Comparative genomics of cytochrome P450 monooxygenases in newly explored pathogenic Oomycetes

By

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Dissertation submitted in fulfilment of the requirements of the degree

Magister Technologiae (M. Tech) in Biomedical Technology

Department of Health Sciences

Central University of Technology, Free State

07 January 2016

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DECLARATION

I, **MOPELI MARSHAL SELLO**, hereby certify that the dissertation submitted by me for the degree MAGISTER TECHNOLOGIAE (M. Tech): BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology (Free State). I hereby declare, that this research project has not been previously submitted before to any university or faculty for the attainment of any qualification. I further waive copyright of the dissertation in favour of the Central University of Technology (Free State).

MOPELI M. SELLO **DATE**

ACKNOWLEDGEMENTS

I pass my sincere gratitude for the success of my study to:

- My supervisor, Dr. Khajamohiddin Syed, for guidance and full support in every aspect. I could not have made it without him.
- Prof. Samson Setheni Mashele for his un-measurable support.
- Profs. David R Nelson, Jae-Hyuk Yu and Dr Wanping Chen for their huge input in this study.
- Research and Innovation Fund, Central University of Technology, Free State for funding my studies.
- Mrs ‘Mamotsoeneng, my wife and my boy Motsoeneng for the endless support and love they provide me with.
- My mother and late father, my sisters and brothers for being present in my time of life, their support and trust in me.
- My colleagues at the Faculty of Health and Environmental Sciences, Department of Biomedical Technology at the Central University of Technology especially my research team for their overwhelming friendship and motivation.
- All my friends I am so grateful for your love and support.
- To my Creator for my existence, protection, guidance and blessings I am provided with.

CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

$	Dollar currency sign
%	Percentage
2Fe	Two-iron
2S	Two-sulfur
Adx	Adrenodoxin
ADR	Adrenodoxin reductase
C	Carbon
C-C	Carbon-carbon bond
ClustalW2	Multiple sequence alignment program
CoA	Co-enzyme A
CPR	Cytochrome P450 reductase
C-terminal	Carbon terminal end
CYP	Cytochrome P450
CXG	Cytochrome P450 signature motif
DNA	Deoxyribonucleic acid
ER	Endoplastic reticulum
EXXR	Cytochrome P450 signature motif
et al.	*Et alia* (and others)
FAD	Flavin adenine dinucleotide
FDR	Ferredoxin reductase

Fdx	Ferredoxin
Fe^{II}	Ferrous iron
Fe^{III}	Ferric iron
Fe-S	Iron-sulphur
Fig	Figure
FAD	flavodoxin-flavin mononucleotide
FMN	Flavin mononucleotide
G	Glycine
H+	Hydrogen ion
Hara	*Hyaloperonospora arabidopsidis*
HEM	Heme group
HMMER	Hidden Markov model sequence alignment and database search tool
iTOL	Interactive tree of life
ID	Identity
MEGA	Molecular Evolutionary Genetics Analysis
NADP	Nicotinamide adenine dinucleotide phosphate
NAD (P) H	Reduced nicotinamide adenine dinucleotide phosphate
NCBI CDD	National Center for Biotechnology Information Conserved Domain Database
NPTL	Number of P450s tandemly localized
NPTL-SF	Number of P450s tandemly localized on the same scaffold

N-terminal	Amino terminal end
nm	Nanometre
O-O	Oxygen - oxygen bond
P450	Cytochrome P450
Paph	*Pythium aphanidermatum*
Pcap	*Phytophthora capsici*
Pfam	Protein families database
Pinf	*Phytophthora infestans*
Pirr	*Pythium irregular*
Piwa	*Pythium awayamai,*
Ppar	*Phytophthora parasitica*
Pram	*Phytophthora ramorum*
Psoj	*Phytophthora sojae*
Pult	*Pythium ultimum*
PQQ	pyrroloquinoline quinone
Pvex	*Pythium vexan*
RH	Substrate
Sdec	*Saprolegnia declina*
Sp	Species
Spar	*Saprolegnia parasitica*.
USA	United States of America
USDA-APHIS	United States Department of Agriculture Animal and Plant Health Inspection Service

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ABSTRACT

Cytochrome P450 monooxygenases (P450s) are heme-thiolate proteins whose role as drug targets against pathogens, as well as in valuable chemical production and bioremediation, has been explored. In this study comprehensive comparative analysis of P450s in 13 newly explored oomycete pathogens performed. Three hundred and fifty-six P450s were identified in the 13 oomycetes species. These P450s were grouped into 15 P450 families and 84 P450 subfamilies. Among those, nine P450 families and 31 P450 subfamilies were newly identified in oomycetes. Research revealed that oomycetes belonging to different orders contain distinct P450 families and subfamilies in their genomes. Evolutionary analysis and sequence homology data revealed P450 family blooms in oomycetes. Tandem arrangement of a large number of P450s belonging to the same family suggested that, P450 family blooming is possibly due to duplications in family members. A unique combination of amino acid patterns was observed at EXXR and CXG motifs for the P450 families; CYP5014, CYP5015 and CYP5017. A novel P450 fusion protein (CYP5619 family) with an N-terminal P450 domain fused to a heme peroxidase/dioxygenase domain was discovered in *Saprolegnia declina*. Oomycetes P450 patterns suggested host influence in shaping their P450 content. This manuscript serves as reference for future P450 annotations in newly explored oomycetes.

The study has been published in Scientific Reports (impact factor 5.6). Manuscript details: **Sello, M. M.**, Jafta, N., Nelson, D. R., Chen, W., Yu, J., Parvez, M., Kgosiemang, I. K. R., Monyaki, R., Raselemane, S. C., Qhanya, L., Mthakathi, N. T., Mashele, S. S., Syed, K. (2015). Diversity and evolution of cytochrome P450 monooxygenases in Oomycetes. *Scientific Reports*, 5:11572. DOI: 10.1038/srep11572.

Also the discovery of novel P450 protein was aired in South African TV channels (https://www.youtube.com/watch?v=VbOdUMTsEyc) and Newspapers.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction on oomycetes

1.1.1 History

Back in 1845 in one week, one summer, oomycete species *Phytophthora infestans* nearly wiped out all the potato crops in Ireland leaving people with almost nothing. In those days potato was the principal food for marginalized people. Close to a million people died due to famine and approximately one and half million fled to America and many different countries. Some of people of Irish blood now living in those countries are descendants of people, who escaped the great Irish potato famine. The potato blight affected not only Ireland but covered the entire Europe (University of California Museum of Paleontology (UCMP), n.d.).

The second oomycete in history books is *Plasmopara viticola* which is involved in downy mildew of grapes. It has severed wine industry economy in the Mediterranean since 1865 and almost wiped out the French vine yards when accidentally introduced to France from America by grape seedlings in 1870s (Department of Botany University of Hawai'I at Manoa (DBUHM). n.d & UCMP, n.d.). The vine plantation was saved unexpectedly by a discovery of a simple mixture called "Bordeaux mixture", a concoction of lime and copper sulfate. This was the first chemical used to control fungi (UCMP, n. d.). The above story is to highlight the relationship between oomycetes and humans.

1.1.2 Characteristics

Oomycetes are a heterogeneous group of fungus like stramanopiles. The other name for oomycetes is water moulds (Kamoun lab@ TSL, n.d.). These microorganisms were first

classified under fungi kingdom due to similarity in the nature of forming mycelium and feeding on decaying matter (UCMP; Kamoun lab@ TSL, n.d.). Later, the incompatibility of oomycetes to true fungi was discovered as illustrated in Table 1.1 below. Oomycetes bear more similarities to golden brown algae, diatoms and brown algae that fall under the kingdom Stramenopila. Therefore, oomycetes are now classified under Stramenopila (Kamoun lab@ TSL, n.d.; Tyler *et al*., 2006).

Table 1.1 The distinctive characteristics between the oomycetes and the true fungi (adapted from Rossman & Palm, 2007).

Character	**Oomycota**	**True Fungi**
Sexual reproduction	Heterogametangia. Fertilisation of oospheres by nuclei from antheridia forming oospores	Oospores not produced; sexual reproduction results in zygospores, ascospores or basidiospores
Nuclear state of vegetative mycelium	Diploid	Haploid or dikaryotic
Cell wall composition	Beta glucans, cellulose	Chitin, cellulose rarely present
Type of flagella on zoospores, if produced	Heterokont, of two types, one whiplash directed posteriorly, the other fibrous, ciliated and directed anteriorly	Usually of only one posterior whiplash type if produced
Mitochondria	With tubular cristae	With flattened cristae

1.1.3 Classification

Oomycetes' taxonomic classification is still controversial among authors. Here is the close example: Van West (2006) describes three oomycete sub classes; *Saprolegniomycetidae*, *Rhipidiomycetidae* and *Peronosporomycetidae* while Kamoun (2015) adds the forth; *Lagenidales*. Under the sub class *Peronosporomycetidae* falls two orders; *Peronosporales* and *Pythiales. Pythiales* comprise of genera *Pythium* and *Phytophthora*. These genera consist of most destructive plant pathogenic species. The order *Peronosporales* consists of *Peronospora* and *Bremia* genera under which fall the downy mildews pathogens, obligate plant pathogens. The sub class *Saprolegniomycetidae* consists mostly of fish and animal pathogens. It is divided in to two orders; *Saprolegniales* and *Leptomitales*. Under *Saprolegniales* three genera are observed; *Saprolegnia*, *Achlya* and *Aphanomyces*. *Saprolegnia* and *Aphanomyces* are fish, shell fish or amphibian pathogens (Van West, 2006). However, the evolutionary studies currently being carried out on ribosomal and mitochondrial sequences may provide authentic classification of oomycetes (Kamoun, 2015). Oomycetes species in this study are from genera *Saprolegnia*, *Peronosporales* and *Pythiales*.

1.1.4 Life style and diversity

Majority of oomycetes are land pathogens and only a small percentage is waterborne (Nigrelli & Thines, 2013). Oomycetes pathogens have a very wide host range from plants, insects, crustaceans, fish, and vertebrates to other microorganisms. However, the survival of oomycetes does not only depend on pathogenic life style but also as saprophytes in different habitats (marine, fresh water, and land) (Beakes & Sekimoto, 2009). Saprophytic oomycetes play a vital role in playing natural recyclers by breaking down decaying matter. Most of these saprobes are found in aquatic and moist soils (Kamoun lab@ TSL, n.d.).

Oomycetes are famous for their destructive behaviour on plants and animals. They have a remarkable negative impact on economy and cause environmental destruction in both natural habitats and farming (Van West, 2006). Together with fungi, oomycetes are dominant members of eukaryotic plant pathogens league (Latijnhouwers *et al*., 2003). Identified oomycetes species count was estimated at 2000 species by Nigrelli & Thines (2013) which has probably increased based on UK Assays (n.d.). More than 60 species of genus *Phytophthora*, numerous genera of biotrophic dawny mildews and over 100 species of *Pythium* have been noticed (Beakes *et al*., 2011; Kamoun lab@ TSL, n.d.). A great number of oomycetes is involved in detrimental diseases to crops and ornamental plants while others are involved in animal diseases (Kamoun lab@ TSL, n.d.). They are second to bacteria in compromising the aquaculture economy. Moreover, oomycete diseases are not easy to control. They have a remarkable ability to adapt to chemicals and build genetic resistance (particularly *Phytophothora* species) (Koc & Ustun, 2012; Kamoun, 2015).

Humans are not excluded in oomycete hosts. *Pythium insidiosum* is a deadly pathogen to mammals that occasionally lands on humans. Although pathogenic oomycetes are such a nuisance they can be of some importance. *Lagenidium giganteum* (insect pathogen) is of benefit in controlling mosquitoes, where it is of current use in California (Kamoun, 2015).

1.1.5 Species of interest

Phytophothora are world's biggest plant pathogens with a vast host range. They play the most notorious character in plant diseases posing disastrous effects to crops (Figure 1.1) and marked impact on economy (Cooke *et al*., 2000; Kamoun lab@ TSL, n.d.). *P. infestans*, *P. sojae* and *P. ramorum* are most noted species (Garnica *et al*., 2006).

Pythium genus consists of vast species number found globally. Over a hundred species have been noted. A greater number of species from this genus live in the soil while others live in different aquatic environments. *Pythium* genus consists of saprophytic and pathogenic species that are involved in a wide range of plant diseases like damping off, field rot, fruit soft rot and post harvest rot (Adhikari *et al*., 2013; Levesque *et al*., 2010; William & Grunwald, 2010). *Pythium* species take chance (opportunistic pathogens) on plants at tender stages and troubled older plants. They do not only invade plants, but are also parasites to humans and some animals (Levesque *et al*., 2010). *Pythium* species can survive in various environmental conditions which make genus species perfect candidates for studies concerning plant decomposition and disease infections (Adhikari *et al*., 2013).

Genus *Saprolegnia* consists of species that attack fish and their eggs. They cause a disease known as Saprolegniosis which is noticeable by naked eye as white or grey patches on fish body or fins (Figure 1.1). Additionally, the zoospores produced by *Saprolegnia parasitica* are also infectious (Van West, 2006). On infection with *Saprolegnia*, death is a sure result. That is how deadly they are (William & Grunwald, 2010).

The migration of organism species results in interaction with other alien species which ends up in hybridisation and emergence of new breeds. This is also observed among oomycetes where newer species have been noted. *P. ramorum* and *Hyaloperonospora arabidopsidis* are results of this organisms' migration (William & Grunwald, 2010).

Based on impact on economy, host diversity and behaviour and deadliness, the following oomycetes are most important pathogens worldwide, pronounced as "noble oomycete pathogens" in William & Grunwald (2010); *P. infestans, P. ramorum, Pytium ultimum, Pythium aphanidermatum, Plasmopara viticola, Phytophthora cinnamomi, Sclerophthora rayssiae* var. *Zeae, Peronosclerospora philippinensis, Pythium insidiosum, Aphanomyces euteiches, Aphanomyces astaci* and *Saprolegnia* species. They earn positions in

the list of dreadful pathogens in the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS). Table 1.2 gives elaborate information on 13 oomycete species used in this study.

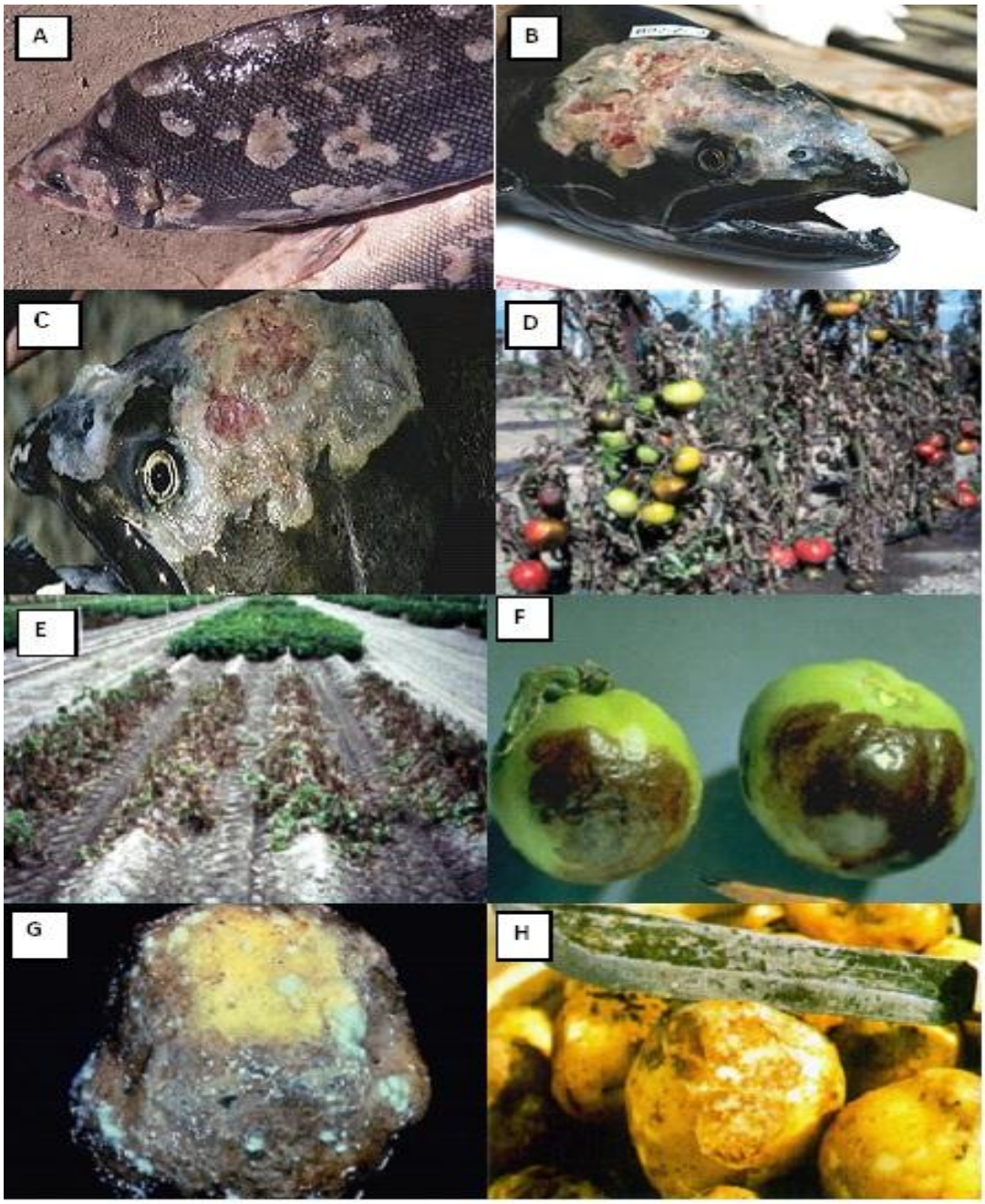


Figure 1.1 Pictures of oomycetes destructions (taken from Schumann & D'Arcy, 2005; Van West, 2006). Pictures A, B and C show Saprolegniosis patches and lethal lesions on the body and head of fish caused by the oomycete species *Sapronelgia declina*. Pictures D and E are tomato and potato plants, F are tomato fruits while G and H are potato tubers. All affected by *Phytophthora infestans* (tomato and potato blight).

Table 1.2 Taxonomic group, life style, host and general information on 13 oomycete species in the study.

Taxonomic Group	Species name	Life style	Host	General information	References
Class: Peronosporomycetidae **Order:** Peronosporales	*Phytophthora sojae* & *Phytophthora ramorum*	Saprophytic and parasitic	Plants	These species are considered as model species for *Phytophthora* genus due to well-developed genetic and genomics resources including genetic maps, BAC libraries, and EST sequences. *P. sojae* is responsible for root and stem rot and dumping off in soya bean plants. It consumes the whole plant, meaning it attacks the plant from roots to leaves, discriminating neither germinating nor older plants. In the United States of America, estimated $1 – 2 million is lost annually owing to *P. sojae*. *P. ramorum* "emerged almost overnight as a very important pathogen". It was identified in California and is responsible for sudden oak death and ramorum blight. It also causes tree stem cankers, and leaf blight or stem dieback on ornamentals and forest under canopy species.	Tyler *et al* 2006; Garnica *et al*., 2006; Tyler, 2007; Grunwald *et al*., 2008; William & Grunwald, 2010

				It has killed different tree and shrub species in Californian forests and advances to cover the whole of North America. *P. ramorum* "list of known hosts continues to expand at the time of writing".	
Class: Peronosporomycetidae **Order:** Peronosporales	*Phytophthora infestans*	Saprophytic and parasitic	Plants	*P. infetans* is well known for the Irish potato famine. It causes a disease called late blight. The disease spreads quickly in cool and moist environment favoured by this organism. It attacks a number of plants including solanaceae plants, tomato and many economically important fruits in the tropics. The world losses estimated $6.7 billion annually in potato production owing to *P. infetans*.	Haas *et al*., 2009; Tyler *et al*., 2006; Lamour *et al*., 2012; University of California Museum of Paleontology, n.d.; Garnica *et al*., 2006
Class: Peronosporomycetidae **Order:** Peronosporales	*Phytophthora parasitica*	Saprophytic and parasitic	Plants	Also a model species for oomycete pathogens with a very long host list and detrimental diseases to various economically important crops, fruits (tobacco, tomato, pepper, eggplant, potato, tobacco, cacao, pineapple,	Broad Institute, n.d.; Tyler *et al*., 2011

				passion fruit, safflower, sesame, common bean, banana, citrus, walnut, almond, pistachio, papaya, peach, plum, apricot, apple, macadamia, pear, avocado, guava, pomegranate) a variety of nursery and ornamental plants, and forest plants.	
Class: Peronosporomycetidae **Order:** Peronosporales	*Phytophthora capsici*	Saprophytic and parasitic	Plants	Attacks the roots, stems, leaves, and plant fruits. It is one of the most economically important *Phytophthora* species worldwide. It is a soil borne plant pathogen that covers a wide range of plant species more importantly pepper. tomato, eggplant, cucumber, watermelon, pumpkin, squash, and cocoa. Others include various fruits and different vegetables. The organism attacks every plant generation causing damping-off, seedling blight and foliar blight. The plant death is preceded by wilting.	Koc & Ustun, 2012; Lamour *et al*., 2012
Class: Peronosporomycetidae	*Hyaloperonospora arabidopsidis*	Obligate parasite	Plant	*Hyaloperonospora arabidopsidis* was earlier known as *Peronospora parasitica.* It is a	Ali *et al*., 2011; William & Grunwald,

Order: Peronosporales	(formerly *Hyaloperonospora parasitica*)			pathogen to *Arabidopsis thaliana*, which is known to be a model host plant. *H. parasitica* causes a downy mildew disease. Because of that, together with others it shares a model plant pathogenic oomycete title.	2010
Class: Peronosporomycetidae **Order:** Pythiales	*Pythium aphanidermatum*	Saprophytic and parasitic	Plants	It is vast host ranged, and favours high temperature conditions. It is common in green houses. Causes damping off, seed, root and stem rots and blights of grasses and fruits, papaya, beets, pepper and cotton. Though known "exclusively" for a plant disease agent, unusual incident occurred in Afghanistan where it showed ability to infect man.	Adhikari *et al*., 2013; Pythium Genome Database, n.d.; William & Grunwald, 2010
Class: Peronosporomycetidae **Order:** Pythiales	*Pythium irregulare*	Saprophytic and parasitic	Plants	*Pythium irregulare* favours cooler environmental conditions hence extremely infectious at these conditions. It is pathogenic on wide range of cereal and leguminous plants.	Adhikari *et al*., 2013
Class:	*Pythium awayamai*	Saprophytic	Plants	It is a monocot grasses pathogen that can	Adhikari *et al*., 2013

Peronosporomycetidae **Order:** Pythiales		and parasitic		survive at remarkably low temperatures. It is responsible for snow rot disease that affects turf grass and barley. It also causes winter wheat disease.	
Class: Peronosporomycetidae **Order:** Pythiales	*Pythium ultimum*	Saprophytic and parasitic	Plants	It is a widely dispersed plant disease agent which is counted among most pathogenic *Pythium* species. It causes dumping off and root rot to a list of crops, ornamental plants and forests.	Lévesque *et al*., 2010
Class: Peronosporomycetidae **Order:** Pythiales	*Pythium vexan*	Saprophytic and parasitic	Plants	Causes canker, damping-off and rot disease to many economically important crops and trees including durian and rubber trees, potato and sugar cane.	Adhikari *et al*., 2013
Class: Saprolegniomycetidae **Order:** Saprolegniales	*Saprolegnia parasitica*	Saprophytic and parasitic	Animals	*Saprolegnia parasitica* is the first species from Saprolegniomycetidae and animal pathogenic oomycete to be sequenced. It falls among the most ruinous oomycete fish pathogens. It is found in fresh waters all over the world and it is a threat to fresh water fish populations. It attacks a wide variety of fish,	Van West, 2006; Broad Institute, n.d.

				amphibians and crustaceans. Millions of Pounds are lost each year in countries in various states of salmon fish farming. Not only cultured fish are affected by *Saprolegnia parasitica,* but also wild salmon populations worldwide are in danger. *Saprolegnia parasitica* is also responsible for catfish ‘‘winter kill’’ that impacts USA at up to 50 percent financial loss.	
Class: Saprolegniomycetidae **Order:** Saprolegniales	*Saprolegnia declina*	Saprophytic and parasitic	Animals	An amphibian, fish and insect pathogen that is accountable for a drop in amphibian populations. It falls among the culprits of amphibian “extinction”. Salmon and trout culturing is under threat in fish hatcheries as this organism is responsible for egg loses.	Broad Institute, n.d.

1.2 Cytochrome P450 monooxygenases

1.2.1 Origin of name "P450"

The trails of cytochrome P450 monooxygenases were spotted as early as 1950s. It was evident that there was indeed a catalyst responsible for oxidation of non-polar xenobiotics in mammals however the enzyme was unknown. The spectrometry of microsomal haem proteins that showed to form carbon monoxide complexes when pre-treated with some reductases provided a green light. In 1958, a breakthrough was made when a membrane bound reduced pigment of absorbance 450nm (nanometre) was observed in experimental animals' microsomal protein fractions that have formed complexes with carbon monoxide. This pigment was later identified as a P450 hemoprotein. That is how these proteins acquired their name; from the peak absorbance when in complex with carbon monoxide. The hypothesis is that the resultant absorbance is a result of the shift between cysteine and carbon monoxide ligands of the haem in the enzyme. However, cytochrome P450 monooxygenases are not the only haem proteins with the peak absorbance of 450nm. Other haem proteins are nitric oxide, chloroperoxidases and protein H450 (Danielson, 2002).

Due to diversity, P450s were to be given names. The standard P450 naming that was established by the P450 Nomenclature Committee is used to give identification to unnamed P450s. The procedure is well explained by Danielson (2002). Since the discovery of P450s their numbers have been escalating. At least over 6500 P450s have been estimated (Danielson, 2002; Lamb *et al*., 2007; Urlacher & Eiben, 2006). On the growing P450 research, the numbers should have dramatically increased.

1.2.2 Catalytic activity

Cytochrome P450 monooxygenases (P450s) is a super family haem-proteins distributed throughout all classified biological kingdoms. P450s are also observed as metalloenzymes in Ellis and Raner (1995) due to their iron constituent in the haem group (metal ion cofactor). They are involved in catalysis of oxidative biotransformation of a wide range of exogenous substrates including drugs, carcinogens and environmental pollutants and endogenous substrates including vitamins and steroids (Graves *et al*., 2013; Hlavica, 2012; Sohl & Guengerich). Table 1.3 shows a list of examples of P450 catalysed reactions. Eukaryotes bear most numbers of P450s as compared to prokaryotes (Pazmino *et al*., 2010).

Table 1.3 Cytochrome P450 monooxygenases reactions (taken from Bernhardt, 2006).

Hydrocarbon hydroxylation	N-Oxide reduction
Alkene epoxidation	Epoxide reduction
Alkyne oxygenase	Reductive beta-scission of alkyl peroxides
Arene epoxidation	NO reduction
Aromatic hydroxylation	Isomerization
N-Dealkylation	Oxidative C-C bond cleavage
S-Dealkylation	Reductive dehalogenation
O-Dealkylation	Dehydratations
N-Hydroxylation	Dehydrogenation
N-oxydation	Alcohol and aldehyde oxidation
S-oxydation	Oxidative dehalogenation
Oxidative deamination	

The following are examples of some of the important roles of P450s in plants and animals. Mammalian P450s are involved in processes including generation of steroids, fatty acids hydroxylation and drug metabolism. However, they can be of some undesirable consequences where they can activate dormant malignant substances (procarcnogens and promutagens). Fungal P450s are crucial for sterol synthesis (Kelly & Kelly, 2013) and aid the organism to acclimatise to host environment during their invasion. In plants they are required for various purposes like synthesis of hormones, colour in their blossoms and defensive mechanisms against pathogens (Danielson, 2002). In arthropods they also play a terminal role in defence against chemicals and aid in development and reproduction (Danielson, 2002).

P450s were thought to be proto-haem IX also known as haem B or Iron protoporphyrin IX containing type of proteins like haemoglobin and myoglobin (Waterman, 1995). Comparison was made and following differences were observed. Haemoglobin produced absorbance 420 nm when reduced with carbon monoxide to 450 nm of the P450s. P450s consist of a thiolate group inherited from cysteine while haemoglobin consists of an imidazole group from histidine at the same position in their haem. Based on function, haemoglobin binds and releases oxygen and shows no reductive properties but P450s on the other hand reduce oxygen. This distinctive property of P450s from haemoglobin is enabled by the thiolate group. The thiolate group attaches iron to cysteine. This configuration is believed to hinder the redox potential of haem iron (Waterman, 1995).

1.2.3 Distribution of P450s and expression in animals

In studied animal species (higher eukaryotes) including humans, most P450s are found in the liver. But there is still other tissue distribution (Graves *et al*., 2013; Capdevila & Falck, 2002). In these higher animals P450s are membrane bound and most reside in the

endoplasmic reticulum while others are found in the mitochondria. Bacterial P450s are found in solution in the cytoplasm (Waterman, 1995). Higher animal P450 expression differs in terms of quantity, and family type in different organs (Graves *et al*., 2013; Capdevila & Falck, 2002). For instance in Human, CYP2J2 is highly expressed in the heart, liver, kidney, and other tissues. In murine, CYP2J5 is mostly detected in the kidney and liver, CYP2J6 is more in small intestine while CYP2J9 is mostly expressed in the brain (Graves *et al*., 2013). P450 expression is age and gender dependant. Different P450 enzymes expression is also determined by drugs, environmental chemicals, hormones, cytokines, diet, starvation, and ailments like diabetes mellitus and hypertension (Capdevila & Falck, 2002).

1.2.4 Where do P450s fall among oxidoreductases family

P450s are oxidoreductase enzymes, also known as redox enzymes. These enzymes are involved in transfer of electrons between molecules (redox reactions). For this to happen, a cofactor (that could be flavins, metal-ions, hemes and pyrroloquinoline quinone (PQQ)) is required to aid the transfer. However, other redox reactions can proceed without a cofactor but most of these reactions need one (Pazmino *et al*., 2010).

Oxidoreductases are divided in to four sub-groups: oxidases, peroxidases, oxygenases/hydroxylases and dehydrogenases/reductases (Figure 1.2) (Pazmino *et al*., 2010). Oxygenases and hydroxylases are involved in insertion of one oxygen or two oxygen atoms into an organic substrate where molecular oxygen is an oxygen donor. The insertion of one oxygen atom to the substrate is done by monooxygenases/hydroxylases, and the insertion of two oxygen atoms, is done by dioxygenases/hydroxylases. These are two types of enzymes observed in sub-group oxygenases and hydroxylases (Figure 1.2). Monooxygenases comprises of seven members (Heme-dependent monooxygenases, Flavin-dependent

monooxygenases, Copper-dependent monooxygenases, Non-heme iron-dependent monooxygenases, Pterin-dependent monooxygenases, other cofactor-dependent monooxygenases and cofactor-independent monooxygenases) (Figure 1.2). Among seven members of monooxygenases, P450s are referred to as "haem-dependant monooxygenases" based on the type of cofactor needed for the enzymes to carry out their catalytic function (Pazmino *et al*., 2010).

