nigricans CBS 389.68	24	26
Choiromyces venosus 120613-1	24	29
Kalaharituber pfeilii F3	20	22
Morchella importuna CCBAS932	33	36
Morchella importuna SCYDJ1-A1	33	36
Pyronema confluens CBS100304	37	43
Sphaerosporella brunnea Sb_GMNB300	38	45
Terfezia boudieri ATCC MYA-4762	16	18
Terfezia claveryi T7	14	16
Tirmania nivea G3	17	19
Trichophaea hybrida UTF0779	31	36
Tuber aestivum var. *urcinatum*	24	26

Tuber borchii Tbo3840	22	27
Tuber brumale	24	27
Tuber indicum	24	28
Tuber magnatum	21	23
Tuber melanosporum Mel28	23	25
Wilcoxina mikolae CBS 423.85	40	49

A comparison of P450 families and P450 subfamilies indicated that saprophytes have more P450 families in their genome than ectomycorrhizal Pezizomycetes (Table 4.1). Five saprophyte Pezizomycetes have 103 P450 families, whereas 14 ectomycorrhizal Pezizomycetes have 89 P450 families (Figure 4.2). 39 P450 families were found in common between saprotrophs and ectomycorrhizal Pezizomycetes (Figure 4.2). Furthermore, 50 and 64 P450 families were found to be unique to ectomycorrhizal and saprophytic Pezizomycetes, respectively (Figure 4.2).

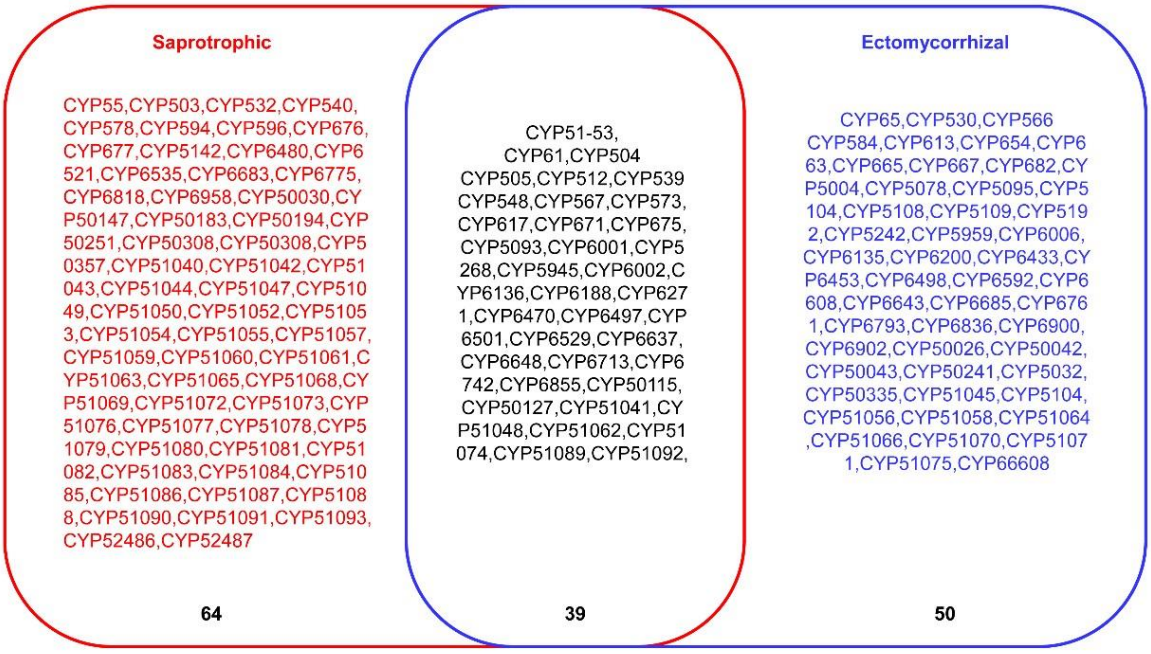

Figure 4.2 Comparative analysis of P450 families between saprotrophic and ectomycorrhizal Pezizomycetes. The number indicates the total number of P450 families.

