Elucidating the evolutionary and structural features of cytochrome P450 genes in *Cryptococcus neoformans* using in-silico approaches

[1] Wadzani Palnam Dauda, [2] Israel Ogwuche Ogra, [3] Peter Abraham, [4] Charles Oluwaseun Adetunji, [5] Elkanah Glen, [6] Daji Morumda, [7] Grace Wabba Peter, [8] Shittu Emmanuel Abraham, and [9] Mawuli Kwamla Azameti

1 Crop Science Unit, Department of Agronomy, Federal University, Gashua, Yobe State, Nigeria
2 UNESCO International Centre for Biotechnology, Nsukka, Nigeria
3 Department of Horticulture, Federal College of Horticulture, Dadin Kowa, Gombe State, Nigeria,
4 Applied Microbiology, Biotechnology and Nanotechnology Laboratory, Department of Microbiology, Edo University Iyamho, PMB 04, Auchi, Edo State, Nigeria
5 Department of Biochemistry, Federal University Lokoja, Kogi State, Nigeria
6 Department of Microbiology, Federal University Wukari, Taraba State, Nigeria
7 Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria
8 Department of Agronomy, Bayero University Kano, Nigeria
9 Division of Molecular Biology and Biotechnology, Indian Agricultural Research Institute, New Delhi, India

Corresponding Author: Wadzanidauda@gmail.com

Abstract

Cytochrome P450 (CYP450) enzymes have been shown to contribute to the fungal exploration of different niches. Elucidation and annotation of the ever-expanding genomic information of CYP450 enzymes is of great importance. A detailed study on the CYP450 genes in *Cryptococcus neoformans* is not yet available. The present study, employed bioinformatics tools to annotate forty identified CYP genes of *C. neoformans* var. *neoformans* JEC21 based on their phylogeny, motif, gene structure, family and clan classification and subcellular localization. It was established during this study that forty CYP450 were phylogenetically grouped into eight clades. A total of five conserved motifs were identified which are valuable as molecular markers for the identification of *C. neoformans*. The number of introns ranges from two to nine while that of exons ranges from three to ten with only CNJ03430 gene sequence shown to possess an untranslated region. *C. neoformans* genes were found to belong to only two Cytochrome P450 families and clans (CYP51 and CYP61) that carry out specifically primary metabolism and ergosterol metabolism in the cell. The cytochrome P450 proteins were distributed across the different organelles of the cell, which shows a wide range of involvement of this enzyme class in cellular functions and activities. The dominant subcellular location is the cytoplasm. The findings from this study have provided the requisite insight on the Cytochrome P450 genes in *Cryptococcus neoformans* which could be harnessed as a basis to genetically manipulate different genes to alter their functions to curb the fatal sickness caused this important pathogen.

Keywords: *Cryptococcus neoformans*, Cytochrome P450, Evolutionary history, Gene duplication, Subcellular localization.

Introduction

Cryptococcus neoformans is an encapsulated fungal pathogen causing fatal meningitis in humans (Vechi et al., 2019). *C. neoformans* is unique among the most common human fungal pathogens from the family basidiomycete (McClelland et al., 2014) and exhibits an evolutionary divergence from more common pathogenic ascomycetes (Yang et al., 2012). *Cryptococcus neoformans* possess two specialized virulence factors. These include polysaccharide capsule, which inhibits phagocytosis, and melanin, which functions as an antioxidant (Perfect, 2006; Gaylord et al., 2020). The normal vegetative form of *C. neoformans* is the yeast form (Loftus et al., 2005). *Cryptococcus neoformans* is an opportunistic disease-causing fungus with global occurrences. It is characterized as soil-borne and reported to be associated with excreta from avians (Li et al., 2010). It is the predominant pathogen causing meningoencephalitis in people with weakened immune systems, such as HIV-AIDS patients however, healthy individuals have also been reported to be infected (Kwon-Chung et al., 2014). The inhalation of air that is contaminated with either basidiospores or haploid yeasts is known to be a major route of infection by *C. neoformans* (Yang et al., 2012). A total of thirty-eight species of *Cryptococcus* from the *Filobasidiella* genus have traditionally grouped into five serotypes namely A, B, C, D and AD. However, two strains of *Cryptococcus neoformans*; *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. grubii (serotype A) have been reported to be pathogenic with the former being more important worldwide (Li et al., 2010). Genetically, four serotypes of *C. neoformans* var. *neoformans* are identified: VNI under serotype A class, VNII under serotype A class, VNIV under serotype D and VNIII under serotype AD class out of which VNI serotype A is known to be the most virulent and globally infectious serotype inciting systemic

