Molecular Phylogenetics and Evolution 206 (2025) 108310

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Deciphering the distribution and types of Multicopper oxidases in Basidiomycota fungi

Gonzalo Molpeceres, Pablo Aza, Iván Ayuso-Fernández, Guillermo Padilla, Francisco Javier Ruiz-Dueñas, Susana Camarero *

Centro de Investigaciones Biológicas Margarita Salas, Consejo Superior de Investigaciones Científicas (CSIC), 28040 Madrid, Spain

ARTICLE INFO

Keywords:
Basidiomycota Fungi
Multicopper oxidase
Phylogeny
Laccase
Classification
Laccase-type enzymes

ABSTRACT

Multicopper oxidases (MCOs) comprise different types of enzymes widely distributed in nature with quite diverse functions. Laccases are the most interesting MCOs from a biotechnological point of view, particularly those secreted by ligninolytic Basidiomycota fungi due to their versatility to oxidize lignin and a variety of aromatic substrates. The term "laccase" has been broadly (but sometimes erroneously) applied due to their low sequence homology and some overlapping activities with other MCO groups.

We examined the distribution and phylogenetic relationships of MCOs in Basidiomycota fungi aiming to provide a complete and precise picture of the different MCO types across the division, including fungal orders phylogenetically distant from those typically studied. The phylogenetic tree revealed eight clusters of MCOs, each sharing common sequence/structural features. With this information we classified the MCOs in eight groups and described their distinctive amino acid residues. These eight MCO types are: laccases (LAC), ferroxidases (FOX), laccase-ferroxidases (LAC-FOX), ascorbate oxidases (AO), fungal pigment MCOs, and three new groups of laccase-like enzymes or "atypical laccases" related to but different from laccases sensu stricto, namely novel laccases (NLAC), new MCO (NMCO) and new laccases with potential ferroxidase activity (NLF). Additionally, several MCOs already described in the literature were reclassified into the updated groups.

1. Introduction

The multicopper oxidase (MCO) superfamily comprises diverse blue copper proteins such as laccases (EC 1.10.3.2), ascorbate oxidases (EC 1.10.3.3) or ferroxidases (EC 1.16.3.1) that share a common structural folding in cupredoxin-type domains. Commonly, they hold a minimum of four catalytic copper ions coordinated to the protein backbone for the oxidation of different types of substrates coupled to reduction of molecular oxygen to water. The coppers are categorized into three types (T1–T3) based on their spectroscopic properties (Kosman, 2010; Solomon et al., 1996). The blue T1 copper catalyzes the oxidation of the reducing substrate. The electrons are transferred via the copper ligands to the trinuclear copper cluster (TNC), which consists of one type 2 (T2) and two type 3 (T3) coppers, where oxygen is reduced to water (Jones & Solomon, 2015; Solomon et al., 1996). MCOs are widely distributed in nature, having been found in bacteria, fungi, plants, arthropods, and vertebrate animals (including humans) (Claus, 2004; Dittmer & Kanost, 2010; Vashchenko & MacGillivray, 2013). Their physiological roles include copper homeostasis, pigment formation, sporulation, morphogenesis, stress defense, virulence, cuticle sclerotization in arthropods, and participation in plant–pathogen interactions, and iron and ascorbate metabolism (Dittmer & Kanost, 2010; Janusz et al., 2020; Mayer & Staples, 2002; Morozova et al., 2007; Vashchenko & MacGillivray, 2013).

Laccases play a fundamental role in the global carbon cycle, participating in lignin biosynthesis (in plants) and lignin biodegradation (in fungi). White-rot Basidiomycota fungi are main actors in the oxidative process of lignin biodegradation during wood decay (Kirk & Farrell, 1987; Martínez et al., 2005), for which they secrete an outstanding repertoire of ligninolytic peroxidases and high-redox potential laccases (Ayuso-Fernández et al., 2019; Eggert et al., 1997; Lundell et al., 2010; Martínez et al., 2005). Fungal laccases have a high biotechnological value as versatile biocatalysts for different industrial sectors due to their promiscuous activity on a range of aromatic substrates and lignin. Moreover, they offer operational advantages (over, for example, ligninolytic peroxidases) due to their low catalytic requirements (only

* Corresponding author.
E-mail address: susanacam@cib.csic.es (S. Camarero).

https://doi.org/10.1016/j.ympev.2025.108310
Received 25 July 2024; Received in revised form 7 February 2025; Accepted 15 February 2025
Available online 22 February 2025

oxygen from air for activation) and the production of water as the only by-product. Laccases from white-rot fungi have been the subject of numerous studies and have traditionally served as reference laccases (Eggert et al., 1996; Uzan et al., 2010; Yaver et al., 1996). These enzymes are biocatalysts of choice for the valorization of industrial lignins into simple aromatic compounds (Rodríguez-Escribano et al., 2023), given their ability to break down linkages with high bond-dissociation energies such as those found in lignins derived from the globally predominant kraft pulping process (Suota et al., 2021).

However, classification of the ever-increasing number of available laccase genes and enzymes is challenging due to their low sequence homology and some overlapping activities with other MCO groups (Aza et al., 2023a; Giardina et al., 2007; Larrondo et al., 2003). Consequently, many MCO entries in databases, or even characterized enzymes, are labeled as "laccases" although they are not laccases sensu stricto. This underlines the need for a comprehensive, accurate, and universally applicable classification system to effectively identify and differentiate the different groups of enzymes within the MCO superfamily, such as the one carried out here.

Only a limited number of phylogenetic studies have directly focused on the interrelationships among the different MCO groups in fungi (Hoegger et al., 2006; Kües & Rühl, 2011; Moiseenko et al., 2016; Valderrama et al., 2003). The conclusions drawn from these studies might be incomplete or overly generalized, largely due to the limited sampling of taxa. Nevertheless, the recent increase in genomic data facilitates more comprehensive phylogenetic analyses. We examine here the distribution and phylogenetic relationships of MCOs across the division of Basidiomycota fungi, including orders that are phylogenetically distant from those typically studied. Based on the clustering in the phylogenetic tree and the sequence/structural features found in each cluster, we defined eight groups of MCOs, including three new groups of enzymes phylogenetically related to but different from laccases sensu stricto, that we recently found in Agaricales species (Ruiz-Dueñas et al., 2021). These eight MCO types are: ferroxidases (FOX), laccase-ferroxidases (LAC-FOX), ascorbate oxidases (AO), fungal pigment MCOs, and the newly introduced laccase-like enzymes. We coin here the term laccase-type enzymes to jointly designate the laccases sensu stricto (hereafter referred to LAC) and the three related groups of laccase-like enzymes or "atypical laccases", namely: novel laccases (NLAC), new MCO (NMCO) and new laccases with potential ferroxidase activity (NLF).

2. Materials and methods

2.1. Selection of fungal species included in the study

We examined 107 species accessible through the MycoCosm JGI DOE Portal, chosen to serve as representatives across diverse orders within the Basidiomycota fungi. This selection aimed to provide a comprehensive overview of MCOs within this fungal division. Our chosen species covered the three subphyla representing the basal divergence in Basidiomycota: Pucciniomycotina, Ustilaginomycotina, and Agaricomycotina, with a particular emphasis on the latter (Table S1). Beyond taxonomic diversity, our selection also aimed to capture ecological diversity, encompassing various nutritional modes. Within Agaricomycotina subphyla, we intentionally overrepresented Agaricales species (42 members). This is because more genomes of these species are available, they have an ampler and diverse set of MCOs (including the recently identified groups of laccase-like MCOs), and they exhibit a broad range of lifestyles, making them particularly relevant for our research. We also included a representative number of Polyporales species (23 members), as this order encompasses to date the most efficient known lignin degraders (white rot fungi). Boletales (8 members), Russulales (6 members) and Hymenochaetales (4 members) were the subsequent most represented orders. Representative members from the Amylocorticiales, Atheliales, Thelephorales, Gloeophyllales, Auriculariales, Sebacinales and Cantharellales orders, and 4 species from Phallomycetidae subclass were also included within Agaricomycetes class. Dracrymycetes and Tremellomycetes classes were represented by two species each. Finally, two species from the Pucciniomycotina and Ustilaginomycotina subphyla were respectively included. We selected as outgroups the Ascomycota species *Aspergillus nomius and Xylaria cubensis* (Table S1).

2.2. Selection of orthologous groups and organismal phylogeny

We selected 20 species widely distributed throughout Basidiomycota and Ascomycota divisions (see Dataset S1, worksheet 1) with the goal of identifying families of orthologous proteins with a single protein per species. To achieve this, we conducted an all-versus-all homology search with the non-redundant predicted proteins from the 20 species (downloaded from the DOE JGI MycoCosm portal (Grigoriev et al., 2014) using Diamond (v2.0.13) (Buchfink et al., 2021), with the following parameters to report an alignment: hits of $>$ 60 % identity and E-Value $<$ 1e-5. Subsequently, the results were clustered using FastOrtho, a reimplementation of the Orthomcl program (Li et al., 2003), with the Markov clustering algorithm (MCL) and an inflation parameter of 1.5. We filtered the output to identify clusters with only one protein per species, including 20/20, 19/20, or 18/20 species (cutoff 10 %), resulting in 872, 377, and 155 protein families, respectively. These 1404 families were individually used as protein reference databases in the final phylogeny of 107 Basidiomycota species.

Once again, homology searches were conducted using Diamond (with the same parameters as described above). This time, each of the 1404 protein families served as an independent protein reference database, and the non-redundant predicted proteins of the 109 species were used as protein query sequences. Within a search, only those pairs of sequences that achieved a minimum match length score of 50 % were chosen (values obtained from orthomclBlastParser). An amino acid sequence is included in the protein family if it aligns with all members of the database.

We applied a cutoff of taxon occupancy, discarding clusters without representation from at least 80 % of the total number of species (87 species). This resulted in 1006 homologous protein families. At this point, we selected a final set of families of single-copy orthologous to resolve our species-level phylogeny. We considered the homologous protein families as orthologous or paralogous based on whether its species contributed to the family with a single protein or a set of proteins. Within these families, 83 clusters of single-copy proteins (defined as true orthologous proteins) were selected for the subsequent phylogenetic analysis. In the remaining 923 clusters, one or more species included more than one protein. We further filtered by selecting only those families with paralogs from five species or fewer, resulting in 472 clusters.