1.2.5 Classification of P450s

There are four classes of P450s observed (Figure 1.2); classes I, II, III and IV (Pazmino *et al*., 2010). The classification is based on their electron sources (NAD (P) H, ferredoxin and ferredoxin reductase). Class I P450s are made up of three non-linked residues being the haem group, ferredoxin and ferredoxine reductase. These are common in bacteria (in cytoplasm solution) and in mitochondria (bound to membrane). Class IIs consist of components; the haem group, and a reductase group with a cofactor flavin adenine dinucleotide/flavin mononucleotide (FAD/FMN). Unlike class I that consists of soluble and bound P450 enzymes, class II are all anchored to membranes at their sites. Class III are the same as class II however, in the case of class III the constituents are tethered in a chain. This feature enhances the reactivity of the class IIIs. The reaction rate is far better than that in separate residues. As class I, this class consists of free and bound members of P450s hence found in prokaryotes and eukaryotes. P450 class IV also consists of three components as class I (the haem group, ferredoxin and ferredoxine reductase just to remind). Nonetheless, in class IV these members are linked in a chain, the similar fashion to that of class III (Pazmino *et al*., 2010).

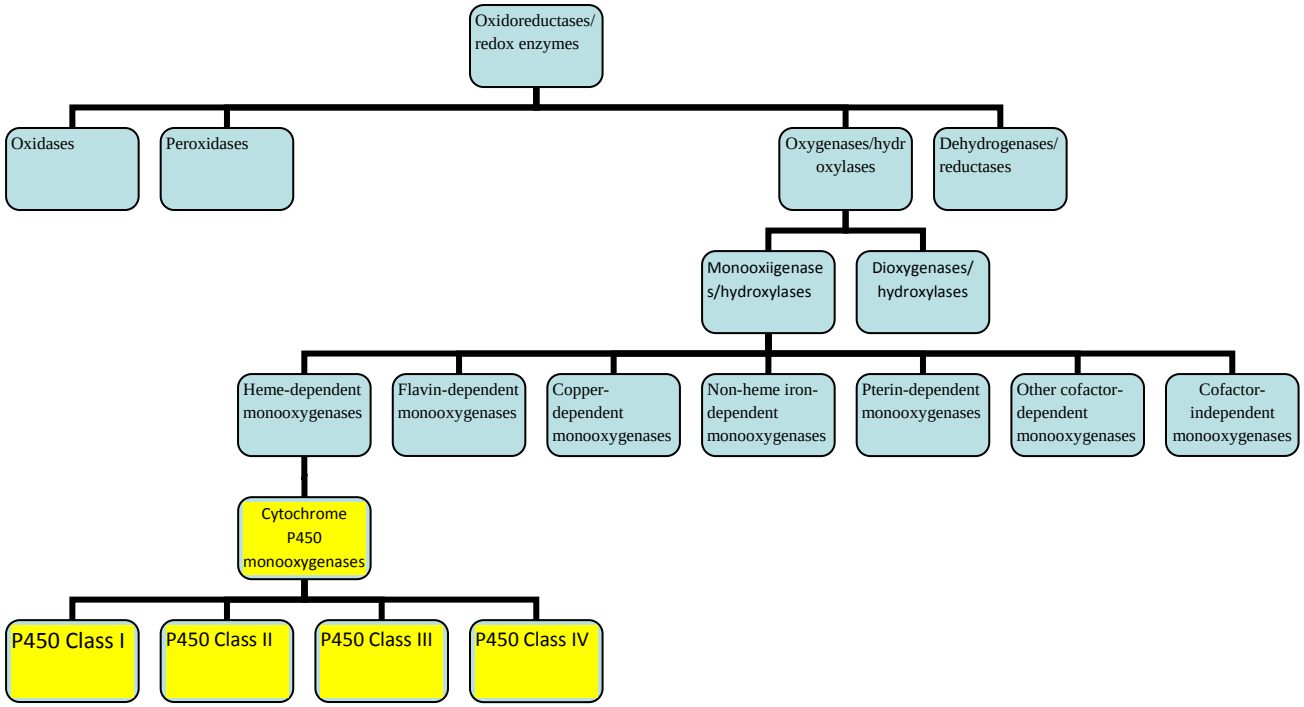


Figure 1.2 A schematic tree diagram that shows the breaking down of oxidoredactases and classification of cytochrome P450 monooxygenases.

1.2.6 Application of cytochrome P450 monooxygenases

Though P450s have been known to exist for quite a long time, it was not long when researchers' interests arose. Researchers were tantalised by P450s' ability to activate dormant carbon atoms and their regio and stereo selective oxygenation of organic substrates. Among opportunities perceived were short cuts to chemical synthesis, pollution reduction and to cut costs. P450s have been successfully applied in processes like synthesis of drugs (antibiotics and anti-cancer), colorants, flavourings, fragrances, chemicals and fighting of pollution (bioremediation) (Caswell *et al*., 2013; Munro *et al*., 2006; Urlacher & Eiben, 2006). The mechanisms for the above processes have been described elsewhere.

Application of P450s as drug targets has also been practised as an alternative to combat microbial infections (Figure 1.3). This has been especially triggered by immergence of new pathogenic microbial species and resistance of known pathogens to commonly used drugs. Therefore, sequenced pathogens genomes are being explored to identify the possibility of using their P450s as drug targets.

For common life threatening pathogens, studies are in progress to explore the possibility of utilizing their P450s as drug targets. One successfully applied P450 is CYP51 in fungi as a target for the azoles (Kelly & Kelly, 2013). The importance of this CYP51 in fungi metabolism provides a good chance to hinder their survival. Recent genome sequencing of *Trypanosoma cruzi* and *Leishmania major*, agents of African sleeping sickness, Chagas disease and leishmaniasis respectively, has also shown the presence of CYP51 in their genome (Lamb *et al*., 2007). This rings a bell that use of azoles on these pathogens could be tried as there is currently no vaccine for prevention. Moreover, currently used drugs are of little impact on these pathogens and they rather result in toxic built up. CYP128A1 has been identified in *Mycobacterium tuberculosis* being vital for growth of this pathogen and its

"transposon disruption" is said to be of some importance as it diminishes the spread of infection (Lamb *et al*., 2007). This disruption was seen to inhibit entrance and infection of murine macrophages (Lamb *et al*., 2007). Therefore a drug that could be targeted to this P450 could be of effective function on combating *M. tuberculosis*.

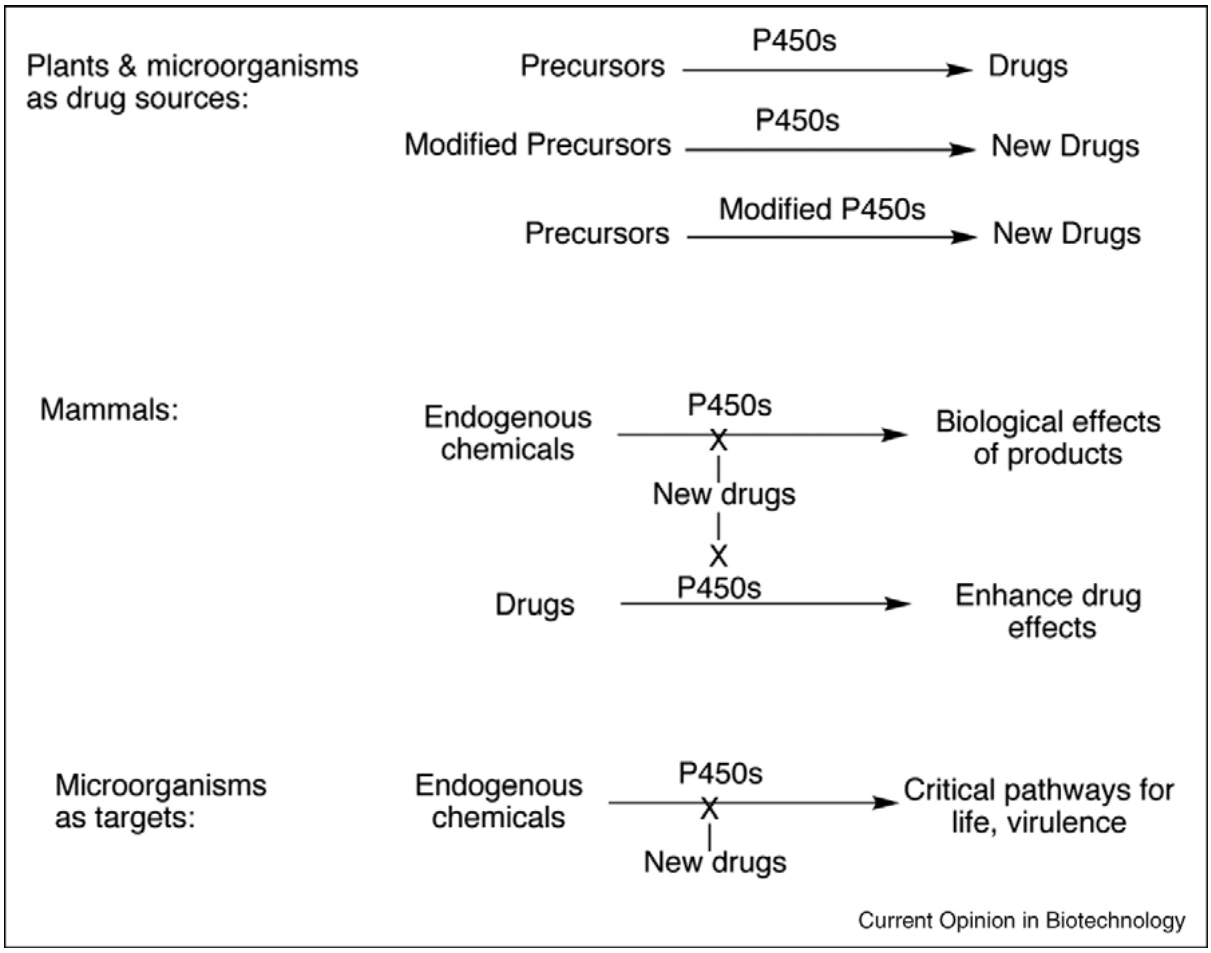


Figure 1.3 Cytochrome P450 monooxigenases in drug targets and new drug discovery processes. This is the summary of roles played by P450s in various applications that have been accomplished that include drug synthesis and drug targets. (Adapted from Lamb *et al*., 2007)

1.2.7 Catalytic mechanism

The catalytic property of P450s is aided by an electron supply from a coenzyme. The typical electron supplier for eukaryotic metabolism is NAD (P) H reductase (reduced Nicotinamide adenine dinucleotide phosphate) while prokaryotes need ferredoxin and ferredoxin reductase (Lamb *et al*., 2007). The general catalytic nature of P450s is shown in figure 1.4.

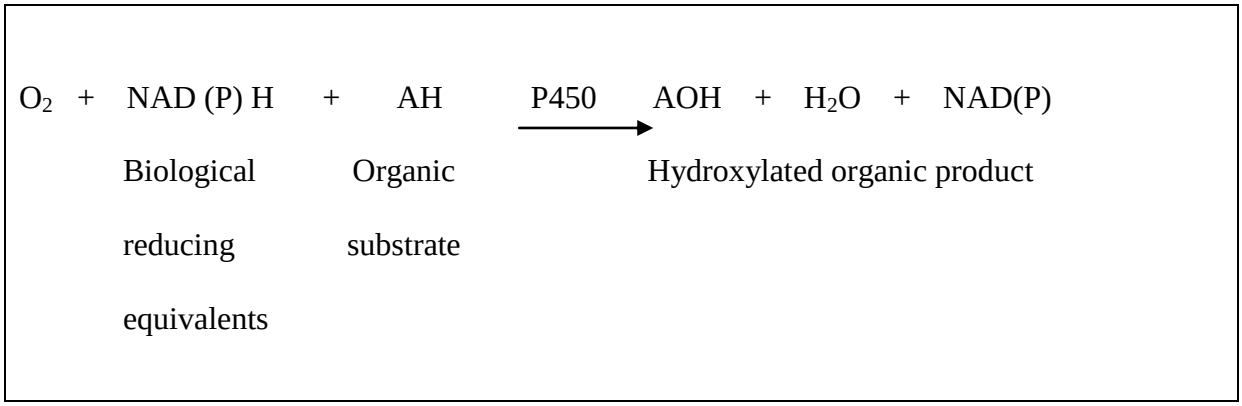


Figure 1.4 General catalytic nature of P450s. The above figure represents a typical electron supply for eukaryotic metabolism reductase. O_2 is oxygen, NAD (P) H is reduced Nicotinamide adenine dinucleotide phosphate and AH is an organic substrate in a reaction catalysed by a P450. The product is the hydroxylated organic product water (H_2O) and NAD (P) oxidized Nicotinamide adenine dinucleotide phosphate (adapted from Waterman, 1995)

Several stages are observed in the catalytic cycle of P450s and intermediate products are identified (Figure 1.5). The following is a brief explanation of the circle: To start with, at non-reactive state of the enzyme, the "catalytic iron" is a ferric iron denoted by Fe^{III}, and without the introduction of a substrate, the enzyme remains dormant. The property is essential for enzyme control to prevent autonomous reaction initiation without a substrate to

work on. This P450 enzyme behaviour is solely on the thiolate group that links cysteine to the haem (Mclntosh *et al*., 2014; Pazmino *et al*., 2010).

On substrate (organic substrate) encounter, the reaction commences. The involved mechanisms enable an electro transfer by the related coenzyme (a reductase) there by reducing ferric iron to ferrous (Fe^{II}). The step that follows is the binding of molecular oxygen to ferrous iron resulting to an oxy-P450 complex intermediate known as "ferric superoxide complex". The second electron transfer by the reductase produces "iron peroxo intermediate".

There is a proton supply to the intermediates produced. The first proton supply is on iron peroxo intermediate (iron peroxo intermediate protonation) which yields what is called iron hydroperoxy intermediate. The second protonation is on the resultant intermediate, iron hydroperoxy intermediate. This protonation results in breaking down of the O-O bond making a potent enzyme compound (compound I). Compound I adds an oxygen atom to the substrate forming compound II and an enzyme product complex. Finally the product is released and the enzyme gets back to original resting state (Mclntosh *et al*., 2014; Pazmino *et al*., 2010).

The main players in P450 catalytic cycle are the associated reductases and the haem group. However, the reaction would not be smooth in the absence of other residues. Threonine facilitates proton addition to iron-peroxo and iron hydroperoxy compounds and enables O-O cleavage. Cysteine, universally conserved P450 residue with some exceptions (Syed & Mashele, 2014), holds haem in place as they are fastened together by a thiolate. It also boosts O-O iron hyroperoxy bond intermediate. Thiolate, as said before, hinders the redox potential of haem group hence regulating the enzyme to wait for substrate (Mclntosh *et al*., 2014).

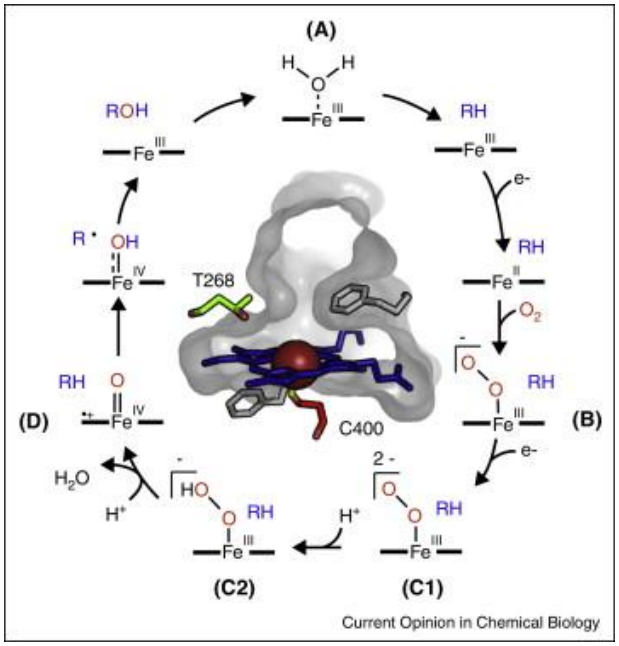


Figure 1.5 The common catalytic cycle stages of cytochrome P450 monooxygenase (Taken from Mclntosh *et al* 2014). At point (A) the enzyme is at resting state. RH is the substrate. B, C1, C2 and D are important intermediates and compounds formed along the circle. An e- and H+ with arrows illustrates addition of electrons and protons respectively. At the centre of the circle, is the three dimensional structure of a P450 enzyme's (CYP102A1) active site. Shown in red and round, is the haem iron lingered to cysteine C400 (red) by a thiolate ligand (yellowish part). Threonine (T268) is in greenish colour.

1.3 Study aim

Knowledge about oomycetes is still scares regardless of evident existence and prominent hazards seen in agriculture. Oomycetes are big threat to world's economy. However, genome sequencing of oomycetes pathogens has provided a better understanding on these pathogens. So far some knowledge has been dug out in various aspects including pathogenesis, evolutionary relation and different genes of pathogenic importance (Broad Institute; Soanes *et al*., 2007). This could also enable further studies on how to control these organisms effective as they have been a hard nut to crack.

The aim of this study was to perform a systemic analysis P450s in 13 oomycete species which 11 and 2 are plants and animal pathogens respectively from oomycete sub classes Peronosporomycetidae and Saprolegniomycetidae. Namely; *P. sojae*, *P. ramorum*, *P. infestans*, *P. parasitica*, *P. capsici*, *P. awayamai*, *P. irregular*, *P. aphanidermatum*, *P. ultimum*, *P. vexan*, *H. parasitica*, *S. declina* and *S. parasitica*.

1.4 References

Adhikari, B.N., Hamilton, J. P., Zerillo, M. M., Tisserat, N., Levesque, C. A., Buell, R. (2013). Comparative Genomics Reveals Insight into Virulence Strategies of Plant Pathogenic Oomycetes. *PLOS ONE*, 8(10): 1-15.

Ali, S. (2011). Identification of effectors with avirulence functions in the pathogenic barley smut fungus, using marker based approaches and comparison among genomes of related species. *The University of British Columbia*. Available from: Circle.ubc.ca/bitstream/handle/2429. [Accessed: 20/04/2015]

Beakes, W. G., Sekimoto, S. (2009). The evolutionary phylogeny of oomycetes - insights gained from studies of holocarpic parasites of algae and invertebrates. Oomycete Genetics and Genomics: Diversity, Interactions, and Research Tools. Eds. Lamour, K., Kamoun, S. John Wiley & Sons, Inc. Available from: http://www.academia.edu. [Accessed: 29/11/2014]

Beakes, W. G., Glockling, S. L., Sekimoto, S. (2011). The evolutionary phylogeny of the oomycete "fungi". *Protoplasma*, 249: 3–19.

Bernhardt, R. (2006). Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology*, 124: 128–145.

Broad Institute. (n. d). *Saprolegnia parasitica* CBS 223.65. Available from: http://www.broadinstitute.org/annotation/genome/Saprolegnia_parasitica/GenomeDescriptions.html#Saprolegnia_declina_VS20 [Accessed: 29/11/2014]

Capdevila, J. H., Falck, J. R. (2002). Biochemical and molecular properties of the cytochrome P450 arachidonic acid monooxygenases. *Prostaglandins & other Lipid Mediators*, 68–69: 325–344.

Caswell, J. M., O'Neill, M., Taylor, S. J. C., Moody, T. S. (2013). Engineering and application of P450 monooxygenases in pharmaceutical and metabolite synthesis. *Chemical Biology*, 17: 271–275.

Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G., Brasier, C. M. (2000). A Molecular Phylogeny of Phytophthora and Related Oomycetes. *Fungal Genetics and Biology* 30: 17–32.

Danielson, P. B. (2002). The Cytochrome P450 Superfamily: Biochemistry, Evolution and Drug Metabolism in Humans. *Current Drug Metabolism*, 3(6): 561 – 596.

Department of Botany University of Hawai'I at Manoa (DBUHM). (n. d). The Origin of Plant Pathology and The Potato Famine, and Other Stories of Plant Diseases. Available from: http://www.botany.hawaii.edu/faculty/wong/BOT135/LECT06.HTM [Accessed: 13/05/2014]

Ellis, W. R. Jr., Raner, G. M. (1995). Metaloenzymes. *Molecular biology and biotechnology* Meyers, R. A. (Ed) VCH Publishers, Inc. 220 East New York, NY 10010 USA. p. 546 – 549.

Garnica, D. P., Pinzon, A. M., Quesada-Ocampo, L. M., Bernal, A. J., Emiliano Barreto, E., Grünwald N. J., Restrepo, S. (2006). Survey and analysis of microsatellites from transcript sequences in *Phytophthora* species: frequency, distribution, and potential as markers for the genus. *BMC Genomics*, 7(245): 1-11.

Graves, J. P, Edin, M. L., Bradbury, J. A., Gruzdev, A., Cheng, J., Lih, F. B., Masinde, T. A., Qu, W., Clayton, N. P., Morrison, J. P., Tomer, K. B., Zeldin, D. C. (2013). Characterization of Four New Mouse Cytochrome P450 Enzymes of the CYP2J Subfamily. *Drug Metab Dispos*, 41: 763–773.

Grunwald, N. J., Goss, E. M., Press, C. M. (2008). *Phytophthora ramorum*: a pathogen with a remarkably wide host range causing sudden oak death on oaks and ramorum blight on woody ornamentals. *Mol Plant Pathol*, 6: 729-40.

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M. V., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, N., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I. B., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P. J., Grenville-Briggs, L. J., Griffith, J., Grünwald, N. J., Horn, K., Horner, N. R., Hu, C., Huitema, E., Jeong, D., Jones, A. M. E., Jones, J. D. G., Jones, R. W., Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, K., Ma, L., MacLean, D., Chibucos, M. C., McDonald, H., McWalters, J., Meijer, H. J. G., Morgan, W., Morris, P. F., Munro, C. A., O'Neill, K., Ospina-Giraldo, M., Pinzón, A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L., Sharpe T.,, Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., van de Vondervoort, P. J. I., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W., Meyers, B. C., van West, P., Ristaino, J., Govers, F., Birch, P. R. J., Whisson, S. C., Judelson S. H., Nusbaum, C. (2009).

Genome sequence and analysis of the Irish potato famine pathogen phytophthora infestans. *Nature*, 461(17): 393-398.

Hlavica, P. (2012). Evaluation of structural features in fungal cytochromes P450 predicted to rule catalytic diversification. *Biochimica et Biophysica Acta*, 1834: 205–220.

Kamoun, S. (2015). Oomycetes. *Salem Press Encyclopedia Of Science*. Available from: http://eds.b.ebscohost.com/eds/detail/detail?sid=d22bebbc-f891-4364-a042-ec91d3ce1201%40sessionmgr115&vid=5&hid=120&bdata=JnNpdGU9ZWRzLWxpdmU%3d#db=ers&AN=89551780. [Accessed 26/05/2015]

Kamoun lab@ TSL. n.d. *The oomycetes: A unique group of eukaryotic pathogens*. Available from: http://kamounlab.dreamhosters.com/oomycetes.htm [Accessed 25/11/2014]

Kelly S. L., Kelly D. E. (2013) Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450come from, what do they do and what can they do for us? *Phil. Trans. R. Soc. B.Biol. Sci*. 368:20120476.

Koc, E., Ustun, A. S. (2012). Influence of *Phytophthora capsici* L. inoculation on disease severity, necrosis length, peroxidase and catalase activity, and phenolic content of resistant and susceptible pepper (*Capsicum annuum* L.) plants. *Turk J Biol*, 36: 357-371.

Lah, L., Krasevec, N., Trontelj, P., Komel, R. (2008). High diversity and complex evolution of fungal cytochrome P450 reductase: Cytochrome P450 systems. *Fungal Genetics and Biology*, 45: 446–458.

Lamb, D. C., Waterman, M. R., Kelly, S. L., Guengerich, F. P. (2007). Cytochromes P450 and drug discovery. *Chemical biotechnology*, 18: 504–512.

Lamour, K. H., Mudge, J., Gobena, D., Hurtado-Gonzales, O. P., Schmutz, J., Kuo, A., Miller, N. A., Rice, B. J., Raffaele, S., Cano, L. M., Bharti, A. L., Donahoo, R. S., Finley, S., Huitema, E., Hulvey, J., Platt, D., Salamov, A., Savidor, A., Sharma,R., Stam, R., Storey, D., Thines, M., Win, J., Haas, B. J., Dinwiddie, D. L.,Jenkins, J., Knight, J. R., Affourtit, J. P., Han, C. S., Chertkov, O., Lindquist, E. A., Detter, C., Grigoriev, I. V., Kamoun, S., Kingsmore, S. F. (2012). Genome Sequencing and Mapping Reveal Loss of Heterozygosity as a Mechanism for Rapid Adaptation in the Vegetable Pathogen *Phytophthora capsici*. *Molecular Plant-Microbe Interactions*, 25(10): 1350–1360.

Latijnhouwers, M., de Wit, P. J. G. M., Govers, F. (2003). Oomycetes and fungi: similar weaponry to attack plants, *TRENDS in Microbiology*, 11: 10.

Lévesque, C. A., Brouwer, H., Cano, L Hamilton, J. P., Hol,t C., Huitema, E., Raffaele, S., Robideau, G. P., Thines, M., Win, J., Zerillo, M. M., Beakes, G. W., Boore, J. L., Busam, D., Dumas, B., Ferriera, S., Fuerstenberg, S. I., Gachon, C. M., Gaulin, E., Govers, F., Grenville-Briggs, L., Horner, N., Hostetler, J., Jiang, R. H., Johnson, J., Krajaejun, T., Lin, H., Meijer, H. J., Moore, B., Morris, P., Phuntmart, V., Puiu, D., Shetty, J., Stajich, J. E., Tripathy, S., Wawra, S., van West, P., Whitty, B. R., Coutinho, P. M., Henrissat, B., Martin, F., Thomas, P. D., Tyler, B. M., De Vries, R. P., Kamoun, S., Yandell, M., Tisserat, N., Buell, C. R. (2010). Genome sequence of the necrotrophic plant pathogen Pythium ultimum reveals original pathogenicity mechanisms and effector repertoire. *Genome Biology*, 11: R73.

Mclntosh, J. A., Farwell, C. C., Arnold, F. H. (2014). Expanding P450 catalytic reaction space through evolution and engineering. *Current Opinion in Chemical Biology*, 19: 126–134.

Munro, A. W., Girvan, H. M., McLean, K. J. (2007). Cytochrome P450–redox partner fusion enzymes. *Biochimica et Biophysica Acta*, 1770: 345–359.

Nigrelli, L., Thines, M. (2013). Tropical oomycetes in the German Bight-Climate warming or overlooked diversity? *fungal ecology*, 6: 152-160.

Park, J., Lee, S., Choi, J., Ahn, K., Park, B., Park, J., Kang, S., Lee, Y. (2008). Fungal cytochrome P450 database. *BMC Genomics*, 9:402 doi: 10.1186/1471-2164-9-402.

Pazmino, D. E. T., Winkler, M., Glieder, A., Fraaije, M. W. (2010). Monooxygenases as biocatalysts: Classification, mechanistic aspects and biotechnological applications. *Journal of Biotechnology*, 146: 9–24.

Pythium Genome Database. (n. d.) *Pyhtium aphanidermatum*. Available from: (http://pythium.plantbiology.msu.edu). [Accessed: 10/06/2014]

Rossman, A.Y. Palm, M. E. (2007). Why Are Phytophthora and Other Oomycota Not True Fungi? *Inoculum*, 58(1): 1-3.

Schumann, G. L., D'Arcy, C. J. (2005).*Late blight of potato and tomato*. The Plant Health Instructor. DOI: 10.1094/PHI-I-2000-0724-01 Available from:

http://www.apsnet.org/edcenter/intropp/lessons/fungi/Oomycetes/Pages/LateBlight.aspx. [Accessed: 09/06/2015]

Soanes, D. M., Richards, T. A., Talbot, N. J. (2007). Insights from Sequencing Fungal and Oomycete Genomes: What CanWe Learn about Plant Disease and the Evolution of Pathogenicity? *The Plant Cell*, 19: 3318–3326.

Sohl, C. D., Guengerich, F. P. (2010). Kinetic Analysis of the Three-step Steroid Aromatase Reaction of Human Cytochrome P450 19A1. *J Biol Chem*, 285(23): 17734–17743.

Syed, K., Mashele, S. S. (2014). Comparative Analysis of P450 Signature Motifs EXXR and CXG in the Large and Diverse Kingdom of Fungi: Identification of Evolutionarily Conserved Amino Acid Patterns Characteristic of P450 Family. *PLoS ONE* 9(4): e95616. doi:10.1371/journal.pone.0095616.

Tyler, B. M., Panabieres, F., Shan, W., Grunwald, N., Machado, M. (2011). *Phytophthora parasitica* INRA-310 Sequencing Project, Broad Institute of Harvard and MIT. Available from: (http://www.broadinstitute.org/) [Accessed: 21/06/2014]

Tyler, B. M., Tripathy, S., Zhang, X. M., Dehal, P., Jiang, R. H. Y., Aerts, A., Arredondo, F. D., Baxter, L., Bensasson, D., Beynon, J. L., Chapman, J., Damasceno, C. M. B., Dorrance, A. E., Dou, D. L., Dickerman, A. W., Dubchak, I. L., Garbelotto, M., Gijzen, M., Gordon, S. G., Govers, F., Grunwald, N. J., Huang, W., Ivors, K. L., Jones, R. W., Kamoun, S., Krampis, K., Lamour, K. H., Lee, M., McDonald, W. H., Medina, M., Meijer, H. J. G., Nordberg, E. K., Maclean, D. J., Ospina-Giraldo, M. D., Morris, P. F., Phuntumart, V., Putnam, N. H.,

Rash, S., Rose, J. K. C., Sakihama, Y., Salamov, A. A., Savidor, A., Scheuring, C. F., Smith, B. M., Sobral, B. W. S., Terry, A., Torto-Alalibo, T. A., Win, J., Xu, Z., Zhang, H., Grigoriev, I. V., Rokhsar, D. S., Boore, J. L. (2006). *Phytophthora* Genome Sequences Uncovered Evolutionary Origins and Mechanisms of pathogenesis. *Science*, 313: 1261-1266.

Tyler, B. M. (2007). *Phytophthora sojae*: root rot pathogen of soybean and model oomycete. *Molecular Plant Pathology*, 8(1): 1.