polysaccharide capsule, which inhibits phagocytosis, and melanin, which functions as an antioxidant (Gaylord et al., 2020; Perfect, 2006). The normal vegetative form of C. neoformans is the yeast form (Loftus et al., 2005). *Cryptococcus neoformans* is an opportunistic disease-causing fungus with global occurrences. It is characterized as soil-borne and reported to be associated with excreta from avians (Li et al., 2010). It is the predominant pathogen causing meningoencephalitis in people with weakened immune systems, such as HIV-AIDS patients however, healthy individuals have also been reported to be infected (Kwon-Chung et al., 2014). The inhalation of air that is contaminated with either basidiospores or haploid yeasts is known to be a major route of infection by *C. neoformans* (Yang et al., 2012). A total of thirty-eight species of *Cryptococcus* from the *Filobasidiella* genus have traditionally grouped into five serotypes namely A, B, C, D and AD. However, two strains of *Cryptococcus neoformans; C. neoformans var. neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A) have been reported to be pathogenic with the former being more important worldwide (Li et al., 2010). Genetically, four serotypes of *C. neoformans* var. *neoformans* are identified: VNI under serotype A class, VNII under serotype A class, VNIV under serotype D and VNIII under serotype AD class out of which VNI serotype A is known to be the most virulent and globally infectious serotype inciting systemic *cryptococcosis* that could severely lead to meningoencephalitis (Hitchcock and Xu, 2022). The available antifungal agents are very few, and there is a higher level of drug-resistant strains.

A robust study on CYP450 genes and their functions was well annotated in several important fungal organisms including *Aspergillus nidulance* (Kelly et al., 2009), *Trichoderma* spp., (Chadha et al., 2018), *Phanerochaete chrysosporium* (Sayedet al., 2012) *Mycosphaerella graminicola* (Newsome et al., 2013), *Grosmannia clavigera* (Lahet al., 2013), *Fusarium* species (Dauda et al., 2021a), Aspergillus species (Dauda et al., 2022a), *Alternaria* spp., (Dauda et al., 2022b), and *Candida tropicalis* (Dauda et al., 2022c). However, this is lacking in Cryptococcus neoformans. A detailed study on the phylogenetic, conserved Motif, intron-exon structure family and clan classification and cellular localization analyses of CYP450s in *Cryptococcus neoformans* could broaden our understanding of the evolutionary processes, structural and functional roles of CYP450 genes in the pathogen for that can be harnessed for its management. Hence, the objective of the present study is to use in-silico approaches to elucidate the evolutionary and structural features of cytochrome P450 genes in *Cryptococcus neoformans* var. *neoformans* JEC21.

Materials and Methods

Retrieval of CYP450 Gene Sequences

Forty CYP540 protein gene sequences out of 6, 475 total genes of *Cryptococcus neoformans* var. neoformans JEC21 were retrieved from the MycoCosm fungal genome database of the Joint Genome Institute (JGI) (https://mycocosm.jgi.doe.gov/pages/search-for-genes.jsf?organism=Cryne_JEC21_1) on 23/10/2020, 4.55 am.

Annotation of CYPs

The conserved domains of CYPs in *Cryptococcus neoformans* were initially determined in the NCBI Conserved Domain Database. The identified CYPs conserved domains were further verified in the Nelson's P450 database against all known fungal CYPs (http://p450.riceblast.snu.ac.kr) (Park et al., 2008) with an E-value of 10-4 (Nelson, 2009). Sequences with up to 40 % similarity were assigned into the same families as rule described in the International P450 Nomenclature Committee Database (https://drnelson.uthsc.edu/Cytochrome P450.html) (Nelson, 1999) and the Fungal Cytochrome P450 Database - FCPD (http://p450.riceblast.snu.ac.kr) (Park et al., 2008).