In the 472 families with paralogs, each cluster was aligned using MAFFT v7.388 (Katoh & Standley, 2013), trimmed with TrimAl v1.2 −gappyout method (Capella-Gutiérrez et al., 2009), and a phylogenetic tree was estimated using FastTree v2 (Price et al., 2010) (MAFFT and FastTree programs with default parameters). We checked whether the duplication proteins formed a monophyletic group in the tree (terminal duplication) or not (deep paralog). To achieve this, we used a customized R script (the original script was kindly provided by Naggy and colleagues (Prasanna et al., 2020) to screen and select clusters with only terminal duplications from the same species (inparalogs sequences), thus discarding clusters containing deep paralogs. With this strategy, we obtained 289 clusters from which paralogous sequences were removed. These clusters, along with the 83 clusters of true orthologous genes, were our final selection of protein families (372), all with $>$50 amino acid sites.

Alignments of the 372 single-copy protein clusters, generated using MAFFT with default parameters and TrimAl with gappyout method, were concatenated into a supermatrix comprising 171,213 sites. We inferred a maximum likelihood organismal phylogeny using RaxML-NG

(Kozlov et al., 2019) and a partitioned model where each data partition represented a single protein family. Prior to this, each cluster alignment was tested using ProtTest 3.4.2 (Darriba et al., 2011) to determine the best-fit evolutionary model based on the Akaike Information Criterion (AIC). The best-scoring ML tree was found after 10 tree searches (5 random and 5 parsimony-based starting trees). To evaluate the branch support, we conducted a bootstrap analysis in RAxML-NG with 120 replicates using the same model.

2.3. Phylogenetic analysis of MCOs

We downloaded the predicted amino acid sequences of MCOs from the 107 genomes from the JGI. The download was based on the EuKaryotic Orthologous Groups (KOG) classification (Tatusov et al., 2003) by searching sequences associated with KOG1263 description ("Multicopper oxidases [Secondary metabolites biosynthesis, transport and catabolism]") in the JGI default database, resulting in a set of 1355 sequences. Sequence names were modified to facilitate later interpretation based on the JGI genome abbreviation and the amino acid sequence ID from JGI. For example, Agrped1|741811 corresponds to the amino acid sequence identified by the number 741,811 from the *Agrocybe pediades* AH 40210 v1.0 genome (Table S1). We removed signal peptides using SignalP 4.0 (Petersen et al., 2011).

It is important to note that the raw dataset of sequences may contain partial sequences resulting from mutation events during species evolution (or potential errors in the automatic annotation process). Under the assumption that these partial sequences did not encode for a functional protein, and they most likely influenced the accuracy of the results, we carried out a filtering of the 1355 predicted multicopper oxidase sequences as follows: i) We chose twenty complete and diverse sequences for each MCO group based on the information previously obtained (Ruiz-Dueñas et al., 2021). This selection ensured a broad taxonomic representation throughout the entire Basidiomycota division (see Dataset S1, worksheet 2). ii) We determined the mean length of each MCO group by the sequence average length of these 20 selected sequences. iii) To filter the initial 1355 predicted sequences, those with a length < 75 % of their respective group's mean length were categorized as partial and eventually excluded after visual confirmation. Following this process, we acquired 1215 putative amino acid sequences for subsequent analyses (see Dataset S1, worksheet 3 and Supplementary Files 1&2). Classification of the identified MCO sequences in different groups was conducted based on the analysis of their features at the amino acid sequence level, and their phylogenetic relationships. We manually revised the 140 discarded partial sequences (see Dataset S1, worksheet 4) to search for putative small fungal laccases. The sequences did not share gaps consistently and uniformly located and many large gaps encompassing the copper-binding motifs were found. Therefore, the existence of small fungal laccases was ruled out.

For the general phylogenetic analysis, these 1215 MCO sequences were aligned using MAFFT (default options) and then trimmed by removing poorly aligned regions using the gappyout method of TrimAl v1.2. A phylogenetic tree was constructed with FastTree 2.1.11, and we color-coded the sequences of each MCO cluster.

Subsequently, to infer more reliable relationships between the eight MCO clusters, we carried out a more robust phylogenetic analysis selecting 65 non-redundant sequences from different and diverse orders of Basidiomycota. Additionally, we included as probes for each cluster representative members of each MCO group found in the literature or previously characterized (Table S2).

The amino acid sequences were aligned with MAFFT (L-INS-i method) and then trimmed by removing poorly aligned regions using the gappyout method of TrimAl v1.2. We tested the alignment with ProtTest 3.4.2 to determine the best-fit evolutionary model (Akaike information criterion). We conducted a Maximum-likelihood analyses using RAXML-NG applying the Whelan and Goldman evolution model with gamma-distributed rate of heterogeneity, empirical amino acid frequencies from the alignment, and invariant sites (WAG + I + G + F). The best-scoring ML tree was found after 20 tree searches (10 random and 10 parsimony-based starting trees), and branch supports of the best ML tree were estimated by 1000 bootstrap replicates. We positioned a midpoint rooting at the node where the AO and MCO pigment cluster diverges.

3. Results and discussion

We selected 107 fungal genomes available in the MycoCosm JGI DOE Portal (Grigoriev et al., 2014) as representative species of different orders within Basidiomycota, aiming to obtain an overview of all types of MCOs distributed in this division (see details on the selected species in Table S1). Our selection covered species from the three subphyla into which the phylum Basidiomycota diverges: Pucciniomycotina, Ustilaginomycotina, and Agaricomycotina, focusing mainly on the latter and particularly in the Agaricomycetes class where, in principle, the greatest diversity of MCO enzymes is to be found. In addition to taxonomic diversity, our selection encompassed ecological diversity, covering a wide range of nutritional modes: symbionts, pathogens, and saprotrophs. The saprotrophic lifestyle involved preferentially, but not exclusively, fungi specialized in lignocellulose degradation, including white-rot, brown-rot, forest-litter and grass-litter, and decayed-wood fungi (Ruiz-Dueñas et al., 2021).

3.1. Topology of the organismal phylogeny

The topology derived from the maximum likelihood (ML) phylogenetic analyses of the 107 fungal species was consistent with previous studies (Kohler et al., 2015; Nagy et al., 2016; Ruiz-Dueñas et al., 2021; Sánchez-García et al., 2020; Varga et al., 2019; Zhao et al., 2017), and most nodes in the tree were robustly supported by bootstrap values (Fig. 1A). The Agaricales order exhibited consistent splitting as a sister group to the clade containing Boletales, Atheliales, and Amylocorticiales. A discrepancy arose regarding the clade encompassing Polyporales, Thelephorales, Gloeophyllales, and Russulales, where previous analyses showed dissimilar results (Kohler et al., 2015; Nagy et al., 2016; Sánchez-García et al., 2020; Varga et al., 2019; Zhao et al., 2017). In our analysis, there was moderate support (bootstrap value of 74) for the sister-group relationship between the Polyporales-Thelephorales clade and Gloeophyllales. The placement of the other clades along the backbone of the Agaricomycotina subphylum also agreed with findings from other analyses (Nagy et al., 2016; Varga et al., 2019; Zhao et al., 2017). The most inconsistent placement of taxa was the branching order of the three Basidiomycota subphyla, in accordance with the historically unresolved basal Basidiomycota relationships (Nagy et al., 2016; Nagy & Szöllősi, 2017). Potential contributors to this situation could be the biased taxon sampling, as occurs in our analysis, where only two species of Pucciniomycotina and Ustilaginomycotina subphyla were sampled. However, the basal split in Basidiomycota remained ambiguous even in studies where the taxon sampling density of these subphyla was increased (Prasanna et al., 2020).

3.2. Distribution of MCOs in Basidiomycota

The distribution of the different MCOs in the 107 fungal species (Fig. 1B) was analysed according to the MCO classification in eight groups obtained in this study (see next subsection, Fig. 2), which completed the results we previously obtained for Agaricales species (Ruiz-Dueñas et al., 2021). The MCOs were classified as laccases (LAC), ferroxidase (FOX), laccase-ferroxidases (LAC-FOX), ascorbate oxidases (AO), fungal pigment MCOs, and the three groups of laccase-like enzymes: novel laccases (NLAC), new MCO (NMCO) and new laccases with potential ferroxidase activity (NLF).

Almost all analyzed species from Agaricomycotina contained **LACs**, except those from earlier diverging clades (Tremellomycetes,

