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Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences

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A phylogenetic analysis of more than 350 multicopper oxidases (MCOs) from fungi, insects, plants, and bacteria provided the basis for a refined classification of this enzyme family into laccases *sensu stricto* (basidiomycetous and ascomycetous), insect laccases, fungal pigment MCOs, fungal ferroxidases, ascorbate oxidases, plant laccase-like MCOs, and bilirubin oxidases. Within the largest group of enzymes, formed by the 125 basidiomycetous laccases, the gene phylogeny does not strictly follow the species phylogeny. The enzymes seem to group at least partially according to the lifestyle of the corresponding species. Analyses of the completely sequenced fungal genomes showed that the composition of MCOs in the different species can be very variable. Some species seem to encode only ferroxidases, whereas others have proteins which are distributed over up to four different functional clusters in the phylogenetic tree.

Multicopper oxidases (MCOs) are a family of enzymes comprising laccases (EC 1.10.3.2), ferroxidases (EC 1.16.3.1), ascorbate oxidase (EC 1.10.3.3), and ceruloplasmin. This family in turn belongs to the highly diverse group of blue copper proteins which contain from one to six copper atoms per molecule and about 100 to > 1000 amino acid residues in the single peptide chain [1]. MCOs have the ability to couple the oxidation of a substrate with a four-electron reduction of molecular oxygen to water. The electron transfer steps in these redox reactions are coordinated in two copper centres that usually contain four copper atoms. In a redox reaction catalyzed by an MCO, electrons from the substrate are accepted in the mononuclear centre (type 1 copper atom) and then transferred to the trinuclear cluster (one type 2 and two type 3 copper atoms), which serves as the dioxygen binding site and reduces the molecular oxygen upon receipt of four electrons. The type 1 copper is bound to the enzyme by two histidine and one cysteine residue in the T1 centre, whereas eight histidine residues in the T2/T3 cluster serve as ligands for the type 2 and type 3 copper atoms [2–5]. Based on the conservation of the amino acid ligands, two consensus patterns (G-X-[FYW]-X-[LIVMFYW]-X-[CST]-X_8-G-[LM]-X_3-[LIVMFYW] and H-C-H-X_3-H-X_3-[AG]-[LM]) were

Abbreviations
ABTS, 2,2′-azinobis(3-ethylbenzo-6-thiazolinesulfonic acid); DHN, 1,8-dihydroxynaphthalene; L-DOPA, 3,4-dihydroxyphenylalanine; LMCO, laccase-like multicopper oxidase; MCO, multicopper oxidase.

defined for the MCOs (PROSITE PDOC00076, http://us.expasy.org/prosite/). Compared with other members of the MCO family, ceruloplasmin, responsible for iron homeostasis in vertebrates, is rather unusual, as it has five to six copper atoms per molecule [6]. Therefore, this enzyme will not be further discussed in this paper.

Laccases in the broader sense by far make up the largest subgroup of MCOs, originating from bacteria, fungi, plants, and insects. Laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera* [7], hence the name. Subsequently, laccases were also found in various basidiomycetous and ascomycetous fungi and, until now, the fungal laccases account for the most important group with respect to number and extent of characterization.

Laccases were found in almost all wood-rotting fungi analyzed so far [8]. It has become evident that laccases can play an important role in lignin degradation [9] even though one of the strongest lignin degrading species, *Phanerochaete chrysosporium*, does not produce a typical laccase [10]. The precise function of the enzyme in this process, however, is still poorly understood [9,11]. Besides delignification, fungal laccases have been associated with various organismal interactions (intra- and interspecific) and developmental processes such as fruiting body formation [12,13], pigment formation during asexual development [14,15], pathogenesis [16–18], competitor interactions [19]. Laccases of saprophytic and mycorrhizal fungi have also been implicated in soil organic matter cycling, e.g. degradation of soil litter polymers or formation of humic compounds [20,21].