UK Assays. (n. d.) *Phytophthora*. Available from: http://www.ukessays.co.uk/essays/environmental-studies/phytophthora.php. [Accessed: 21/05/2015]

University of California Museum of Paleontology (UCMP). (n. d.) .*Introduction to the Oomycota*. Available from: http://www.ucmp.berkeley.edu/chromista/oomycota.html. [Accessed: 30/07/2014]

Urlacher, V. B., Eiben, S. (2006). Cytochrome P450 monooxygenases: perspectives for synthetic application *TRENDS in Biotechnology*, 24(7): 324 – 330.

Van West, P. (2006). Saprolegnia parasitica, an oomycete pathogen with a fishy appetite: new challenges for an old problem. *Mycologist*, 20: 99–104.

Waterman, M. R. (1995). Cytochrome P450. *Molecular biology and biotechnology*. Meyers, R. A. (Ed) VCH Publishers, Inc 220 East New York, NY 10010 USA. p. 197 – 199.

William, E. F., Grunwald, J. G. (2010). Introduction to Oomycetes. *The Plant Health Instructor*. DOI:10.1094/PHI-I-2010-1207-01. Available from: http://www.apsnet.org/edcenter/intropp/PathogenGroups/Pages/IntroOomycetes.aspx# [Accessed: 25/04/2015]

CHAPTER 2

GENOME DATA-MINING, ANNOTATION AND PHYLOGENETIC ANALYSIS OF CYTOCHROME P450 MONOOXYGENASES IN OOMYCETES

2.1 Introduction

2.1.1 Genome sequencing

The availability of genome sequencing data nowadays made researchers life easy in finding a way of answering difficult biological questions that arise daily and to uncover the secrets in the genomes of various organisms. The first DNA sequencing technique was invented by Gilbert and Maxam followed by Sanger back in 1970s. From there new creativities sprang up (Wessner *et al*., 2013).

Previously this science of genome sequencing was not as simple to perform as nowadays, the techniques used were time consuming and expensive. However, with new improvements, this process has been performed relatively faster and with high accuracy. Among genome sequencing techniques early researchers developed, commonly used was Sanger sequencing technique. With time, technology advancement transformed this method dramatically. Newer techniques that involve automation have improved genome sequencing and made it easier to perform within reasonable time and with high throughput. Accumulation of genome sequences and development of genome databases has been archived, thanks to new technology (Wessner *et al*., 2013). This escalated dramatically after human genome was sequenced. Many prokaryotic organisms' genomes were sequenced and

comparative genomics of various organisms started (Lee & Kim, 2008). Today, we have repositories of genomic data accessible on the internet worldwide (Wessner*et al*., 2013).

2.1.2 Genome data mining

Harvesting the genome sequences was big a problem due limited data availability. This was the problem of yesterday, as sequencing methods advanced and now genomes of many organisms have been available (Huttenhower & Hofmann, 2010). Many genome sequencing projects are still continuing to bridging up that gap even today (Soanes, 2007). However, it seems the "bottle neck" has shifted to data analysis. Nonetheless, there is a relief as there is escalating invention of analytical tools to augment on that matter (Huttenhower & Hofmann, 2010).

On genome sequencing of various organisms revealed presence of P450s in their genomes (Nelson 2013). The availability of genome sequences has been of tremendous benefit to researchers as analysis of huge genome data sets has enabled establishment of relationships between microorganisms and their physiological characteristics also their environmental conditions. Hypothesis can now be made relating to organisms based on their genome and eventually be proven by experimental procedures (Wessner *et al*., 2013). Most annotated P450s are those in fungi and a co-relation on fungal P450s and their role in adaptation to different ecological niches has been reported (Syed *et al*. 2014). Genome sequencing now provides opportunity to explore oomycetes species genomes that have been so far under studied. Therefore, this study was aimed to perform genome-wide annotation and phylogenetic analysis of P450s in oomycete species.

2.2 Methods

2.2.1 Oomycete species for P450 analysis

Thirteen oomycete species belonging to two different classes and three different orders were used in this study. Oomycete species used in this study, their taxonomic group and general information like host and diseases were listed in table 1.2 of Chapter 1. As listed in the table, 11 species (*P. sojae*, *P. ramorum*, *P. infestans*, *P. parasitica*, *P. capsici*, *H. arabidopsidis* (formerly. *H. parasitica*), *P. aphanidermatum*, *P. irregular*, *P. awayamai*, *P. ultimum* and *P. vexan*) belonging to class Peronosporomycetidae and two species (*Saprolegnia parasitica* and *S. declina*) belonging to Class Saprolegniomycetidae were used for comparative analysis of P450s. It is noteworthy that Peronosporomycetidae contain plant pathogens whereas Saprolegniomycetidae contain animal pathogens.

2.2.2 Genome data-mining and identification of P450s

Genomes of oomycete species in this study have been published and are publicly available. The whole proteomes of oomycete species were downloaded from the databases listed in table 2.1. Identification of P450 proteins in whole proteome was carried out using the procedure described elsewhere (Syed *et al*., 2014 (a, b)). Briefly, the downloaded protein sequences were grouped into different protein families using the National Centre for Biotechnology and Information (NCBI) Conserved Domain Database: NCBI Batch Web CD-search tool (Marchler-Bauer *et al*., 2011). The proteins grouped under the cytochrome P450 monooxygenases superfamily were selected for further study.

Table 2.1 Genome databases where whole oomycete species proteome was retrieved.

Species	**Database**
Phytophthorasojae	http://genome.jgi-psf.org/Physo3/Physo3.home.html
Phytophthora parasitica	http://www.broadinstitute.org/annotation/genome/PhytophthoraparasiticaMultiHome.html
Phytophthora ramorum	http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html
Pythium irregular	http://pythium.plantbiology.msu.edu/download.shtml
Pythium iwayamai	http://pythium.plantbiology.msu.edu/download.shtml
Pythium aphanidermatum	http://pythium.plantbiology.msu.edu/
Pythium ultimum	http://pythium.plantbiology.msu.edu/
Pythium vexan	http://pythium.plantbiology.msu.edu/
Phytophthora infestans	http://www.broadinstitute.org/annotation/genome/Saprolegnia_parasitica/GenomesIndex.html
Phytophthora capsici	http://p450.riceblast.snu.ac.kr/species.php?a=intro&spe_id=2785&ref_id=3424
Saprolegnia parasitica CBS 223.65	http://www.broadinstitute.org/annotation/genome/Saprolegnia_parasitica/GenomesIndex.html
Saprolegnia declina VS20	http://www.broadinstitute.org/annotation/genome/Saprolegnia_parasitica/GenomesIndex.html
Hyaloperonopora parasitica	http://www.broadinstitute.org/annotation/genome/Saprolegnia_parasitica/GenomesIndex.html

2.2.3 Assigning a family and subfamily to orphan P450s

Identified P450s were subjected to BLAST analysis against all named Protists sequences on the Cytochrome P450 Webpage (Nelson, 2009). Based on percentage identity, i.e., family members share more than 40% amino acid identity and members of subfamilies share more than 55% amino acid homology (Syed & Mashele, 2014), families and subfamilies were assigned to oomycete P450s. P450s that showed less than 40% identity were assigned to a new family. In addition, evolutionary analysis of P450s was performed in order to authenticate the annotation. P450s that showed less than 40% identity were assessed for their position on the phylogenetic tree and based on their location/alignment with other P450s they were assigned to different P450 families. Annotated and publicly available *P. sojae* and *P. ramorum* P450s were retrieved from the database and used in this study (Nelson, 2009).

2.2.4 Phylogenetic analysis of oomycete P450s

The phylogenetic tree was constructed for evolutionary analysis of oomycete P450s. Firstly, the protein sequences were aligned by adjusting them to the hidden Markov model of P450s in the Pfam protein families database (http://pfam.xfam.org/family/PF00067) with HMMER package 3.1 (http://hmmer.janelia.org/) (Eddy, 2011; Finn *et al*., 2014). Then, the phylogenetic tree from the alignment of protein sequences was inferred by Fast Tree version 2.1.4 using the maximum-likelihood method (http://www.microbesonline.org/fasttree/) (Price *et al*., 2009). The generated tree data was submitted to iTOL (http://itol. embl.de/upload.cgi) for viewing phylogenetic trees and making figures (Letunic & Bork, 2007).

2.3 Results and Discussion

2.3.1 Oomycetes P450omes

Genome-wide identification and annotation of P450s in 13 oomycetes belonging to two different classes and three different orders (Table 1.2, Chapter 1) revealed the presence of a moderate number of P450s in their genomes. Three hundred and fifty-six P450s were found in 13 oomycetes genomes (Table 2.2). The P450 count in oomycete genomes ranged from 7-41. Among the oomycetes selected for the study, *H. arabidopsidis* showed the lowest number of P450s (7) and *P. iwayamai* showed the highest number of P450s (41) in their genome. Except *H. arabidopsidis*, all oomycete genomes had 19 or more P450s. On average, Peronosporales showed a lower number of P450s (27), (excluding *H. arabidopsis*,) compared to Pythiales that showed 31 P450s. Comparison of oomycete P450omes with other lower eukaryotes such as fungi revealed that, the number of P450s observed in oomycetes is most similar to fungal species belonging to the sub-phylum saccharomycotina and least similar among species belonging to the rest of the fungal kingdom, with few exceptions, as shown in table 2.3.

Table 2.2 Comparative analysis of P450 in 13 oomycete species.

Species name	**No. of P450s**	**No. of P450 families**	**No. of P450 subfamilies**
Phytophthora sojae	30	4	18
Phytophthora parasitica	31	4	18
Pythium irregular	41	3	17
Pythium iwayamai	42	3	19
Phytophthora ramorum	24	4	17
Phytophthora infestans	20	3	14
Pythium aphanidermatum	31	4	18
Pythium ultimum	19	3	12
Pythium vexan	20	4	15
Phytophthora capsici	28	3	17
Saprolegnia parasitica	24	6	16
Saprolegnia declina	39	9	26
Hyaloperonospora arabidopsidis	7	2	7
Total	**356**	**52**	**222**

Table 2.3 Comparative analysis of P450s between Oomycota and different fungal phyla.

Species name	P450 count	No. of P450 families
Saccharomyces cerevisiae	3	3
Candida glabrata	3	3
Klyveromyces lactis	5	5
Kluyveromyces waltii	3	3
Pichia anomala	6	6
Ashbya gossypii	3	3
Dekkera bruxellensis	4	4
Pichia pastoris	4	4
Candida lusitaniae	8	6
Kluyveromyces polysporus	4	3
Candida albicans	10	6
Candida guillermondii	10	6
Candida dublinensis	10	6
Pichia stipites	10	6
Debaryomyces hansenii	9	5
Lodderomyces elongisporus	10	5
Candida parpsilosis	14	6
Yarrowiali polytica	17	6
Candida tropicalis	21	5
Neurospora crassa	41	39
Neurospora crassa	41	39
Neurospora discreta	43	39
Coccidioides immitis	40	31

Uncinocarpus reesii	38	29
Aspergillus fumigatus	74	56
Mycosphaerella fijiensis	89	66
Aspergillus clavatus	92	66
Thielavia terrestris	70	50
Fusariumoxy sporum	140	100
Histoplasma capsulatum	47	32
Fusarium graminearum	109	72
Aspergillus terreus	124	80
Myceliophthora thermophila	79	49
Aspergillus oryzae	142	85
Aspergillus flavus	162	95
Aspergillus niger	154	87
Tremella mesenterica	8	7
Cryptococcus neoformans	8	5
Serpulala crymans	159	47
Phlebiopsis gigantea	127	34
Agaricus bisporus	115	27
Phanerochate chrysosporium	149	33
Postia placenta	190	42
Ganoderma lucidium	197	42
Phlebia brevispora	209	42
Ganoderma sp.	209	41
Bjerkandera adusta	199	39
Ceriporiopsis subvermispora	205	32

Phanerochaete carnosa	266	36
Mucor circinelloides	43	16
Phycomyces blakesleeanus	55	15
Rhizopus oryzae	53	14
Batrachochytrium dendrobatidis	9	7
Phytophthora sojae	30	4
Phytophthora parasitica	31	4
Phytophthora ramorum	24	4
Phytophthora infestans	20	3
Phytophthora capsici	28	3
Hyaloperonospora arabidopsidis	7	2
Pythium irregulare	41	3
Pythium aphanidermatum	31	4
Pythium ultimum	19	3
Pythium iwayamai	42	3
Pythium vexan	21	4
Saprolegnia parasitica	24	6
Saprolegnia declina	38	9

2.3.2 P450 families and subfamilies in oomycetes

Annotation of P450 families and subfamilies in 13 oomycete genomes revealed the presence of 15 P450 families (Figure 2.1) and 84 P450 subfamilies (Table 2.4). Nine new P450 families and 31 new P450 subfamilies were identified in oomycetes. Those nine new P450 families were CYP5613, CYP5614, CYP5615, CYP5616, CYP5617, CYP5618, CYP5619, CYP5620 and CYP5621. New subfamilies were confined to four P450 families: CYP5014

showed 15 new subfamilies, followed by the CYP5015 and CYP5017 families each with seven new subfamilies, and CYP558 with two new subfamilies. A detailed analysis of P450 families and subfamilies and their member P450s was listed in table 2.4.

Figure 2.1 Comparative analysis of P450s in 13 oomycete animal and plant pathogens. Three hundred and fifty-six P450s were grouped under 15 P450 families. The P450 family name, number of member P450s and their percentage in the total number of P450s are shown in the figure.

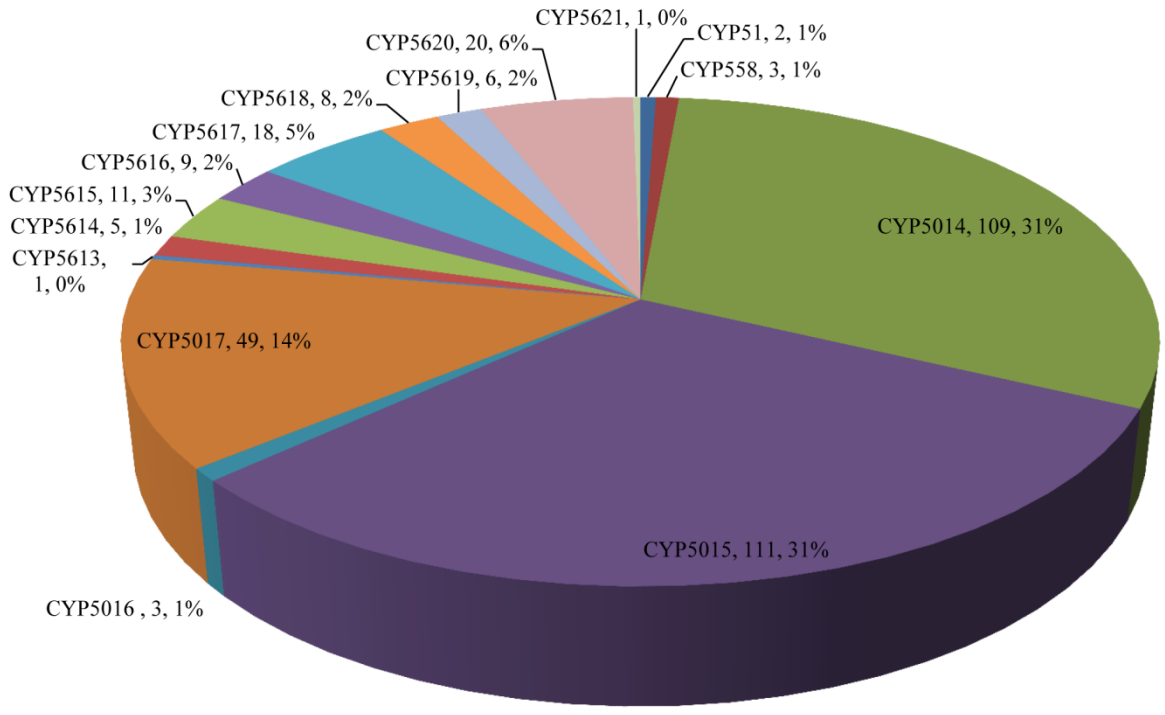


Table 2.4 Comparative P450 analysis at family and subfamily level in the 13 oomycete species.

		Peronosporales						**Pythiales**					**Saprolegniales**		
Family	**Subfamily**	***Psoj***	***Ppar***	***Pram***	***Pinf***	***Pcap***	***Hara***	***Pirr***	***Paph***	***Pult***	***Piwa***	***Pvex***	***Spar***	***Sdec***	**Total**
CYP51	C												1	1	2
CYP558	B													1	3
	C													2	
CYP5014	A	1													110
	B		1	1	1	1									
	C	1	1	1		1									
	D	3	4	3	1	3		1	2	3	2	2			
	E	1	1	1		1									
	F	2	2	2	2	2	1		1	1					
	G	1	1	1	1	1									
	H	1	1	1	1	2	1								
	J	1				1									
	K	1	1	1	1	1	1								

	L		2		2	1									
	M							1			1				
	N										1				
	P							2			1				
	Q							1		1	1				
	R								2						
	S							7		1	2				
	T							2		1	1				
	U								1						
	V											1			
	W											1			
	X											2			
	Y											2			
	Z											1			
	AA1											1			
CYP5015	A	1	1	1	1	2			1	1	1	1			111

	B	1	1	1								1			
	C	1	1	1	2		1								
	D	1	1	1	1	1			1	1	1	1			
	E	3	4	3	4	4	1								
	F	2	2	2	1	2	1			1					
	G	6	4	2	1	2	1					1			
	H							1		3	2				
	J							4	1						
	K							3			4				
	L							4			7				
	M								1						
	N											3			
	P					1						1			
CYP5016	A	1	1	1											3
CYP5017	A	2	2	1	1	2		2	1	1	2	1			49
	B									1	1				

	C							1			1				
	D							6			8				
	E							3			1				
	F							1		4	4				
	G							1			1				
	H							1							
CYP5613	A													1	1
CYP5614	A												2	1	5
	B												1	1	
CYP5615	A												5	6	11
CYP5616	A												1	1	9
	B												1	2	
	C												2	2	
CYP5617	A												2	2	18
	B													1	
	C												1	1	

	D												1	1	
	E												1	1	
	F												1	2	
	G													1	
	H												1	1	
	J													1	
CYP5618	A												1	1	8
	B												2	2	
	C												1	1	
CYP5619	A													1	6
	B													2	
	C													1	
	D													2	
CYP5620	A								2						20
	B								3						
	C								1						

	D								3						
	E								2						
	F								1						
	G								4						
	H								2						
	J								2						
CYP5621	A											1			1
15	**84**	30	31	24	20	28	7	41	31	19	42	20	24	39	**356**

Abbreviations: *Psoj*, *Phytophthora sojae*; *Ppar*, *Phytophthora parasitica*; *Pram*, *Phytophthora ramorum*; *Pinf*, *Phytophthora infestans*; *Pcap*, *Phytophthora capsici*; *Hara*, *Hyaloperonospora arabidopsidis*; *Pirr*, *Pythium irregular*; *Paph*, *Pythium aphanidermatum*; *Pult*, *Pythium ultimum*; *Piwa*, *Pythium iwayamai*; *Pvex*, *Pythium vexan*; *Spar*, *Saprolegnia parasitica*; *Sdec*, *Saprolegnia declina*.

Comparative analysis of P450 members across 13 P450 families revealed that the CYP5014, CYP5015 and CYP5017 P450 families are dominant P450 families in oomycetes with 109, 111 and 49 members respectively, making 76% of total P450s (Figure 2.1). This suggests a high level of P450 blooming (Feyereisen, 2011) of these families. A single member was found in CYP5613 and CYP5621 families (Figure 2.1). Analysis of P450 families, particularly their member P450s and their contribution to the total number of P450s, is shown in figure. 2.1.

2.3.4 P450 family and subfamily dynamics in oomycetes

After annotation of families and subfamilies, further study was carried out to assess the dynamics of P450 families and subfamilies (loss or gain of P450 families/subfamilies) in these organisms. Among oomycetes, Saprolegniales showed the highest number of P450 families compared to Peronosporales and Pythiales (Table 2.2). The number of P450 families in oomycetes ranged from two to nine. Peronosporales showed a minimum of two and a maximum of four families in their genomes. Pythiales showed three to four P450 families in their genomes. Species belonging to Saprolegniales showed six (*S. parasitica*) and nine (*S. declina*) P450 families in their genomes (Table 2.2).

Comparative analysis of P450 families revealed no common P450 family across the oomycetes used in this study (Figure 2.2). However, the CYP5016 family was present only in Peronosporales and the CYP5014, CYP5015 and CYP5017 families were present in both Peronosporales and Pythiales. Saprolegniales had eleven P450 families (CYP51, CYP558 and CYP5613-CYP5621). Nine of them (CYP5613-CYP5621) were new P450 families only found in Saprolegniales. The answer to the presence of the highest number of P450 families and particularly the presence of new P450 families in Saprolegniales compared to

Peronosporales and Pythiales can be obtained from a recently published genome sequencing study (Jiang *et al*., 2013). Genome sequencing analysis of *S. parasitica* revealed that loss of heterozygosity is an efficient mechanism for new variant genes to adapt to a distinct animal pathogenic lifestyle (Jiang *et al*., 2013). The presence of distinct P450 families (new P450 families) in Saprolegniales compared to Peronosporales and Pythiales (Figure 2.2) suggested that P450s in these organisms play a key role in their adaptation to a pathogenic lifestyle (animal host). One interesting observation was that the CYP51 family, involved in membrane sterols biosynthesis (Kelly & Kelly, 2013), was only found in Saprolegniales (Figure 2.1). The loss of CYP51 in other oomycetes implied dependence on the host sterols. The distinct pattern observed in P450 families among oomycete species was also reflected in P450 subfamilies (Figure 2.3 and Table 2.4).

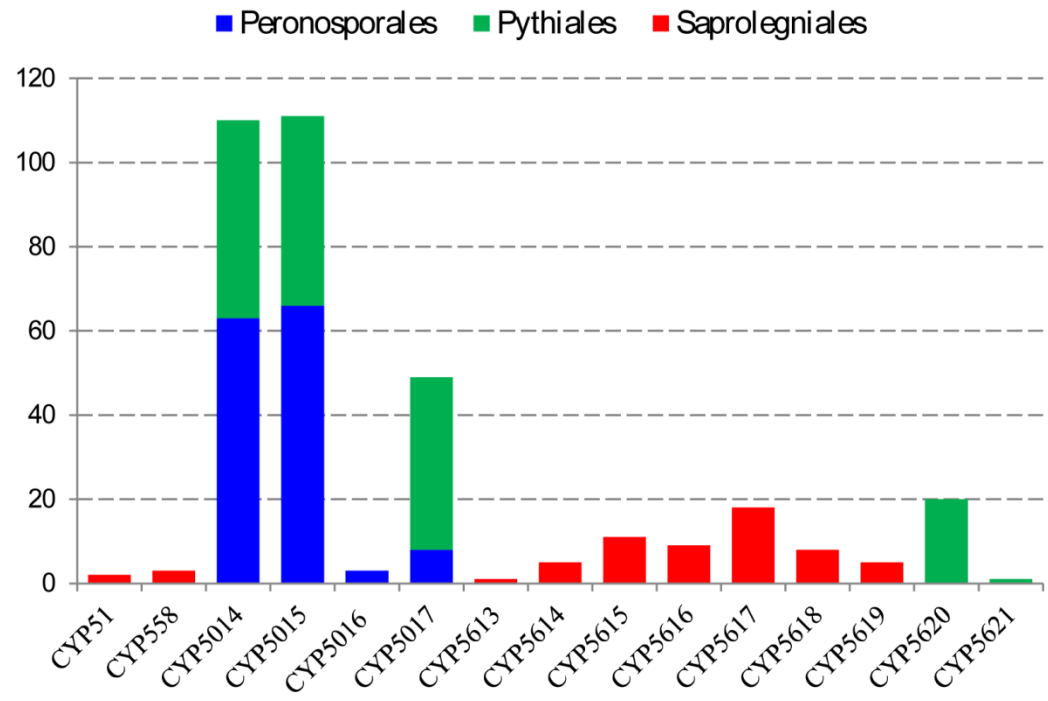


Figure 2.2 P450 family-level comparative analysis between three oomycete orders: Peronosporales, Pythiales and Saprolegniales. The Y-axis represents number of P450s.

Comparative analysis of subfamilies revealed that only nine subfamilies were shared between Peronosporales and Pythiales. Analysis of P450 subfamilies revealed that all the subfamilies shared between Peronosporales and Pythiales were found in CYP5014 (2), CYP5015 (6) and CYP5017 (1) (Figure. 2.3). This suggests that distinct pathogenic lifestyles (host and site of infection) of Peronosporales and Pythiales (Table 1.2, Chapter 1) influence the P450 content in their genomes, as species belonging to these orders show distinct P450 subfamilies (Figure 2.3 and Table 2.4).

The above results revealed that oomycetes that belong to different orders show distinct P450 families and P450 subfamilies in their genomes. This strongly suggests that oomycetes belonging to different orders retain or evolve distinct P450 families in their genomes possibly to adapt to a pathogenic lifestyle in different hosts. In other ways, as recently suggested by researchers (Jiang *et al*., 2013), host cellular environment has driven distinct patterns of gene expansion and loss in the genomes of plant and animal pathogens. From the above results it is clear that the host environment plays a key role in the development of distinct/new variants of P450 families in oomycetes.

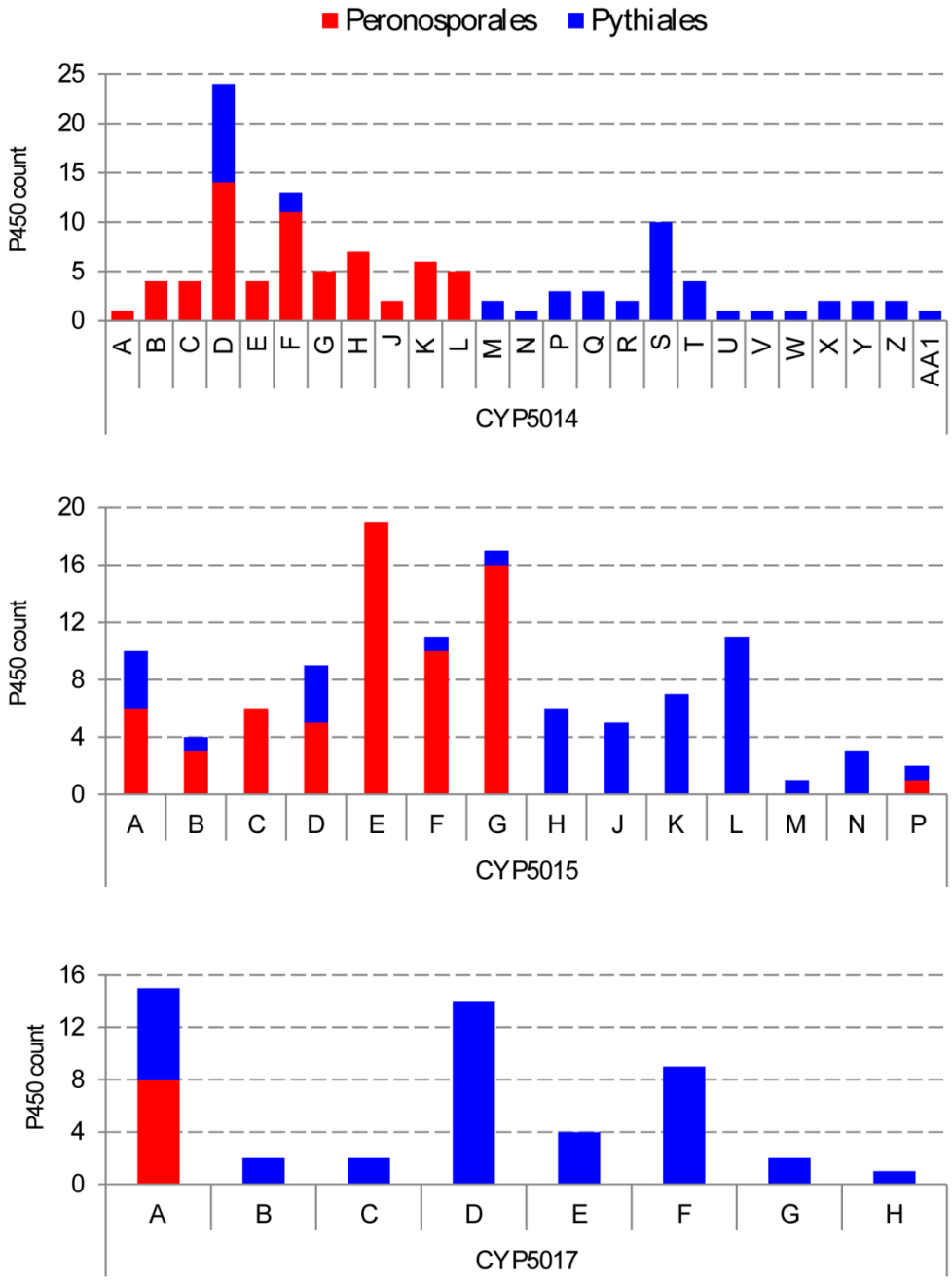


Figure 2.3 Comparative analysis of CYP5014, CYP5015 and CYP5017 families between Peronosporales and Pythiales.

2.3.5 Evolutionary analysis of oomycete P450s

The presence of distinct P450 families, particularly new families and subfamilies, in oomycetes necessitated the performance of evolutionary analysis to allow grouping of P450s into different clades, a higher level P450 classification (Nelson, 1998). Furthermore, evolutionary analysis of oomycetes P450s played a key role in the annotation of oomycete P450s into different families.

Hence in this study a phylogenetic tree of oomycete P450s was constructed for their evolutionary analysis (Figure. 2.4). The results showed that the phylogenetic relationship of oomycete P450s is related with their family and species taxonomy. On the whole, the P450s of the order Saprolegniales showed a very distant phylogenetic relationship to those of the order Pythiales and Peronosporales; they were clearly separated in the tree, while the P450s from the order Pythiales and Peronosporales were phylogenetically close. This is in agreement with the taxonomy relationship between the orders Saprolegniales, Pythiales and Peronosporales, which suggests that the evolution of oomycete P450s is closely related with their species' evolution.

Based on phylogenetic relationships, oomycete P450s were classified into six clades (Figure. 2.4) and the distribution of CYP families and oomycete taxonomy was investigated in these clades (Table 2.5). Only clade 5 had members from all three orders. Clade 6 was a very large branch, suggesting blooming in the order Pythiales and Peronosporales. Especially CYP5014 and CYP5015 members ware not only very frequently presented in the order Pythiales and Peronosporales, but also maintained a high gene number in their genomes (Figures 2.1, 2.3 and Table 2.4). This suggests that CYP5014 and CYP5015 family members may play a pivotal role in the physiological function of order Pythiales and Peronosporales.

Table 2.5 Distribution of P450 families in the six oomycete P450 Clades.