Structural Feature Analysis of CYPs in *C. neoformans*

The Conserved Domain Database was used to identify Cytochrome P450 conserved domains present in *Cryptococcus neoformans* (Marchler-Bauer et al., 2011). Structural features were then explored in order to reveal the phylogenetic relationship in relation to the conserved domain patterns of the CYPs.

Phylogenetic Analysis of Cytochrome P450s in *Cryptococcus neoformans*

The evolutionary relationship was analyzed using 40 amino acid sequences after their alignment by MUSCLE (Edgar, 2004). The pairwise deletion was used to clear off all ambiguous positions on each sequence. The Phylogenetic tree was conducted in MEGA X (Kumar et al., 2018) as described by Dauda et al. (2021b) using the Neighbour-Joining method (Saitou and Nei, 1987; Tomczyk et al., 2016). The Poisson correction method was used to compute the evolutionary distances (Zuckerkandl and Pauling, 1965; Tomczyk et al., 2016).

Identification of motif in *Cryptococcus neoformans* CYP450 genes

Motif analysis was carried out with the aid of MEME online software (http://meme-suite.org/) (Bailey and Elkan, 1994; Bailey et al., 2006; Bailey et al., 2009). Attributes employed in carrying out the analysis are the expected number of motifs: 5, lowest and highest with of motif: 6 and 50 amino acids respectively, motif occurrence distribution: 0 or 1 per sequence. The discovery mode was set at 'classic' and E-value set at default while any repetition number was selected for site distribution.

Analysis of cytochrome P450 genes structure in *Cryptococcus neoformans*

The Cryptococcus neoformans CYP450 gene structures were annotated in an online server (http://gsds.gao-lab.org/index.php) (Hu et ol. 2014). Sequences were

imputed in FASTA format. The transcript FASTA was imputed in the first layout, followed by the Genomic FASTA. Other features to display were set at default. Output (phylogenetic tree/order) was also set at default.

Identification of CYP Clans and Families in Cryptococcus neoformans

The clans and families of CYP genes in Cryptococcus neoformans was identified using the online server of FCPD (http://p450.riceblast.snu.ac.kr/index.php?a=view) on Cytochrome P450 homepage (https://drnelson.uthsc.edu/CytochromeP450.html) (Moktali et al., 2012)

Sub-cellular localization analysis of Cryptococcus neoformans cytochrome P450 gene sequence

An online integrative subcellular location predictor tool was utilized to identify the determined the cellular localization of CYP450 genes in C. neoformans. These predictor tools include Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) (Kuo-Chen Chou and Hong-Bin Shen, 2010), BUSCA (http://busca.biocomp.unibo.it/) (Savojardo et al., 2018), YLoc (https://abi-services.informatik.uni-tuebingen.de/yloc/webloc.cgi) (Sebastian et al., 2010) and SherLoc (https://abi-services.informatik.uni-tuebingen.de/sherloc2/webloc.cgi) (Briesemeister et al., 2009). The protein FASTA sequence was uploaded into the server, and ambiguous parts of the sequence ID were deleted to suit the specifications of the server. All settings were left at default and the analysis was conducted in batches.

Results and Discussion

Phylogenetic Tree of Cryptococcus neoformans cytochrome P450, clade features and evolutionary events

The phylogeny of all the cytochrome P450s in Cryptococcus neoformanswas constructed based on their consensus sequences and features of their respective clades were analyzed. The results obtained (Figure 1) showed a level of evolutionary divergence indicated by the branches of the genes in the phylogenetic tree. Each of the clades was presented as monophyletic members of a given ancestor. Clade H has the largest number of members. Clade B and D have the least number with just three (3) members.