Several lines of evidence (capacity to oxidize lignin precursors, localization in lignifying xylem cell walls, higher expression in xylem compared to other tissues) suggest the involvement of plant laccases in the lignification process [22–25]. However, given the complexity of the laccase gene families in plant species, additional, so far not specified functions unrelated to lignin formation have been proposed [26]. Due to the ferroxidase activity of the MCO LAC2-2 from *Liriodendron tulipifera* and expression studies of the *Arabidopsis thaliana* laccase gene family, the term 'laccase-like multicopper oxidases' or LMCOs was introduced in order to account for their potential multiplicity of functions [27,28]. All 17 of the *A. thaliana* LMCOs were shown to be expressed and the expression patterns suggested that LMCO function in *A. thaliana* probably extends well beyond lignification [28].

In insects, laccases seem to play an important role in cuticular sclerotization [29,30]. In *Drosophila melanogaster*, a role in the melanization pathway during the insect's immune response [31] and in *Manduca sexta* a role in the oxidation of toxic compounds in the diet and/or in the iron metabolism has been proposed [32].

Laccases have only recently been discovered in bacteria and their classification and function are still controversial. The first report of a bacterial laccase was from the Gram-negative soil bacterium *Azospirillum lipoferum* [33] and the enzyme was suggested to be involved in melanization [34]. The *Bacillus subtilis* endospore coat protein CotA is a laccase required for the formation of spore pigment [35] and was recently shown to have also bilirubin oxidase (EC 1.3.3.5) activity [36]. Other bacterial MCOs like the copper efflux protein CueO from *Escherichia coli* and the copper resistance protein CopA from *Pseudomonas syringae* and *Xanthomonas campestris* were considered pseudo-laccases due to the dependence of the 2,6-dimethoxyphenol oxidation on Cu^{2+} addition [37].

This plethora of functions of the various laccases implicates the capability of oxidizing a wide range of substrates, which by the use of mediators (oxidizable low-molecular-weight compounds) can even be greatly extended [38]. Therefore, laccases are very interesting enzymes for various biotechnological applications. Most of the proposed uses for laccases are based on the ability to produce a free radical from a suitable substrate. The multifaceted consecutive secondary reactions of the radicals are responsible for the versatility of possible applications [39].

A novel MCO with weak laccase and strong ferroxidase activity was identified in *P. chrysosporium* [10]. Ferroxidase activity was also detected in a heterologously expressed laccase from *Cryptococcus neoformans* [40]. The role of ferroxidase has been analyzed extensively in *Saccharomyces cerevisiae*. The yeast ferroxidase Fet3p is a plasma membrane protein that, along with the iron permease Ftr1p, is part of a high affinity iron uptake system [41]. Next to its function in iron metabolism, a protective role by suppressing copper and iron cytotoxicity has been suggested [42].

Ascorbate oxidase catalyzes the oxidation of ascorbic acid to monodehydroascorbate. However, its specificity is not as strict, as it was shown to oxidize also phenolic substrates typical for laccases [43]. Despite extensive studies on structure, biochemistry, and expression of ascorbate oxidase in plant cells, the physiological roles remained uncertain [44]. Ascorbate oxidase was suggested to modify the apoplastic redox state and thereby regulate growth and defence [44]. De Tullio *et al.* [45] proposed a function in dioxygen management during photosynthesis, fruit ripening, and wound healing.

With the availability of genomic sequences, a multitude of genes putatively coding for MCOs has been