Clade	CYP family	Taxonomy
1	CYP5619	Saprolegniales
2	CYP5615	Saprolegniales
3	CYP51, CYP5613, CYP5614	Saprolegniales
4	CYP558, CYP5616, CYP5617	Saprolegniales
5	CYP5618, CYP5017	Saprolegniales, Pythiales and Peronosporales
6	CYP5621, CYP5016, CYP5620, CYP5014, CYP5015	Pythiales and Peronosporales

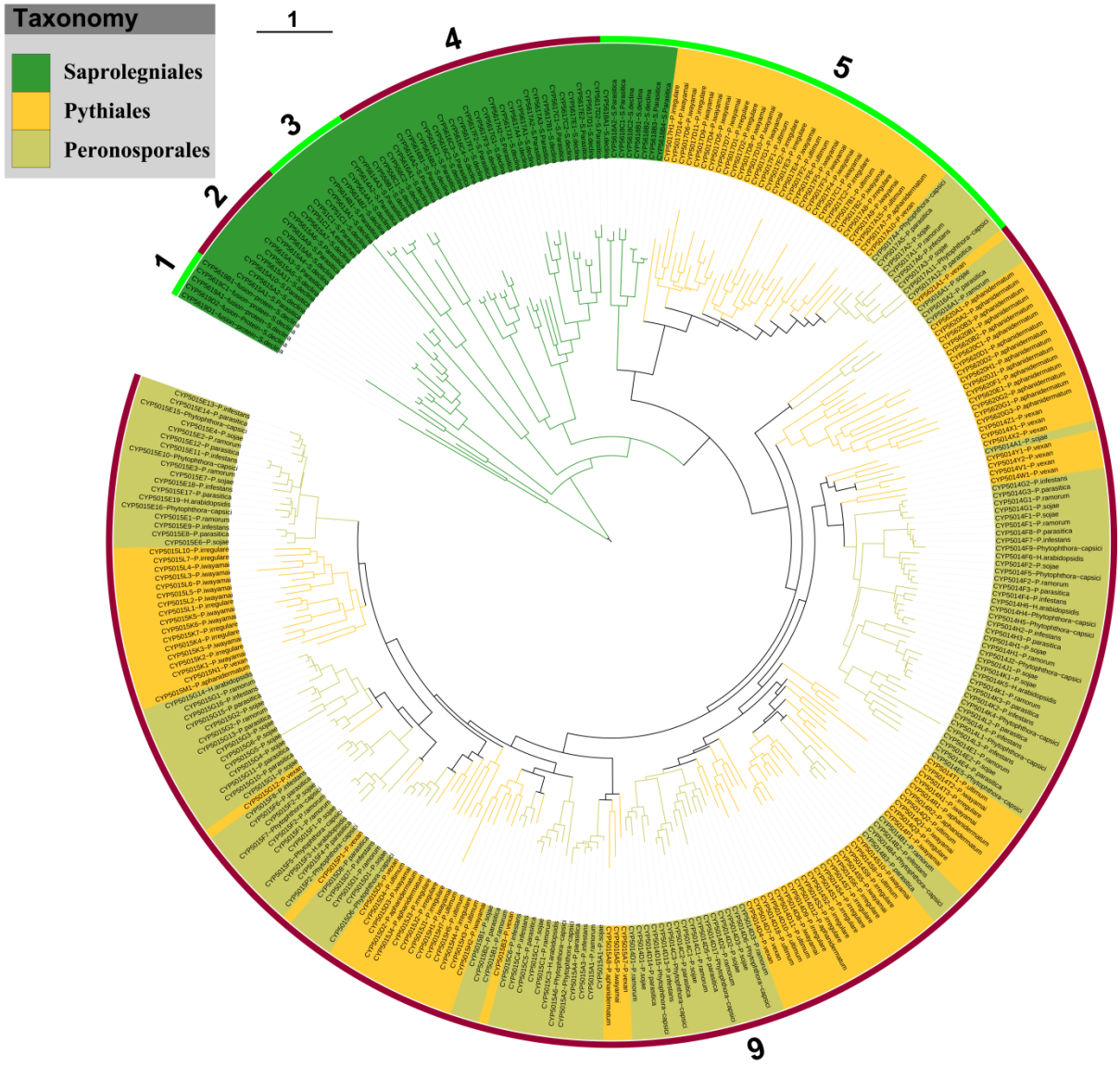


Figure 2.4 Phylogenetic tree of P450s in 13 oomycete species. The inner circle is the phylogenetic tree of annotated oomycete P450s. The branches with different colours show their taxonomic groups, as indicated in the legend. Ancestral branches with children that had identical colours were assigned the same colour as the children. The middle circle is the taxon represented as P450 family, followed by the corresponding oomycetespecies name, which is covered by different colours to show its taxonomic group, as the legend indicates. Eachtaxon links the branch with a dotted line. The outer numbers indicate the six clades derived in this studyand their ranges are marked by alternating reddish brown and green.

2.4 Conclusion

This study revealed presence of low number of P450s in oomycetes. Large number of new P450 families was found in oomycetes. Comparative analysis of P450s revealed that plant and animal pathogenic oomycetes have unique P450 families in their genomes. This study also revealed number of P450s in oomycetes genomes is matched with P450s numbers observed in fungal species belong to Saccharomycotina.

2.5 References

Chen, W., Lee, M., Jefcoate, C., Kim, S., Chen, F., Yu, J. (2014). Fungal Cytochrome P450 Monooxygenases: Their Distribution, Structure, Functions, Family Expansion, and Evolutionary Origin.*Genome Biol. Evol*, 6(7): 1620–1634.

Danielson, P. B. (2002).The Cytochrome P450 Superfamily: Biochemistry, Evolution and DrugMetabolism in Humans. *Current Drug Metabolism*, 3(6): 561 – 596.

Eddy, S. R. (2011). Accelerated profile HMM searches. *PLoS Comp. Biol*,7:e1002195.

Feyereisen, R. (2011). Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochim.Biophys.Acta*, 1814: 19–28.

Finn, R. D., Bateman, A., Jody Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E. L. L., Tate, J., Punta, M. (2014).The Pfam protein families database. *Nucleic Acids Res*, 42: D222–D230.

Huttenhower, C., Hofmann, O.(2010).A Quick Guide to Large Scale Genomic Data Mining.*PLoS Computational Biology*, 6(5): 1 – 6.

Higgins, D. G. (2007). Clustal W and Clutal X version 2.0.*Bioinformatics* 23(21):2947–2948.

Jiang, R. H. Y. de Bruijn, I., Haas, B. J., Belmonte, R., Lo bach, L., Christie,J., Ackerveken, G., Bottin, A., Bulone, V., Dı´az-Moreno, S. M., Dumas, B., Fan, L., Gaulin, E., Govers, F., Grenville-Briggs, L. J., Horner, N. R., Levin, J. Z., Mammella, M., Meijer, H. J. G., Morris, P., Nusbaum, C., Oome, S., Phillips, A. J., van Rooyen, D., Rzeszutek, E., Saraiva, M.,

Secombes, C. J., Seidl, M. F., Snel, B., Stassen, J. H. M., Sykes, S., Tripathy, S., van den Berg, H., Vega-Arreguin, J. C., Wawra, S., Young, S. K., Zeng, Q., Dieguez-Uribeondo, J., Russ, C., Tyler, B. M., van West, P. (2013). Distinctive expansion of potential virulence genes in the genome of the oomycete fish pathogen *Saprolegniaparasitica.PLOS Genet*, 9: e1003272.

Kelly, S. L., Kelly, D. E. (2013). Microbial cytochrome P450: Biodiversity and biotechnology, where do cytochrome P450 come from, what do they do and what can they do for us? *Phil. Trans. R. Soc. B. Biol. Sci*, 368: 161220120430.

Larkin, M. A.,Blackshields,G., Brown, N. P., Chenna, R., McGettigan, P. A.,McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., Letunic, I., Bork, P. (2007). Interactive Tree of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*, 23:127–128.

Marchler-Bauer, A., Lu, S., Anderson, J. B.,ChitsazF.,Derbyshire, M. K.,DeWeese-Scott, C., Fong, J. H., Geer, L. Y.,Geer, R. C., Gonzales, N. R.,Gwadz, M., Hurwitz, D. I., Jackson, J. D.,Ke, Z.,Lanczycki, C. J., Fu Lu, Marchler, G. H.,Mullokandov, M.,Omelchenko, M. V., Robertson, C. L., James S. Song, J. S.,Thanki,N., Yamashita, R. A., Zhang, D.,Zhang, N., Zheng, C., Bryant, S. H.(2011). CDD: A conserved domain database for the functional annotation of proteins. *Nucleic Acids Res*, 39: D225–9.

McLean, K. J., Belcher, J., Driscoll, M. D. (2010).The Mycobacterium tuberculosis cytochromes P450: physiology, biochemistry & molecular intervention. *Future Med Chem*, 2(8): 1339 – 53.

Meadows, R. (2011). Why Biotrophs Can't Live Alone.*PLoS Biol*. 9(7), e1001097.

Nelson, D. R. (1998). Metazoan cytochrome P450 evolution. *Comp.Biochem. Physiol. C Pharmacol.Toxicol.Endocrinol*, 121:15–22.

Nelson, D. R. (2009). Cytochrome P450 monooxygenases homepage. *Human Genomics*, 4(1): 59 – 65.

Nelson, D. (2013). A world of P450s. *Phil Trans R Soc B*, 368:20120430. http://dx.doi.org/10.1098/rstb.2012.0430.

Price, M. N., Dehal, P. S., Arkin, A. P. (2009). FastTree: Computing large minimum evolution trees with profiles instead of a distancematrix. *Mol. Biol. Evol*, 26:1641–1650.

Soanes, D. M. (2007). Insights from Sequencing Fungal and Oomycete Genomes: What Can We Learn about Plant Disease and the Evolution of Pathogenicity? *The Plant Cell*, 19(1) 1:3318-3326.

Syed, K., Shale, K., Nazir, K. H. M. N. H., Krasevec, N., Mashele, S. S., Pagadala, N. S. (2014a). Genome-wide identification, annotation and characterization of novel thermostable cytochrome P450monooxygenases from the thermophilic biomass-degrading fungi *Thielavia terrestris* and *Myceliophthora thermophila*. *Genes Genom*, 36:321–333.

Syed, K., Shale, K., Pagadala, N. S., Tuszynski, J. (2014b). Systematic identification and evolutionary analysis of catalytically versatilecytochrome P450 monooxygenase families enriched in model basidiomycete fungi. *PLoS ONE*, 9(1):e86683.

Syed, K., Mashele, S. S. (2014). Comparative Analysis of P450 Signature Motifs EXXR andCXG in the Large and Diverse Kingdom of Fungi: Identification of Evolutionarily Conserved Amino Acid Patterns Characteristic of P450 Family. *PLOS ONE*, 9(4).

Wessner, D. R., Dupont, C., Charles, T.C. (2013). *Microbiology*: Microbial genomics. John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ, USA. p. 302 – 323.

Zhang, S., Widemann, E., Bernard, G., Lesot, A., Pinot, F., Pedrini, N., Nemat, O., Keyhani, N. O. (2012). CYP52X1, Representing New Cytochrome P450 Subfamily, Displays Fatty Acid Hydroxylase Activity and Contributes to Virulence and Growth on Insect Cuticular Substrates in Entomopathogenic Fungus *Beauveria bassiana*. *J Biol Chem*, 13(287)16: 13477–13486.

CHAPTER 3

INSIGHTS ON OOMYCETES P450S: HOMOLOGY, DIVERSITY, DUPLICATIONS AND SIGNATURE SEQUENCES

3.1 Introduction

Information on oomycetes, their life style and pathogenesis has not been explored (Kamoun, 2003) and recently researchers across the world turned their focus on this deadliest pathogens. The present chapter is focused on oomycetes cytochrome P450 monooxygenase enzymes (P450s) aspects that include homology, duplications, diversity and signature. These aspects are very important in revealing evolution of P450s in an organism.

3.1.1 Homology

Homology is a key aspect in evolutionary biology applied to deduce the relationship between organisms and to "examine the evolutionary processes driving evolution at a molecular level". This is based on similarities in characteristics. Homology analysis can be done in nucleic acids or proteins (National Centre for Science Education, 2008). With many genome sequences now available it is easy to classify and predict protein or gene function by comparative analysis based on sequence pattern and structure (Loewenstein *et al*., 2009).

3.1.2 P450 diversity and duplications

As documented on other organisms that include insects, plants and fungi, more exploration to their genome has been done and variable numbers of P450s have been observed in which many P450 families and subfamilies have been named (Table 3.1) (Nelson, 2011). The study of P450s in arthropods showed increased P450 counts. However they were only classified in to less than a hand full of P450 families (Feyereisen, 2010; Tijet *et al*., 2001). This was suggested to be a result of P450 duplication of these proteins. This increased numbers of P450s in a family was termed "P450 family bloom" (Figure 3.1). However, the P450 bloom identified was not generalised to all P450 families (Feyereisen, 2010).

Table 3.1 Named Cytochrome P450s in March 2010 (taken from Nelson, 2011).

		P450 families [a]	Sequenced genomes [b]	P450s/genomes
Animals	4088			
Insects	2137	67	11	37–178[c]
Vertebrates	1461	18	8	57–102[d]
Invertebrates (not insects)	490	71	4	76–89[e]
Plants	4267	126	14	10–332[f]
Fungi	2784	399	53	1–153[g]
Protists	247	62	22	1–55[h]
Bacteria	1042	333	212	1–52[i]
Archaea	26	13	17	1–4
Viruses (Mimivirus)	2	2	1	2
Total	12, 456			

P450 families [a]: Includes all named P450 families not just those from sequenced genomes. Sequenced genomes [b]: includes only genomes that were in the nomenclature by the time of Nelson, 2011, Progress in tracing the evolutionary paths of cytochrome P450 writing.

Bacterial, archaeal or viral counting exclude genomes without any P450s. Small letters ([c, d, e, f, g, h and i]) identify the number of P450s per genomes for *Aedes aegypti*, Mouse, *Tetranychus urticae* (two-spotted spider mite), Rice, *Aspergillus oryzae*, *Dictyostelium discoideum*, and *Frankia* sp. EAN1pec respectively.

Figure 3.1 A phylogenetic tree of 46 P450 genes of *Apismellifera* (Western honey bee) (taken from Feyereisen, 2011).

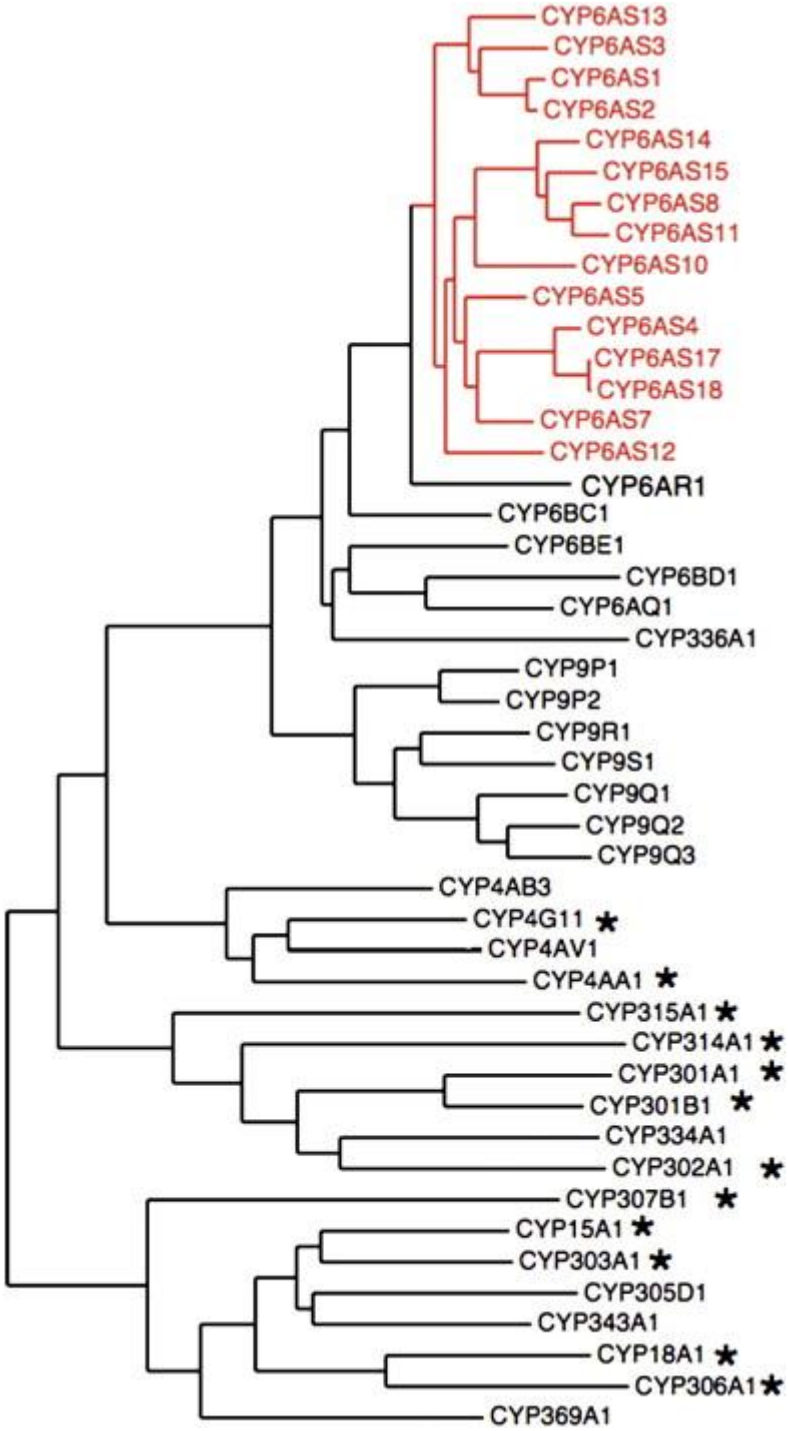


Figure 3.1 shows the P450 family bloom in *Apismellifera* with CYP6AS sub family (highlighted in red) showing increased members (bloom). Another finding was exclusiveness

of certain P450 families to other organism classes while others were observed to be dispersed across several organism classes or species. CYP6 is confined in insects whereas CYP4 spreads to vertebrates (Tijet *et al*., 2001). CYP 56 and CYP 61 are limited to fungi (Cresna & Petric, 2010). Figure 3.1 also shows orthologous P450s in insects (marked with an asterisk) (Feyereisen, 2011). Not only have the distribution of P450s been determined but also the function. CYP51 is widely spread in animals and plants, but not recognised in insects and nematodes, it is important for sterol biosynthesis (Lepesheva & Waterman, 2004; Tijet *et al*., 2001).

3.1.4 P450 signature sequences

P 450s possess two unique amino acids patterns in their protein sequences. These are referred to as P450 signature motifs (FXXGXRXCXG/CXG and EXXR). The CXG is found on the heme-binding group while the EXXR is found in the K-helix. They are the key markers for identification of P450s (Gotoh, 1992; Sirim *et al*., 2010). In these motifs especially CXG, among nine amino acid that form the in the beta bulge region, cysteine is the same in all P450s, whereas two glycines and a single phenylalanine are "generally" the same but not in all cases (Gotoh, 1992; Sirim *et al*., 2010). In the EXXR motif, glutamic acid and arginine are generally the same (Rupasinghe *et al*., 2006; Li *et al*., 2013).

Recent study from Syed and Mashele (2014) revealed that the amino acid patterns in these two signature motifs are characteristic of a P450 family. Authors further suggested that during the divergence of P450 families from a common ancestor these amino acids patterns evolve and are retained in each P450 family as a signature of that family.

3.2 Methods

3.2.1 Analysis of homology

Percentage identity between P450s was determined using ClustalW2 multiple sequence analysis (Larkin *et al* 2007). The ClustalW2 result file designated as percentage identity matrix was downloaded and checked for the percentage identity between P450s.

3.2.2 P450 diversity percentage

The percentage contribution of the number of P450 families in the totalnumber of P450s in an organism was considered as P450 diversity percentage. P450 count and P450 familiesin fungal species were retrieved from published literature (Kgosiemang, 2014; Syed *et al*., 2013; Syed *et al*., 2014b).

3.2.3 Analysis of tandem arrangement of P450s

P450s localized in proximity on the genome wereidentified by scanning manually in the respective genome databases for each oomycete (Chapter 2, Table 2.2). P450s localized on the same scaffold/contig/supercontig were noted. P450s that were tandemly localized and belonged to the same family were expressed as percentage in the total number of P450s in an organism. Tandem arrangement of P450s was omitted for *P. irregular* and *P. Iwayamai* with the shorter length of scaffold/contig/supercontigbeing the reason.

3.2.4 Analysis of EXXR and CXG motifs

Identification of P450 family-specific amino acid patterns at EXXR and CXG motifs was carried out using the procedure described elsewhere (Syed & Mashele, 2014). Briefly, P450 members were subjected to ClustalW multiple alignment using Molecular Evolutionary

Genetics Analysis (MEGA5.2.2) (Schneider & Stephens, 1990). After ClustalW alignment of P450s, amino acids in the EXXR and CXG motifs were selectedand used for generation of WebLogos and calculation of percentage contribution by an amino acid ateach position in the motifs. Only four amino acids were selected for EXXR motif analysis, whereas forCXG motifs upstream seven amino acids were included in the analysis, exactly as previously described (Syed & Mashele, 2014).

3.2.5 Generation of sequence logos

Sequence logos for EXXR and CXG motifs were generated using the published method (Syed & Mashele, 2014). Briefly, WebLogo, a sequence logo generator programme (http://weblogo.threeplusone.com/create.cgi) (Schneider & Stephens, 1990; Crooks *et al*., 2004), was used to create sequence logos at EXXR and CXG motifs. After ClustalW alignment of member P450s, the EXXR and CXG (FXXGXRXCXG) motifs' amino acids were selected and pasted in the WebLogo program. As a selection parameter, image format was selected as PDF and 32symbols per line were selected. The generated EXXR and CXG sequence logos were used for the analysis.

3.3 Results and Discussion

3.3.1 P450 blooming in oomycetes

As defined earlier, in comparative analysis of P450s in arthropods, mainly insects, P450 families with the high number of members in their genomes were revealed and authors termed this nature of the highest number of members for a P450 family "P450 family blooming" (Feyereisen, 2011). A recent study on fungal P450s also revealed the blooming nature of a large number of P450 families in fungi (Syed *et al*., 2014a). Blooming of P450

families might play a key role in an organism's metabolism or its adaptation to diverse ecological niches, for example fungal colonization of wood substrates (Syed *et al*., 2014a).

In order to analyse P450 bloom or the direct opposite P450 diversity in oomycetes, a comprehensive comparison of P450 count and P450 families between Oomycota and different fungal phyla was performed (Chapter 2, Table 2.3). Another reason for using fungal organisms for comparison, apart from what is mentionedin the introduction, is that for a long time oomycetes were regarded as true fungi; it was only recently that these organisms were grouped under the biological kingdom "Stramenopile". Furthermore, analysis of P450 diversity/blooming between these organisms will provide insights in evolution of P450 families, considering the primitive nature of oomycetes.

Comparative analysis of P450 families across Oomycota and other fungal phyla revealed that a number of P450 families present in oomycetes are to some extent matched with species belonging toAscomycota, particularly the subphylum Saccharomycotina (Chapter 2, Table 2.3). In order to identify the P450 diversity/blooming in oomycetes, an average number of P450s and an average number of P450 families across different phyla and the average P450 diversity percentage were measured (Figure 3.2 and Chapter 2, Table 2.3).

Figure 3.2 Comparative P450 diversity analysis between Oomycota and other lower eukaryote phyla

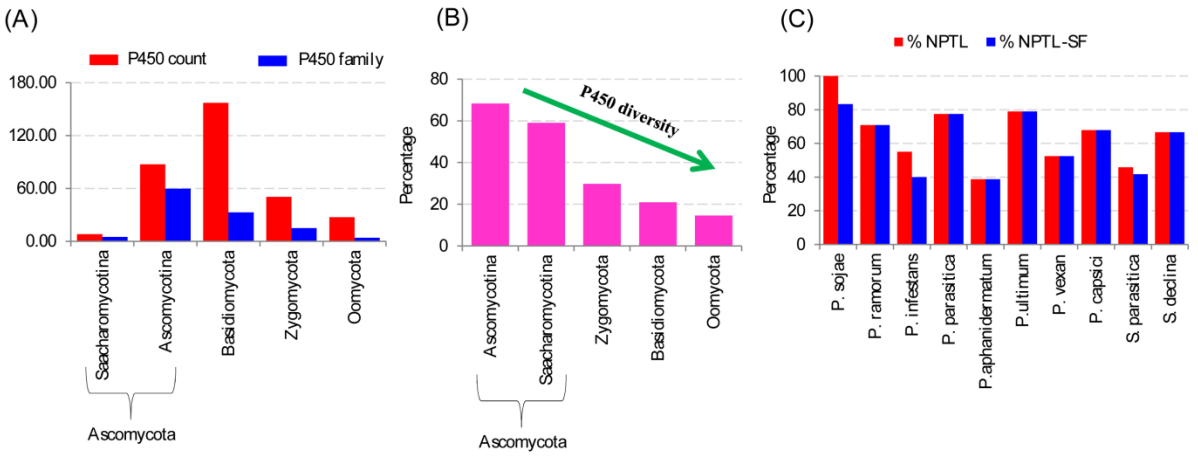


Figure 3.2 shows the comparative P450 diversity analysis between Oomycota and other lower eukaryote phyla (A, B) and P450 bloom analysis in oomycetes (C). A comparative analysis of the average number of P450sand P450 families (A) and P450 diversity percentage (B) between different phyla is shown in the figure. Detailed analysis of P450 count, families and measured P450 diversity percentage has been presented in Chapter 2, Table 2.3. As shown in Panel B of figure 3.2, oomycota showed the lowest diversity compared to different fungal phyla, indicating P450 blooming in these organisms. P450 family blooming in oomycetes was measured (i) percentage of number of P450s tandemly localized on the same scaffold (%NPTL) and (ii) percentage of NPTL belongingto the same family (%NPTL-SF) in the total number of P450s in a species. Detailed analysis of P450s that are tandemly localized on scaffolds in each species and %NPTL and %NPTL-SF is presented in table 3.3 that will follow.

In figure 3.1 (A) shows the average P450 count and average P450 families in oomycota that were found to be lowest (excluding Saccharomycotina species in Ascomycota) compared to different fungal phyla. This indicates the low diversity of P450s in oomycetes.

On the other hand, this implies the highest blooming of P450 families in oomycetes. To identify the blooming nature of oomycota P450ome the average P450 diversity percentage between oomycota and other fungal phyla was measured (Figure 3.1B). As in Figure 3.1B, oomycota showed the lowest P450 diversity percentage (15%) compared to other fungal phyla indicating the highest P450 blooming in oomycetes or the lowest diversity.

A contribution of 76% of P450s by three P450 families CYP5014, CYP5015 and CYP5017 (Chapter 2, Figure 2.1) resulted in the lowest diversity in oomycetes. This suggests the blooming of CYP5014, CYP5015 andCYP5017 families in oomycetes. The blooming nature of P450 families is attributed by tandem duplication of their members (Syed *et al*., 2014a; Feyereisen, 2011). The duplicating nature of member P450s is easily identified either by the highest identity at protein level or analysis of the gene structure (analysis of introns and exons) between members. Since the oomycetes, genes show the lowest number of introns in their structure (Kamoun, 2003), it was notideal to perform gene structure analysis to identify genome-duplicated P450s. Hence, in this study, protein percentage identity criteria were used to identify P450s that were possibly duplicated in oomycetes.

Detailed analysis of P450 count, families and measured P450 diversity percentage has been presented previously (Chapter 2, Table 2.4.)As shown in Panel B (Figure 3.1), Oomycota showed the lowest diversity compared to different fungal phyla, indicating P450 blooming in these organisms. P450 family blooming in oomycetes was measured (i) percentage of number of P450s tandemly localized on the same scaffold (%NPTL) and (ii) percentage of NPTL belonging to the same family (%NPTL-SF) in the total number of P450s in a species. Detailed analysis of P450s that are tandemly localized on scaffolds in each species and %NPTL and %NPTL-SF is presented in table 3.2.

To assess the duplicate nature of P450s, oomycete P450omes were subjected to ClustalW2 analysis(Larkin, 2007).The percentage identity between oomycete P450s was

analysed and the proteins showing more than 70%identity were selected and presented in table 3.1. As shown in table 3.1, a large number of P450s (115) showed more than 70% identity and 82 P450s showed more than 80% identity. This indicates that the majority of the oomycete P450s are highly conserved in their primary structure. Analysis of P450s withrespect to the P450 families revealed that all of the P450s that showed more than 70% identity belong tothree P450 families, i.e. CYP5014, CYP5015 and CYP5017, except three P450s belonging to the CYP5016family and two P450s belong to CYP5619 family (Table 3.1). This suggests that member P450s in theseP450 families possibly increased their number through duplication, which resulted in blooming of theseP450 families.