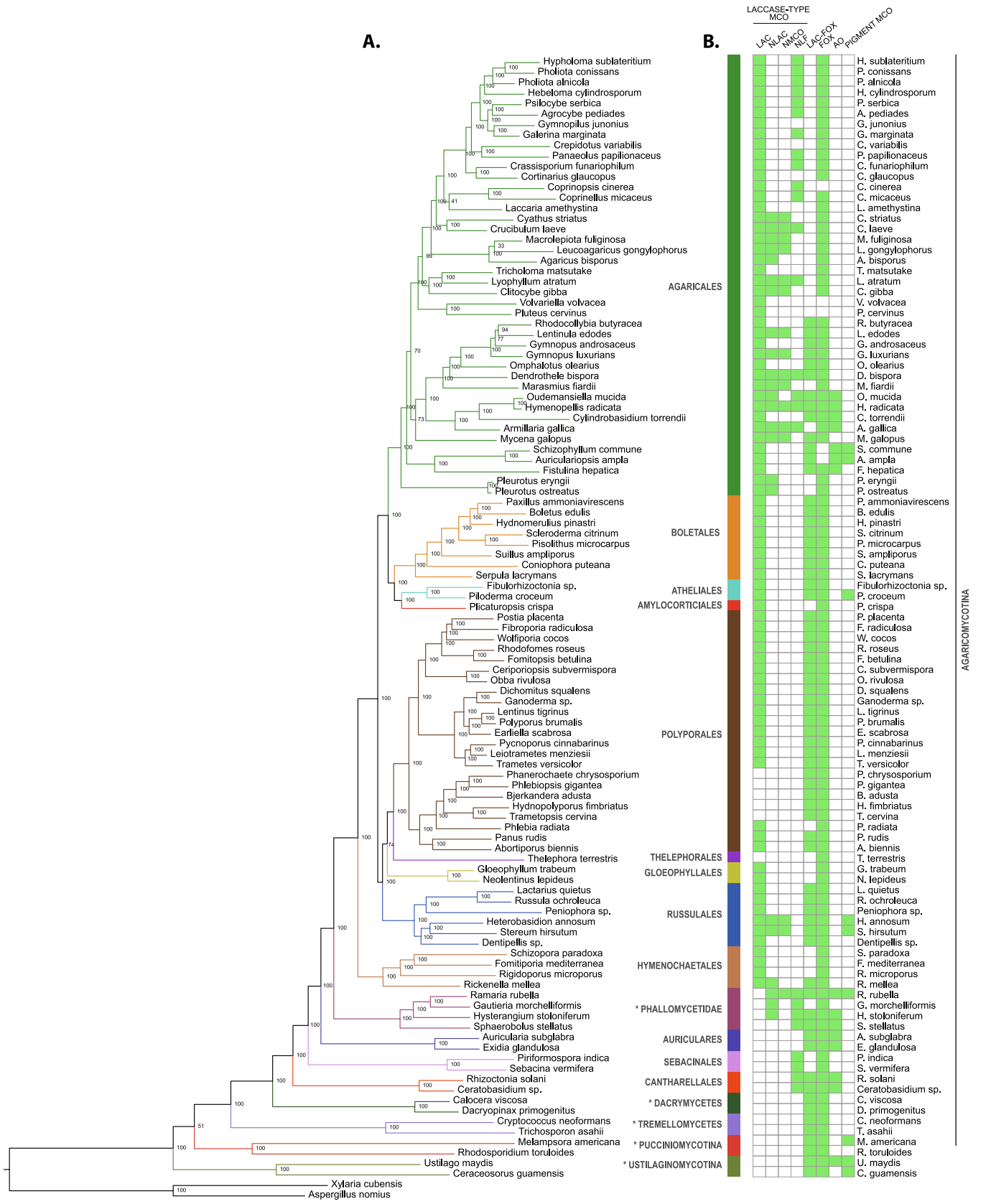

Fig. 1. A) Phylogeny of the 107 Basidiomycota analyzed species. Numbers next to branches represent ML bootstrap values. The Ascomycota species *Aspergillus nomius* and *Xylaria cubensis* were selected as outgroups. Key clades are colored to denote relevant taxonomic groups (also depicted in B). **B)** Presence (green) or absence (white) of the different MCO groups in each fungal species (see Table S1 for detailed information).

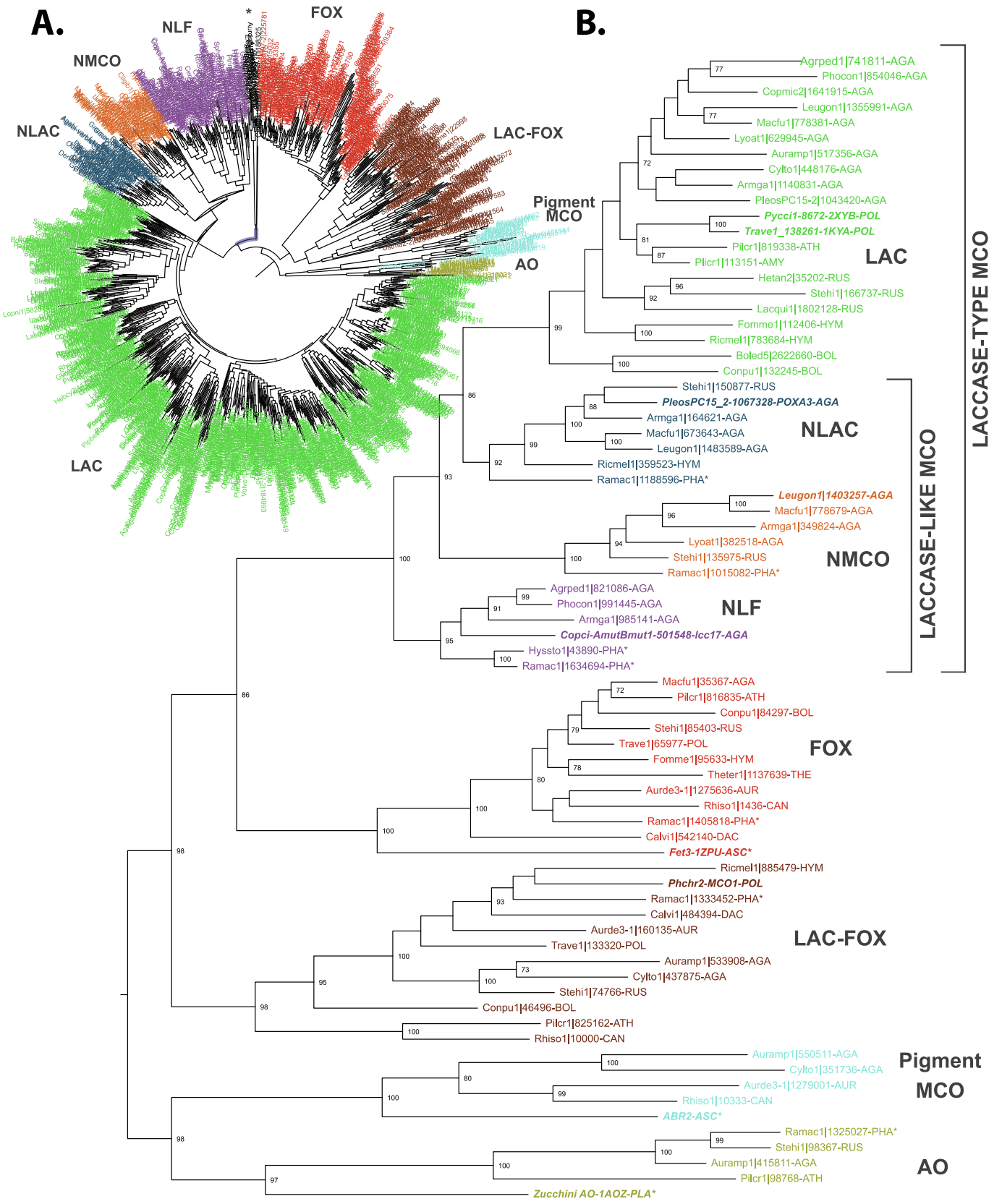

Fig. 2. ML phylogenetic trees for **A)** the complete set of MCO sequences (1215) from the 107 Basidiomycota species; and **B)** a selection of 74 representative members of all MCO groups, including previously described enzymes from each group as probes (highlighted in bold). In both trees, MCO clusters are represented by colors: LAC (green), NLAC (blue), NMCO (orange), NLF (violet), FOX (red), LAC-FOX (brown), pigment MCO (cyan) and AO (olive green). Sequence labels consist of the JGI species abbreviation, sequence number (JGI ID#) and the taxonomic order (AGA; Agaricales, BOL; Boletales, POL; Polyporales, RUS; Russulales, AMY; Amylocorticiales, ATH; Atheliales, THE; Thelephorales, HYM; Hymenochaetales, AUR; Auriculariales; CAN; Cantharellales, DAC; Dacrymycetes, TRE; Tremellomycetes) or other taxonomic levels (PHA*; Phallomycetidae, ASC*; Ascomycota, PLA*; Plant) to which the species belongs. Bootstrap values ≥0.7 are indicated.

Dacrymycetes, Cantharellales, Sebacinales, Auriculariales, and Phallomycetidae) (Table S1, Fig. 1B). From the divergence of the latter onwards, all species contained LAC genes, except for *Thelephora terrestris* (Thelephorales) and one Polyporales clade that includes the intensively studied white rot *Phanerochaete chrysosporium*. Most **NLACs** were predominantly found in Agaricales (Table S1), but they were also observed in Russulales (*Stereum hirsutum* and *Heterobasidion annosum*), Hymenochaetales (*Rickenella mellea*), Hysteranginales (*Hysterangium stoloniferum*), and Phallomycetidae (*Gautieria morchelliformis* and *Ramaria rubella*). **NMCOs** were exclusively found in species that also possessed NLACs, mostly within the Agaricales order, but also in Russulales species (*Stereum hirsutum* and *Heterobasidion annosum*), and *Ramaria rubella* from Phallomycetidae. **NLFs** were the only laccase-type MCOs found in orders that diverged early in the Basidiomycota division, where species do not possess LAC. Examples included species from Cantharellales, Sebacinales, and orders from the Phallomycetidae subclass. **LAC-FOXs** exhibited a great heterogeneous distribution throughout the Basidiomycota division. While Polyporales and Russulales species contained at least one LAC-FOX, and other species from orders such as Agaricales lack LAC-FOX genes, many species from orders that diversified early in the Basidiomycota division held numerous LAC-FOXs (Table S1).

FOXs exhibited the most widespread distribution among MCOs in Basidiomycota, typically with only a single or two member per species. They were present in almost every analyzed genome, with a few exceptions in Agaricales species that lack FOX: *Coprinopsis cinerea*, *Pluteus cervinus*, *Cylindrobasidium torrendii*, *Schizophyllum commune* and *Auriculariopsis ampla*. Furthermore, there were species randomly dispersed throughout the division that increased the amount of FOX genes, attributable to duplication events (e.g. *Mycena galopus* and *Hymenopellis radicata* with five and four copies, respectively). In addition, *Auricularia subglabra* and *Exidia glandulosa*, the selected species from the Auriculariales order, possessed six isoforms of an MCO closely related to FOX but forming a distinct cluster (depicted in black and named with * in Fig. 2A, and included in Table S1 in ** column). These MCOs exhibit notable differences at the amino acid sequence level compared to FOX genes. It will be necessary to conduct more in-depth studies on these MCOs when a larger number of members will be available. The two species from Schizophyllaceae family were the only Agaricales possessing both AO and fungal pigment MCOs. There is very little representation of AOs and fungal pigment MCOs in our analysis and their members are randomly distributed in Basidiomycota. There are also some sequences phylogenetically close to both groups but due to their limited representation, we have not been able to classify them in any MCO group (they are classified in the column ** in Table S1).

3.3. Main MCO groups in Basidiomycota

Initially, we obtained 1355 predicted MCO amino acid sequences from the 107 fungal species, from which we excluded 140 partial sequences by using a sequence-length filtering criterion (see Materials and Methods for a full description of sequence selection and filtering).

We then performed a phylogenetic analysis of the remaining 1215 MCO sequences. The resulting phylogenetic tree revealed eight well-defined clusters of sequences that grouped according to their putative function (Fig. 2A, Supplementary Files 1&2). More than half of the MCO sequences identified in the 107 genomes (711) gathered within the large **LAC** cluster, which contained sequences of almost all analyzed species from Agaricomycotina, except for the earlier diverged Basidiomycota clades (Fig. 1B). Three small clusters were distinguished alongside the LAC cluster, corresponding to the novel groups of laccase-like enzymes (NLACs, NMCOs, and NLFs). **NLAC** (48 sequences) emerged as the closest cluster to LAC (Fig. 2B). Interestingly, **NMCOs** (49 sequences) were exclusively found in species that also possess NLACs (Fig. 1B). Our results indicate that the early diverged species from the Sebacinales and Cantharellales orders contain laccase-like MCOs, more in particular NLFs (Fig. 1B). In fact, **NLF** cluster (94 sequences) was the earliest laccase-like MCO group to emerge in evolution. LAC enzymes, which have a confirmed role in lignin degradation, did not appear until the Hymenochaetales order (although the precise determination of LAC origins could be limited by the reduced sampling of taxa in these early-diverged orders). Previous studies have suggested that the oxidative enzyme families involved in lignin degradation experienced significant diversification within the class Agaricomycetes, likely occurring in the common ancestor of the Auriculariales and other mushroom-forming fungi (Floudas et al., 2012; Nagy et al., 2016).