4.3. A few P450 families are conserved in Pezizomycetes

Analysis of P450 family conservation revealed out of 153 P450 families, only four P450 families: CYP51, CYP61, CYP5093, and CYP6001, were conserved across 19 Pezizomycetes (Figure 4.3). CYP539 and CYP548 were found to be conserved in 18 Pezizomycetes, followed by CYP567 in 17 species and CYP52 in 16 species (Figure 4.3 and Table S3). Detailed analysis of P450 family conservation across the 19 Pezizomycetes is presented in Table S3. Conservation of four P450 families across 19 Pezizomycetes indicates these P450 families might be involved in critical functions. It is well-known that CYP51 and CYP61 are involved in sterol biosynthesis (Lamb *et al*., 2021, Kelly *et al*., 1997, Chen *et al*., 2014), the essential components of cell wall membranes, and CYP6001 members were also shown to be involved in the oxidation of fatty acids (Brodhun *et al*., 2009). The reactions performed by these three P450 families are important in the physiology of these species, and thus, these families are conserved. The function of CYP5093 family members is not identified, and thus based on its conservation; one can assume it might be involved in critical functions as well.

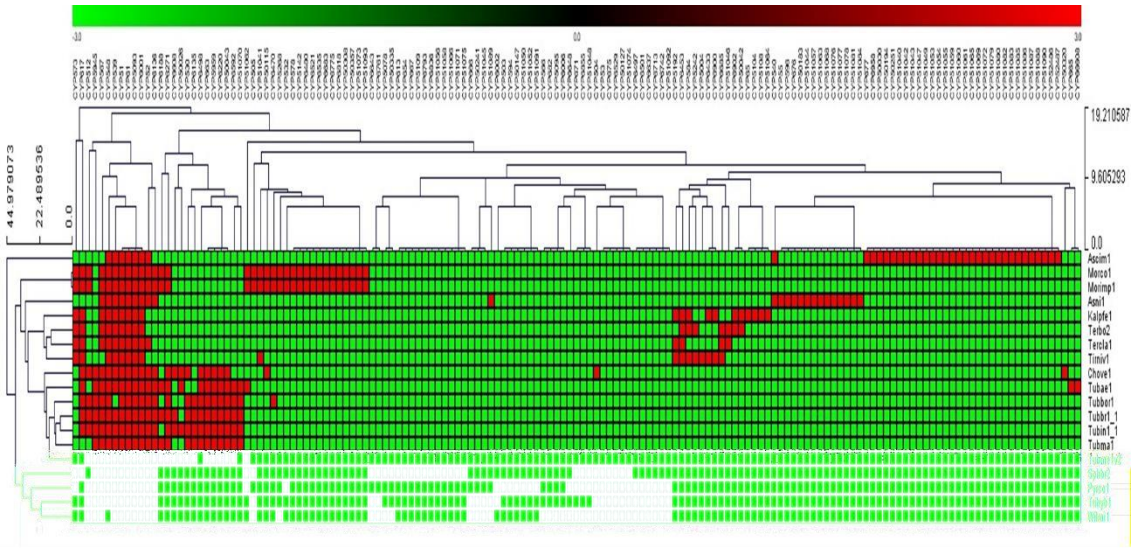

Figure 4.3 P450 family conservation analysis in 19 Pezizomycetes. The heat map represents the presence (red) or absence (green) of the P450 family in Pezizomycetes. Pezizomycetes form the vertical axis, and P450 families form the horizontal axis. Detailed analysis of P450 family conservation in Pezizomycetes is presented in Table S3.

4.4. Terpene biosynthetic gene clusters are dominant in Pezizomycetes

A natural metabolite biosynthetic gene clusters (BGCs) analysis across 12 Pezizomycetes revealed the presence of 142 clusters belonging to 13 cluster types (Figure 4.4 and Table S4). Among the BGC types, terpene was dominant with 50 clusters, followed by NRPS-like with 29 clusters and T1PKS with 18 clusters (Figure 4.4 and Table S4). Analysis of most similar BGCs revealed that six Pezizomycetes have a terpene BGC that has 100% similarity to clavaric acid, and one species has an NRPS, T1PKS BGCs that have 100% similarity to ACT-Toxin II (Table S5) indicating these BGCs certainly involved in the production of these metabolites.