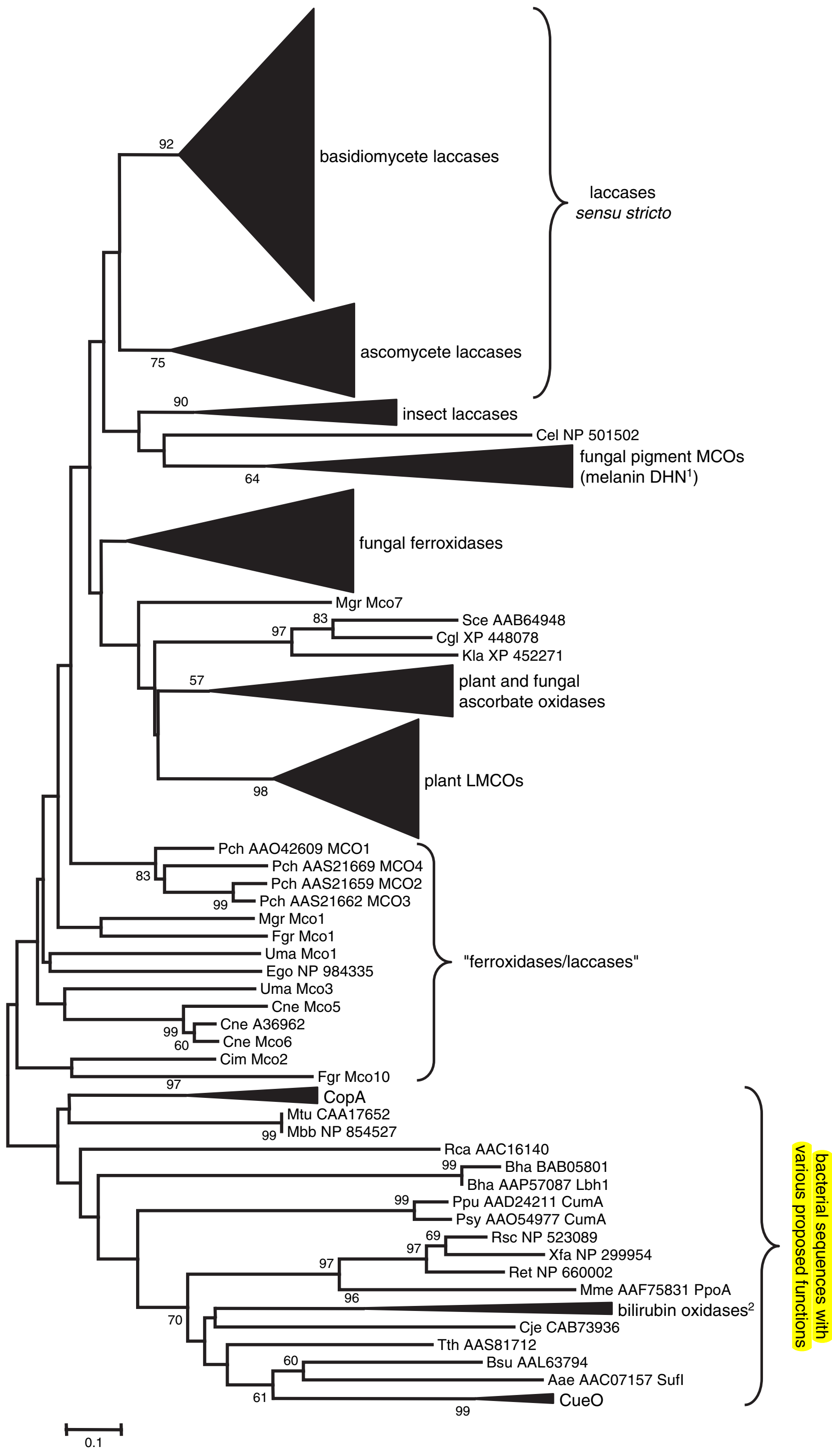


Fig. 1. Neighbour joining tree of multicopper oxidase amino acid sequences. Sequences without accession number were derived from the genome sequences (see Experimental procedures). Bootstrap values are from 500 replications, only values ≥ 50% are shown ([1]) including enzymes involved in melanin synthesis by the 1,8-dihydroxy-naphtalene (DHN) pathway, and ([2]) including two sequences from ascomycetes.