Table 3.2 Analysis of sequence identity between oomycete P450s**.**

CYP name	Species Name	% identity	CYP name	Species name
CYP5015E8	*P. parasitica*	84	CYP5015E6	*P. sojae*
CYP5015D8	*P. parasitica*	81	CYP5015D1	*P. sojae*
CYP5015C5	*P. parasitica*	78	CYP5015C1	*P. ramorum*
CYP5015F4	*P. parasitica*	89	CYP5015F1	*P. sojae*
CYP5016A2	*P. parasitica*	71	CYP5016A1	*P. ramorum*
CYP5017A5	*P. parasitica*	82	CYP5017A2	*P. sojae*
CYP5015A4	*P. parasitica*	87	CYP5015A1	*P. sojae*
CYP5014B3	*P. parasitica*	73	CYP5014B1	*P. ramorum*
CYP5014K3	*P. parasitica*	91	CYP5014K1	*P. ramorum*
CYP5014D14	*P. parasitica*	83	CYP5014D1	*P. ramorum*
CYP5014G3	*P. parasitica*	85	CYP5014G1	*P. ramorum*
CYP5015G15	*P. parasitica*	93	CYP5015G2	*P. sojae*
CYP5015E12	*P. parasitica*	77	CYP5015E7	*P. sojae*
CYP5015G10	*P. parasitica*	83	CYP5015G1	*P. sojae*
CYP5015B2	*P. parasitica*	82	CYP5015B1	*P. sojae*
CYP5014C2	*P. parasitica*	79	CYP5014C1	*P. ramorum*
CYP5015E17	*P. parasitica*	86	CYP5015E1	*P. ramorum*
CYP5017A12	*P. parasitica*	72	CYP5017A2	*P. sojae*
CYP5014H3	*P. parasitica*	86	CYP5014H1	*P. ramorum*
CYP5014D16	*P. parasitica*	80	CYP5014D3	*P. sojae*
CYP5015G11	*P. parasitica*	82	CYP5015G1	*P. sojae*
CYP5015E14	*P. parasitica*	89	CYP5015E2	*P. ramorum*
CYP5014D5	*P. parasitica*	72	CYP5014D2	*P. sojae*
CYP5015G13	*P. parasitica*	89	CYP5015G2	*P. ramorum*
CYP5014F8	*P. parasitica*	92	CYP5014F1	*P. sojae*
CYP5015F6	*P. parasitica*	83	CYP5015F2	*P. ramorum*
CYP5014F3	*P. parasitica*	86	CYP5014F2	*P. ramorum*
CYP5014G2	*P. infestans*	84	CYP5014G1	*P. ramorum*
CYP5015E9	*P. infestans*	84	CYP5015E6	*P. sojae*
CYP5015A3	*P. infestans*	89	CYP5015A1	*P. ramorum*

CYP5017A6	*P. infestans*	79	CYP5017A2	*P. sojae*
CYP5015F8	*P. infestans*	79	CYP5015F2	*P. ramorum*
CYP5014B2	*P. infestans*	70	CYP5014B1	*P. ramorum*
CYP5014D13	*P. infestans*	80	CYP5014D1	*P. ramorum*
CYP5014H2	*P. infestans*	85	CYP5014H1	*P. ramorum*
CYP5014K2	*P. infestans*	91	CYP5014K1	*P. ramorum*
CYP5015D7	*P. infestans*	81	CYP5015D1	*P. sojae*
CYP5015C4	*P. infestans*	81	CYP5015C1	*P. ramorum*
CYP5014F7	*P. infestans*	90	CYP5014F1	*P. sojae*
CYP5014F4	*P. infestans*	87	CYP5014F2	*P. ramorum*
CYP5015D7	*P. infestans*	81	CYP5015D1	*P. sojae*
CYP5015C6	*P. infestans*	80	CYP5015C1	*P. ramorum*
CYP5015E11	*P. infestans*	87	CYP5015E2	*P. ramorum*
CYP5015E18	*P. infestans*	86	CYP5015E1	*P. ramorum*
CYP5015G16	*P. infestans*	92	CYP5015G1	*P. ramorum*
CYP5014F5	*P. capsici*	82	CYP5014F2	*P. ramorum*
CYP5014F9	*P. capsici*	87	CYP5014F1	*P. ramorum*
CYP5015F7	*P. capsici*	79	CYP5015F2	*P. ramorum*
CYP5015G17	*P. capsici*	87	CYP5015G1	*P. ramorum*
CYP5015A6	*P. capsici*	74	CYP5015A1	*P. ramorum*
CYP5015A2	*P. capsici*	89	CYP5015A1	*P. sojae*
CYP5017A4	*P. capsici*	72	CYP5017A2	*P. sojae*
CYP5017A11	*P. capsici*	78	CYP5017A2	*P. sojae*
CYP5015G18	*P. capsici*	81	CYP5015G1	*P. sojae*
CYP5015E15	*P. capsici*	86	CYP5015E4_	*P. sojae*
CYP5015E16	*P. capsici*	83	CYP5015E1	*P. ramorum*
CYP5014C3	*P. capsici*	84	CYP5014C1	*P. ramorum*
CYP5014H5	*P. capsici*	88	CYP5014H1	*P.sojae*
CYP5014K4	*P. capsici*	86	CYP5014K1	*P. ramorum*
CYP5014G4	*P. capsici*	85	CYP5014G1	*P. ramorum*
CYP5015E10	*P. capsici*	77	CYP5015E7	*P.sojae*
CYP5015E20	*P. capsici*	90	CYP5015E4	*P.sojae*
CYP5014D17	*P. capsici*	73	CYP5014D2	*P. ramorum*
CYP5014D15	*P. capsici*	82	CYP5014D1	*P. ramorum*
CYP5014D6	*P. capsici*	76	CYP5014D3	*P. ramorum*
CYP5014B4	*P. capsici*	70	CYP5014B1	*P. ramorum*
CYP5015F5	*P. capsici*	87	CYP5015F1	*P.sojae*
CYP5015E19	*H. parasitica*	84	CYP5015E1	*P. ramorum*
CYP5015F3	*H. parasitica*	86	CYP5015F1	*P. ramorum*
CYP5014F6	*H. parasitica*	74	CYP5014F2	*P. ramorum*
CYP5015C3	*H. parasitica*	72	CYP5015C2P	*P.sojae*
CYP5014H6	*H. parasitica*	74	CYP5014H1	*P. ramorum*
CYP5014K5	*H. parasitica*	83	CYP5014K1	*P. ramorum*
CYP5015G14	*H. parasitica*	88	CYP5015G1	*P. ramorum*
CYP5015F3	*H. parasitica*	86	CYP5015F1	*P.sojae*
CYP5014C1	*P.sojae*	79	CYP5014C1	*P. ramorum*
CYP5014D1	*P.sojae*	81	CYP5014D1	*P. ramorum*
CYP5014D2	*P.sojae*	72	CYP5014D2	*P. ramorum*
CYP5014D3	*P.sojae*	75	CYP5014D3	*P. ramorum*
CYP5014F1	*P.sojae*	89	CYP5014F1	*P. ramorum*
CYP5014G1	*P.sojae*	84	CYP5014G1	*P. ramorum*
CYP5014H1	*P.sojae*	89	CYP5014H1	*P. ramorum*
CYP5014K1	*P.sojae*	86	CYP5014K1	*P. ramorum*
CYP5015A1	*P.sojae*	90	CYP5015A1	*P. ramorum*
CYP5015B1	*P.sojae*	82	CYP5015B1	*P. ramorum*
CYP5015C1	*P.sojae*	82	CYP5015C1	*P. ramorum*

CYP5015D1	*P.sojae*	82	CYP5015D1	*P. ramorum*
CYP5015E4	*P.sojae*	88	CYP5015E2	*P. ramorum*
CYP5015E7	*P.sojae*	77	CYP5015E1	*P. ramorum*
CYP5015E7	*P.sojae*	76	CYP5015E2	*P. ramorum*
CYP5015F1	*P.sojae*	89	CYP5015F1	*P. ramorum*
CYP5015F2	*P.sojae*	84	CYP5015F2	*P. ramorum*
CYP5015G2	*P.sojae*	89	CYP5015G1	*P. ramorum*
CYP5015G3	*P.sojae*	87	CYP5015G2	*P. ramorum*
CYP5016A1	*P.sojae*	74	CYP5016A1	*P. ramorum*
CYP5017A2	*P.sojae*	82	CYP5017A1	*P. ramorum*
CYP5014C1	*P. ramorum*	79	CYP5014C1	*P.sojae*
CYP5014D1	*P. ramorum*	81	CYP5014D1	*P.sojae*
CYP5014D2	*P. ramorum*	72	CYP5014D2	*P.sojae*
CYP5014D3	*P. ramorum*	75	CYP5014D3	*P.sojae*
CYP5014F1	*P. ramorum*	89	CYP5014F1	*P.sojae*
CYP5014F2	*P. ramorum*	86	CYP5014F2	*P.sojae*
CYP5014G1	*P. ramorum*	84	CYP5014G1	*P.sojae*
CYP5014H1	*P. ramorum*	89	CYP5014H1	*P.sojae*
CYP5014K1	*P. ramorum*	86	CYP5014K1	*P.sojae*
CYP5015A1	*P. ramorum*	90	CYP5015A1	*P.sojae*
CYP5015B1	*P. ramorum*	82	CYP5015B1	*P.sojae*
CYP5015C1	*P. ramorum*	82	CYP5015C1	*P.sojae*
CYP5015D1	*P. ramorum*	82	CYP5015D1	*P.sojae*
CYP5015E1	*P. ramorum*	81	CYP5015E4	*P.sojae*
CYP5015E3	*P. ramorum*	75	CYP5015E7	*P.sojae*
CYP5015F1	*P. ramorum*	89	CYP5015F1	*P.sojae*
CYP5015F2	*P. ramorum*	84	CYP5015F2	*P.sojae*
CYP5017A1	*P. ramorum*	78	CYP5017A3	*P.sojae*
CYP5619D1	*S. declina*	76	CYP5619D2	*S. declina*

3.3.2 Tandem localization of oomycete P450s

Tandem localization of P450s, particularly P450s belonging to the same P450 family, is a good indication of P450 duplications. In order to analyse P450 duplications in oomycetes further analysis on localization of P450s was performed. As shown in figure. 3.1C and table 3.2, a large number of P450s were found to be tandemly arranged in oomycetes. Tandem arrangement of P450sin oomycetes ranged from 39% to 100% in the total number of P450s (Figure 3.1C). The highest number of tandemly arranged P450s were found in *P. sojae*, where all the P450s (100%) were tandemly arranged. *P.aphanidermatum* showed the lowest number of tandemly arranged P450s (30%) in its genome. *H. Arabidopsidis* showed no tandemly arranged P450s possibly due to low copy of P450s in its genome. Analysis of tandemly arranged P450s revealed that all the P450s that were tandemly arranged belonged to

the same P450 family in all analyzed organisms except in *P. infestans*, where only 40% of P450s belonged tothe same family (Figure 3.1C and Table 3.3).

Family level analysis of tandemly localized P450s revealed that all of the tandemly localized P450sbelonged to the P450 families that showed blooming in the respective species (as discussed above). Forexample, CYP5014-CYP5017, CYP5615-CYP5620 and CYP558 family members were found tandemly arranged in oomycetes. It was interesting to note that the P450 family CYP558 in *S. Declina* had its two members found tandemly localized (Table 3.2). Based on evolutionary analysis, sequence identity data and tandem arrangement, it was concluded that many P450 families in oomycetes were bloomed, owing to tandem duplication of their members.

Table 3.3 Tandem localization of P450s in oomycetes. Abbreviations: NPTL, number of P450s tandemly localized on the same scaffold; NPTL-SF, NPTL and belongs to the same P450 family.

P. sojae			
P450 name	**Scaffold**	**NPTL**	**NPTL-SF**
CYP5015C1	1:4692948-4694507	30	25
CYP5015B1	1:4694621-4696276		
CYP5015D1	1:4696577-4698232		
CYP5016A1	14:1122147-1123784		
CYP5014A1	14:445774-448154		
CYP5015G5	2:2294781-2296295		
CYP5015G6	2:2294781-2296295		
CYP5014H1	3:10425244-10426914		
CYP5014K1	3:10436487-10438012		
CYP5014J1	3:748780-750381		
CYP5014F2	3:752921-754234		
CYP5014F1	3:755754-757340		
CYP5015F2	3:8028502-8029848		
CYP5014C1	4:3153518-3155140		
CYP5014D2	4:3158086-3159726		
CYP5014D1	4:3182143-3183675		
CYP5014D3	4:3187712-3189298		
CYP5014E2	4:5695842-5698363		
CYP5015F1	5:4408528-4410078		

CYP5014G1	5:465521-466667		
CYP5015G1	6:1365977-1367784		
CYP5015G2	6:1373214-1374788		
CYP5015G3	6:1375336-1377079		
CYP5015G4	6:1377256-1378824		
CYP5017A2	6:3343502-3345194		
CYP5017A3	6:3357870-3359688		
CYP5015E7	9:2244328-2245944		
CYP5015E4	9:2372009-2373643		
CYP5015A1	9:370937-372642		
CYP5015E6	9:583115-584585		
P. ramorum			
P450 name	**Scaffold**	**NPTL**	**NPTL-SF**
CYP5015G1	11:344098-345675	17	17
CYP5015G2	11:346204-347784		
CYP5014B1	20:181911-183491		
CYP5014D3	20:473485-475095		
CYP5014D1	20:475271-476830		
CYP5014D2	20:482094-483344		
CYP5014C1	20:484076-484498		
CYP5015B1	27:365533-365883		
CYP5015D1	27:386496-388172		
CYP5014F1	36:333016-334587		
CYP5014F2	36:334989-336623		
CYP5014E1	36:393966-395543		
CYP5015E3	41:111359-112975		
CYP5015E2	41:128803-130419		
CYP5015E1	41:135039-135858		
CYP5014H1	6:590429-592072		
CYP5014K1	6:592794-594401		
P. infestans			
P450 name	**Supercontig**	**NPTL**	**NPTL-SF**
CYP5014L4	8: 2262001-2263972	11	8
CYP5015A3	8: 2802697-2806822		
CYP5015E18	8: 2811799-2815690		
CYP5014G2	11: 621945-623639		
CYP5015E9	11: 3191768-3193330		
CYP5014D13	30: 1958109-1959675		
CYP5014B2	30: 966101-967645		
CYP5014F7	44: 665896-667467		
CYP5014F4	44: 674710-676035		
CYP5014K2	395: 7452-9416		
CYP5014H2	395: 11045-12942		
P. parasitica			
P450 name	**Supercontig**	**NPTL**	**NPTL-SF**
CYP5015E8	18: 245717-247498	24	24
CYP5015A4	18: 325700-327449		

CYP5017A12	3: 2403114-2405127		
CYP5017A5	3: 2488209-2490070		
CYP5014D14	3: 467827-469543		
CYP5014D5	3: 474355-476168		
CYP5014D19	3: 481982-482988		
CYP5014B3	3: 48202-50243		
CYP5014D16	3: 483346-484501		
CYP5014C2	3: 487372-488985		
CYP5015D8	32: 37878-39181		
CYP5015C5	32: 65415-67158		
CYP5014H3	35: 325524-327290		
CYP5014K3	35: 328298-330157		
CYP5015G11	37: 539130-540772		
CYP5015E12	37: 55526-57326		
CYP5015G13	37: 559163-560810		
CYP5015G15	37: 561176-562953		
CYP5015G10	37: 562982-564837		
CYP5015E14	37: 57808-59820		
CYP5015E17	37: 65526-67416		
CYP5014F8	8: 536377-537948		
CYP5014F3	8: 538235-540173		
CYP5014L2	8: 548190-549779		
P.aphanidermatum			
P450 name	**Scaffold**	**NPTL**	**NPTL-SF**
CYP5620F1	138:10660 -12225	12	12
CYP5620B2	138:27734 -29498		
CYP5620B3	138:31354 -32908		
CYP5620C1	138:33338 -34882		
CYP5620B1	138:34985 -36583		
CYP5015J4	144:12585 -14159		
CYP5015A8	144:14252-15814		
CYP5620G3	177:3808-5875		
CYP5620G2	177:6038-7554		
CYP5620G1	177:890-1862		
CYP5620A1	864:2604-3659		
CYP5620A2	864:5089-6748		
P.ultimum			
P450 name	**Scaffold**	**NPTL**	**NPTL-SF**
CYP5015H3	1117875582023:818634-820244	15	15
CYP5015H7	1117875582023:821985-822842		
CYP5015D4	1117875582023:823909-825393		
CYP5014-fragment1	1117875582028:106159-107640		
CYP5014T1	1117875582028:108463-109667		
CYP5014D11	1117875582028:115894-117111		
CYP5014D10	1117875582028:126097-127350		
CYP5014D18	1117875582028:208033-208944		
CYP5014Q2	1117875582028:343435-344763		

CYP5017A15	1117875582033:20208-21107		
CYP5017F1	1117875582033:29594-31038		
CYP5017B1	1117875582040:330182-331012		
CYP5017F7	1117875582040:351574-353698		
CYP5017F6	1117875582040:372970-373647		
CYP5017F2	1117875582040:376104-377849		
P. vexan			
P450 name	**Scaffold**	**NPTL**	**NPTL-SF**
CYP5015N1	1115:1758-3381	11	11
CYP5015N-fragment1	1115:179-1757		
CYP5015N-fragment2	1115:6014-6808		
CYP5015D5	211:25683-27287		
CYP5015B3	211:28383-29954		
CYP5017A10	259:8663-10355		
CYP5014W1	357:8439-10055		
CYP5014AA1	372:7692-9299		
CYP5014D4	40:217-2073		
CYP5014D7	40:3018-4307		
CYP5014Y2	583:15736-17339		
CYP5014Y1	583:259-1835		
CYP5014X1	583:7438-9235		
CYP5014X2	583:9985-11071		
P. capsici			
P450 name	**Scaffold**	**NPTL**	**NPTL-SF**
CYP5015A2	10:558729-560373	19	19
CYP5015A6	10:603866-605413		
CYP5015P2	100:54458-56114		
CYP5015G17	100:56638-58218		
CYP5015G18	100:58445-60028		
CYP5014F9	12:307421-309004		
CYP5014F5	12:309235-310566		
CYP5014L1	12:320975-322546		
CYP5014H4	15:665195-666990		
CYP5014K4	15:667766-669567		
CYP5015E10	24:401441-402907		
CYP5015E15	24:411842-413470		
CYP5015E16	24:448260-450227		
CYP5017A4	5:781530-783170		
CYP5017A11	5:794369-795550		
CYP5014C3	55:106837-108386		
CYP5014D17	55:113826-115400		
CYP5014D15	55:130493-132055		
CYP5014D6	55:132132-133691		
S. parasitica			
P450 name	**Contig**	**NPTL**	**NPTL-SF**
CYP5618B3	18: 172697-174184	11	10
CYP5615A6	18: 225246-226760		

CYP5615A1	18: 238646-240184		
CYP5615A10	18: 246228-247715		
CYP5618B4	18: 375093-376574		
CYP5616C3	35: 144918-146460		
CYP5616C1	35: 146781-148300		
CYP5617E2	8: 303172-305005		
CYP5617D2	8: 305455-306983		
CYP5617C1	8: 312501-314611		
CYP5616A2	8: 509567-511081		
S. declina			
P450 name	**Supercontig**	**NPTL**	**NPTL-SF**
CYP5618B1	14: 453794-455275	26	26
CYP5615A11	14: 587491-589118		
CYP5615A2	14: 591523-593236		
CYP5615A7	14: 604570-606323		
CYP5618B2	14: 657390-658877		
CYP5617H1	18: 35461-37977		
CYP5617G1	18: 549369-551291		
CYP558C1	3: 1038264-1039523		
CYP558B1	3: 1235049-1236631		
CYP5616C4	30: 149571-151248		
CYP5616C2	30: 151524-153132		
CYP5617A4	32: 525178-527253		
CYP5617A1	32: 527419-529373		
CYP5616B3	67: 111142-112094		
CYP5616A1	67: 113069-114553		
CYP5619C1	69: 202902-205979		
CYP5619B2	69: 220501-222939		
CYP5619D2	69: 227112-230164		
CYP5619A1	69: 230636-233888		
CYP5619B1	69: 234432-237510		
CYP5617C2	7: 166635-168753		
CYP5617B1	7: 169094-171218		
CYP5617D1	7: 172665-174636		
CYP5617E1	7: 174992-177017		
CYP5617F3	7: 890360-892347		
CYP5617F1	7: 904925-906951		
Species name	**P450 count**	**% NPTL**	**% NPTL-SF**
P. sojae	30	100	83
P. ramorum	24	71	71
P. infestans	20	55	40
P. parasitica	31	77	77
P.aphanidermatum	31	39	39
P.ultimum	19	79	79

P. vexan	21	52	52
P. capsici	28	68	68
S. parasitica	24	46	42
S. declina	39	67	67

3.3.3 Oomycete P450 family characteristic amino acid patterns at EXXR and CXG motifs

A recent study revealed that a certain combination of amino acid patterns at EXXR and CXG motifs are characteristic of a P450 family (Syed & Mashele, 2014). Authors have suggested that these amino acid patterns are evolved during theP450 family divergence from a common ancestor and are retained in family members as a characteristic of the family (Syed & Mashele, 2014). Considering the large number of member P450s, in this study, amino acidcombinations for P450 families such as CYP5014, CYP5015 and CYP5017were analysed (Figure 3.3 and Table 3.3). Analysis of the EXXR motif revealed that the first and fourth amino acids of this motif "E" and "R" is conserved in all P450 families CYP5014, CYP5015 and CYP5017 with rare exceptions. CYP5017F8 showed "K" instead of "E" and CYP5014N1 and CYP5015L showed "W" and "H" instead of "R" (Figure 3.3) below. Non-conservation of "E" and "R" amino acids at the EXXR motif are reported rarely (Sezutsu *et al*., 2013). Leucine is the major amino acid appearing at the third position in this motif in all three oomycetes P450 families. Threonine is the predominant amino acid at the second position in P450 families CYP5014 and CYP5017, whereas serine and aspargine are the predominant amino acids at this position in the CYP5015 family (Figure 3.3). Compared to P450 families across the biological kingdoms (Syed & Mashele, 2014), oomycete P450 families CYP5014 and CYP5017 alsoshowed ETLR as predominant amino pattern. However, the E-S/N-L-R amino acid pattern where "S/N"is the predominant amino acid at the second

position is unique to the CYP5015 family and this patternwas not found in P450 families published in the literature(Syed & Mashele, 2014). Analysis of the CXG motif (FXXGXRXCXG) across the three P450 families revealed conservation of amino acids such as “F”, “G” and “C” at the first, fourth and eighth positions. These amino acids at these positions are well known to be conserved inthe P450s across the biological kingdoms (Syed & Mashele, 2014; Gotoh, 1992; Sirim *et al*., 2010), with some P450s showing variant amino acids at these positions (Sezutsu, 3013). The canonical amino acids “R” and “G” at the sixth and tenth positions are conserved in theCYP5014 family and are predominant in CYP5015 and CYP5017 (Figure 3.3). The amino acid pattern at the CXG motif of the CYP5017 family is to some extent matched with the CYP94 and CYP704 families (Syed & Mashele, 2014) where “Q” is dominant at the second position in all these P450 families. However, differences were found at the seventh and ninth position amino acids among the three P450 families CYP5017, CYP94and CYP704. Comparison of CXG motif amino acid patterns for CYP5014 and CYP5015 with publishedP450 families CXG motif amino acid patterns (Syed & Mashele, 2014) suggested that these families have unique amino acid patterns. This strongly supports the hypothesis previously proposed (Syed &Mashele, 2014) that the amino acid pattern at these motifs is unique for a P450 family. Overall, amino acid patterns at the EXXR and CXG motifs of the three oomycete P450 families CYP5014, CYP5015 and CYP5017 are unique and these amino acid patterns (Figure 3.2) can be considered characteristics of these P450 families.

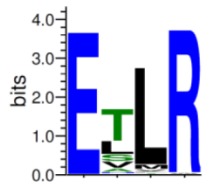
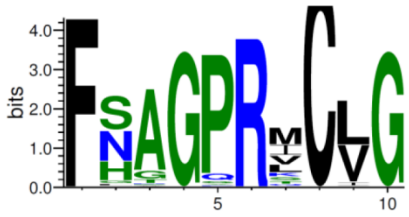
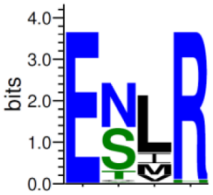
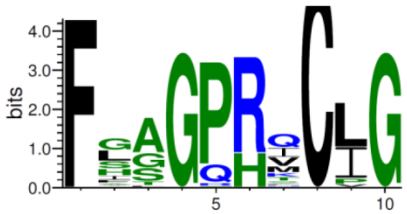
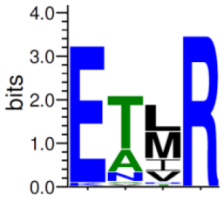
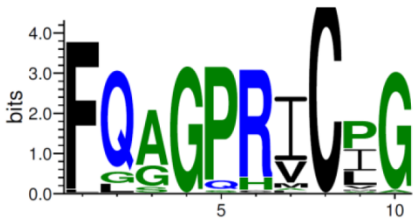
CYP5014	
	
E-T(49.4)/L(20.2)-L(91.0)-R(99.0)	**F**-S(38.2)/N(33.7)/H(21.3)-A(83.1)-**G**-P(89.9)-**R**-M(29.2)/I(17.9)/V(15.7)-**C**-L(51.6)/V(42.7)-**G**
CYP5015	
	
E-N(44.8)/S(42.5)-L(65.5)-R(97.7)	**F**-G(25.3)/L(21.8)/S(16.1)-A(50.6)/G(21.8)-**G**-P(81.6)-R(73.6)-Q(26.4)/V(17.2)/I(17.2)/M(11.5)-**C**-L(52.9)/I(36.8)-**G**
CYP5017	
	
E(97.4)-T(59.0)/A(25.6)-L(33.3)/M(33.3)-**R**	**F**-Q(82.1)-A(66.7)-**G**-P(89.7)-R(87.1)-I(66.7)/V(23.0)-**C**-P(38.5)/I(31.0)/l(20.5)-G(97.4)

Figure 3.3 Analysis of amino acid combinations at EXXR and CXG motifs in CYP5014, CYP5015 and CYP5017 families.The P450s used for deducing amino acid combinations are shown in table 3.3. Sequence logos were constructed as described in Methods. The amino acids and their percentage occurrence at each of this domain are also presented. The invariant residues at these motifs were shown in bold with red font.

Table 3.4 List of P450s used to deduce amino acid combinations at EXXR and CXG motifs in P450 families, CYP5014, CYP5015 and CYP5017. In the list, the P450s from the same family and subfamily belong to *P. sojae* and *P. ramorum*. The number in the parenthesis indicates number of P450s used to deduce the amino acid combinations for a family.

CYP5014 (89)	**CYP5015 (84)**	**CYP5017 (38)**
CYP5014	CYP5015A1	CYP5015E4
CYP5014AA1	CYP5015A1	CYP5015E6
CYP5014B1	CYP5015A2	CYP5015E7
CYP5014B2	CYP5015A3	CYP5017A1
CYP5014B3	CYP5015A4	CYP5017A10
CYP5014B4	CYP5015A5	CYP5017A11
CYP5014C1	CYP5015A6	CYP5017A12
CYP5014C1	CYP5015A7	CYP5017A13
CYP5014C2	CYP5015A8	CYP5017A14
CYP5014C3	CYP5015B1	CYP5017A15
CYP5014D1	CYP5015B2	CYP5017A3
CYP5014D1	CYP5015B3	CYP5017A4
CYP5014D10	CYP5015C1	CYP5017A5
CYP5014D11	CYP5015C3	CYP5017A6
CYP5014D12	CYP5015C4	CYP5017A8
CYP5014D13	CYP5015C5	CYP5017B1
CYP5014D14	CYP5015D1	CYP5017B2
CYP5014D15	CYP5015D1	CYP5017C1
CYP5014D16	CYP5015D2	CYP5017C2
CYP5014D17	CYP5015D3	CYP5017D10
CYP5014D18	CYP5015D4	CYP5017D11
CYP5014D2	CYP5015D5	CYP5017D14
CYP5014D2	CYP5015D7	CYP5017D2
CYP5014D3	CYP5015E1	CYP5017D3
CYP5014D3	CYP5015E10	CYP5017D4
CYP5014D4	CYP5015E11	CYP5017D5
CYP5014D5	CYP5015E12	CYP5017D6
CYP5014D6	CYP5015E13	CYP5017D9
CYP5014D7	CYP5015E14	CYP5017E4
CYP5014D8	CYP5015E15	CYP5017F1
CYP5014D9	CYP5015E17	CYP5017F2
CYP5014E1	CYP5015E18	CYP5017F4
CYP5014E4	CYP5015E19	CYP5017F5
CYP5014E5	CYP5015E2	CYP5017F6
CYP5014F1	CYP5015E20	CYP5017F7
CYP5014F1	CYP5015E3	CYP5017F8

CYP5014F2	CYP5015E8	CYP5017F9
CYP5014F2	CYP5015E9	CYP5017G1
CYP5014F3	CYP5015F1	
CYP5014F4	CYP5015F1	
CYP5014F5	CYP5015F2	
CYP5014F6	CYP5015F2	
CYP5014F7	CYP5015F3	
CYP5014F8	CYP5015F4	
CYP5014F9	CYP5015F5	
CYP5014G1	CYP5015F6	
CYP5014G2	CYP5015F7	
CYP5014G3	CYP5015F8	
CYP5014H1	CYP5015G1	
CYP5014H1	CYP5015G1	
CYP5014H2	CYP5015G10	
CYP5014H3	CYP5015G11	
CYP5014H4	CYP5015G12	
CYP5014H6	CYP5015G13	
CYP5014J1	CYP5015G14	
CYP5014J2	CYP5015G15	
CYP5014K1	CYP5015G16	
CYP5014K1	CYP5015G2	
CYP5014K2	CYP5015H1	
CYP5014K3	CYP5015H3	
CYP5014K4	CYP5015H4	
CYP5014K5	CYP5015H5	
CYP5014L1	CYP5015J1	
CYP5014L2	CYP5015J2	
CYP5014L3	CYP5015J3	
CYP5014L5	CYP5015J4	
CYP5014M1	CYP5015K1	
CYP5014M2	CYP5015K2	
CYP5014N1	CYP5015K4	
CYP5014P	CYP5015K5	
CYP5014P1	CYP5015K6	
CYP5014Q1	CYP5015K7	
CYP5014R	CYP5015L	
CYP5014R2	CYP5015L1	
CYP5014S1	CYP5015L2	
CYP5014S10	CYP5015L4	
CYP5014S2	CYP5015L5	
CYP5014S3	CYP5015L6	
CYP5014S4	CYP5015L7	
CYP5014S5	CYP5015M1	
CYP5014S6	CYP5015N	
CYP5014S7	CYP5015N1	
CYP5014S9	CYP5015P1	

CYP5014T2	CYP5015P2	
CYP5014T3		
CYP5014U1		
CYP5014V1		
CYP5014W1		
CYP5014Z1		

3.4 Conclusion

Though they have shown lower statistics in comparison with fungi in this study, oomycetes are not left behind in the P450 evolutionary race, thereby keeping up with the ecosystem. The increased percentage identity suggesting duplication and hence family bloom is observed in their P450s.The P450 signature motifs are highly conserved across all P450 families in all organisms however, the amino acids patterns at these regions are totally different among P450 families, and hence each family has its own amino acids display.

3.5 References

Cresnar, B., Petric, S. (2011). Cytochrome enzymes in the fungal kingdom. *Biochimica et BiophysicaActa*, 1814: 29–35.

Crooks, G. E., Hon, G., Chandonia, J. M., Brenner, S. E. (2004). WebLogo: A sequence logo generator. *Genome Research*,14:1188–1190.

Feyereisen, R. (2011). Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochim.Biophys.Acta*, 1814: 19–28.

Gotoh, O. (1992). Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J. Biol. Chem*, 267: 83–90.

Kamoun, S. (2003). Molecular genetics of pathogenic oomycetes.*Eukaryot. Cell*, 2: 191–199.

Kgosiemang, I. K. R., Mashele, S. S., Syed, K. (2014). Comparative genomics and evolutionary analysis of cytochrome P450monooxygenases in fungal subphylum saccharomycotina.*J. Pure Appl. Microbiol*, 8:291–302.