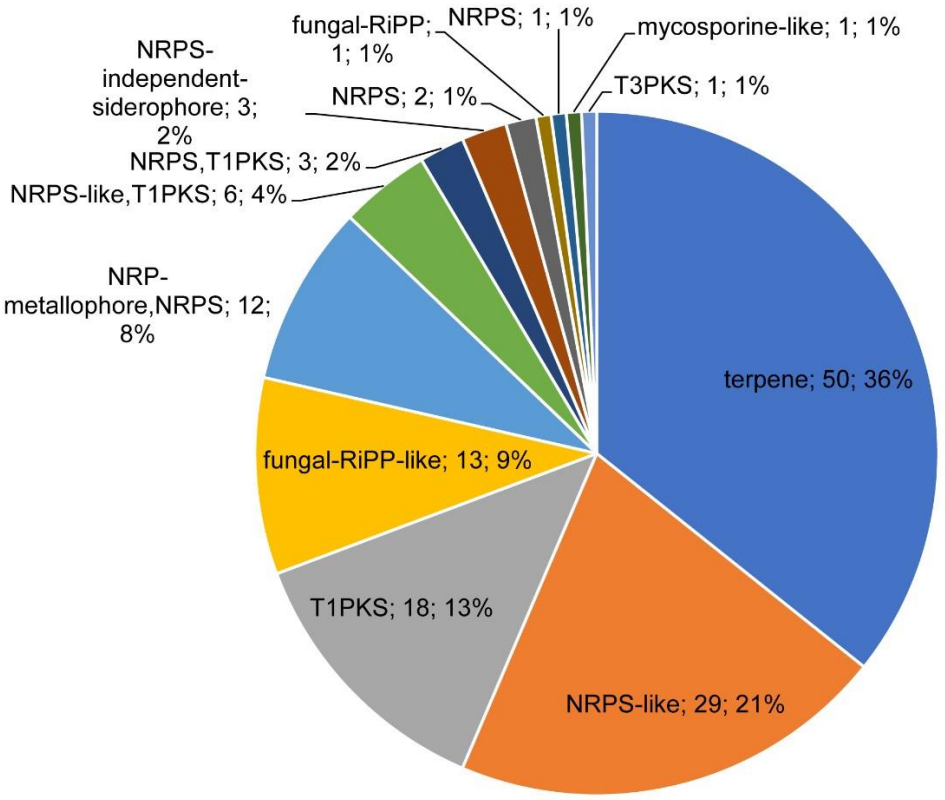

Figure 4.4 Comparative analysis of natural metabolite biosynthetic gene clusters (BGCs) in 19 Pezizomycetes. The number next to BGCs indicates the number of BGCs and their percentage in the total number of BGCs. Standard abbreviations representing the BGCs as indicated in anti-SMASH (antibiotics & Secondary Metabolite Analysis Shell) (Blin *et al*., 2021) were used in the figure. Detailed information is presented in Table S4.

Comparative analysis of BGCs revealed the highest number of BGCs in *W. mikolae* CBS 423.85 (21 BGCs), followed by *Sphaerosporella brunnea* (18 BGCs) and *Trichophaea hybrida* (16 BGCs) (Table 4.4). The average number of BGCs in 12 Pezizomycetes was 12. Some BGCs in a few species were found to have P450s (Table 4.4), indicating the possible involvement of P450s in the synthesis of natural metabolites, as fungal P450s are known to be involved in the production of various natural metabolites (Durairaj *et al*., 2016, Zhang *et al*., 2021). A total of 9 P450s were found to be part of different BGCs, with five of them being part of the terpene BGC (Table 4.4). CYP6637B2 was found to be common between *T. hybrida* and *W. mikolae* CBS 423.85 as part of a terpene BGC (Table 4.4), indicating this P450 is involved in the production of terpene metabolite in both species. Compared to the number of P450s found in Pezizomycetes (668 P450s), the number of P450s (9 P450s) present in the BGCs seems to be very few (1% only). This suggests that most of the Pezizomycetes P450s possibly play a role in primary metabolism.