identified. However, from only a small part of these genes the product has been identified or even characterized. McCaig *et al.* [28] proposed to categorize plant LMCOs on the basis of sequence similarity and phylogenetic analysis until specific physiological functions are defined. They presented a classification of plant LMCO sequences and, together with expression profiles, provided strong evidence that most LMCOs from *A. thaliana* are not involved in lignification but may play a role in iron or other metal metabolisms. In order to characterize plant and fungal laccases into distinct subgroups based on signature sequences, Kumar *et al.* [46] analyzed over 100 laccase-like sequences. Here we present phylogenetic analyses and a classification of over 350 MCO sequences, including laccases, ascorbate oxidases, ferroxidases, and other, not clearly assigned proteins from the animal, plant, fungal, and bacterial kingdom.

Results and discussion

MCO phylogenetic tree overview

After the different search and selection processes, a total of 271 MCO amino acid sequences were obtained from the NCBI GenBank. Another 90 sequences were retrieved from the publicly available genomic sequences of basidiomycetous and ascomycetous fungi (see Experimental procedures), resulting in a total number of 361 sequences. The sequences cover various taxonomic groups. The 258 fungal sequences make up more than two thirds of all sequences. They were derived from 38 different basidiomycete, 30 ascomycete, and one zygomycete species. Further, a total of 62 plant sequences (from one gymnosperm, 12 dicotyledon angiosperms, and two monocotyledon angiosperms), 12 animal (from one nematode and four insect species), and 29 prokaryotic sequences (from one archaea, 17 Gram-negative, and six Gram-positive bacteria) were included in the analysis. In order to analyze the similarities among these sequences, we used the neighbour joining method with different distance estimation models (see Experimental procedures) to construct phylogenetic trees based on the manually adjusted ClustalX alignment. Clades consistent among trees were assigned and named according to included sequences with known functions and/or enzymatic characteristics (Fig. 1, only tree based on the JTT model shown). Based on the main clusters we propose the following classification of MCOs (see below): laccases *sensu stricto* (basidiomycetous and ascomycetous), insect laccases, fungal pigment MCOs, fungal ferroxidases, ascorbate oxidases, plant LMCOs, bilirubin oxidases. Nakamura and Go [47] recently presented a comparison of blue copper proteins (including the MCOs) and proposed an evolutionary scenario creating the molecular diversity in this diverse assemblage of proteins. Focusing on the MCOs only, our analysis yielded a more resolved phylogeny of the MCO sequences, providing the base for the (putative) functional assignment of sequences.

One of the most obvious features of the tree was that the laccase *sensu stricto* sequences clustered according to the taxonomical association of the corresponding species. The fungal laccases were clearly separated in two clusters containing either exclusively homobasidiomycete or filamentous ascomycete sequences, respectively (Fig. 1). The former cluster included all the well characterized basidiomycete laccases (e.g. from *Coprinopsis cinerea*, *Pleurotus ostreatus*, *Pycnoporus cinnabarinus*, *Rhizoctonia solani*, *Trametes* sp., Fig. 2A, for references see Table 1) referred to as *bona fide* laccases [48]. The latter contained most of the reported ascomycete laccases (from *Aspergillus terreus* [49], *Botrytis cinerea* [50], *Cryphonectria parasitica* [18], *Gaeumanomyces graminis* [51], *Melanocarpus albomyces* [52], *Neurospora crassa* [53], and *Podospora anserina* [54], as well as several previously undescribed sequences we deduced from whole genome sequences (Fig. 2B). Similarly, all insect sequences grouped together (Fig. 2C). Although the enzymatic activity-sequence link has been established for none of these animal sequences yet, expression data suggest that some of the enzymes included here are involved in cuticular sclerotization [32].