The rest of MCO groups, FOX, LAC-FOX, fungal pigment MCO, and AO, were also identified in the phylogenetic tree. **LAC-FOX** cluster gathered a total of 116 sequences from different species (with a varying number of members from each species), whereas **FOXs** (136 sequences) were broadly distributed across the division, typically with a single member per species. Finally, **pigment MCOs** (27 sequences) and **AOs** (15 sequences) were scarcely found (Fig. 1B, Table S1).

To better evaluate the phylogenetic relationships between clades in Basidiomycota MCOs, we reconstructed a robust phylogenetic tree with 74 amino acid sequences selected from all MCO groups (Fig. 2B, Supplementary Files 3&4). The selection was intended to realistically represent the number of members of each MCO group, with a minimum of five sequences from the less represented groups. In addition, to emulate MCO distribution across Basidiomycota, we chose sequences from different taxonomic groups. We also selected species holding a wide variety of MCO types to confirm their sequences coherently clustered in the corresponding MCO group, together with sequences from other species (even if they are phylogenetically distant). Finally, we included in the analysis representative members (characterized or reported in the literature) as probes for each MCO type (see Table S2). All the studied MCO sequences consistently clustered within their respective groups and probes in the phylogenetic tree, even when some probes came from notably distant organisms (Fig. 2B).

In summary, our study provides a well-supported and revised classification of MCOs in Basidiomycota fungi, encompassing MCO groups that have been partially defined in previous reports (Hoegger et al., 2006; Kües & Rühl, 2011; Ruiz-Dueñas et al., 2021). The topology of the tree clearly differentiates the five major MCO groups traditionally defined (AOs, pigment MCOs, FOXs, LAC-FOXs, and LACs), as well as the three new groups of laccase-like enzymes which are phylogenetically related to LACs (NLACs, NMCOs, and NLFs). The latter three, along with LACs, are categorized here as laccase-type enzymes and incorporated into the updated MCO classification proposed in this work.

3.4. Key structural elements of the different MCO groups

We found remarkable differences in key features (at the sequence and structure level) among the eight MCO groups defined in the phylogenetic tree built with the 1215 sequences. These key features are associated with: i) the coordination of copper ions, ii) disulfide bridges, iii) the positioning and oxidation of the substrate in the T1 site, and iv) the redox potential of the enzyme. These differences are illustrated in Fig. 3 for representative members of each MCO group, while the sequence logos obtained from between-group comparisons within the 1215 MCO sequences are shown in Fig. 4.

The regions including the residues coordinating the copper ions, defined as L1-L4 motifs in laccases (Kumar et al., 2003), are conserved across all MCO groups. The eleven residues (ten His and one Cys) that coordinate the catalytic coppers are fully conserved, except in NMCOs. Laccases sensu stricto, such as *Pycnoporus cinnabarinus* laccase (PDB 2XYB used here as reference (Fig. 3A,B), typically feature four cysteine residues involved in two disulfide bridges important for protein stability (Piontek et al., 2002). The amino acid residue at position 206 plays a crucial role in the positioning and oxidation of phenols and aryl amines by laccases (Bertrand et al., 2002; Galli et al., 2013; Mehra et al., 2018). Although the structural determinants influencing the MCO redox potential are yet to be completely elucidated, the presence or absence of a

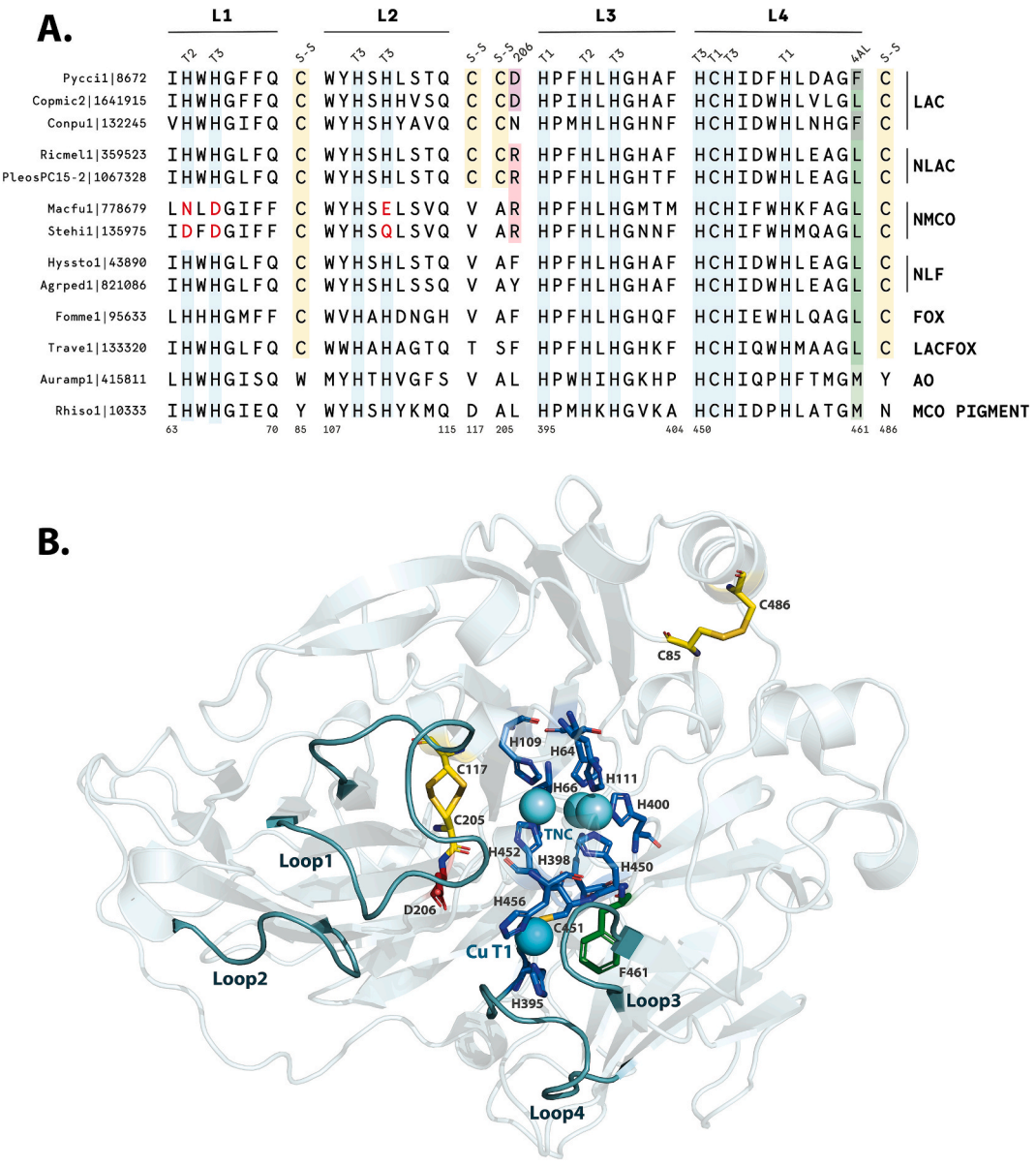

Fig. 3. A) Sequence comparison after multiple sequence alignment of representative members of the different MCO groups based on distinctive key residues. Residues coordinating the catalytic copper ions (contained in L1-L4 motifs) are depicted in blue; cysteines that potentially form disulfide bridges (S-S) are highlighted in yellow; the residue at position 206, involved in the positioning and oxidation of phenols and aryl amines by laccases, is reddish marked, and the residues in the position of the fourth axial ligand (AL) of T1 Cu are depicted in green. The absence of residues coordinating the catalytic copper ions in NMCO (L1 and L2 motifs) are denoted in red. Sequence labels include fungal species abbreviations according to JGI, followed by the unique sequence number (JGI ID#). Numbering of *Pycnoporus cinnabarinus* laccase (JGI: Pycci1|8672; PDB: 2XYB) (Camarero et al., 2012). **B)** Cartoon representation of the crystal structure of *P. cinnabarinus* laccase (PDB: 2XYB) with the key residues aforementioned shown as colored sticks, and the loops delimiting the substrate binding pocket colored in deep green.

fourth axial ligand of the T1 copper is described as an important contributor to the T1 redox potential (Braunschmid et al., 2020; Xu et al., 1999). In plant and bacterial laccases, a methionine residue weakly coordinating T1 copper results in a tetrahedral coordination geometry (Jones & Solomon, 2015), while a non-coordinating leucine or phenylalanine residue at this position implies a trigonal geometry of the T1 copper, typical for fungal laccases which have higher redox potential (Piontek et al., 2002).

3.5. Description of the different types of MCOs

Below we describe the main distinctive characteristics of the four laccase-type groups (LAC, NLAC, NMCO and NLF), and of the rest of MCO groups (FOX, LAC-FOX, AO, and Pigment MCO).

LACs, the sensu stricto laccases

The LAC group typically holds two disulfide bonds and an acidic residue at 206 position, together with a Phe or Leu residue commonly occupying the position of the fourth axial ligand of T1 copper (Fig. 3A,B, Fig. 4).