Table 4.4 Comparative analysis of natural metabolite gene clusters and P450s in the clusters in 12 Pezizomycetes. Detailed information is presented in Table S4.

Species name	Number Of Clusters	Cluster having P450	Cluster type	P450(s) part of the cluster
Ascobolus immersus	14			
Morchella importuna	12			
Sphaerosporella brunnea	18	2	Terpene	CYP654C8, CYP667F1
		11	Fungal-RiPP	CYP51F1
		12	NRPS	CYP5109B1, CYP6836A1
		16	NRPS	CYP613S1
Terfezia claveryi	8			
Tirmania nivea	9			
Trichophaea hybrida	16	14	Terpene	CYP6637B2
Tuber borchii	8			
Tuber brumale	8			
Tuber indicum	10			
Tuber magnatum	10			
Tuber melanosporum	8			
Wilcoxina mikolae CBS 423.85	21	9	Terpene	CYP51048A1

		11	Terpene	CYP6637B2

Note: Standard abbreviations representing the BGCs as indicated in anti-SMASH (antibiotics & Secondary Metabolite Analysis Shell) (Blin et al., 2021) were used in the table.

Chapter 5 : CONCLUSIONS AND FUTURE PERSPECTIVES

In this post-genomic era understanding the molecular basis behind fundamental aspects such as adaptation to diverse ecological niches by organisms is gaining momentum. This study observed that ectomycorrhizal Pezizomycetes have the lowest number of P450s, P450 families, and P450 subfamilies compared to saprotrophic Pezizomycetes. Furthermore, we also identified the development of many unique P450 families in ectomycorrhizal Pezizomycetes.

Our study results strongly support previous studies that the transition from saprophytic to ectomycorrhizal lifestyle resulted in the loss of specific gene complements and enrichment of novel genes in Pezizomycetes, indicating the genome level changes for adaptation (Murat *et al*., 2018, Miyauchi *et al*., 2020). This phenomenon seems universal as it was observed in bacteria (Parvez *et al*., 2016, Senate *et al*., 2019, Padayachee *et al*., 2020, Msomi *et al*., 2021, Msweli *et al*., 2022, Zondo *et al*., 2022) and a few fungal species (Kgosiemang *et al*., 2014, Jawallapersand *et al*., 2014, Qhanya *et al*., 2015, Ngwenya *et al*., 2018, Akapo *et al*., 2019) where the transition from saprophytic to pathogenic or simple lifestyles resulted in the loss of P450s or the development of unique P450s.

More fungal genomes from different fungal groups need to be investigated to get conclusive evidence on changes in P450 compliments between saprotrophs and mycorrhizal lifestyle. Furthermore, future research involves identifying the role of P450s in adaptation, especially the unique P450 families of ectomycorrhizal Pezizomycetes.

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ANNEXURE

The following supporting information can be downloaded at:

https://www.mdpi.com/2309-608X/9/8/830

Figure S1. A high-resolution figure of the phylogenetic analysis of P450s of Pezizomycetes. P450 families that are populated in Pezizomycetes are highlighted in different colors.

Table S1. Analysis of P450 families and subfamilies in 19 Pezizomycetes.

Table S2. Comparative analysis of P450 families and subfamilies in Pezizomycetes.

Table S3. P450 family conservation analysis in 19 Pezizomycetes.

Table S4. Comparative analysis of natural metabolite biosynthetic gene clusters (BGCs) in 12 Pezizomycetes. BGCs and P450s part of these BGCs were predicted as indicated in Section 3.6. Standard abbreviations representing the BGCs as indicated in anti-SMASH (Antibiotics and Secondary Metabolite Analysis Shell) were used in the table.

Supplementary Dataset S1. P450s identified and annotated in Pezizomycetes are presented with their assigned name, followed by protein ID from the Joint Genome Institute MycoCosm database as indicated in Table 4 and species code. P450 fragments identified in Pezizomycetes are also listed.