The fungal pigment MCO cluster included sequences from filamentous ascomycetes, ascomycetous yeasts and from basidiomycetes (Fig. 2D). It contained the enzymes YA from *Aspergillus nidulans* and Abr2p from *A. fumigatus*, both of which are required in conidial pigment biosynthesis [14,15]. More specifically, Abr2p was suggested to be involved in a DHN-melanin (named for the pathway intermediate 1,8-dihydroxynaphthalene) biosynthesis pathway [15]. YA has been named a laccase because of its ability to oxidize typical laccase substrates such as *p*-phenylenediamines, pyrogallol, and gallic acid, however, no data on enzyme kinetics are available [14].

The fungal ferroxidase cluster comprised sequences from ascomycetous yeasts, filamentous ascomycetes and basidiomycetes (Fig. 2E). It included the characterized Fet3 ferroxidases from the yeasts *Arxula adeninivorans*, *Candida albicans*, and *S. cerevisiae* [55–57] and the sequence from gene *abr1* neighbouring the putative laccase gene *abr2* in a gene cluster for conidial pigment synthesis in *Aspergillus fumigatus* [15]. In the neighbour joining tree based on p-distances, the ferroxidase cluster included three additional sequences (Ego_NP_984335, Fgr_Mco1, Mgr_Mco1) compared to the PAM and JTT trees (not shown). These three sequences belong to a grade of sequences whose grouping was not consistently supported between the different trees. We marked them 'ferroxidases/laccases' (in quotes to differentiate this grade from clusters/clades) due to the presence of Mco1 from *P. chrysosporium* [10] and a laccase from *C. neoformans*, shown to polymerize 3,4-dihydroxyphenylalanine (L-DOPA) in melanin synthesis [17,58]. These two enzymes were shown to have both strong ferroxidase and weak laccase activities and are thus not typical laccases [10,40]. This grade also included sequences from filamentous ascomycetes (Fig. 1).

Plant and fungal ascorbate oxidase sequences grouped together separate from the laccase or ferroxidase clusters (Fig. 1). These sequences were further divided into three closely related subclusters: one with characterized and predicted plant ascorbate oxidases [4,59,60], the second with predicted sequences from the zygomycete *Rhizopus oryzae*, and the third with the so far sole reported fungal ascorbate oxidase Asom from *Acremonium* sp. HI-25 [61]. Further sequences in the latter subcluster originated from other filamentous ascomycetes and from the basidiomycete *Ustilago maydis* (Fig. 2F).

The cluster with the sequences of characterized laccases or LMCOs from the plants *Acer pseudoplatanus*, *L. tulipifera*, and *Populus trichocarpa* [23,62,63] included exclusively plant sequences (Fig. 2G).