LACs are monomeric glycoproteins that have been by far the most extensively studied MCO enzymes with the largest applicability as biocatalysts in various industrial sectors (Aza & Camarero, 2023; Rodríguez Couto & Toca Herrera, 2006). This is due to their ability to oxidize lignin and various organic compounds such as phenols, aryl amines, benzenethiols, N-heterocycles, etc. (Baldrian, 2006; Claus, 2004; Mayer & Staples, 2002; Xu, 1996). The broad presence of LAC genes in saprotrophic fungi highlights their role in the biodegradation of organic

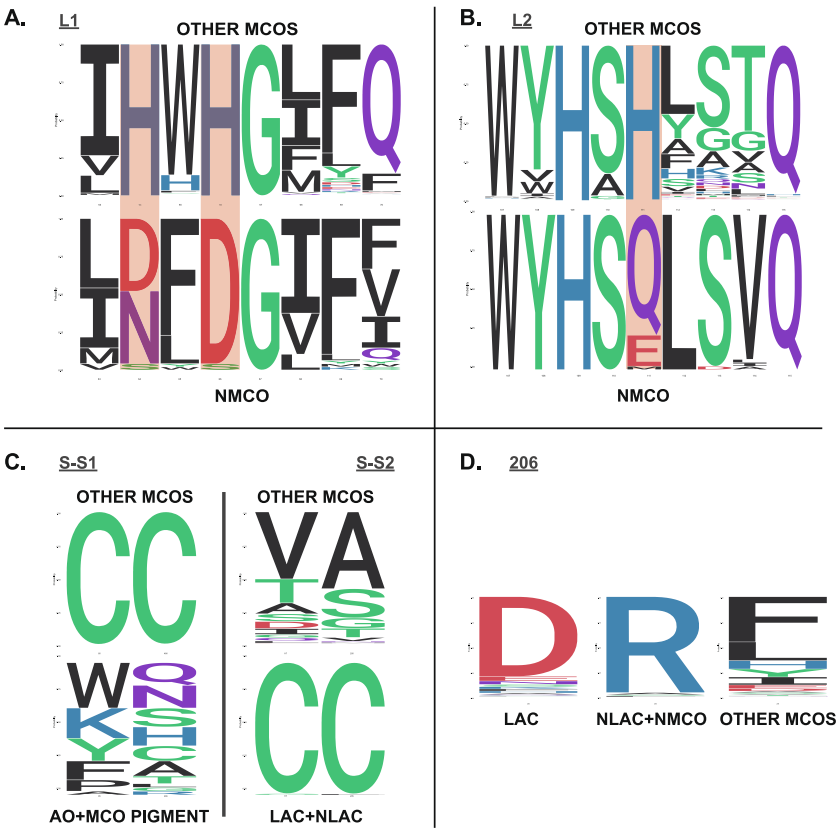

Fig. 4. Sequence logos obtained from all MCO sequences (1215) analysed in this study in relevant between-group comparisons. A) L1 and B) L2 motifs (Kumar et al., 2003) showing the lack of three of the His residues coordinating T2 and T3 coppers in NMCO versus the rest of MCOs. C) Conservation of the two putative disulfide bridges in MCOs; S-S1 logo compares AOs and pigment MCOs versus the rest of MCOs, and S-S2 logo compares LAC and NLAC versus the rest of MCOs. D) Conservation of residue at position 206 in LAC, NLAC and NMCO, and rest of MCOs.

matter (Ruiz-Dueñas et al., 2021). LAC members are found in abundance in litter decomposer fungi, especially those inhabiting forest soils with a high lignin content (Blackwood et al., 2007). A high copy number of genes coding for these enzymes can be also found in white rot ligninolytic species, being laccases from white-rot Polyporales thoroughly studied due to their role in lignin biodegradation (Hatakka, 1994; Youn et al., 1995). In addition, other physiological roles have been described for these fungal enzymes including defense/protection, pathogenesis and pigmentation (Eggert, 1997; Velázquez-Cedeño et al., 2007), as well as fruiting body formation (Wang et al., 2015). The distinct regulation of isoenzymes in mycelium or fruiting body supports the functional diversification of LACs within the same species (Sierra-Patev et al., 2023).

NLACs form heterodimers with small proteins of unknown function

NLACs are the only MCOs showing an unusual heterodimeric structure consisting of a large catalytic subunit and a small subunit (ss) with unknown function. The large subunit shares high homology with LAC, and it remains biologically active even without the small subunit binding, whereas no activity has been detected for the small subunit (Aza et al., 2023a; Giardina et al., 2007). The catalytic subunit (NLAC) holds two disulfide bridges typical of canonical laccases, a conserved Arg residue at the position 206, and a Leu residue at position of the fourth axial ligand of T1 copper (Fig. 3A, Fig. 4).

We analyzed the presence of small subunits in the 107 fungal species studied here and we found 31 representatives in 22 genomes. All genomes containing NLACs encode at least one small subunit, except for those from *Phallomycetidae*, the earlier diverged species covered in our analysis that hold NLACs. Conversely, genomes lacking NLACs do not possess a small subunit (except in two cases, Table S3).

The most extensively studied members of the NLAC group are POXA3 from *Pleurotus ostreatus* (Faraco et al., 2008; Giardina et al., 2007; Palmieri et al., 2003), and its homologous enzyme from *Pleurotus eryngii* (Aza et al., 2021; Aza et al., 2023a; Muñoz et al., 1997a, 1997b; Zhang et al., 2021), both first described as laccases. The small subunit of NLACs significantly contributes to the activity and stability of the heterodimer (Aza et al., 2023a; Faraco et al., 2008; Zhang et al., 2021) and seems to facilitate NLAC secretion (Zhang et al., 2021). Furthermore, the NLAC-ss complex can be formed in vivo and in vitro (Aza et al., 2023a; Zhang et al., 2021). Recently, we resolved for the first time the structure of a small subunit (JGI ID: Pleery1|1468497, PDB ID: 8PAQ), from *P. eryngii* NLAC (Aza et al., 2023a).

The biological functions of NLACs remain unclear, although a role in lignocellulose degradation has been suggested based on their remarkable presence in secretomes of fungi grown on lignocellulose (Fernández-Fueyo et al., 2016; Peña et al., 2021). Furthermore, we found in a transcriptome analysis of *Lentinula edodes* that the NLAC (JGI: Lentinedodes1-15576) exhibited up-regulation in mycelium compared to fruiting body (Sierra-Patev et al., 2023). When *Agaricus bisporus* is incubated in the presence of lignin dimers, two enzymes (JGI: Agabi_varbisH97_2|139148; 146228), that we label here as NLACs, emerged as the 1st and 5th most abundant proteins (Duran et al., 2023). However, these data contrast with the poor oxidation of lignin phenols by *P. eryngii* and *P. citrinopileatus* NLACs (Aza et al., 2023a; Zerva et al., 2021). The fully conserved presence of Arg in NLACs instead of Asp 206 in LACs is most likely related to this fact, as in the concerted electron/proton transfer process that occurs during phenol oxidation by laccase, the phenolic proton is transferred to Asp 206 (Galli et al., 2013).

NMCOs, the rare MCO enzymes not containing all copper ligands

All the enzymes grouped in the NMCO cluster are characterized by the absence of three of the eight His residues that usually coordinate the three catalytic coppers at the TNC. They also contain a single disulfide bridge, a conserved Arg at position 206 like NLACs, and a Leu in the fourth axial ligand position (Fig. 3A, Fig. 4). It is worth mentioning that NMCOs were exclusively found in species that also contain NLACs (Table S3), opening the possibility that NMCOs could also interact with NLACs or their small subunits.

In the search for laccase-like enzymes in fungal species other than those studied here, we identified NLACs and NMCOs in *Leucocoprinus gongylophorus*, the primary agent responsible for leaf decomposition in the fungus-garden of *Acromyrmex echinatior* leaf-cutting ants (Grell et al., 2013). The lgLcc8 (that we label here as a NMCO) and a small subunit were the most highly expressed genes in the top section of the fungus-garden, where new substrate is continually added by the ants. Both genes, together with lgLcc5 (that we reclassify as a NLAC), reached their maximum expression in the top section, gradually decreasing in the middle and bottom sections. Considering the premise that fresh leaves require more phenol detoxification, this correlation may support the idea that the laccase-like activities of both NLACs and NMCOs could be directly or indirectly involved in this process (Grell et al., 2013), since laccase activity mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts for the detoxification of secondary plant compounds (De Fine Licht et al., 2013). We also reclassify as a NLAC the lcc1-2 gene, and as a NMCO the lcc2-5 gene found in the fungus comb of termites-symbiotic fungi, that are respectively described as laccase and pseudolaccase in the literature (Taprab et al., 2005).

Likewise, in the pathogenic fungus *Heterobasidion irregulare*, transcript levels of hiLcc6 (that we labeled here as NLAC) and hiLcc13 (a NMCO) increased in woody substrates (Yakovlev et al., 2013). Both enzymes were up-regulated in the reaction zone wood (a xylem defense tissue rich in phenolic compounds) in comparison to heartwood wood. This suggests again that these laccase-like enzymes play a role in metabolizing or detoxifying phenolic compounds, thereby eliminating host tree defenses in the reaction zone wood. However, to our knowledge, there is no evidence supporting the activity of these enzymes, as no NMCO has been characterized.

NLFs contain residues that might be related with Fe (II) binding

All NLFs feature a Leu in the T1 fourth axial ligand position and a Tyr at 206 position, along with only one disulfide bridge (Fig. 3A, Fig. 4). We named this MCO group as novel laccases with potential ferroxidase activity (NLFs) because of the presence of one or two residues equivalent to those essential for Fe(II) binding and oxidation in FOX enzymes (Ruiz-Dueñas et al., 2021). However, this activity has not been verified experimentally. We found in the present work that the residue equivalent to E185 in Fet3 protein is mostly conserved in NLFs, with some members also featuring an acidic residue equivalent to D283. However, none of the NLFs exhibit an acidic residue equivalent to D409 (a characteristic found in LAC-FOXs).

We identify as NLFs four enzymes typically described as laccases (Lcc1-Lcc4) in the plant pathogenic fungus *Rhizoctonia solani* (Wahleithner et al., 1996). The authors classified them into two distinct subgroups (Lcc1-3 and Lcc4) based on their sequence homology and transcriptional regulation. Notably, the expression of Lcc4 increased when the fungus grew in the presence of *p*-anisidine. Interestingly, the redox potential of a NLF from *R. solani* is 0.73 V, which is considered as notably high for a laccase with medium redox potential (Xu et al., 1998). We also identify as NLFs the lac5 and lac9 genes of *Flammulina velutipes* (Wang et al., 2015). Finally, *Coprinopsis cinerea* contains seventeen laccases of which two of them formed a second subgroup (Kilaru et al., 2006). We found that the latter (lcc16 and lcc17) are NLFs. The authors observed that both enzymes shared specific amino acid residues with FOXs and LAC-FOXs, suggesting potential ferroxidase and laccase activities (in accordance with our nomination as NLFs).