This analysis shows that most of the cytochrome P450 families in Cryptococcus neoformans have a close phylogenetic relationship. The diversity must have mainly been a result of gene duplication (Deng et al., 2007; Chadha et al., 2018). The occurrence of multiple identical members depicts their common ancestral origin and can be due to a recent gene duplication event

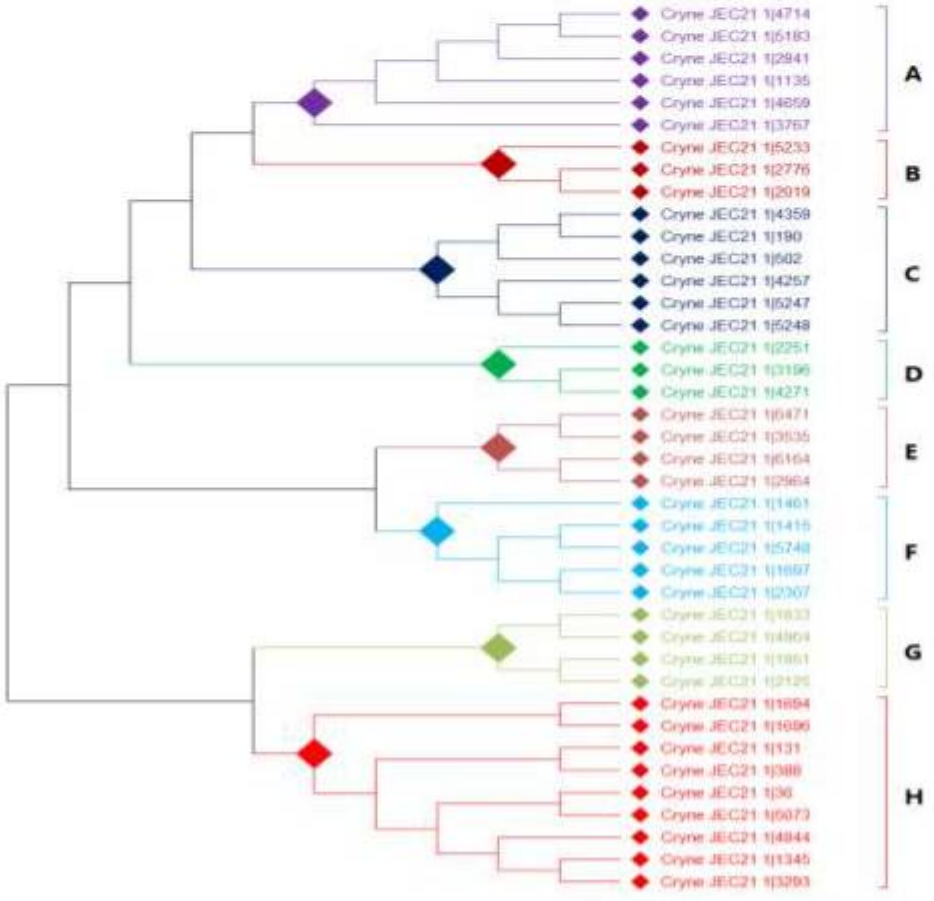


Figure 1: Evolutionary relationship of cytochrome P450 proteins in *Cryptococcus neoformans*.

(Sezutsu et al., 2013). On the other hand, over time, these duplications gave rise to redundant genes, which diverged into new families of cytochrome P450. The close relationships among the cytochrome P450 families are a possible indication of duplication events that occurred in the fungal evolutionary history. Generally, it has been shown that the blooms and great diversity of cytochrome P450s could be due to gene duplication (Feyereisen, 2011). The ability of fungal species to possess several CYP450 genes has been reported to serve as the adaptive strategy providing superior metabolic requirements for survival in various ecological niches (Pratiwi et al., 2022). Rampersad, (2020) and Dauda et al. (2021a) also opined that the prevalence and expansion of CYPs along the timeline of evolutionary descents could to be associated with the development of several fungal traits, including host specificity and pathogenicity.

Conserved Motifs identification among cytochrome P450 genes of *Cryptococcus neoformans*

Moreover, it was discovered that Motif 1, 2, 3, and 4 in figures 2a & 2b are conserved on the CNJ03430, CNC00020, CNF00010, and CNF04900 sequences. In the second sequence, motifs 1 and 4 are also conserved. Motif 5 occurred singly on the CNM00470, CNE00710, CNF02510, CNG00600, and CNG00600 sequences. There is not much discrepancy in the conserved motifs between the first five gene sequences, and the last five gene sequences have the same conserved motif.