Larkin, M. A., Blackshields, G., Brown, N. P., henna, R., McGettigan1, H. McWilliam, P.A., Valentin, F., Wallace, I.M.,Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. (2007). Clustal W and Clutal X version 2.0. *Bioinformatics*, 23: 2947–2948.

Li, Z. Z., Li, X. F., Yang, W., Dong, X., Yu, J. (2013). Identification and functional analysis of cytochrome P450 complement in Streptomyces virginiae IBL14. *BMC Genomics,* 14: 130. doi: 10.1186/1471-2164-14-130.

Loewenstein, Y., Raimondo, D., Redfern, O. C., Watson, J., Frishman, D., Linial, M., Orengo, C., Thornton, J., Tramontano, A. (2009). Protein function annotation by homology-based inference. *Genome Biology,* 10:207 doi:10.1186/gb-2009-10-2-207.

National Centre for Science Education (NCSE), (2008). Molecular homology. Available from: http://ncse.com/creationism/analysis/molecular-homology. [Accessed: 02/10/2015]

Nelson, D. (2011). Progress in tracing the evolutionary paths of cytochrome P450. *Biochimica et Biophysica Acta* 1814: 14–18.

Rupasinghe, S., Schuler, M. A., Kagawa, N., Yuan, H., Lei, L., Zhao, B., Kelly, S. L., Waterman, M. R., Lamb, D. C. (2006). The cytochrome P450 gene family CYP157 does not contain EXXR in the K-helix reducing the absolute conserved P450 residues to a single cysteine. *FEBS Lett*, 580: 6338–6342. doi: 10.1016/j.febslet.2006.10.043.

Schneider, T. D., Stephens, R. M. (1990). Sequence logos: A new way to display consensus sequences. *Nucleic Acids Res*, 18:6097–6100.

Sezutsu, H., Le Goff, G., Feyereisen, R. (2013). Origins of P450 diversity. *Phil. Trans. R. Soc. B. Biol. Sci.* 368, 20120428.

Sirim, D., Widmann, M., Wagner, F., Pleiss, J. (2010). Prediction and analysis of the modular structure of cytochrome P450 monooxygenases. *BMC Struct, Biol.* 10: 34.

Syed, K., Nelson, D. R., Riley, R., Yadav, J. S. (2013). Genome-wide annotation and comparative genomics of cytochrome P450monooxygenases (P450s) in the Polyporale species *Bjerkanderaadusta*, *Ganoderma*sp.And*Phlebiabrevispora*.*Mycologia*, 105:1445–145.

Syed, K., Shale, K., Pagadala, N. S., Tuszynski, J. (2014a). Systematic identification and evolutionary analysis of catalytically versatile cytochrome P450 monooxygenase families enriched in model basidiomycete fungi. *PLoS ONE,* 9(1): e86683.

Syed, K., Shale, K., Nazir, K. H. M. N. H., Krasevec, N., Mashele, S. S.,Pagadala, N. S .(2014b). Genome-wide identification, annotation and characterization of novel thermostablecytochrome P450monooxygenases from the thermophilic biomass-degrading fungi *Thielavia terrestris* and *Myceliophthora thermophila*. *GeneGenom*, 36:321–333.

Syed, K., Mashele, S. S. (2014). Comparative analysis of P450 signature motifs EXXR and CXG in the large and diverse kingdom of fungi: Identification of evolutionarily conserved amino acid patterns characteristic of P450 family. *PLoS ONE*, 9(4):e95616.

Tamura, K., .Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, andmaximum parsimony methods. *Mol. Biol. Evol*, 28:2731–2739.

CHAPTER 4

GENOME DATA MINING, ANNOTATION AND PHYLOGENETIC ANALYSIS OF P450 FUSED PROTEINS

4.1 Introduction

The general knowledge about P450s is that they are very versatile redox enzymes, consisting of a heme group tethered to a thiolate group hence why called heme-thiolate proteins and they can activate dormant molecular oxygen. However, P450s are also involved in catalytic reactions that are not of redox nature. The "mixed function" of P450s has been elucidated in Guengerich & Munro (2013). Before the discovery of unusual P450 properties, they were divided into two classes. Class I found in prokaryotes' cytosol in three component form (P450–Fdx–FDR electron chain) and class II which is in eukaryotes' endoplasmic reticulum (ER) with P450–CPR electron chain. The classification took a turn after discovery of a reductase partner system consisting of two-iron, two-sulfur cluster/protein, bovine adrenodoxin and a flavin adenine dinucleotide containing adrenodoxin reductase (2Fe–2S-Adx-FAD-ADR) (Lamb & Waterman, 2013).

In both prokaryotes and eukaryotes, some P450s have been identified to be attached to reductases by a covalent bond. This is believed to be of advantage over the non-linked P450s as it enhances efficacy (The European Bioinformatics Institute. n.d.; McLean *et al*., 2007). Usually the fusion proteins comprise of only functional groups "domains" of the two joined proteins not the other remnants of the involved proteins (Fisher *et al*., 2003). This P450

fusion system has sketched a direction on how to utilise P450s for synthesis of various products. Nowadays P450s have been manipulated by fusion with other enzymes to produce catalysts of desired function (McLean *et al*., 2007).

The first P450 protein that was identified to be fused with a reductase was CYP102 also known as P450BM3 from a bacterium *Bacillus megaterium* (Kitazume *et al*., 2000). CYP102 is attached to the cytochrome P450 reductase (CPR) that contains flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) at its C-terminal end (Lamb & Waterman, 2013). The catalytic rate of CYP102 has never been matched by any P450 enzyme known since the discovery of P450 fused proteins. This has made it the enzyme of interest as it has been engineered to produce many of its variants for different desired out comes. Other reductase fused P450s have been identified also and manipulation of these proteins, particularly CYP116B1 and CYP116B2 resulted in identification of the binding pattern or arrangement of constituents that form the P450 fused protein (Guengerich & Munro, 2013). Eventually, the "diversity of P450 redox system" was established (Figure 4.1) (Lamb & Waterman, 2013; Guengerich & Munro, 2013).

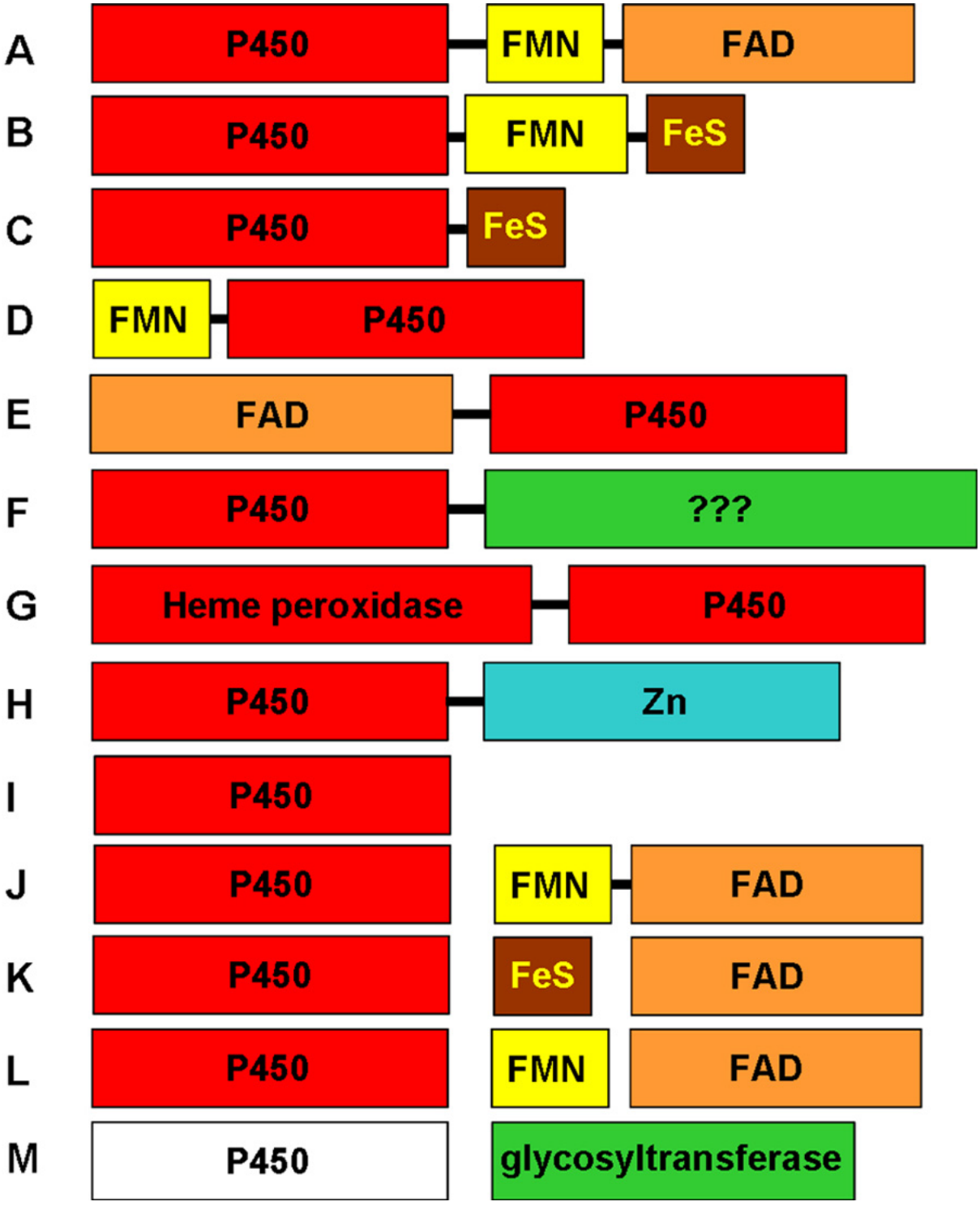


Figure 4.1 Diversity of P450 redox systems and P450 fusion proteins (adapted from (Guengerich & Munro, 2013). The redox partner systems of selected P450 enzymes listed in alphabetical order from A to M. The block sizes represent the protein lengths. Different

constituents are shown in varying colours. In "I" the P450 is called a "stand alone" P450 as it acts independently of a partner. A, P450-CPR fusion (CYP102A1), the arrangement resembles that of fungal CYP505. B is P450-phthalate dioxygenase reductase fusion (CYP116B). C is P450-FDx fusion CYP51FX in *M. capsulatus*. D, flavodoxin-P450 fusion XplA, seen in *R. rhodochrous*. E, *Pseudomonas fluorescens* PfO-1 acyl-CoA dehydrogenase-P450 fusion (CYP222A1). F, *Mimivirus* CYP5253A1, fused to unknown protein believed to be involved in protein modelling after translation. G, PpoA dioxygenase/peroxidase-P450 fusion enzyme from *A. nidulans*. H, CYP631B5-hydrolase fusion. J, eukaryotic Class II P450s system (P450-CPR partner). K, Class I P450s system with iron-sulfur protein ADx that is reduced by ADR. L, is the same arrangement as in K but iron-sulfur protein is substituted by a flavodoxin-flavin mononucleotide (CYP176A1). M, shows a "heme-free EryCII", a protein similar to a P450 but without cysteine.

4.2 Methods

Publicly available lower eukaryote genomes, especially basidiomycetes (Table 4.1) (Grigoriev *et al*., 2014) were mined for P450 fused proteins (heme dioxygenase/peroxidase domain fused to P450 domain proteins). BLAST was performed using P450 fused protein CYP6001A1 and CYP6002A1 from *Aspergillus nidulans* (Brodhun *et al*., 2009) against lower eukaryote genomes. The whole protein with both heme dioxygenase/peroxidase and P450 domain and the protein sequence with only heme dioxygenase/peroxidase domain were used for BLAST. The resulting hit proteins were subjected to NCBI Batch Web CD-search (Marchler-Bauer *et al*., 2011). Proteins with both domains were selected as P450 fused proteins. The selected P450 fused proteins were assigned to different P450 families or

subfamilies following the Cytochrome P450 Nomenclature Committee standard explained in section 2.2.3 of chapter 2. The domain organization in the P450 fused protein was recorded using the NCBI Batch Web CD-search (Marchler-Bauer *et al*., 2011). The ascomycete P450 fused proteins were retrieved from recently published literature (Chen *et al*., 2014). Phylogenetic analysis of P450 fused protein was carried out using the minimum evolution method (Rzhetsky & Nei, 1992) and the phylogenetic tree was constructed using MEGA 5.2.2. (Tamura *et al*., 2011).

Table 4.1 Basidiomycota species and respective genome database pages where their fused P450 proteins were mined.

Species	**Web Address**
Auricularia subglabra v2.0	http://genome.jgi.doe.gov/Aurde3_1/Aurde3_1.home.html
Agaricus bisporus var bisporus	http://genome.jgi.doe.gov/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html
Serpula lacrymans S7.3 v2.0	http://genome.jgi.doe.gov/SerlaS7_3_2/SerlaS7_3_2.home.html
Ganoderma sp. 10597 SS1 v1.0	http://genome.jgi.doe.gov/Gansp1/Gansp1.home.html
Bjerkandera adusta v1.0	http://genome.jgi.doe.gov/Bjead1_1/Bjead1_1.home.html
Phlebia brevispora HHB-7030 SS6 v1.0	http://genome.jgi.doe.gov/Phlbr1/Phlbr1.home.html
Phanerochaete carnosa HHB-10118-Sp v1.0	http://genome.jgi.doe.gov/Phaca1/Phaca1.home.html

Wolfiporia cocos MD-104 SS10 v1.0	http://genome.jgi.doe.gov/Wolco1/Wolco1.home.html
Trametes versicolor v1.0	http://genome.jgi.doe.gov/Trave1/Trave1.home.html
Fomitopsis pinicola FP-58527 SS1 v3.0	http://genome.jgi.doe.gov/Fompi3/Fompi3.home.html
Fomitiporia mediterranea v1.0	http://genome.jgi.doe.gov/Fomme1/Fomme1.home.html
Dacryopinax sp. DJM 731 SSP1 v1.0	http://genome.jgi.doe.gov/Dacsp1/Dacsp1.home.html
Dichomitus squalens v1.0	http://genome.jgi.doe.gov/Dicsq1/Dicsq1.home.html
Stereum hirsutum FP-91666 SS1 v1.0	http://genome.jgi.doe.gov/Stehi1/Stehi1.home.html
Coniophora puteana v1.0	http://genome.jgi.doe.gov/Conpu1/Conpu1.home.html

4.3 Results and discussion

4.3.1 Novel P450 fused proteins in oomycetes

P450s fused to redox partners and different proteins are well documented in the literature (Guengerich & Munro, 2013; Lamb & Waterman, 2013). Two different types of P450 fused proteins were reported in lower eukaryotes (fungi). These two different types were: (i) P450 fused to CPR at the C-terminal end, which is well-known as P450foxy (CYP505 family) (Kitazume *et al*., 2000) and (ii) P450 fused to heme peroxidase/dioxygenase at the N-terminal end (CYP6001 family) (Brodhun *et al*., 2009).

Analysis of P450s in oomycetes revealed the presence of P450 fused proteins. The new P450 family CYP5619 with six members found in *S. declina* is fused to heme peroxidase/dioxygenase protein. However, the combination of fusion is different compared to

the reported combination of P450 fused proteins in lower eukaryotes (Guengerich & Munro, 2013; Lamb & Waterman, 2013). In oomycetes, the heme peroxidase/dioxygenase protein is fused at the C-terminal end to P450 (Figure 4.2). This combination, i.e. N-terminal P450 domain fused to heme peroxidase/dioxygenase at its C-terminal, is a novel combination and it is not reported in the literature (Guengerich & Munro, 2013; Lamb & Waterman, 2013).

To confirm the novelty of this P450 fused protein, performed was a comprehensive genome data mining to identify fused P450s, particularly heme peroxidase/dioxygenase protein fused to P450 in the publicly available lower eukaryote genomes (Grigoriev *et al*., 2014). A total number of 61 P450 fused proteins were identified (Figure 4.2 and Table 4.2). The identified P450 fused proteins were grouped under five different P450 families namely CYP6001-CYP6005. Interestingly, the CYP6005 family is found only in Basidiomycota, whereas Ascomycota showed four different P450 fused families, CYP6001-CYP6004 (Figure 4.2 and Table 4.2). The identified P450 fused proteins were subjected to heme peroxidase/dioxygenase and P450 domain analysis. As shown in Figure 4.2, all the P450 fused proteins (CYP6001-CYP6005 family) identified in fungi showed an N-terminal heme peroxidase/dioxygenase domain and a P450 domain at the C-terminal end. This clearly confirms that the combination identified in CYP5619 family members is novel. Considering the nature of P450 fused protein, it can be concluded that CYP5619 family members are possibly involved in the oxidation of fatty acids, like CYP6001A1 (Brodhun *et al*., 2009). However, experimental analysis is needed to unravel the difference between the two different domains' combinations.

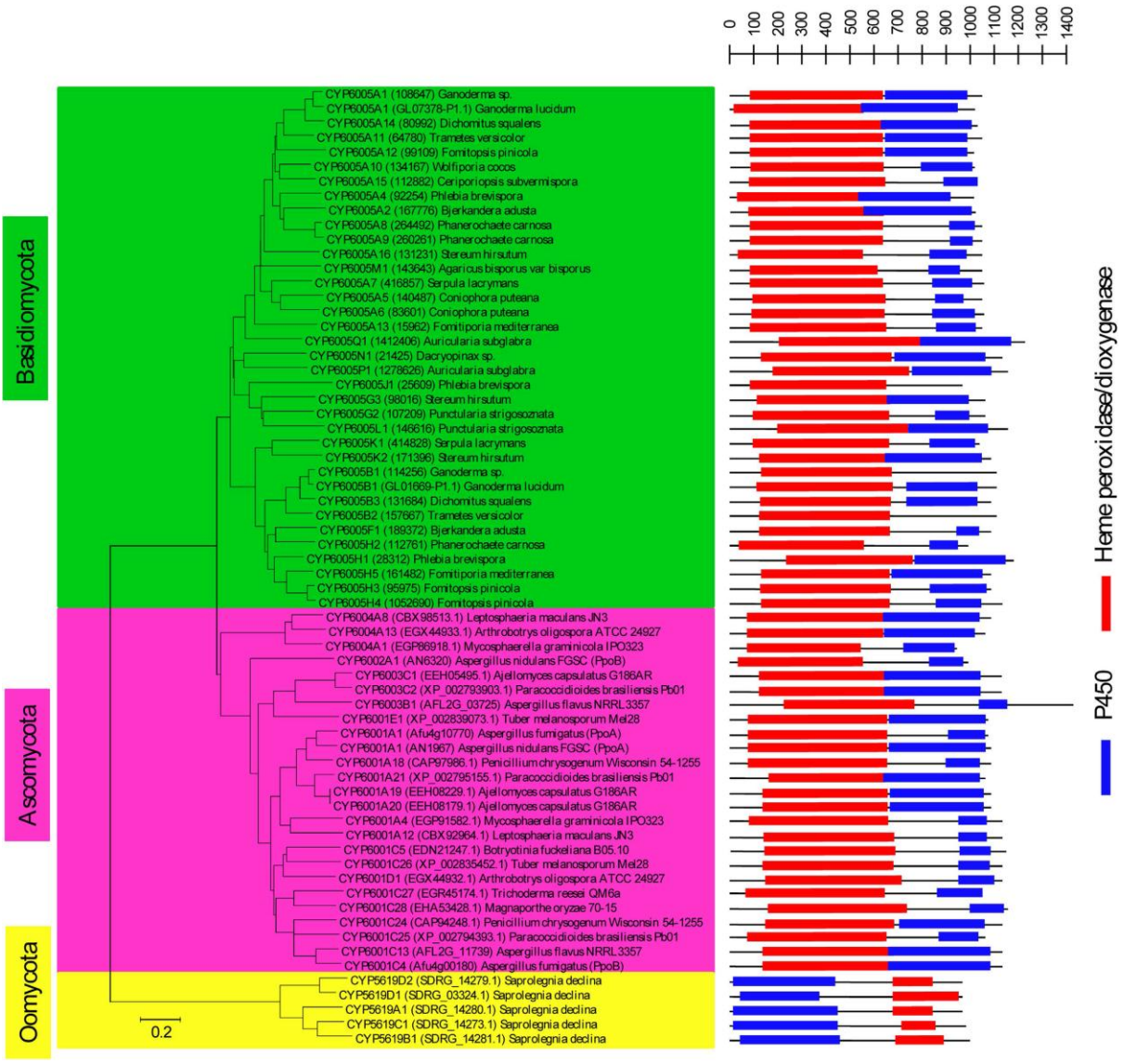


Figure 4.2 Phylogenetic and structural analysis of P450 fused proteins (heme peroxidase/dioxygenase fused to P450) between Oomycota and different fungal phyla. Sixty-six P450 fused proteins were used for the construction of a phylogenetic tree. Structural analysis of P450 fused proteins were carried out as described in 'Methods'. The heme

peroxidase/dioxygenase and P450 domain boxes is indicative of the domain length. For three P450 fused proteins (CYP6005J1, CYP6005B1 and CYP6005B2) NCBI CDD (Marchler-Bauer *et al*., 2011) did not identify the P450 domain length, suggesting the presence of non-variant amino acids at P450 signature motifs in these proteins. A detailed analysis of the P450 fused proteins, species, size of the proteins and size of each of the domains is presented in table 4.2.

Table 4.2 Comparative analysis and structural analysis of P450 fused proteins in Oomycota and different fungal phyla. Heme peroxidase/dioxygenase and P450 domains in the P450 fused proteins were identified as described in 'Methods'. The size of each of the domain is presented in the table below.

CYP NAME	PROTEIN ID	SPECIES NAME	Protein size (AA)	N-terminal to C-terminal	
				Heme peroxidase/dioxygenase	P450
CYP6001A4	EGP91582.1	*Mycosphaerella graminicola*	1113	96-664	941-1058
CYP6004A1	EGP86918.1	*Mycosphaerella graminicola*	939	88-536	727-916
CYP6004A8	CBX98513.1	*Leptosphaeria maculans* JN3	1099	88-638	635-1040
CYP6001A12	CBX92964.1	*Leptosphaeria maculans* JN3	1134	135-696	958-1088
CYP6001C13	AFL2G_11739	*Aspergillus flavus* NRRL3357	1139	144-694	661-1092
CYP6003B1	AFL2G_03725	*Aspergillus flavus* NRRL3357	1419	228-784	1032-1173
CYP6001A1	Afu4g10770(PpoA)	*Aspergillus fumigatus*	1079	98-647	909-1050
CYP6001C4	Afu4g00180(ppoB)	*Aspergillus fumigatus*	1136	141-691	686-1089
CYP6001A1	AN1967 (ppoA)	*Aspergillu nidulans* FGSC	1081	98-647	654-1050
CYP6002A1	AN6320 (ppoB)	*Aspergillu nidulans* FGSC	997	22-546	828-971
CYP6001A18	CAP97986.1	*Penicillium chrysogenum* Wisconsin 54-1255	1074	93-642	900-1031
CYP6001C24	CAP94248.1	*Penicillium chrysogenum* Wisconsin 54-1255	1118	145-695	704-1068
CYP6001C5	EDN21247.1	*Botryotinia fuckeliana* B05.10	1128	131-685	957-1085
CYP6001A19	EEH08229.1	*Ajellomyces capsulatus* G186AR	1084	134-652	659-1055
CYP6001A20	EEH08179.1	*Ajellomyces capsulatus* G186AR	1084	134-652	659-1055
CYP6003C1	EEH05495.1	*Ajellomyces capsulatus* G186AR	1138	109-674	649-1049

CYP6001A21	XP_002795155.1	*Paracoccidioides brasiliensis* Pb01	1066	160-634	624-1035
CYP6001C25	XP_002794393.1	*Paracoccidioides brasiliensis* Pb01	1059	88-641	890-1028
CYP6003C2	XP_002793903.1	*Paracoccidioides brasiliensis* Pb01	1121	109-651	649-1054
CYP6004A13	EGX44933.1	*Arthrobotrys oligospora* ATCC24927	1062	88-638	641-1015
CYP6001D1	EGX44932.1	*Arthrobotrys oligospora* ATCC24927	1138	155-706	967-1100
CYP6001E1	XP_002839073.1	*Tuber melanosporum* Mel28	1079	86-646	655-1052
CYP6001C26	XP_002835452.1	*Tuber melanosporum* Mel28	1119	128-691	979-1065
CYP6001C27	EGR45174.1	*Trichoderma reesei* QM6a	1046	79-633	886-1045
CYP6001C28	EHA53428.1	*Magnaporthe oryzae* 70-15	1153	170-726	1000-1128
CYP6005J1	140487	*Coniophora puteana*	1051	100-650	866-959
CYP6005A6	83601	*Coniophora puteana*	1067	99-649	847-1010
CYP6005A7	416857	*Serpula lacrymans*	1061	97-637	858-1018
CYP6005K1	414828	*Serpula lacrymans*	1035	100-646	815-1012
CYP6005A1	108647	*Ganoderma* sp	1054	88-636	644-988
CYP6005B1	114256	*Ganoderma* sp.	1102	116-689	Not detected
CYP6005A2	167776	*Bjerkandera adusta*	1061	89-643	599-1024
CYP6005F1	189372	*Bjerkandera adusta*	1088	113-669	936-1015
CYP6005A4	92254	*Phlebia brevispora*	996	21-568	524-939
CYP6005J1	25609	*Phlebia brevispora*	967	86-650	Not detected
CYP6005H1	28312	*Phlebia brevispora*	1192	232-761	769-1153
CYP6005A8	264492	*Phanerochaete carnosa*	1050	84-634	912-1013
CYP6005H2	112761	*Phanerochaete carnosa*	990	29-546	828-946
CYP6005A9	260261	*Phanerochaete carnosa*	1050	84-634	912-1013
CYP6005A10	134167	*Wolfiporia cocos*	1061	90-643	791-1024
CYP6005A11	64780	*Trametes versicolor*	1052	82-632	640-986
CYP6005B2	157667	*Trametes versicolor*	1102	110-691	Not detected
CYP6005G2	107209	*Punctularia strigosoznata*	1070	101-670	875-998
CYP6005L1	146616	*Punctularia strigosoznata*	1155	200-742	734-1084

CYP6005A12	99109	*Fomitopsis pinicola*	1055	86-639	648-993
CYP6005H3	95975	*Fomitopsis pinicola*	1093	119-671	817-1054
CYP6005H4	1052690	*Fomitopsis pinicola*	1111	119-671	864-1071
CYP6005H5	161482	*Fomitiporia mediterranea*	1094	114-662	676-1032
CYP6005A13	15962	*Fomitiporia mediterranea*	1057	90-646	866-1019
CYP6005M1	143643	*Agaricus bisporus var bisporus*	1057	92-609	821-957
CYP6005N1	21425	*Dacryopinax* sp.	1129	132-688	696-1075
CYP6005A14	80992	*Dichomitus squalens*	1060	91-643	616-994
CYP6005B3	131684	*Dichomitus squalens*	1099	120-687	737-1035
CYP6005A15	112882	*Ceriporiopsis subvermispora*	1058	84-639	910-1056
CYP6005A1	GL07378-P1.1	*Ganoderma lucidum*	1006	38-585	558-940
CYP6005B1	GL01669-P1.1	*Ganoderma lucidum*	1101	116-688	738-1022
CYP6005A16	131231	*Stereum hirsutum*	1037	42-584	835-992
CYP6005G3	98016	*Stereum hirsutum*	1089	107-671	664-999
CYP6005K2	171396	*Stereum hirsutum*	1092	112-658	654-1043
CYP6005P1	1278626	*Auricularia subglabra*	1166	197-730	747-1094
CYP6005Q1	1412406	*Auricularia subglabra*	1215	200-795	795-1177
CYP5619A1	SDRG_14280.1	*Saprolegnia declina*	970	693-831	15-432
CYP5619D2	SDRG_14279.1	*Saprolegnia declina*	968	667-863	32-370
CYP5619C1	SDRG_14273.1	*Saprolegnia declina*	988	710-883	87-435
CYP5619D1	SDRG_03324.1	*Saprolegnia declina*	971	693-962	44-371
CYP5619B1	SDRG_14281.1	*Saprolegnia declina*	997	696-892	30-453
CYP5619B2	SDRG_14277.1	*Saprolegnia declina*	795	685-784	35-784

4.4 Conclusion

P450 fused proteins may have been a result of protein evolution to improve the metabolic processes of the organisms as this system has been recognised to have improved speed and efficiency in undertaking the catalytic functions as compared to non-fused system, or to augment the vulnerability of organisms to different ecological niches as one of P450s significance. The P450 fused proteins is of novel discovery to the industry world. Though function of other P450 fused proteins has not been established, their applications in various processes in industry have been of value.

Different arrays of P450 fusion systems and diversity are well documented in literature and identification of P450 fused proteins in oomycetes especially that of a non-common arrangement is intriguing. It raises a perception that there are yet other P450 proteins and their other remarkable properties yet to be discovered. May be there is still other enzymes out there that could out match the capacity of P450BM3 (CYP102A1).

4.5 References

Brodhun, F., Gobel, C., Hornung, E., Feussner, I. (2009). Identification of PpoA from *Aspergillus nidulans* as a fusion protein of a fatty acid heme dioxygenase/peroxidase and a cytochrome P450. *J. Biol. Chem*, 284: 11792–11805.

Chen, W., Lee, M., Jefcoate, C., Kim, S., Chen, F., Yu Y. (2014). Fungal cytochrome P450 monooxygenases: Their distribution, structure, functions, family expansion, and evolutionary origin. *Genome Biol. Evol*, 6: 1620-34.

Fisher, C. W., Barnes, H. J., Estabrook, R. W. (2003). FUSION PROTEINS COMPRISING EUKARYOTIC CYTOCHROME P450 FUSED TO A REDUCTASE. Patent: EP0649472. Available from: http://www.freepatentsonline.com/EP0649472B1.html. [Accessed: 31/08/2015]

Guengerich, F. P., Munro, A. W. (2013). Unusual cytochrome p450 enzymes and reactions. *J. Biol. Chem*, 288: 17065-73.

Grigoriev, I. V. Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., Riley, R., Salamov, A., Zhao, X., Korzeniewski, F., Smirnova, T., Nordberg, H., Dubchak, I., Shabalov, I (2014). MycoCosm portal: Gearing up for 1000 fungal genomes. *Nucleic Acids Res*, 42: D699-704.

Kitazume, T., Takaya, N., Nakayama, N., Shoun, H. (2000). *Fusarium oxysporum* fatty-acid subterminal hydroxylase (CYP505) is a membrane bound eukaryotic counterpart of *Bacillus megaterium* cytochrome P450BM3. *J. Biol. Chem*, 275: 39734–39740.

Lamb, D. C., Waterman, M. R. (2013). Unusual properties of the cytochrome P450 superfamily. *Phil. Trans. R. Soc. Lond. B. Biol. Sci*, 368: 20120434.