FOXs, main source of ferroxidase activity in fungi

FOXs are enzymes homologous to the well-known Fet3p enzyme of *Saccharomyces cerevisiae* (Stearman et al., 1996), constituting the primary source of ferroxidase activity in fungi. The existence of a Fe-binding site, consisting of three conserved acidic residues (E185, D283, and D409 according to Fet3p numbering − PDB 1ZPU) within the substrate pockets of FOXs is essential for the oxidation of Fe(II) to Fe(III). The negatively charged side chains of E185 and E409 are hydrogen-bonded to the His ligands of the T1 copper, playing a dual role in both iron binding and electron transfer to the T1 site (Stoj et al., 2006; Taylor et al., 2005). Fe(II) oxidation is coupled to the reduction of O_2 to H_2O and the resulting product, Fe(III), is transferred for later import into the cytosol (Taylor et al., 2005).

Fungal FOX are also characterized by the presence of a Leu in the fourth axial ligand position and a single disulfide bridge (Fig. 3A, Fig. 4). The strictly presence of FOXs in almost every fungal genome we analyzed proves their important physiological role (iron uptake and metal homeostasis) in nature (Jung et al., 2009).

LAC-FOXs, hybrid enzymes with putative dual ferroxidase-laccase activity

LAC-FOX group is characterized by the presence of some metal-binding structural determinants found in FOX (Kües & Rühl, 2011; Ruiz-Dueñas et al., 2021), and have been described as MCO enzymes with putative hybrid ferroxidase-laccase activity (Aza et al., 2023b; Larrondo et al., 2003). They hold either two acidic residues (equivalent to E185 and D409 in Fet3p) or only one (equivalent to D409) (Aza et al., 2023b).

The first MCOs identified as hybrid enzymes (MCO1 − MCO4) were found in the white-rot Polyporales fungus *Phanerochaete chrysosporium* (Larrondo et al., 2003). MCO1, apparently exhibits efficient Fe(II) oxidation and also oxidized typical laccase substrates. The transcripts of MCO1- MCO4 genes display unusual splicings (Larrondo et al., 2004), aligning with our observations of pronounced heterogeneity at the amino acid sequence level within the LAC-FOX group compared to other MCOs. The enzyme Pfal from *Phanerochaete flavido-alba* also shows hybrid laccase-ferroxidase activity, although poorer ferroxidase activity than MCO1 (Rodríguez-Rincón et al., 2010), most probably due to the presence of only one acidic residue compared to MCO1 that holds two (Aza et al., 2023b). We also identify as LAC-FOXs two MCOs from the pathogenic fungus *Cryptococcus neoformans* (CNLAC1 and CNLAC2) initially classified as laccases (Liu et al., 1999; Missall et al., 2005; Zhu & Williamson, 2004). These enzymes have been extensively studied as crucial virulence factors of many fungal pathogens in human infections. They contribute significantly to melanin production using L-DOPA and also exhibit iron oxidase activity, potentially aiding reduction of antifungal hydroxyl radicals during infection (Liu et al., 1999; Missall et al., 2005; Zhu & Williamson, 2004).

We recently characterized a LAC-FOX from *Heterobasidion annosum s. l.*, HaLF, with only one acidic residue equivalent to D409 in Fet3p. The enzyme oxidized typical laccase substrates but did not oxidize Fe(II) (Aza et al., 2023b). Only a mutated HaLF variant with the three acidic residues exhibited ferroxidase activity, while maintained some laccase activity (Aza et al., 2023b). HaLF has been found to be up-regulated in the transcriptome of the fungus growing on lignin and heartwood wood (Yakovlev et al., 2013). It also appears to play a role as a virulence factor during Scots pine seedling colonization (Kuo et al., 2015).

Fungal pigment MCOs and AOs are scarcely found in Basidiomycota

Fungal pigment MCOs and AOs are the only fungal MCOs in our analysis that do not contain disulfide bridges and have a conserved Met

as fourth axial ligand of the T1 copper (Fig. 3A, Fig. 4). The biological roles of both types of enzymes in Basidiomycota fungi remain elusive, with very limited information in the literature.

The classification of the pigment MCO group is based on enzymes involved in the biosynthesis of melanin-related pigments (Hoegger et al., 2006; Kües & Rühl, 2011; Langfelder et al., 2003). The available information focuses primarily on the *Aspergillus* genus within the Ascomycota division. In fact, YA from *Aspergillus nidulans* and Abr2 from *Aspergillus fumigatus* are used as references for fungal pigment MCOs (Clutterbuck, 1972; Tsai et al., 1999; Sugareva et al., 2006). It is important to note that other types of MCOs also play roles in these synthesis pathways, such as abr1 (FOX), which, together with abr2, forms part of a large gene cluster involved in pigment biosynthesis in *A. fumigatus* (Tamayo Ramos et al., 2011; Tsai et al., 1999). In *A. nidulans*, other pigment MCOs have been described: TILA located at the growing fungal hyphal tip (suggesting a potential association with melanin biosynthesis), and LccD secreted outside the fungus (Mander et al., 2006; Scherer & Fischer, 2001). Finally, overexpression of the pigment MCOs genes mcoA, mcoB and mcoC from *Aspergillus niger* revealed distinct substrate activity patterns, suggesting biochemical and/or functional differences (Tamayo Ramos et al., 2011).

The AO group has not been experimentally studied in Basidiomycota, and most of the reports of this subfamily come from investigations in plants (Messerschmidt et al., 1992). These enzymes have a high substrate specificity, catalyzing the oxidation of ascorbate to dehydroascorbate (DHA), although their biological functions are still unclear. In plants, AOs might be involved in signaling pathways or in the reduction of oxygen levels (De Tullio et al., 2013). Af_AO1, the characterized fungal AO from *Aspergillus flavus*, displayed high specificity for ascorbate and limited activity for ABTS, with no activity towards other typical laccase substrates. This narrow substrate range is likely attributed to the low redox potential of AOs (Braunschmid et al., 2020), in accordance with the presence of Met as fourth axial ligand of the T1 copper. Another putative fungal AO is ASOM from the ascomycete *Acremonium sp.*, able to oxidize ascorbate but apparently lacking laccase activity (Hirose et al., 1994).

4. Conclusions

This work addresses the challenge of sorting the growing number of fungal MCO genes and enzymes with laccase-like activity available through their comprehensive analysis and their phylogenetic relationships in Basidiomycota division. The identification of the protein determinants distinctive for each MCO group allowed us to revise and update the MCO classification and description of the different types of MCO enzymes. This work will serve as a guide to future studies to facilitate the complex classification of the ever-increasing number of members of the MCO superfamily in Basidiomycota fungi, particularly of laccase-type enzymes.

CRediT authorship contribution statement

Gonzalo Molpeceres: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Pablo Aza:** Investigation. **Iván Ayuso-Fernández:** Writing – review & editing, Validation. **Guillermo Padilla:** Software, Data curation. **Francisco Javier Ruiz-Dueñas:** Writing – review & editing, Funding acquisition. **Susana Camarero:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the LIG2PLAST Project Grant PID2021-126384OB-I00 funded by MICIU/AEI/10.13039/501100011033/ and by FEDER/EU, the PIE 202320E018 project funded by CSIC, and the ROBUSTOO Project funded by the European Union under grant agreement n° 101135119. G. Molpeceres thanks the Tatiana Pérez de Guzman el Bueno Foundation for his Predoctoral Environment Grant. We extend our sincere gratitude to Dr LG Nagy and his team for generously sharing the script for discarding deep paralogous sequences.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2025.108310.

Data availability

Dataset S1 and Supplementary Files 1-4

References

Ayuso-Fernández, I., Rencoret, J., Gutiérrez, A., Ruiz-Dueñas, F.J., Martínez, A.T., 2019. Peroxidase evolution in white-rot fungi follows wood lignin evolution in plants. PNAS 116 (36), 17900–17905.
Aza, P., Camarero, S., 2023. Fungal laccases: fundamentals, engineering and classification update. Biomolecules 13 (12).
Aza, P., De Salas, F., Molpeceres, G., Rodríguez-Escribano, D., De La Fuente, I., Camarero, S., 2021. Protein engineering approaches to enhance fungal laccase production in S. cerevisiae. Int. J. Mol. Sci. 22 (3), 1–19.
Aza, P., Linde, D., Molpeceres, G., Vind, J., Medrano, F.J., Camarero, S., 2023a. Role and structure of the small subunit forming heterodimers with laccase-like enzymes. Protein Sci. 32 (9).
Aza, P., Molpeceres, G., Vind, J., Camarero, S., 2023b. Multicopper oxidases with laccase-ferroxidase activity: classification and study of ferroxidase activity determinants in a member from Heterobasidion annosum s. l. Comput. Struct. Biotechnol. J. 21, 1041–1053.
Baldrian, P., 2006. Fungal laccases – occurrence and properties. FEMS Microbiol. Rev. 30 (2), 215–242.
Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., Mougin, C., 2002. Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. Biochemistry 41 (23), 7325–7333.
Blackwood, C.B., Waldrop, M.P., Zak, D.R., Sinsabaugh, R.L., 2007. Molecular analysis of fungal communities and laccase genes in decomposing litter reveals differences among forest types but no impact of nitrogen deposition. Environ. Microbiol. 9 (5), 1306–1316.
Braunschmid, V., Fuerst, S., Perz, V., Zitzenbacher, S., Hoyo, J., Fernandez-Sanchez, C., Tzanov, T., Steinkellner, G., Gruber, K., Nyanhongo, G.S., Ribitsch, D., 2020. A fungal ascorbate oxidase with unexpected laccase activity. Int. J. Mol. Sci. 21 (16), 5754.
Buchfink, B., Reuter, K., Drost, H.G., 2021. Sensitive protein alignments at tree-of-life scale using DIAMOND. Nat. Methods 18 (4), 366–368.
Camarero, S., Pardo, I., Cañas, A.I., Molina, P., Record, E., Martínez, A.T., Martínez, M.J., Alcalde, M., 2012. Engineering platforms for directed evolution of laccase from pycnoporus cinnabarinus. Appl. Environ. Microbiol. 78 (5), 1370.
Capella-Gutiérrez, S., Silla-Martínez, J.M., Gabaldón, T., 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25 (15), 1972–1973.
Claus, H., 2004. Laccases: structure, reactions, distribution. Micron 35 (1–2), 93–96.
Clutterbuck, A.J., 1972. Absence of laccase from yellow-spored mutants of Aspergillus nidulans. J. Gen. Microbiol. 70 (3), 423–435.
Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2011. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27 (8), 1164–1165.
De Fine Licht, H.H., Schiøt, M., Rogowska-Wrzesinska, A., Nygaard, S., Roepstorff, P., Boomsma, J.J., 2013. Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. PNAS 110 (2), 583–587.
De Tullio, M.C., Guether, M., Balestrini, R., 2013. Ascorbate oxidase is the potential conductor of a symphony of signaling pathways. Plant Signal. Behav. 8 (3).
Dittmer, N.T., Kanost, M.R., 2010. Insect multicopper oxidases: diversity, properties, and physiological roles. Insect Biochem. Mol. Biol. 40 (3), 179–188.
Duran, K., Magnin, J., America, A.H.P., Peng, M., Hilgers, R., de Vries, R.P., Baars, J.J.P., van Berkel, W.J.H., Kuyper, T.W., Kabel, M.A., 2023. The secretome of Agaricus bisporus: temporal dynamics of plant polysaccharides and lignin degradation. Iscience 26 (7), 107087.
Eggert, C., 1997. Laccase-catalyzed formation of cinnabarinic acid is responsible for antibacterial activity of Pycnoporus cinnabarinus. Microbiol. Res. 152 (3), 315–318.