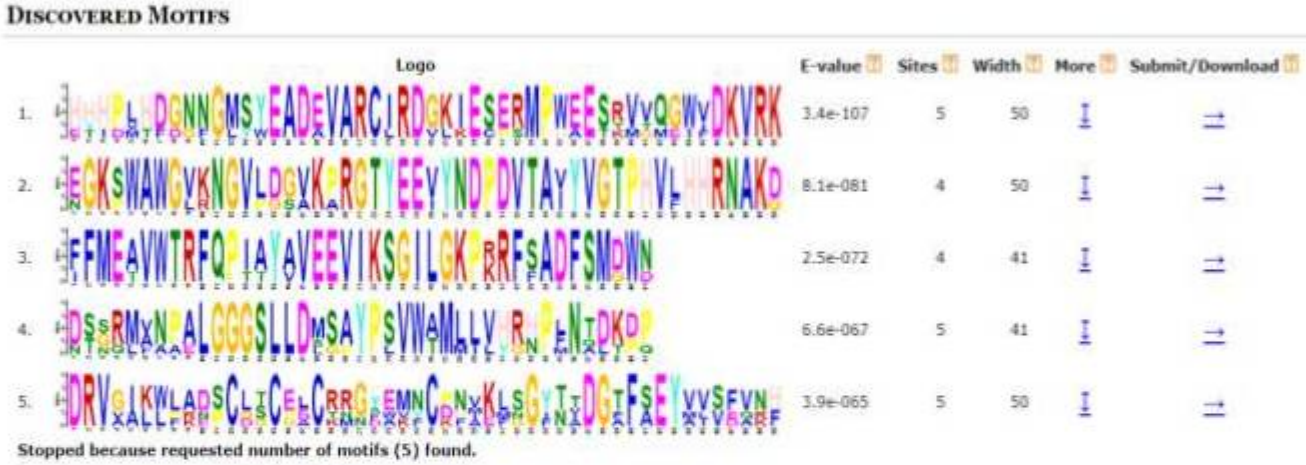


Figure 2a: *Discovered motifs logo among cytochrome P450 genes of Cryptococcus neoformans, showing their E-value, width and number of sites they can be found using MEME Motif discovery analysis*