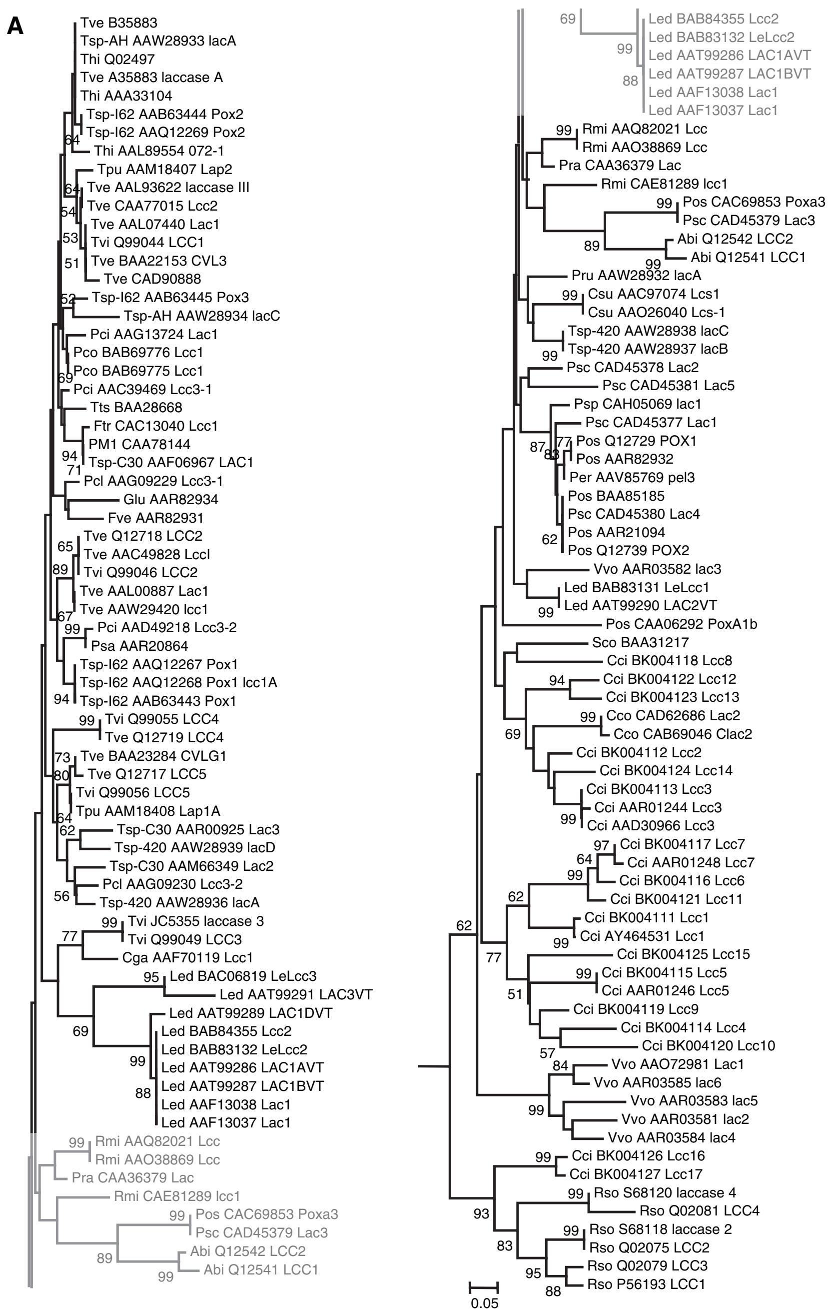
The bacterial sequences grouped clearly separate from almost all eukaryotic proteins. Two clusters were obvious among the *Eubacteria* sequences, consisting of copper resistance proteins (CopA, Fig. 2H) and copper efflux proteins (CueO, Fig. 2J), respectively [64]. Only one *Archaea* and two fungal sequences were among the eubacterial sequences: the undescribed MCO from the hyperthermophilic *Pyrobaculum aerophilum*, the bilirubin oxidase from the ascomycete *Myrothecium verrucaria* [65], and the closely related phenol oxidase from the ascomycete *Acremonium murorum* [66]. The two fungal sequences belong to the third cluster among the bacterial sequences assigned as bilirubin oxidases (Fig. 2I) due to the corresponding activities described for CotA from *B. subtilis* [36] and bilirubin oxidase from *M. verrucaria* [65]. The latter enzyme is a MCO oxidizing bilirubin to biliverdin, but also typical laccase substrates like ABTS [2,2′-azinobis(3-ethylbenzo-6-thiazolinesulfonic acid)] or syringaldazine [67]. It was found in a screen of microorganisms for decolourization of urine and faeces (containing bilirubin) in raw sewage [68]. The biological role of bilirubin oxidase activity, however, is not known. Biliverdin is the chromophore of bacteriophytochromes, homologues of which were found in fungi, and it is also a precursor molecule in chromophore synthesis of plant and cyanobacterial phytochromes [69,70]. Due to the lack of experimental data, however, any connection between the chromophores (synthesis or degradation) and bilirubin oxidase remains purely speculative.