Eggert, C., Temp, U., Eriksson, K.E.L., 1996. The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase. Appl. Environ. Microbiol. 62 (4), 1151–1158.
Eggert, C., Temp, U., Eriksson, K.E.L., 1997. Laccase is essential for lignin degradation by the white-rot fungus Pycnoporus cinnabarinus. FEBS Lett. 407 (1), 89–92.
Faraco, V., Ercole, C., Festa, G., Giardina, P., Piscitelli, A., Sannia, G., 2008. Heterologous expression of heterodimeric laccase from Pleurotus ostreatus in Kluyveromyces lactis. Appl. Microbiol. Biotechnol. 77 (6), 1329–1335.
Fernández-Fueyo, E., Ruiz-Dueñas, F.J., López-Lucendo, M.F., Pérez-Boada, M., Rencoret, J., Gutiérrez, A., Pisabarro, A.G., Ramírez, L., Martínez, A.T., 2016. A secretomic view of woody and nonwoody lignocellulose degradation by Pleurotus ostreatus. Biotechnol. Biofuels 9 (1), 1–18.
Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R.A., Henrissat, B., Martínez, A. T., Otillar, R., Spatafora, J.W., Yadav, J.S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P.M., De Vries, R.P., Ferreira, P., Findley, K., Hibbett, D.S., 2012. The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336 (6089), 1715–1719.
Galli, C., Madzak, C., Vadalà, R., Jolivalt, C., Gentili, P., 2013. Concerted electron/proton transfer mechanism in the oxidation of phenols by laccase. Chembiochem 14 (18), 2500–2505.
Giardina, P., Autore, F., Faraco, V., Festa, G., Palmieri, G., Piscitelli, A., Sannia, G., 2007. Structural characterization of heterodimeric laccases from Pleurotus ostreatus. Appl. Microbiol. Biotechnol. 75 (6), 1293–1300.
Grell, M.N., Linde, T., Nygaard, S., Nielsen, K.L., Boomsma, J.J., Lange, L., 2013. The fungal symbiont of Acromyrmex leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and other plant cell wall polysaccharides. BMC Genomics 14 (1), 1–11.
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 (D1), D699–D704.
Hatakka, A., 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. FEMS Microbiol. Rev. 13 (2–3), 125–135.
Hirose, J., Sakurai, T., Imamura, K., Watanabe, H., Iwamoto, H., Hiromi, K., Itoh, H., Shin, T., Murao, S., 1994. Characterization of ascorbate oxidase from acremonium sp. HI-25. J. Biochem. 115 (5), 811–813.
Hoegger, P.J., Kilaru, S., James, T.Y., Thacker, J.R., Kües, U., 2006. Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. FEBS J. 273 (10), 2308–2326.
Janusz, G., Pawlik, A., Świderska-Burek, U., Polak, J., Sulej, J., Jarosz-Wilkołazka, A., Paszczyński, A., 2020. Laccase properties, physiological functions, and evolution. Int. J. Mol. Sci. 21 (3), 966.
Jones, S.M., Solomon, E.I., 2015. Electron transfer and reaction mechanism of laccases. Cell. Mol. Life Sci. 72 (5), 869–883.
Jung, W.H., Hu, G., Kuo, W., Kronstad, J.W., 2009. Role of ferroxidases in iron uptake and virulence of Cryptococcus neoformans. Eukaryot. Cell 8 (10), 1511–1520.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4), 772–780.
Kilaru, S., Hoegger, P.J., Kües, U., 2006. The laccase multi-gene family in Coprinopsis cinerea has seventeen different members that divide into two distinct subfamilies. Curr. Genet. 50 (1), 45–60.
Kirk, T.K., Farrell, R.L., 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. 41, 465–505.
Kohler, A., Kuo, A., Nagy, L.G., et al., 2015. Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. Nat. Genet. 47 (4), 410–415.
Kosman, D.J., 2010. Multicopper oxidases: a workshop on copper coordination chemistry, electron transfer, and metallophysiology. J. Biol. Inorg. Chem. 15 (1), 15–28.
Kozlov, A.M., Darriba, D., Flouri, T., Morel, B., Stamatakis, A., 2019. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics 35 (21), 4453–4455.
Kües, U., Rühl, M., 2011. Multiple multi-copper oxidase gene families in basidiomycetes – what for? Curr. Genomics 12 (2), 72.
Kumar, S.V.S., Phale, P.S., Durani, S., Wangikar, P.P., 2003. Combined sequence and structure analysis of the fungal laccase family. Biotechnol. Bioeng. 83 (4), 386–394.
Kuo, H.C., Détry, N., Choi, J., Lee, Y.H., 2015. Potential roles of laccases on virulence of Heterobasidion annosum s.s. Microb. Pathog. 81, 16–21.
Langfelder, K., Streibel, M., Jahn, B., Haase, G., Brakhage, A.A., 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet. Biol. 38 (2), 143–158.
Larrondo, L.F., González, B., Cullen, D., Vicuña, R., 2004. Characterization of a multicopper oxidase gene cluster in Phanerochaete chrysosporium and evidence of altered splicing of the mco transcripts. Microbiology 150 (8), 2775–2783.
Larrondo, L.F., Salas, L., Melo, F., Vicuña, R., Cullen, D., 2003. A Novel extracellular multicopper oxidase from phanerochaete chrysosporium with ferroxidase activity. Appl. Environ. Microbiol. 69 (10), 6257–6263.
Li, L., Stoeckert, C.J., Roos, D.S., 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13 (9), 2178–2189.
Liu, L., Tewari, R.P., Williamson, P.R., 1999. Laccase protects Cryptococcus neoformans from antifungal activity of alveolar macrophages. Infect. Immun. 67 (11), 6034–6039.
Lundell, T.K., Mäkelä, M.R., Hildén, K., 2010. Lignin-modifying enzymes in filamentous basidiomycetes – ecological, functional and phylogenetic review. J. Basic Microbiol. 50 (1), 5–20.
Mander, G.J., Wang, H., Bodie, E., Wagner, J., Vienken, K., Vinuesa, C., Foster, C., Leeder, A.C., Allen, G., Hamill, V., Janssen, G.G., Dunn-Coleman, N., Karos, M., Lemaire, H.G., Subkowski, T., Bollschweiler, C., Turner, G., Nüsslein, B., Fischer, R., 2006. Use of laccase as a novel, versatile reporter system in filamentous fungi. Appl. Environ. Microbiol. 72 (7), 5020–5026.
Martínez, A.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A., del Río, J.C., 2005. Biodegradation of lignocellu-losics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin Lignocellulosic materials. Int. Microbiol. 8 (3), 195–204.
Mayer, A.M., Staples, R.C., 2002. Laccase: new functions for an old enzyme. Phytochemistry 60 (6), 551–565.
Mehra, R., Muschiol, J., Meyer, A.S., Kepp, K.P., 2018. A structural-chemical explanation of fungal laccase activity. Sci. Rep. 8 (1), 17285.
Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A., Finazzi-Agró, A., 1992. Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. J. Mol. Biol. 224 (1), 179–205.
Missall, T.A., Moran, J.M., Corbett, J.A., Lodge, J.K., 2005. Distinct stress responses of two functional laccases in Cryptococcus neoformans are revealed in the absence of the thiol-specific antioxidant Tsa1. Eukaryot. Cell 4 (1), 202–208.
Moiseenko, K.V., Maloshenok, L.G., Vasina, D.V., Bruskin, S.A., Tyazhelova, T.V., Koroleva, O.V., 2016. Laccase multigene families in Agaricomycetes. J. Basic Microbiol. 56 (12), 1392–1397.
Morozova, O.V., Shumakovich, G.P., Gorbacheva, M.A., Shleev, S.V., Yaropolov, A.I., 2007. "Blue" laccases. Biochemistry (Moscow) 72 (10), 1136–1150.
Muñoz, C., Guillén, F., Martínez, A.T., Martínez, M.J., 1997a. Induction and characterization of laccase in the ligninolytic fungus Pleurotus eryngii. Curr. Microbiol. 34 (1), 1–5.
Muñoz, C., Guillén, F., Martínez, A.T., Martínez, M.J., 1997b. Laccase isoenzymes of Pleurotus eryngii: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn2+ oxidation. Appl. Environ. Microbiol. 63 (6), 2166–2174.
Nagy, L.G., Riley, R., Tritt, A., Adam, C., Daum, C., Floudas, D., Sun, H., Yadav, J.S., Pangilinan, J., Larsson, K.H., Matsuura, K., Barry, K., Labutti, K., Kuo, R., Ohm, R.A., Bhattacharya, S.S., Shirouzu, T., Yoshinaga, Y., Martin, F.M., Hibbett, D.S., 2016. Comparative genomics of early-diverging mushroom-forming fungi provides insights into the origins of lignocellulose decay capabilities. Mol. Biol. Evol. 33 (4), 959–970.
Nagy, L.G., Szöllősi, G., 2017. Fungal phylogeny in the age of genomics: insights into phylogenetic inference from genome-scale datasets. Adv. Genet. 100, 49–72.
Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G., Giardina, P., 2003. Atypical laccase isoenzymes from copper supplemented Pleurotus ostreatus cultures. Enzyme Microb. Technol. 33 (2–3), 220–230.
Peña, A., Babiker, R., Chaduli, D., Lipzen, A., Wang, M., Chovatia, M., Rencoret, J., Marques, G., Sánchez-Ruiz, M.I., Kijpornyongpan, T., Salvachúa, D., Camarero, S., Ng, V., Gutiérrez, A., Grigoriev, I.V., Rosso, M.N., Martínez, A.T., Ruiz-Dueñas, F.J., 2021. A multiomic approach to understand how pleurotus eryngii transforms non-woody lignocellulosic material. Journal of Fungi 7 (6), 426.
Petersen, T.N., Brunak, S., Von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8 (10), 785–786.
Piontek, K., Antorini, M., Choinowski, T., 2002. Crystal structure of a laccase from the fungustrametes versicolor at 1.90-Å resolution containing a full complement of coppers. J. Biol. Chem. 277 (40), 37663–37669.
Prasanna, A.N., Gerber, D., Kijpornyongpan, T., Aime, M.C., Doyle, V.P., Nagy, L.G., 2020. Model choice, missing data, and taxon sampling impact phylogenomic inference of deep basidiomycota relationships. Syst. Biol. 69 (1), 17–37.
Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. PLoS One 5 (3), e9490.
Rodríguez Couto, S., Toca Herrera, J.L., 2006. Industrial and biotechnological applications of laccases: a review. Biotechnol. Adv. 24 (5), 500–513.
Rodríguez-Escribano, D., de Salas, F., Pliego, R., Marques, G., Levée, T., Suonpää, A., Gutiérrez, A., Martínez, Á.T., Ihalainen, P., Rencoret, J., Camarero, S., 2023. Depolymerisation of Kraft lignin by tailor-made Alkaliphilic fungal laccases. Polymers 15 (22), 4433.
Rodríguez-Rincón, F., Suarez, A., Lucas, M., Larrondo, L.F., De La Rubia, T., Polaina, J., Martínez, J., 2010. Molecular and structural modeling of the Phanerochaete flavido-alba extracellular laccase reveals its ferroxidase structure. Arch. Microbiol. 192 (11).
Ruiz-Dueñas, F.J., Barrasa, J.M., Sánchez-García, M., Camarero, S., Miyauchi, S., Serrano, A., Linde, D., Babiker, R., Drula, E., Ayuso-Fernández, I., Pacheco, R., Padilla, G., Ferreira, P., Barriuso, J., Kellner, H., Castanera, R., Alfaro, M., Ramírez, L., Pisabarro, A.G., Martínez, A.T., 2021. Genomic analysis enlightens agaricales lifestyle evolution and increasing peroxidase diversity. Mol. Biol. Evol. 38 (4), 1428–1446.
Sánchez-García, M., Ryberg, M., Khan, F.K., Varga, T., Nagy, L.G., Hibbett, D.S., 2020. Fruiting body form, not nutritional mode, is the major driver of diversification in mushroom-forming fungi. PNAS 117 (51), 32528–32534.
Scherer, M., Fischer, R., 2001. Molecular characterization of a blue-copper laccase, TILA, of Aspergillus nidulans. FEMS Microbiol. Lett. 199 (2), 207–213.
Sierra-Patev, S., Min, B., Naranjo-Ortiz, M., Looney, B., Konkel, Z., Slot, J.C., Sakamoto, Y., Steenwyk, J.L., Rokas, A., Carro, J., Camarero, S., Ferreira, P., Molpeceres, G., Ruiz-Dueñas, F.J., Serrano, A., Henrissat, B., Drula, E., Hughes, K.W., Mata, J.L., Hibbett, D., 2023. A global phylogenomic analysis of the shiitake genus Lentinula. PNAS 120 (10), e2214076120.
Solomon, E.I., Sundaram, U.M., Machonkin, T.E., 1996. Multicopper oxidases and oxygenases. Chem. Rev. 96 (7), 2563–2605.
Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D., Dancis, A., 1996. A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science 271 (5255), 1552–1557.