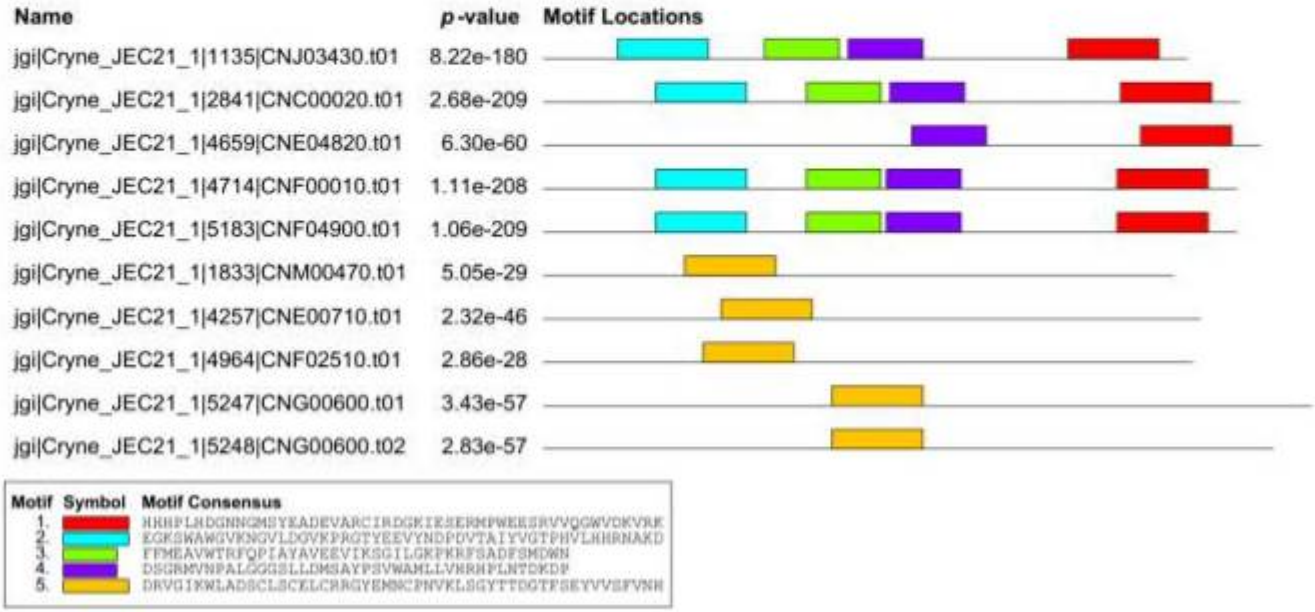


Figure 2b: *Conserved Motif identified among cytochrome P450 genes of Cryptococcus neoformans. The name of the gene and E-value are indicated on the left side, every colour signifies a particular motif and the length of the block indicates motif length.*

The conserved domains observed here are few which are most likely responsible for the significant characteristics of the cytochrome P450 proteins and thus preserving its tertiary structure and enzyme function (Deng et al., 2007: Moktali et al., 2012).

Fungal species possessing cyp genes belonging to CYP51 and CYP61 are reported to be highly stabilized in the conservation of ancestral fungal traits through evolution (Doddapaneni et al., 2005) as expressed in motif signatures. The motif signatures elucidated in this study that are common across or most cyps sequences of *C. neoformans* play a major role as housekeeping genes that could be explored as molecular markers (primers) for its identification and characterization (Dauda et al., 2022a).

Gene structure analysis in Cryptococcus neoformans Cytochrome P450

The intron-exon structure was analyzed to observe the structural relationships among cytochrome P450 genes in *Cryptococcus neoformans* as shown in Figure 3. The results showed that all of the gene sequences have introns and the CNJ03430 gene sequence has an untranslated region. The CNG00600 and CNI04380 gene sequences have the lowest exons (three) and introns (two) number, while the CNC04570, CNM00470, and CNM01710 gene sequences have the highest number of exons (ten) and introns (nine).

Therefore, in *Cryptococcus neoformans* CYP450 genes, the intron number counts range from two to nine while that of exons ranges from three to ten, implying a high possibility of gene product manipulation. The forty Cytochrome P450 genes of *Cryptococcus neoformans* have a total of two hundred and eleven introns. The ranges of introns across the forty genes vary from two to nine. These findings agree with the earlier report of Harshavardhan et al. (2005) who discovered that the fully formed P450 genes have numerous exons that are frequently intercepted by short introns and only eukaryotes are known to have introns in their gene structure.

The observed variation in the CYP450s of *C. neoformans* based on their number of exon/intron or length of genes affects the number of genetic traits they possess and the extent of functions they perform (Rose, 2019) which implies that longer cyp gene sequences contain more genetic information and functions than those with shorter cyp gene sequences. A higher number of introns in gene sequence has been reported to increase gene length and shown to play a significant role in the regulation; expression of gene functions, genetic variation and multiple encoding of proteins through gene duplication, deletion and recombination during gene splicing process (Wong et al., 2015; Rose, 2019).

Identification of Family and Clan classification of CYP450 Genes in *Cryptococcus neoformans*

Furthermore, it was discovered in the present study that CYP450 genes in *Cryptococcus neoformans* belong to CYP51 and CYP61 families and the putative functions of these genes are primary metabolism and ergosterol metabolism as shown in Table 1. These findings agree with Harshavardhan et al. (2005) where the CYP51 family was found as one of the most functionally well-featured and conserved P450 gene families in fungi that encodes the cell wall ergosterol biosynthesis reaction.