Madoui, M. A., Bertrand-Michel, J., Gaulin, E., Dumas, B. (2009). Sterol metabolism in the oomycete *Aphanomyces euteiches*, a legume root pathogen. *New Phytol*, 183: 291-300.

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Lu, F., Marchler, G. H., Mullokandov, M., Omelchenko, M. V., Robertson, C. L., Song, J. S., Thanki, N., Yamashita, R. A., Zhang, D., McLean, K. J., Girvan, H. M., Munro, A. W. (2007). Cytochrome P450/redox partner fusion enzymes: biotechnological and toxicological prospects. 3(6): 847-863.

Moktali, V., Park, J., Fedorova-Abrams, N. D., Park**,** B.**,** **C**hoi**,** J.**,** Lee, Y., Kang, S. (2012). Systematic and searchable classification of cytochrome P450 proteins encoded by fungal and oomycete genomes. *BMC Genomics*, 13: 525.

Rzhetsky, A., Nei, M. (1992). A simple method for estimating and testing minimum evolution trees. *Mol. Biol. Evol*, 9: 945–967.

Tamura, K. Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*, 28: 2731–2739.

The European Bioinformatics Institute. n.d. Cytochrome P450. Available from: http://www.ebi.ac.uk/interpro/potm/2006_10/Page2.htm. [Accessed: 31/08/2015]

Warrilow, A. G., Hulla, C. M., Rolleya, N. J., Parkera, J. E., Nesb, W. D., Smitha, S. N., Kelly D. E., Kelly, S. L. (2014). Clotrimazole as a potent agent for treating the oomycete fish pathogen *Saprolegnia parasitica* through inhibition of sterol 14*α*-demethylase (CYP51). *Appl. Environ. Microbiol*, 80: 6154-66.

Zhang, N., Zheng, C., Bryant, S. H. (2011). CDD: A conserved domain database for the functional annotation of proteins. *Nucleic Acids Res*, 39: D225–9.

CHAPTER 5

CONCLUSION

In conclusion, oomycetes are very important organisms in terms of their hard-wired parasitism leading to the loss of billions of dollars in agriculture and aquaculture. Analysis of P450s in these organisms provided insights into the evolutionary pattern of P450s. Genome-wide analysis of P450s revealed the presence of moderate number of P450s in these organisms. Despite presence of a large number of new P450 families and subfamilies, P450 family blooming resulted in a low P450 diversity in oomycetes. CYP51 and novel P450 fusion proteins with different combination of heme peroxidase/dioxygenase and P450 domain were common between oomycetes and lower eukaryote fungi. The speciation and adaption to diverse ecological niches or lifestyle of oomycetes and fungi resulted in generation of distinct P450 families in both groups. Furthermore, at order level, oomycetes showed distinct P450 families and subfamilies. This confirms that the host influence is a major factor in shaping the oomycetes genomic content and thus also reflected in terms of P450s. Presence of unique combination of amino acid patterns at EXXR and CXG motifs in oomycetes P450 families strongly supported previously proposed hypothesis that the amino acid patterns at these motifs are characteristic of a P450 family. This study serves as a reference and will open new vistas for future genome-wide annotation of P450s in oomycetes. Future genome sequencing of more oomycetes will provide a broader picture of P450 evolution in these organisms and also possibly results in discovery of novel P450 families.

SCIENTIFIC REPORTS

OPEN

Diversity and evolution of cytochrome P450 monooxygenases in Oomycetes

Received: 19 March 2015
Accepted: 27 May 2015
Published: 01 July 2015

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Cytochrome P450 monooxygenases (P450s) are heme-thiolate proteins whose role as drug targets against pathogens, as well as in valuable chemical production and bioremediation, has been explored. In this study we performed comprehensive comparative analysis of P450s in 13 newly explored oomycete pathogens. Three hundred and fifty-six P450s were found in oomycetes. These P450s were grouped into 15 P450 families and 84 P450 subfamilies. Among these, nine P450 families and 31 P450 subfamilies were newly found in oomycetes. Research revealed that oomycetes belonging to different orders contain distinct P450 families and subfamilies in their genomes. Evolutionary analysis and sequence homology data revealed P450 family blooms in oomycetes. Tandem arrangement of a large number of P450s belonging to the same family indicated that P450 family blooming is possibly due to its members' duplications. A unique combination of amino acid patterns was observed at EXXR and CXG motifs for the P450 families CYP5014, CYP5015 and CYP5017. A novel P450 fusion protein (CYP5619 family) with an N-terminal P450 domain fused to a heme peroxidase/dioxygenase domain was discovered in *Saprolegnia declina*. Oomycete P450 patterns suggested host influence in shaping their P450 content. This manuscript serves as reference for future P450 annotations in newly explored oomycetes.

Ongoing genome sequencing momentum has resulted in genome sequencing of a large number of species from different biological kingdoms. Lower eukaryotic kingdoms occupy a special place among biological kingdoms because of the presence of a large number of species and their adaptation to diverse ecological niches. Genome sequencing of lower eukaryotes such as fungi revealed high diversity in their genomes compared to other biological kingdoms. For example, not only the presence of a large number of cytochrome P450 monooxygenases (P450s) was detected in many of their genomes, but also high diversity in terms of the number of P450 families[1].

P450s are heme-thiolate proteins and ubiquitously present in species across the biological kingdoms[2]. These proteins are well known to perform enzymatic reactions in a stereo- and regio-specific manner[3,4]. Because of this characteristic these enzymes become critical in organisms' primary and secondary metabolism, drug development, generation of human valuables and xenobiotic compound degradation[2,5]. Progress has been made in understanding P450s from lower eukaryotic organisms, such as their

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genome-wide annotation and comparative analysis[6–11], heterologous expression and characterization[12–14], that eventually resulted in identification of catalytically versatile P450s[15,16], their engineering[17] and further unraveling of their potential as anti-fungal drug targets[5,18,19].

However, lower eukaryotes belonging to the kingdom Stramenopile, especially phylum Oomycota species P450s, have been underexplored. Oomycetes live as saprophytes or parasites[20,21]. These are organisms considered "hard-wired parasites"[22]. Oomycetes cause diseases in both plants and animals[20,21,23]. Oomycetes are counted among the most widespread and deadliest disease-causing agents of plants and crops worldwide. Their destructive behaviour lies in their ability to breach the host surface and break it down, promptly resulting in extensive destruction that hinders agricultural growth[24]. There has been a huge impairment of aquaculture owing to oomycetes and as for plants, serious diseases are caused not only in agriculturally and ornamentally important plants, but also other plants in the environment. A summary of diseases caused by oomycetes is listed in Table S1. To date, oomycetes remain a serious problem in agriculture and aquaculture[20,21,23]. Oomycete diseases are not commonly easy to control. Moreover, some oomycete species, particularly Phytophthora species, have the ability to build up resistance against chemicals by producing new genetically tougher strains. Plants are also very sensitive to oomycete attacks owing to their weak disease resistance.

The impact of oomycete species on the economy triggered various investigations on pathogenesis and control methods for these pathogens. In the quest to find a remedy, genome sequencing of oomycetes was carried out[25–31]. Genome sequencing analysis of oomycetes revealed the presence of a moderate number of P450s[27]. However, the genome sequencing studies were limited to mentioning the count of P450s in oomycetes[27]. A comparative P450 genomics study was limited to P450 analysis in a few species[7]. The study showed that the CYP51 of oomycetes can be a good drug target against these pathogens[32,33]. Despite this great importance, analysis of P450 enzymes in oomycetes has been underexplored. The recent public availability of quite a number of oomycete genomes[25–31] gives us an opportunity to perform comprehensive comparative analysis of P450s in these species. In this study we performed systematic analysis of P450s across 13 oomycete species. Furthermore, considering that the lower eukaryote fungi P450s are well annotated and the poor availability or unavailability of other lower eukaryotic P450s, in this study we compared Oomycota P450s with different fungal phyla P450s.

Methods

Oomycete species for P450 analysis. Thirteen oomycete species belonging to two different classes and three different orders were used in this study. The oomycete species used in this study, their taxonomic group and general information such as their host and diseases caused by these organisms were listed in Table S1. As listed in Table S1, 11 species (*Phytophthora sojae*, *P. ramorum*, *P. infestans*, *P. parasitica*, *P. capsici*, *Hyaloperonospora arabidopsidis* (formerly *Hyaloperonospora parasitica*), *Pythium aphanidermatum*, *P. irregular*, *P. awayamai*, *P. ultimum* and *P. vexan*) belonging to class Peronosporomycetidae and two species (*Saprolegnia parasitica* and *S. declina*) belonging to Class Saprolegniomycetidae are used for comparative analysis of P450s. It is noteworthy that Peronosporomycetidae contain plant pathogens whereas Saprolegniomycetidae contain animal pathogens.

Genome data-mining and identification of P450s. Oomycete species genomes whose details have been published (Table S1) and are publicly available (Table S2) were used in this study. The whole proteomes of oomycete species were downloaded from the databases listed in Table S2. Identification of P450 proteins in whole proteome is carried out using the procedure described elsewhere[11,16]. Briefly, the downloaded protein sequences were grouped into different protein families using the National Centre for Biotechnology and Information (NCBI) Conserved Domain Database: NCBI Batch Web CD-search tool[34]. The proteins grouped under the cytochrome P450 monooxygenases superfamily were selected for further study.

Assigning a family and subfamily to orphan P450s. The above selected P450s were subjected to BLAST analysis against all named protist sequences on the Cytochrome P450 Webpage[35]. Based on percentage identity, i.e., family members share more than 40% amino acid identity and members of subfamilies share more than 55% amino acid homology, families and subfamilies were assigned to oomycete P450s. P450s that showed less than 40% identity were assigned to a new family. In addition, evolutionary analysis of P450s was performed in order to authenticate the annotation. P450s that showed less than 40% identity were assessed for their position on the phylogenetic tree and based on their location/alignment with other P450s they were assigned to different P450 families. The annotated and publicly available *P. sojae* and *P. ramorum* P450s were retrieved from the database[35] and used in this study.

Phylogenetic analysis of oomycete P450s. The phylogenetic tree was constructed for evolutionary analysis of oomycete P450s. Firstly, the protein sequences were aligned by adjusting them to the hidden Markov model of P450s in the Pfam protein families database (http://pfam.xfam.org/family/PF00067) with HMMER package 3.1 (http://hmmer.janelia.org/)[36,37]. Then, the phylogenetic tree from the alignment of protein sequences was inferred by FastTree version 2.1.4 using the maximum-likelihood method (http://www.microbesonline.org/fasttree/)[38]. The generated tree data were submitted to iTOL (http://itol.embl.de/upload.cgi) for viewing phylogenetic trees and making figures[39].

Analysis of homology. Percentage identity between P450s was determined using ClustalW2 multiple sequence analysis[40]. The ClustalW2 result file designated as percentage identity matrix was downloaded and checked for the percentage identity between P450s.

P450 diversity percentage. The percentage contribution of the number of P450 families in the total number of P450s in an organism is considered as P450 diversity percentage. P450 count and P450 families in fungal species were retrieved from published literature[9–11,35].

Analysis of tandem arrangement of P450s. P450s localized in proximity on the genome were identified by scanning manually in the respective genome databases for each oomycete (Table S2). P450s localized on the same scaffold/contig/supercontig were noted. P450s that were tandemly localized and belonged to the same family were expressed as percentage in the total number of P450s in an organism. Tandem arrangement of P450s was not carried out for *P. irregulare* and *P. iwayamai* because of the shorter length of scaffold/contig/supercontig.

Analysis of EXXR and CXG motifs. Identification of P450 family-specific amino acid patterns at EXXR and CXG motifs was carried out using the procedure described elsewhere[41]. Briefly, P450 members were subjected to ClustalW multiple alignment using Molecular Evolutionary Genetics Analysis (MEGA 5.2.2)[42]. After ClustalW alignment of P450s, amino acids in the EXXR and CXG motifs were selected and used for generation of WebLogos and calculation of percentage contribution by an amino acid at each position in the motifs. Only four amino acids were selected for EXXR motif analysis, whereas for CXG motifs upstream seven amino acids were included in the analysis, exactly as previously described[41].

Generation of sequence logos. Sequence logos for EXXR and CXG motifs were generated using the published method[41]. Briefly, WebLogo, a sequence logo generator programme (http://weblogo.threeplusone.com/create.cgi)[43,44], was used to create sequence logos at EXXR and CXG motifs. After ClustalW alignment of member P450s, the EXXR and CXG (FXXGXRXCXG) motifs' amino acids were selected and pasted in the WebLogo program. As a selection parameter, image format was selected as PDF and 32 symbols per line were selected. The generated EXXR and CXG sequence logos were used for the analysis.

Genome data mining, annotation and phylogenetic analysis of P450 fused proteins. Publicly available lower eukaryote genomes, especially basidiomycetes (http://genome.jgi-psf.org/programs/fungi/index.jsf)[45], were mined for P450 fused proteins (heme dioxygenase/peroxidase domain fused to P450 domain proteins). BLAST was performed using P450 fused protein CYP6001A1 and CYP6002A1 from *Aspergillus nidulans*[46] against lower eukaryote genomes. The whole protein with both heme dioxygenase/peroxidase and P450 domain and the protein sequence with only heme dioxygenase/peroxidase domain were used for BLAST. The resulting hit proteins were subjected to NCBI Batch Web CD-search[34]. Proteins with both domains were selected as P450 fused proteins. The selected P450 fused proteins were assigned to different P450 families or subfamilies following the above described criteria. The domain organization in the P450 fused protein is recorded using the NCBI Batch Web CD-search[34]. The ascomycete P450 fused proteins were retrieved from recently published literature[8]. Phylogenetic analysis of P450 fused protein was carried out using the minimum evolution method[47]. The phylogenetic tree was constructed using MEGA 5.2.2[42].

Results and Discussion

Oomycetes P450omes. Genome-wide identification and annotation of P450s in 13 oomycetes belonging to two different classes and three different orders (Table S1) revealed the presence of a moderate number of P450s in their genomes (Table 1). Three hundred and fifty-six P450s were found in 13 oomycetes genomes (Table S3). The P450 count in oomycete genomes ranged from 7-41. Among the oomycetes selected for the study, *H. arabidopsidis* showed the lowest number of P450s (7) and *P. iwayamai* showed the highest number of P450s (41) in their genome. Except *H. arabidopsidis*, all oomycete genomes had 19 or more P450s (Table 1). On average, Peronosporales showed a lower number of P450s (27), excluding *H. arabidopsis*, compared to Pythiales that showed 31 P450s. Comparison of oomycete P450omes with other lower eukaryotes such as fungi revealed that the number of P450s observed in oomycetes is most similar to fungal species belonging to the subphylum saccharomycotina and least similar among species belonging to the rest of the fungal kingdom, with a few exceptions, as shown in Table S4.

P450 families and subfamilies in oomycetes. Annotation of P450 families and subfamilies in 13 oomycete genomes revealed the presence of 15 P450 families (Fig. 1) and 84 P450 subfamilies (Table S5). Nine new P450 families and 31 new P450 subfamilies were found in oomycetes. The nine new P450 families are CYP5613, CYP5614, CYP5615, CYP5616, CYP5617, CYP5618, CYP5619, CYP5620 and CYP5621. New subfamilies were confined to four P450 families: CYP5014 showed 15 new subfamilies, followed by the CYP5015 and CYP5017 families each with seven new subfamilies, and CYP558 with two new subfamilies. A detailed analysis of P450 families and subfamilies and their member P450s was listed in Table S5.

Species name	No. of P450s	No. of P450 families	No. of P450 subfamilies
Phytophthora sojae	30	4	18
Phytophthora parasitica	31	4	18
Phytophthora ramorum	24	4	17
Phytophthora infestans	20	3	14
Phytophthora capsici	28	3	17
Hyaloperonospora arabidopsidis	7	2	7
Pythium irregulare	41	3	17
Pythium aphanidermatum	31	4	18
Pythium ultimum	19	3	12
Pythium iwayamai	42	3	19
Pythium vexan	20	4	15
Saprolegnia parasitica	24	6	16
Saprolegnia declina	39	9	26

Table 1. Comparative P450 analysis in 13 oomycete species.

Comparative analysis of member P450s across 13 P450 families revealed that the CYP5014, CYP5015 and CYP5017 P450 families are the dominant P450 families in oomycetes with 109, 111 and 49 members comprising 76% of the total P450s (Fig. 1). This suggests a high level of P450 blooming[48] of these families. A detailed analysis of P450 bloom in oomycetes is discussed in the next section of this manuscript. A single member was found in CYP5613 and CYP5621 families (Fig. 1). Analysis of P450 families, particularly their member P450s and their contribution to the total number of P450s, is shown in Fig. 1.

P450 family and subfamily dynamics in oomycetes. After annotation of families and subfamilies, further study was carried out to assess the dynamics of P450 families and subfamilies (loss or gain of P450 families/subfamilies) in these organisms. Among oomycetes, Saprolegniales showed the highest number of P450 families compared to Peronosporales and Pythiales (Table 1). The number of P450 families in oomycetes ranged from two to nine. Peronosporales showed a minimum of two and a maximum of four families in their genomes. Pythiales showed three to four P450 families in their genomes. Species belonging to Saprolegniales showed six (*S. parasitica*) and nine (*S. declina*) P450 families in their genomes (Table 1).

Comparative analysis of P450 families revealed no common P450 family across the oomycetes used in this study (Fig. 2). The CYP5016 family is present only in Peronosporales and the CYP5014, CYP5015 and CYP5017 families are present in both Peronosporales and Pythiales. Saprolegniales has eleven P450 families (CYP51, CYP558 and CYP5613-CYP5621). Nine of them (CYP5613-CYP5621) are new P450 families only found in Saprolegniales. The answer to the presence of the highest number of P450 families and particularly the presence of new P450 families in Saprolegniales compared to Peronosporales and Pythiales can be obtained from a recently published genome sequencing study[31]. Genome sequencing analysis of *S. parasitica* revealed that loss of heterozygosity is an efficient mechanism for new variant genes to adapt to a distinct animal pathogenic lifestyle[31]. The presence of distinct P450 families (new P450 families) in Saprolegniales compared to Peronosporales and Pythiales (Fig. 2) suggested that P450s in these organisms play a key role in their adaptation to a pathogenic lifestyle (animal host). One interesting observation is that the CYP51 family, involved in membrane sterols biosynthesis[5], is only found in Saprolegniales (Fig. 1). The loss of CYP51 in other oomycetes implied dependence on the host sterols.

The distinct pattern observed in P450 families among oomycete species was also reflected in P450 subfamilies (Fig. 3 and Table S5). Comparative analysis of subfamilies revealed that only nine subfamilies were shared between Peronosporales and Pythiales. Analysis of P450 subfamilies revealed that all the subfamilies shared between Peronosporales and Pythiales were found in CYP5014 (2), CYP5015 (6) and CYP5017 (1) (Fig. 3). This suggests that distinct pathogenic lifestyles (host and site of infection) of Peronosporales and Pythiales (Table S1) influenced the P450 content in their genomes, as species belonging to these orders show distinct P450 subfamilies (Fig. 3 and Table S5).

The above results revealed that oomycetes belonging to different orders show distinct P450 families and P450 subfamilies in their genomes. This strongly suggests that oomycetes belonging to different orders retained or evolved distinct P450 families in their genomes possibly to adapt to a pathogenic lifestyle in different hosts. In other ways, as recently suggested by researchers[31], host cellular environment has driven distinct patterns of gene expansion and loss in the genomes of plant and animal pathogens. From the above results it is clear that the host environment played a key role in the development of distinct/new variants of P450 families in oomycetes.

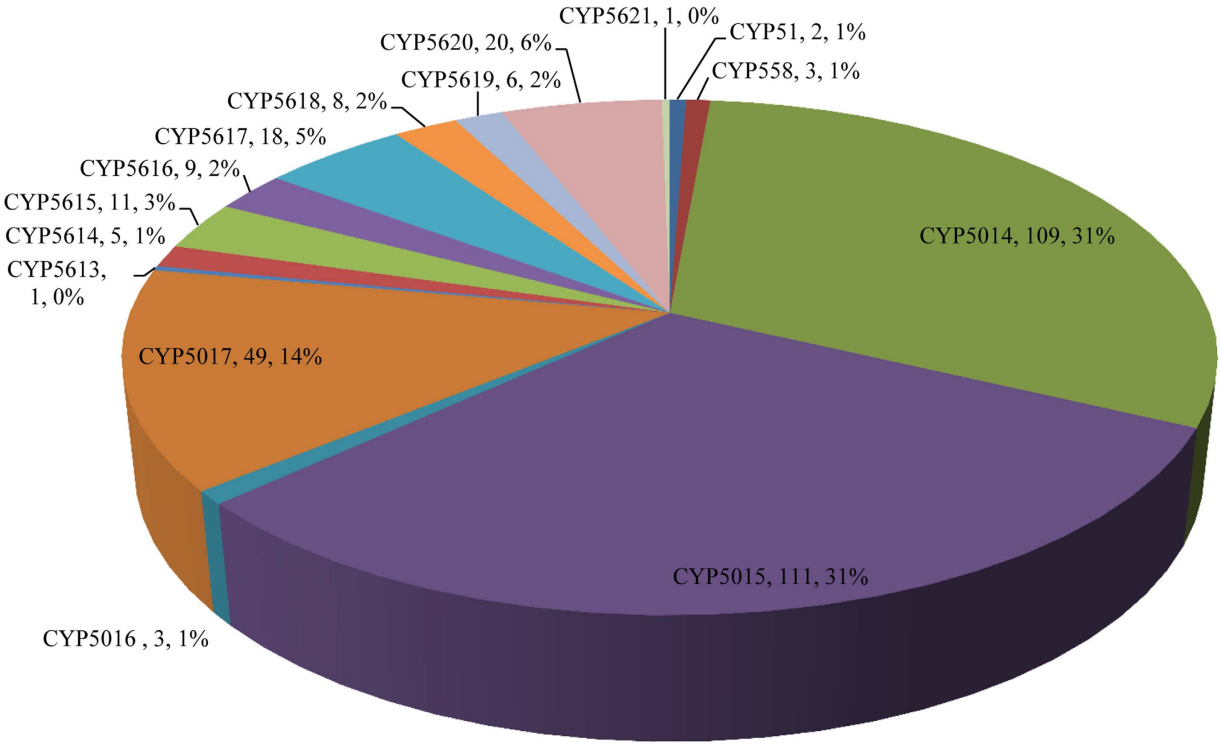


Figure 1. Comparative analysis of P450s in 13 oomycete animal and plant pathogens. Three hundred and fifty-six P450s were grouped under 15 P450 families. The P450 family name, number of member P450s and their percentage in the total number of P450s are shown in the figure.

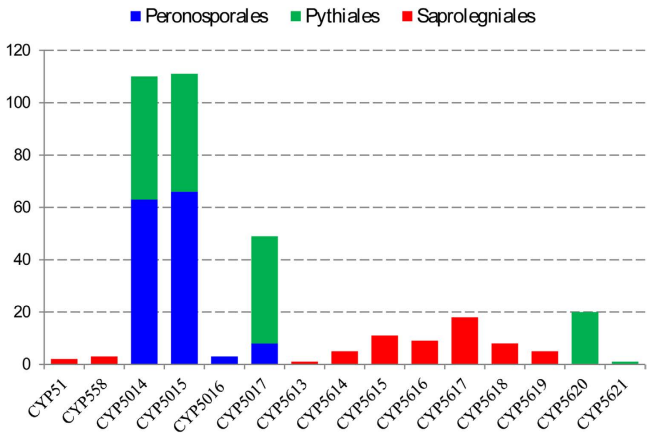


Figure 2. P450 family-level comparative analysis between three oomycete orders: Peronosporales, Pythiales and Saprolegniales. The Y-axis represents number of P450s.

Evolutionary analysis of oomycete P450s. The presence of distinct P450 families, particularly new families and subfamilies, in oomycetes necessitated the performance of evolutionary analysis to allow grouping of P450s into different clades, a higher level P450 classification[49]. Furthermore, evolutionary analysis of oomycetes P450s played a key role in the annotation of oomycete P450s into different families.

Hence in this study a phylogenetic tree of oomycete P450s was constructed for their evolutionary analysis (Fig. 4). The results showed that the phylogenetic relationship of oomycete P450s was related with their family and species taxonomy. On the whole, the P450s of the order Saprolegniales showed a very distant phylogenetic relationship to those of the order Pythiales and Peronosporales; they were clearly separated in the tree, while the P450s from the order Pythiales and Peronosporales were phylogenetically close. This is in agreement with the taxonomy relationship between the orders Saprolegniales, Pythiales and Peronosporales, which suggested that the evolution of oomycete P450s was closely related with their species' evolution.

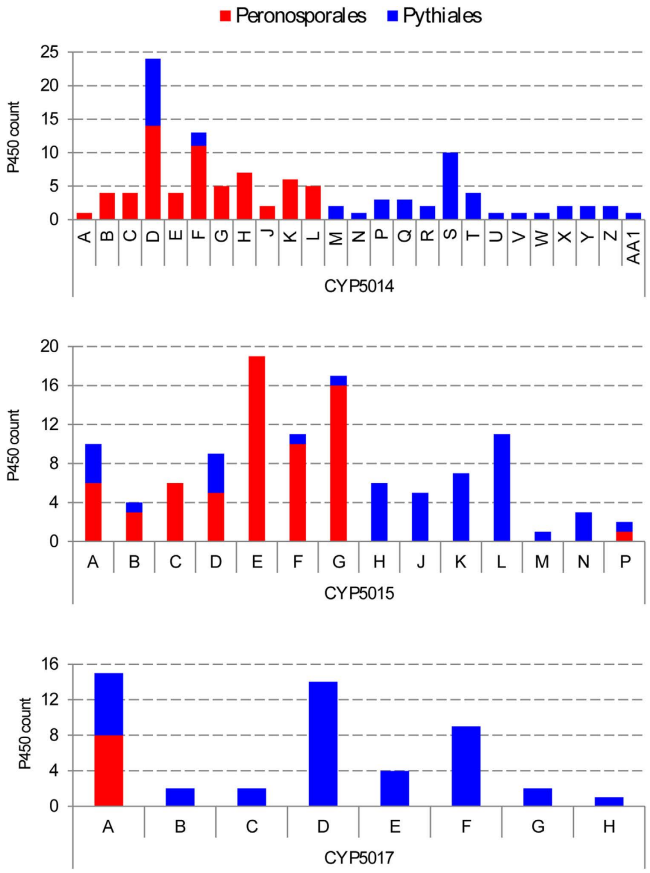


Figure 3. Comparative analysis of CYP5014, CYP5015 and CYP5017 families between Peronosporales and Pythiales.

Based on phylogenetic relationships, oomycete P450s are classified into six clades (Fig. 4), and the distribution of CYP families and oomycete taxonomy is investigated in these clades (Table S6). Only clade 5 has members from all three orders. Clade 6 is a very large branch, suggesting it is blooming in the order Pythiales and Peronosporales. Especially CYP5014 and CYP5015 members are not only very frequently presented in the order Pythiales and Peronosporales, but also maintain a high gene number in their genomes (Figs 1,3 and Table S5). This suggests that CYP5014 and CYP5015 family members may play a pivotal role in the physiological function of order Pythiales and Peronosporales.

P450 blooming in oomycetes. Comparative analysis of P450s in arthropods, mainly insects, revealed the presence of P450 families with the highest number of members in their genomes and authors termed this nature of the highest number of members for a P450 family "P450 family blooming"[48]. A recent study on fungal P450s also revealed the blooming nature of a large number of P450 families in fungi[16]. Blooming of P450 families might play a key role in an organism's metabolism or its adaptation to diverse ecological niches, for example fungal colonization of wood substrates[16].

In order to analyse P450 bloom or its direct opposite P450 diversity in oomycetes, we performed a comprehensive comparison of P450 count and P450 families between Oomycota and different fungal phyla (Table S4). Another reason for using fungal organisms for comparison, apart from what is mentioned in the introduction, is that for a long time oomycetes were regarded as true fungi; it was only recently that these organisms were grouped under the biological kingdom "Stramenopile". Furthermore, analysis of P450 diversity/blooming between these organisms will provide insights in evolution of P450 families, considering the primitive nature of oomycetes.

Comparative analysis of P450 families across Oomycota and other fungal phyla revealed that a number of P450 families present in oomycetes are to some extent matched with species belonging to Ascomycota, particularly the subphylum Saccharomycotina (Table S4). In order to identify the P450 diversity/blooming in oomycetes we measured an average number of P450s and an average number of P450 families across different phyla and measured the average P450 diversity percentage (Fig. 5 and Table S4). As shown in Fig. 5A, the average P450 count and average P450 families in Oomycota were found to be lowest (excluding Saccharomycotina species in Ascomycota) compared to different fungal phyla. This indicates the low diversity of P450s in oomycetes. On the other hand, this implies the highest blooming of P450 families in oomycetes. To identify the blooming nature of Oomycota P450ome we measured

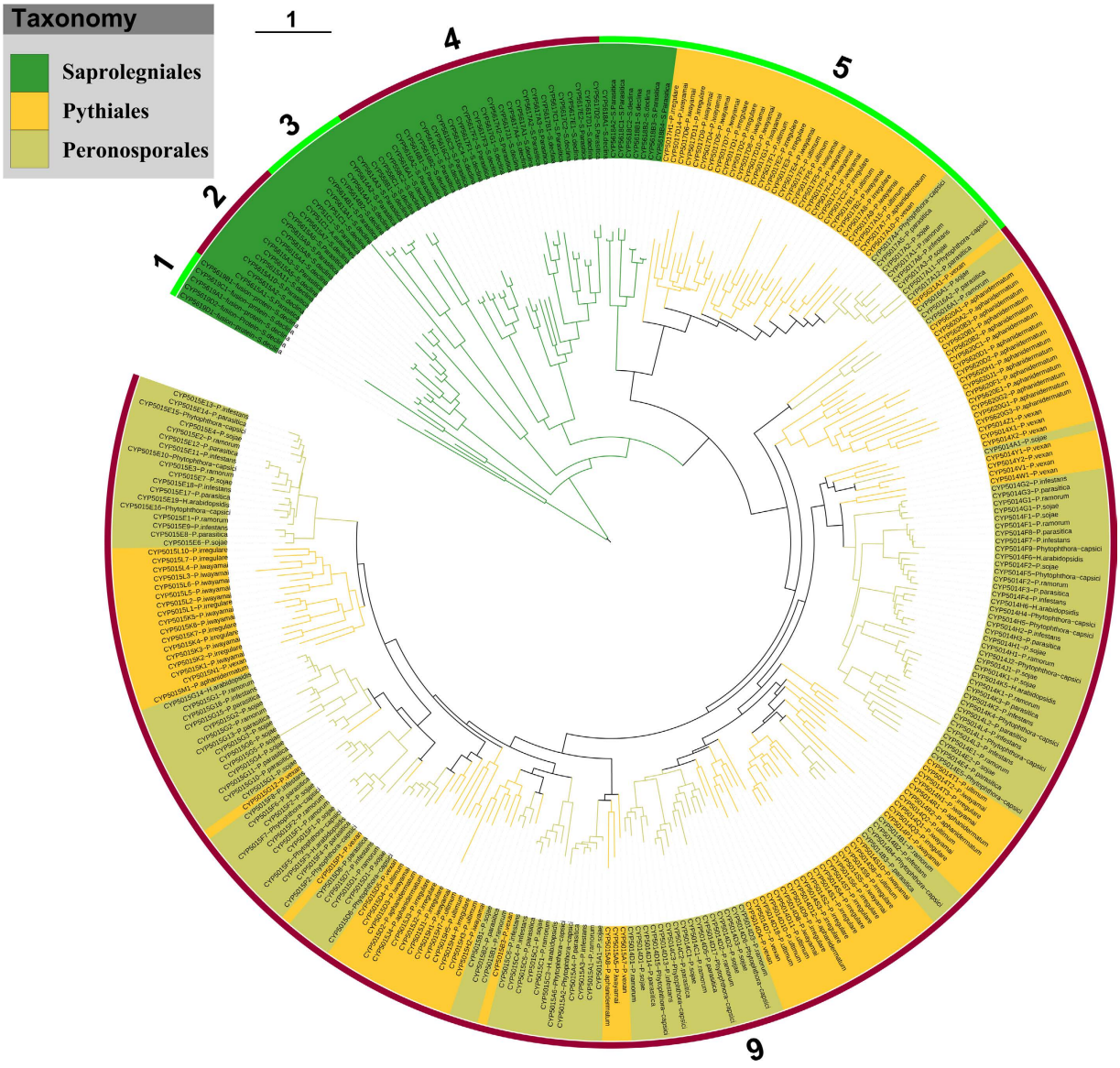


Figure 4. Phylogenetic tree of P450s in 13 oomycete species. The inner circle is the phylogenetic tree of annotated oomycete P450s. The branches with different colors show their taxonomic groups, as indicated in the legend. Ancestral branches with children that had identical colors were assigned the same color as the children. The middle circle is the taxon represented as P450 family, followed by the corresponding oomycete species name, which is covered by different colors to show its taxonomic group, as the legend indicates. Each taxon links the branch with a dotted line. The outer numbers indicate the six clades derived in this study and their ranges are marked by alternating reddish brown and green. A high-resolution phylogenetic tree is provided in the Supplementary data (Fig. S1).

the average P450 diversity percentage between Oomycota and other fungal phyla (Fig. 5B). As shown in Fig. 5B, Oomycota showed the lowest P450 diversity percentage (15%) compared to other fungal phyla indicating the highest P450 blooming in oomycetes or the lowest diversity.