Stoj, C.S., Augustine, A.J., Zeigler, L., Solomon, E.I., Kosman, D.J., 2006. Structural basis of the ferrous iron specificity of the yeast ferroxidase, Fet3p. Biochemistry 45 (42), 12741–12749.
Sugareva, V., Härtl, A., Brock, M., Hübner, K., Rohde, M., Heinekamp, T., Brakhage, A.A., 2006. Characterisation of the laccase-encoding gene abr2 of the dihydroxynaphthalene-like melanin gene cluster of Aspergillus fumigatus. Arch. Microbiol. 186 (5), 345–355.
Suota, M.J., da Silva, T.A., Zawadzki, S.F., Sassaki, G.L., Hansel, F.A., Paleologou, M., Ramos, L.P., 2021. Chemical and structural characterization of hardwood and softwood LignoForceTM lignins. Ind. Crop. Prod. 173, 114138.
Tamayo Ramos, J.A., Barends, S., Verhaert, R.M.D., De Graaff, L.H., 2011. The Aspergillus Niger multicopper oxidase family: analysis and overexpression of laccase-like encoding genes. Microb. Cell Fact. 10 (1), 1–12.
Taprab, Y., Johjima, T., Maeda, Y., Moriya, S., Trakulnaleamsai, S., Noparatnaraporn, N., Ohkuma, M., Kudo, T., 2005. Symbiotic fungi produce laccases potentially involved in phenol degradation in fungus combs of fungus-growing termites in Thailand. Appl. Environ. Microbiol. 71 (12), 7696.
Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V., Krylov, D.M., Mazumder, R., Smirnov, S., Nikolskaya, A.N., Rao, B.S., Mekhedov, S. L., Sverlov, A.V., Vasudevan, S., Wolf, Y.I., Yin, J.J., Natale, D.A., 2003. The COG database: an updated vesion includes eukaryotes. BMC Bioinf. 4 (1), 1–14.
Taylor, A.B., Stoj, C.S., Ziegler, L., Kosman, D.J., Hart, P.J., 2005. The copper-iron connection in biology: structure of the metallo-oxidase Fet3p. PNAS 102 (43), 15459–15464.
Tsai, H.F., Wheeler, M.H., Chang, Y.C., Kwon-Chung, K.J., 1999. A developmentally regulated gene cluster involved in conidial pigment biosynthesis in Aspergillus fumigatus. J. Bacteriol. 181 (20), 6469–6477.
Uzan, E., Nousiainen, P., Balland, V., Sipila, J., Piumi, F., Navarro, D., Asther, M., Record, E., Lomascolo, A., 2010. High redox potential laccases from the ligninolytic fungi Pycnoporus coccineus and Pycnoporus sanguineus suitable for white biotechnology: from gene cloning to enzyme characterization and applications. J. Appl. Microbiol. 108 (6), 2199–2213.
Valderrama, B., Oliver, P., Medrano-Soto, A., Vazquez-Duhalt, R., 2003. Evolutionary and structural diversity of fungal laccases. Anton. Leeuw. Int. J. Gen. Mol. Microbiol. 84 (4), 289–299.
Varga, T., Krizsán, K., Földi, C., Dima, B., Sánchez-García, M., Sánchez-Ramírez, S., Szöllősi, G.J., Szarkándi, J.G., Papp, V., Albert, L., Andreopoulos, W., 2019. Megaphylogeny resolves global patterns of mushroom evolution. Nat. Ecol. Evol. 3 (4), 668–678.
Vashchenko, G., MacGillivray, R.T.A., 2013. Multi-copper oxidases and human iron metabolism. Nutrients 5 (7), 2289.
Velázquez-Cedeño, M., Farnet, A.M., Billette, C., Mata, G., Savoie, J.M., 2007. Interspecific interactions with Trichoderma longibrachiatum induce Pleurotus ostreatus defence reactions based on the production of laccase isozymes. Biotechnol. Lett 29 (10), 1583–1590.
Wahleithner, J.A., Xu, F., Brown, K.M., Brown, S.H., Golightly, E.J., Halkier, T., Kauppinen, S., Pederson, A., Schneider, P., 1996. The identification and characterization of four laccases from the plant pathogenic fungus Rhizoctonia solani. Curr. Genet. 29 (4), 395–403.
Wang, W., Liu, F., Jiang, Y., Wu, G., Guo, L., Chen, R., Chen, B., Lu, Y., Dai, Y., Xie, B., 2015. The multigene family of fungal laccases and their expression in the white rot basidiomycete Flammulina velutipes. Gene 563 (2), 142–149.
Xu, F., 1996. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35 (23), 7608–7614.
Xu, F., Berka, R.M., Wahleithner, J.A., Nelson, B.A., Shuster, J.R., Brown, S.H., Palmer, A.E., Solomon, E.I., 1998. Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. Biochem. J 334 (1), 63–70.
Xu, F., Palmer, A.E., Yaver, D.S., Berka, R.M., Gambetta, G.A., Brown, S.H., Solomon, E. I., 1999. Targeted Mutations in a Trametes villosa Laccase. J. Biol. Chem. 274 (18), 12372–12375.
Yakovlev, I.A., Hietala, A.M., Courty, P.E., Lundell, T., Solheim, H., Fossdal, C.G., 2013. Genes associated with lignin degradation in the polyphagous white-rot pathogen Heterobasidion irregulare show substrate-specific regulation. Fungal Genet. Biol. 56, 17–24.
Yaver, D.S., Xu, F., Golightly, E.J., Brown, K.M., Brown, S.H., Rey, M.W., Schneider, P., Halkier, T., Mondorf, K., Dalbøge, H., 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete Trametes villosa. Appl. Environ. Microbiol. 62 (3), 834–841.
Youn, H.-D., Hah, Y.C., Kang, S.-O., 1995. Role of laccase in lignin degradation by white-rot fungi. FEMS Microbiol. Lett. 132 (3), 183–188.
Zerva, A., Pentari, C., Termentzi, A., America, A.H.P., Zouraris, D., Bhattacharya, S.K., Karantonis, A., Zervakis, G.I., Topakas, E., 2021. Discovery of two novel laccase-like multicopper oxidases from Pleurotus citrinopileatus and their application in phenolic oligomer synthesis. Biotechnol. Biofuels 14 (1), 1–16.
Zhang, Q., Yuan, C., Wang, F., Xu, S., Li, Y., Shi, G., Ding, Z., 2021. Roles of small subunits of laccase (ssPOXA3a/b) in laccase production by Pleurotus eryngii var. ferulae. J. Agric. Food Chem. 69 (44), 13113–13124.
Zhao, R.L., Li, G.J., Sánchez-Ramírez, S., Stata, M., Yang, Z.L., Wu, G., Dai, Y.C., He, S.H., Cui, B.K., Zhou, J.L., Wu, F., 2017. A six-gene phylogenetic overview of Basidiomycota and allied phyla with estimated divergence times of higher taxa and a phyloproteomics perspective. Fungal Divers. 84, 43–74.
Zhu, X., Williamson, P.R., 2004. Role of laccase in the biology and virulence of Cryptococcus neoformans. FEMS Yeast Res. 5 (1), 1–10.