The above-mentioned work also confirmed the involvement of the CYP61 clan in the later stages of the ergosterol pathway. Nelson (2018), has previously reported fungal P450s to be clustered into 805 families.CYP61 has been reported by Venegas et al., (2020) to be responsible for the coding of sterol 22 desaturase, which plays a significant role in the advanced phase of the ergosterol pathway in metabolizing Ergosta-5,7,24 (28)-trienol to Ergosta5,7,22,24(28)-tetra enol by introducing a C-22(23) double bond in the sterol side chain. CYP51 is known to be involved in sterol biosynthesis in basidiomycetes and ascomycetes and is known as housekeeping CYP. Several cytochrome P450 have been reported to be linked with pathogen virulence due to their ability to neutralize antibiotics produced by the host (Chadha et al., 2018)

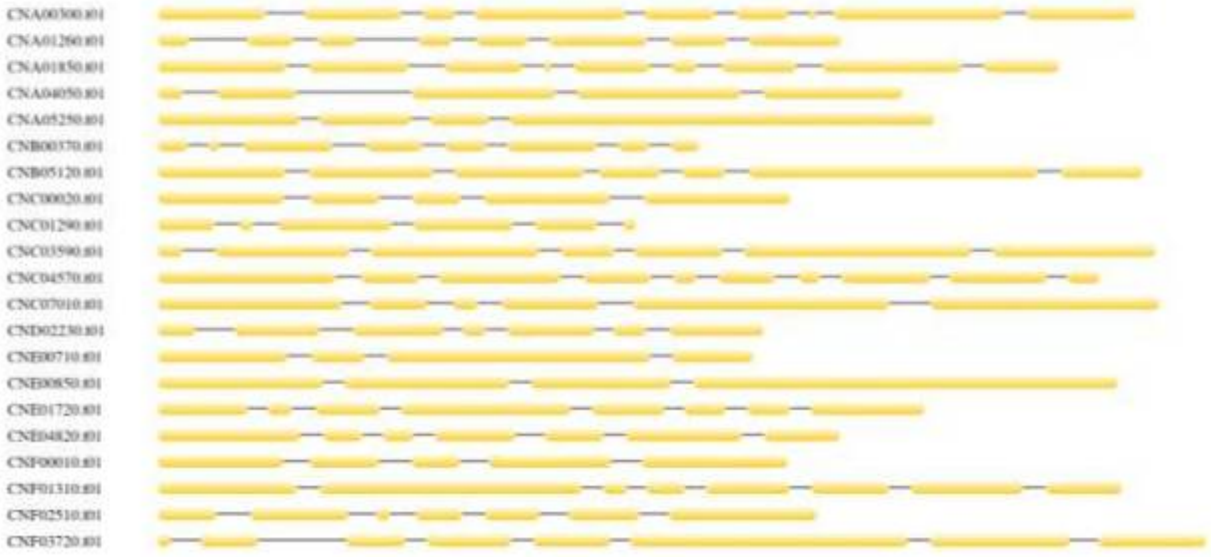




Figure 3: *Gene map (introns and exons) of CYP450 genes in C. neoformans. The scale is designed based on the largest gene. Exon region of the gene is shown by brown color, while intron portion is shown by black line.*

These features have made the two clans a major target for most antifungal control of fungal diseases in human (Shin et al., 2018) which could also be applicable in the queried fungal species.

Sub-cellular localization of Cryptococcus neoformans cytochrome p450 gene sequence:

The sub-cellular localization analysis (Table 2) revealed that the entire *Cryptococcus neoformans* P450 proteins are distributed in the cytoplasm, mitochondrion, nucleus, endoplasmic reticulum, chloroplast, peroxisome, cell membrane, and extracellularly unlike the CaDCL proteins of *Coffea Arabica*, which are distributed in the nucleus, cytoplasm, and plasma membrane (Parvez et al., 2019).

The wide distribution of this enzyme across different organelles suggests its importance and level of involvement in cellular activities. The predominant localization of the enzyme family in the cytoplasm compared with other subcellular locations may play a crucial role in the pathogenicity of this fungus as previously reported by Kizawa et al. (1991) where cytosolic cytochrome P450 from *F. oxysporum* was first isolated and characterized and recently confirmed by Dauda et al. (2021a).