A contribution of 76% of P450s by three P450 families CYP5014, CYP5015 and CYP5017 (Fig. 1) resulted in the lowest diversity in oomycetes. This suggests the blooming of CYP5014, CYP5015 and CYP5017 families in oomycetes. The blooming nature of P450 families is attributed by tandem duplication of their members[16,48]. The duplicating nature of member P450s is easily identified either by the highest identity at protein level or analysis of the gene structure (analysis of introns and exons) between members. Since the oomycetes, genes show the lowest number of introns in their structure[20], it is not ideal to perform gene structure analysis to identify genome-duplicated P450s. Hence, in this study, we used protein percentage identity criteria to identify P450s that were possibly duplicated in oomycetes.

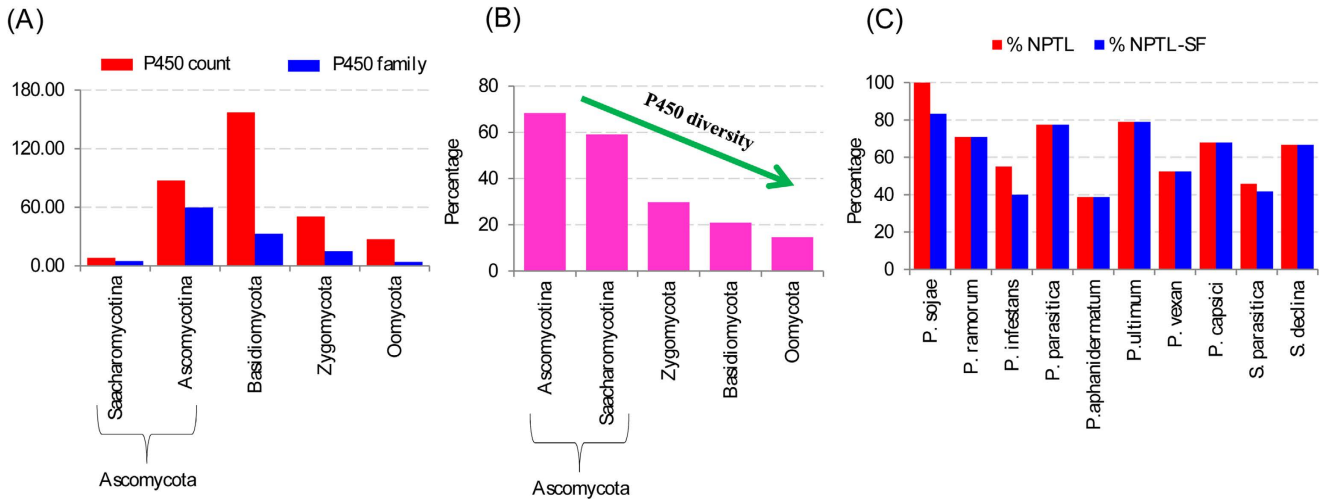


Figure 5. Comparative P450 diversity analysis between Oomycota and other lower eukaryote phyla (**A,B**) and P450 bloom analysis in oomycetes (**C**). A comparative analysis of the average number of P450s and P450 families (**A**) and P450 diversity percentage (**B**) between different phyla is shown in the figure. Detailed analysis of P450 count, families and measured P450 diversity percentage is represented in Table S4. As shown in Panel B, Oomycota showed the lowest diversity compared to different fungal phyla, indicating P450 blooming in these organisms. P450 family blooming in oomycetes was measured (i) percentage of number of P450s tandemly localized on the same scaffold (%NPTL) and (ii) percentage of NPTL belonging to the same family (%NPTL-SF) in the total number of P450s in a species. Detailed analysis of P450s that are tandemly localized on scaffolds in each species and %NPTL and %NPTL-SF is presented in Table S8.

To assess the duplicate nature of P450s, oomycete P450omes were subjected to ClustalW2 analysis[40]. The percentage identity between oomycete P450s was analysed and the proteins showing more than 70% identity were selected and presented in Table S7. As shown in Table S7, a large number of P450s (115) showed more than 70% identity and 82 P450s showed more than 80% identity. This indicates that the majority of the oomycete P450s are highly conserved in their primary structure. Analysis of P450s with respect to the P450 families revealed that all of the P450s that showed more than 70% identity belong to three P450 families, i.e. CYP5014, CYP5015 and CYP5017, except three P450s belonging to the CYP5016 family and two P450s belong to CYP5619 family (Table S7). This suggests that member P450s in these P450 families possibly increased their number through duplication, which resulted in blooming of these P450 families.

Tandem localization of oomycete P450s. Tandem localization of P450s, particularly P450s belonging to the same P450 family, is a good indication of P450 duplications. In order to analyse P450 duplications in oomycetes we proceeded to analyse the localization of P450s. As shown in Fig. 5C and Table S8, a large number of P450s were found to be tandemly arranged in oomycetes. Tandem arrangement of P450s in oomycetes ranged from 39% to 100% in the total number of P450s (Fig. 5C). The highest number of tandemly arranged P450s were found in *P. sojae*, where all the P450s (100%) were tandemly arranged. *P. aphanidermatum* showed the lowest number of tandemly arranged P450s (30%) in its genome. *H. arabidopsidis* showed no tandemly arranged P450s possibly due to low copy of P450s in its genome. Analysis of tandemly arranged P450s revealed that all the P450s that were tandemly arranged belonged to the same P450 family in all analyzed organisms except in *P. infestans,* where only 40% of P450s belonged to the same family (Fig. 5C and Table S8).

Family level analysis of tandemly localized P450s revealed that all of the tandemly localized P450s belonged to the P450 families that showed blooming in the respective species (as discussed above). For example, CYP5014-CYP5017, CYP5615-CYP5620 and CYP558 family members were found tandemly arranged in oomycetes. It is interesting to note that the P450 family CYP558 in *S. declina* its two members were found tandemly localized (Table S8). Based on evolutionary analysis, sequence identity data and tandem arrangement, we conclude that many P450 families in oomycetes are bloomed, owing to tandem duplication of their members.

Oomycete P450 family characteristic amino acid patterns at EXXR and CXG motifs. A recent study revealed that a certain combination of amino acid patterns at EXXR and CXG motifs are characteristic of a P450 family[41]. Authors have suggested that these amino acid patterns are evolved during the P450 family divergence from a common ancestor and are retained in family members as a characteristic

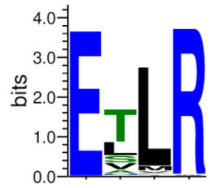
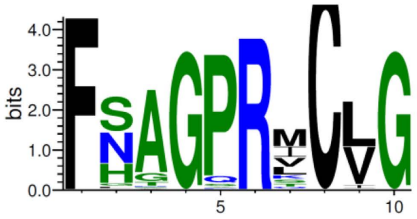
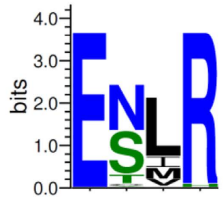
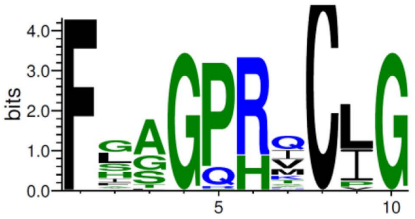
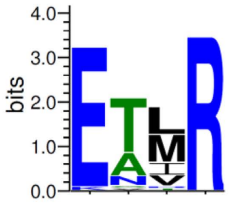
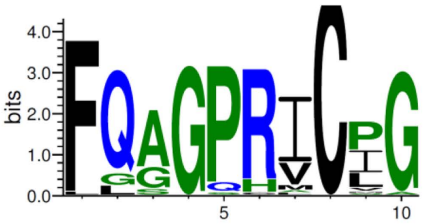
CYP5014	
	
E-T(49.4)/L(20.2)-L(91.0)-R(99.0)	**F**-S(38.2)/N(33.7)/H(21.3)-A(83.1)-**G**-P(89.9)-**R**-M(29.2)/I(17.9)/V(15.7)-**C**-L(51.6)/V(42.7)-**G**
CYP5015	
	
E-N(44.8)/S(42.5)-L(65.5)-R(97.7)	**F**-G(25.3)/L(21.8)/S(16.1)-A(50.6)/G(21.8)-**G**-P(81.6)-R(73.6)-Q(26.4)/V(17.2)/I(17.2)/M(11.5)-**C**-L(52.9)/I(36.8)-**G**
CYP5017	
	
E(97.4)-T(59.0)/A(25.6)-L(33.3)/M(33.3)-**R**	**F**-Q(82.1)-A(66.7)-**G**-P(89.7)-R(87.1)-I(66.7)/V(23.0)-**C**-P(38.5)/I(31.0)/l(20.5)-G(97.4)

Figure 6. Analysis of amino acid combinations at EXXR and CXG motifs in CYP5014, CYP5015 and CYP5017 families. The P450s used for deducing amino acid combinations are shown in Table S9. Sequence logos were constructed as described in Methods. The amino acids and their percent occurrence at each of this domain are also presented. The invariant residues at these motifs were shown in bold with red font.

of the family[41]. Considering the large number of member P450s, in this study, we analysed amino acid combinations for P450 families such as CYP5014, CYP5015 and CYP5017 (Fig. 6 and Table S9). Analysis of the EXXR motif revealed that the first and fourth amino acids of this motif "E" and "R" is conserved in all P450 families CYP5014, CYP5015 and CYP5017 with rare exceptions. CYP5017F8 showed "K" instead of "E" and CYP5014N1 and CYP5015L showed "W" and "H" instead of "R" (Fig. 6). Non-conservation of "E" and "R" amino acids at the EXXR motif are reported rarely[50]. Leucine is the major amino acid appearing at the third position in this motif in all three oomycete P450 families. Threonine is the predominant amino acid at the second position in P450 families CYP5014 and CYP5017, whereas serine and aspargine are the predominant amino acids at this position in the CYP5015 family (Fig. 6). Compared to P450 families across the biological kingdoms[41], oomycete P450 families CYP5014 and CYP5017 also

showed ETLR as predominant amino pattern. However, the E-S/N-L-R amino acid pattern where "S/N" is the predominant amino acid at the second position is unique to the CYP5015 family and this pattern was not found in P450 families published in the literature[41]. Analysis of the CXG motif (FXXGXRXCXG) across the three P450 families revealed conservation of amino acids such as "F", "G" and "C" at the first, fourth and eighth positions. These amino acids at these positions are well known to be conserved in the P450s across the biological kingdoms[41,51,52], with some P450s showing variant amino acids at these positions[50]. The canonical amino acids "R" and "G" at the sixth and tenth positions are conserved in the CYP5014 family and are predominant in CYP5015 and CYP5017 (Fig. 6). The amino acid pattern at the CXG motif of the CYP5017 family is to some extent matched with the CYP94 and CYP704 families[41] where "Q" is dominant at the second position in all these P450 families. However, differences were found at the seventh and ninth position amino acids among the three P450 families CYP5017, CYP94 and CYP704. Comparison of CXG motif amino acid patterns for CYP5014 and CYP5015 with published P450 families CXG motif amino acid patterns[41] suggested that these families have unique amino acid patterns. This strongly supports the hypothesis previously proposed[41] that the amino acid pattern at these motifs is unique for a P450 family. Overall, amino acid patterns at the EXXR and CXG motifs of the three oomycete P450 families CYP5014, CYP5015 and CYP5017 are unique and these amino acid patterns (Fig. 6) can be considered characteristics of these P450 families.

Novel P450 fused proteins in oomycetes. P450s fused to redox partners and also to different proteins are well documented in the literature[53,54]. Two different types of P450 fused proteins were reported in lower eukaryotes. These two different types were: (i) P450 fused to CPR at the C-terminal end, which is well-known as P450foxy (CYP505 family)[55] and (ii) P450 fused to heme peroxidase/dioxygenase at the N-terminal end (CYP6001 family)[46].

Analysis of P450s in oomycetes revealed the presence of P450 fused proteins. The new P450 family CYP5619 with six members found in *S. declina* is fused to heme peroxidase/dioxygenase protein. However, the combination of fusion is different compared to the reported combination of P450 fused proteins in lower eukaryotes[53,54]. In oomycetes, the heme peroxidase/dioxygenase protein is fused at the C-terminal end to P450 (Fig. 7). This combination, i.e. N-terminal P450 domain fused to heme peroxidase/dioxygenase at its C-terminal, is a novel combination and not reported in the literature[53,54]. To confirm the novelty of this P450 fused protein, we performed comprehensive genome data mining to identify fused P450s, particularly heme peroxidase/dioxygenase protein fused to P450 in the publicly available lower eukaryote genomes[45]. A total number of 61 P450 fused proteins were identified (Fig. 7 and Table S10). The identified P450 fused proteins were grouped under five different P450 families namely CYP6001-CYP6005. Interestingly, the CYP6005 family is found only in Basidiomycota, whereas Ascomycota showed four different P450 fused families, CYP6001-CYP6004 (Fig. 7 and Table S10). The identified P450 fused proteins were subjected to heme peroxidase/dioxygenase and P450 domain analysis. As shown in Fig. 7, all the P450 fused proteins (CYP6001-CYP6005 family) identified in fungi showed an N-terminal heme peroxidase/dioxygenase domain and a P450 domain at the C-terminal end. This clearly confirms that the combination identified in CYP5619 family members is novel. Considering the nature of P450 fused protein, it can be concluded that CYP5619 family members are possibly involved in the oxidation of fatty acids, like CYP6001A1[46]. However, experimental analysis is needed to unravel the difference between the two different domains' combinations.

Functional analysis of oomycete P450s. All the oomycete P450s (except CYP51) were orphans, as functional data on oomycete P450s have not been reported. However, based on homology to characterized P450 proteins possible functional role(s) for oomycete P450s can be predicted. The CYP51 family identified in Saprolegniales plays a key role in synthesis of membrane sterols[32,33]. A recent study showed that CYP51 in these organisms can serve as a novel drug target[32,33]. Based on functional analysis of CYP6001A1 of *A. nidulans*[46] it is tempting to speculate that CYP5619 family members play a role in fatty acid hydroxylation. Based on homology to fatty acid hydroxylases CYP5014-CYP5017, family members may play a role in fatty acid metabolism[7]. The above predictions were based on homology data, as mentioned in this article (for CYP51 and CYP5169) and one published prediction (for CYP5014-CYP5017)[7]. Future study will involve unravelling the P450s' role, if any, in successful adaption to the parasitic and saprophytic lifestyle of oomycetes.

In conclusion, oomycetes are very important organisms in terms of their hard-wired parasitism leading to the loss of billions of dollars in agriculture and aquaculture. Analysis of P450s in these organisms provided insights into the evolutionary pattern of P450s. Genome-wide analysis of P450s revealed the presence of moderate number of P450s in these organisms. Despite presence of a large number of new P450 families and subfamilies, P450 family blooming resulted in a low P450 diversity in oomycetes. CYP51 and novel P450 fusion proteins with different combination of heme peroxidase/dioxygenase and P450 domain were common between oomycetes and with lower eukaryote fungi. The speciation and adaption to diverse ecological niches or lifestyle of oomycetes and fungi resulted in generation of distinct P450 families in both groups. Furthermore, at order level, oomycetes showed distinct P450 families and subfamilies. This confirms that the host influence is a major factor in shaping the oomycetes genomic content and thus also reflected in terms of P450s. Presence of unique combination of amino acid patterns at EXXR and CXG motifs in oomycete P450 families strongly supported previously proposed hypothesis

Figure 7. Phylogenetic and structural analysis of P450 fused proteins (heme peroxidase/dioxygenase fused to P450) between Oomycota and different fungal phyla. Sixty-six P450 fused proteins were used for the construction of a phylogenetic tree. Structural analysis of P450 fused proteins were carried out as described in 'Methods'. The heme peroxidase/dioxygenase and P450 domain boxes is indicative of the domain length. For three P450 fused proteins (CYP6005J1, CYP6005B1 and CYP6005B2) NCBI CDD[34] did not identify the P450 domain length, suggesting the presence of non-variant amino acids at P450 signature motifs in these proteins. A detailed analysis of the P450 fused proteins, species, size of the proteins and size of each of the domains is presented in Table S8.

that the amino acid patterns at these motifs are characteristic of a P450 family. This study serves as a reference and opened new vistas for future genome-wide annotation of P450s in oomycetes. Future genome sequencing of more oomycetes provides a broader picture of P450 evolution in these organisms and also possibly results in discovery of novel P450 families.

References

1. Nelson, D. R. Progress in tracing the evolutionary paths of cytochrome P450. *Biochim. Biophys. Acta.* **1814,** 14–18 (2011).
2. Nelson, D. R. A world of cytochrome P450s. *Phil. Trans. R. Soc. B. Biol. Sci.* **368,** 161220120430 (2013).
3. Sono, M., Roach, M. P., Coulter, E. D. & Dawson, J. H. Heme-containing oxygenases. *Chem. Rev.* **96,** 2841–2888 (1996).
4. Bernhardt, R. Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.* **124,** 128–145 (2006).

5. Kelly, S. L. & Kelly, D. E. Microbial cytochrome P450: Biodiversity and biotechnology, where do cytochrome P450 come from, what do they do and what can they do for us? *Phil. Trans. R. Soc. B. Biol. Sci.* **368,** 161220120430 (2013).
6. Deng, J., Carbone, I. & Dean, R. A. The evolutionary history of cytochrome P450 genes in four filamentous Ascomycetes. *BMC Evol. Biol.* **7,** 30 (2007).
7. Moktali, V. *et al.* Systematic and searchable classification of cytochrome P450 proteins encoded by fungal and oomycete genomes. *BMC Genomics.* **13,** 525 (2012).
8. Chen, W. *et al.* Fungal cytochrome P450 monooxygenases: Their distribution, structure, functions, family expansion, and evolutionary origin. *Genome Biol. Evol.* **6,** 1620–34 (2014).
9. Kgosiemang, I. K. R., Mashele, S. S. & Syed, K. Comparative genomics and evolutionary analysis of cytochrome P450 monooxygenases in fungal subphylum saccharomycotina. *J. Pure Appl. Microbiol.* **8,** 291–302 (2014).
10. Syed, K., Nelson, D. R., Riley, R. & Yadav, J. S. Genome-wide annotation and comparative genomics of cytochrome P450 monooxygenases (P450s) in the Polyporale species *Bjerkandera adusta*, *Ganoderma* sp. and *Phlebia brevispora*. *Mycologia* **105,** 1445–1455 (2013).
11. Syed, K. *et al.* Genome-wide identification, annotation and characterization of novel thermostable cytochrome P450 monooxygenases from the thermophilic biomass-degrading fungi *Thielavia terrestris* and *Myceliophthora thermophila*. *Genes Genom.* **36,** 321–333 (2014).
12. Syed, K., Doddapaneni, H., Subramanian, V., Lam, Y. W. & Yadav, J. S. Genome-to-function characterization of novel fungal P450 monooxygenases oxidizing polycyclic aromatic hydrocarbons (PAHs). *Biochem. Biophys. Res. Commun.* **399,** 492–497 (2010).
13. Hirosue, S. *et al.* Insight into functional diversity of cytochrome P450 in the white-rot basidiomycete *Phanerochaete chrysosporium*: Involvement of versatile monooxygenase. *Biochem. Biophys. Res. Commun.* **407,** 118–23 (2011).
14. Ide, M., Ichinose, H. & Wariishi, H. Molecular identification and functional characterization of cytochrome P450 monooxygenases from the brown-rot basidiomycete *Postia placenta*. *Arch. Microbiol.* **194,** 243–53 (2012).
15. Syed, K., Porollo, A., Lam, Y. W., Grimmett, P. E. & Yadav, J. S. CYP63A2, a catalytically versatile fungal P450 monooxygenase capable of oxidizing higher-molecular-weight polycyclic aromatic hydrocarbons, alkylphenols, and alkanes. *Appl. Environ. Microbiol.* **79,** 2692–7020 (2013).
16. Syed, K., Shale, K., Pagadala, N. S. & Tuszynski, J. Systematic identification and evolutionary analysis of catalytically versatile cytochrome P450 monooxygenase families enriched in model basidiomycete fungi. *PLoS ONE* **9(1),** e86683 (2014).
17. Syed, K., Porollo, A., Miller, D. & Yadav, J. S. Rational engineering of the fungal P450 monooxygenase CYP5136A3 to improve its oxidizing activity toward polycyclic aromatic hydrocarbons. *Protein Eng. Des. Sel.* **26,** 553–557 (2013).
18. Yoshida, Y. Cytochrome P450 of fungi: Primary target for azole antifungal agents. *Cur. Top. Med. Mycol.* **2,** 388–418 (1988).
19. Jawallapersand, P. *et al.* Cytochrome P450 monooxygenase CYP53 family in fungi: Comparative structural and evolutionary analysis and its role as a common alternative anti-fungal drug target. *PLoS ONE* **9(9),** e107209 (2014).
20. Kamoun, S. Molecular genetics of pathogenic oomycetes. *Eukaryot. Cell* **2,** 191–199 (2003).
21. Lamour, K. & Kamoun, S. *Oomycete genetics and genomics: Diversity, interactions, and research tools*. John Wiley & Sons, Inc., Hoboken, New Jersey, USA (2009).
22. Beakes, G. W., Clocking, S. & Sekimoto, S. The evolutionary phylogeny of oomycete "fungi". *Protoplasma* **249,** 3–19 (2012).
23. Phillips, A. J., Anderson, V. L., Robertson, E. J., Secombes, C. J. & van West, P. New insights into animal pathogenic oomycetes. *Trends Microbiol.* **16,** 13–9 (2008).
24. Soanes, D. M., Richards, T. A. & Talbot, N. J. Insights from sequencing fungal and oomycete genomes: What can we learn about plant disease and the evolution of pathogenicity? *Plant Cell* **19,** 3318–3326 (2007).
25. Tyler, B. M. *et al.* Phythophthora genome sequencing uncovers evolutionary origins and mechanisms of pathogenesis. *Science* **313,** 1261–1266 (2006).
26. Haas, B. J. *et al.* Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* **461,** 393–8 (2009).
27. Lévesque, C. A. *et al.* Genome sequence of the necrotrophic plant pathogen *Pythium ultimum* reveals original pathogenicity mechanisms and effector repertoire. *Genome Biol.* **11,** R73 (2010).
28. Baxter, L. *et al.* Signatures of adaptation to obligate biotrophy in the *Hyaloperonospora arabidopsidis* genome. *Science* **330,** 1549–51 (2010).
29. Lamour, K. H., Stam, R., Jupe, J. & Huitema, E. The oomycete broad-host-range pathogen *Phytophthora capsici*. *Mol. Plant. Pathol.* **13,** 329–37 (2012).
30. Adhikari, B. N., Hamilton, J. P. & Zerillo, M. M. Comparative genomics reveals insight into virulence strategies of plant pathogenic oomycetes. *PLoS ONE* **8(10),** e75072 (2013).
31. Jiang, R. H. Y. *et al.* Distinctive expansion of potential virulence genes in the genome of the oomycete fish pathogen *Saprolegnia parasitica*. *PLOS Genet.* **9,** e1003272 (2013).
32. Madoui, M. A., Bertrand-Michel, J., Gaulin, E. & Dumas, B. Sterol metabolism in the oomycete *Aphanomyces euteiches*, a legume root pathogen. *New Phytol.* **183,** 291–300 (2009).
33. Warrilow, A. G. *et al.* Clotrimazole as a potent agent for treating the oomycete fish pathogen *Saprolegnia parasitica* through inhibition of sterol 14α-demethylase (CYP51). *Appl. Environ. Microbiol.* **80,** 6154–66 (2014).
34. Marchler-Bauer, A. *et al.* CDD: A conserved domain database for the functional annotation of proteins. *Nucleic Acids Res.* **39,** D225–9 (2011).
35. Nelson, D. R. The cytochrome P450 homepage. *Human Genomics* **4,** 59–65 (2009).
36. Eddy, S. R. Accelerated profile HMM searches. *PLoS Comp. Biol.* **7,** e1002195 (2011).
37. Finn, R. D. *et al.* The Pfam protein families database. *Nucleic Acids Res.* **42,** D222–D230 (2014).
38. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree: Computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* **26,** 1641–1650 (2009).
39. Letunic, I. & Bork, P. Interactive Tree of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* **23,** 127–128 (2007).
40. Larkin, M. A. *et al.* Clustal W and Clutal X version 2.0. *Bioinformatics* **23,** 2947–2948 (2007).
41. Syed, K. & Mashele, S. S. Comparative analysis of P450 signature motifs EXXR and CXG in the large and diverse kingdom of fungi: Identification of evolutionarily conserved amino acid patterns characteristic of P450 family. *PLoS ONE* **9(4),** e95616 (2014).
42. Tamura, K. *et al.* MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28,** 2731–2739 (2011).
43. Schneider, T. D. & Stephens, R. M. Sequence logos: A new way to display consensus sequences. *Nucleic Acids Res.* **18,** 6097–6100 (1990).
44. Crooks, G. E., Hon, G., Chandonia, J. M. & Brenner, S. E. WebLogo: A sequence logo generator. *Genome Research* **14,** 1188–1190 (2004).
45. Grigoriev, I. V. *et al.* MycoCosm portal: Gearing up for 1000 fungal genomes. *Nucleic Acids Res.* **42,** D699–704 (2014).

46. Brodhun, F., Göbel, C., Hornung, E. & Feussner, I. Identification of PpoA from *Aspergillus nidulans* as a fusion protein of a fatty acid heme dioxygenase/peroxidase and a cytochrome P450. *J. Biol. Chem.* **284,** 11792–11805 (2009).
47. Rzhetsky, A. & Nei, M. A simple method for estimating and testing minimum evolution trees. *Mol. Biol. Evol.* **9,** 945–967 (1992).
48. Feyereisen, R. Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochim. Biophys. Acta.* **1814,** 19–28 (2011).
49. Nelson, D. R. Metazoan cytochrome P450 evolution. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **121,** 15–22 (1998).
50. Sezutsu, H., Le Goff, G. & Feyereisen, R. Origins of P450 diversity. *Phil. Trans. R. Soc. B. Biol. Sci.* **368,** 20120428 (2013).
51. Gotoh, O. Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J. Biol. Chem.* **267,** 83–90 (1992).
52. Sirim, D., Widmann, M., Wagner, F. & Pleiss, J. Prediction and analysis of the modular structure of cytochrome P450 monooxygenases. *BMC Struct. Biol.* **10,** 34 (2010).
53. Guengerich, F. P. & Munro, A. W. Unusual cytochrome p450 enzymes and reactions. *J. Biol. Chem.* **288,** 17065–73 (2013).
54. Lamb, D. C. & Waterman, M. R. Unusual properties of the cytochrome P450 superfamily. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* **368,** 20120434 (2013).
55. Kitazume, T., Takaya, N., Nakayama, N. & Shoun, H. *Fusarium oxysporum* fatty-acid subterminal hydroxylase (CYP505) is a membrane bound eukaryotic counterpart of *Bacillus megaterium* cytochrome P450BM3. *J. Biol. Chem.* **275,** 39734–39740 (2000).

Acknowledgements

K.S. and S.S.M. express their sincere gratitude to the Central University of Technology (CUT), Bloemfontein, Free State, South Africa for a grant from the University Research and Innovation Fund and Emerging Researcher Award (to K.S.). K.S. and S.S.M. thank the Department of Higher Education and Training (DHET), South Africa for a research grant. S.S.M. also thanks the National Research Foundation (NRF), South Africa for the research grant. K.S. thanks Dr. C. Robin Buell, Department of Plant Biology, Michigan State University, Michigan, USA for allowing the use of Pythium species genome sequencing data. The authors also want to extend their thanks to Ms Barbara Bradley, Pretoria, South Africa for English language editing.

Author Contributions

K.S. and S.S.M. conceived and designed the experiments, M.M.S., N.J., D.R.N., W.C., J.-H.Y., M.P., I.K.R.K., L.Q., N.T.M., R.M., S.C.R., S.S.M. and K.S. performed the experiments, analysed the data, contributed reagents/materials/analysis tools and were involved in writing the manuscript. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Sello, M. M. *et al.* Diversity and Evolution of Cytochrome P450 Monooxygenases in Oomycetes. *Sci. Rep.* **5**, 11572; doi: 10.1038/srep11572 (2015).