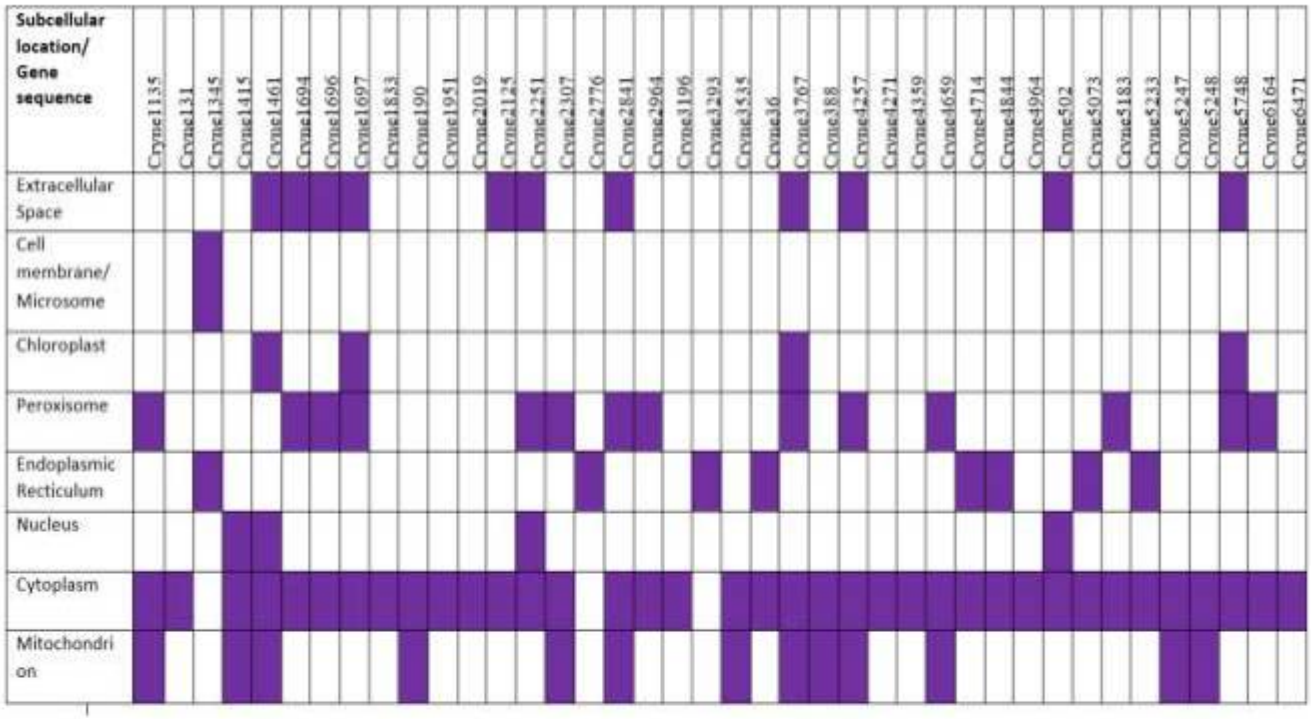
Conclusion

It was established in this study that forty cytochrome P450 genes were available in *Cryptococcus neoformans*. The phylogenetic analysis shows that cyp genes were clustered into eight clades. CYP450 genes in *C. neoformans* possess characteristic conserved motifs that could be valuable as housekeeping genes and molecular markers for the identification of the pathogen. Introns and exons of cyp genes in *C. neoformans* range from two to nine and three to ten respectively, implying a high possibility of gene product manipulation and functions. CYP51 and CYP61 were identified as the two clans and families of cyp genes in *C. neoformans* playing primary metabolic function which could be a major target for the control of this pathogen. The cytochrome P450 proteins were distributed across the different organelles of the cell, but showed a wide range of localization in the cytoplasm, suggesting their crucial function including pathogenicity. The findings of this study could provide the requisite knowledge required to genetically manipulate different genes in this family as a way of altering their functions to curb the fatal sickness caused by *Cryptococcus neoformans*.

Table 1: *Family and Clan classification of CYP450 Genes in Cryptococcus neoformans*

Clan	Family	Putative Functions
CYP51	CYP51	Primary metabolism Ergosterol Metabolism
CYP61	CYP61	Primary Metabolism Ergosterol Metabolism

Table 2: *Prediction of subcellular localization of Cryptococcus neoformans gene sequence*



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