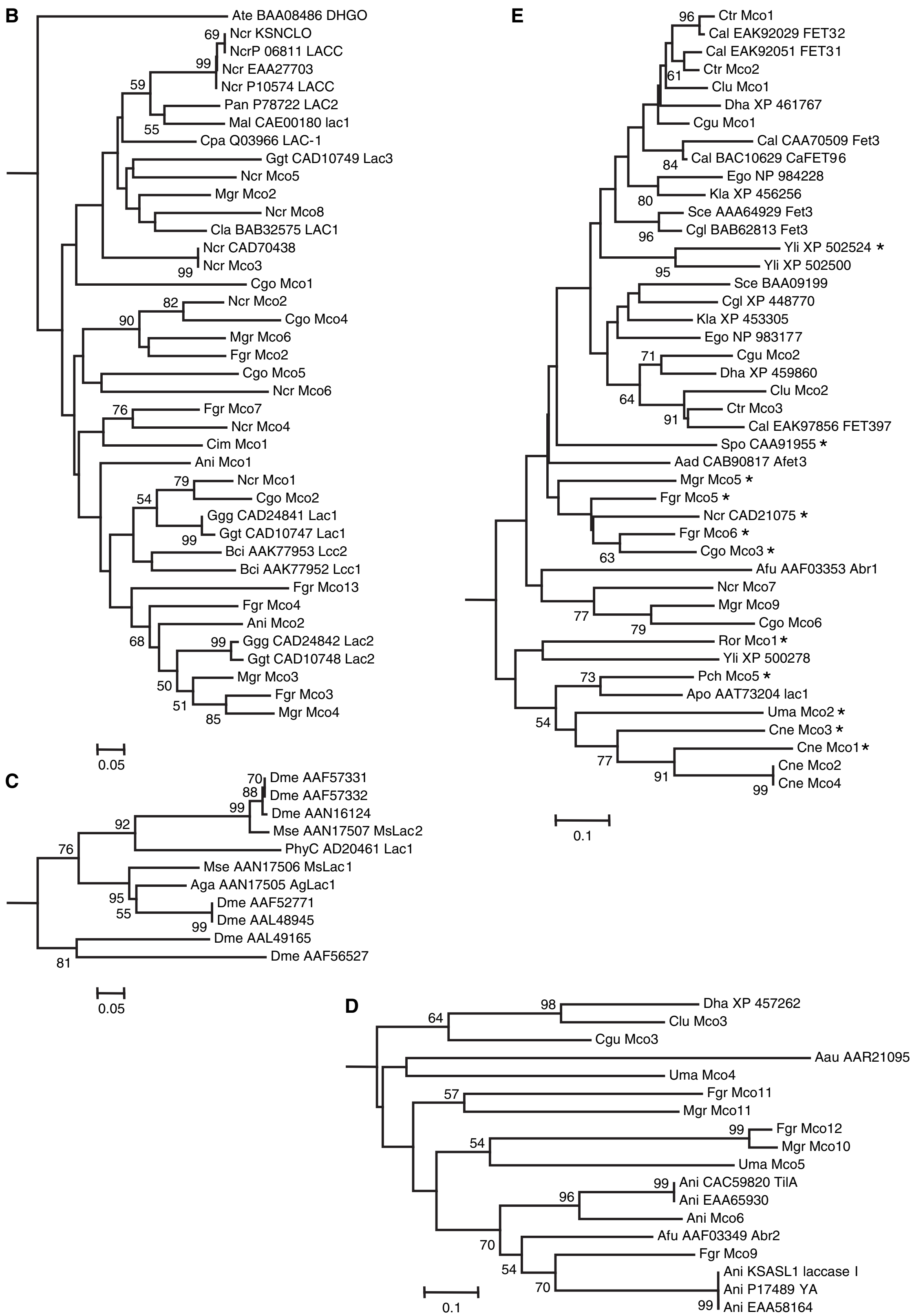
Fig. 2. Details of clusters from Fig. 1. Sequences without accession number were derived from the genome sequences (see Experimental procedures). Bootstrap values are from 500 replications, only values ≥ 50% are shown. (A) Basidiomycete laccases, (B) ascomycete laccases, (C) insect laccases, (D) fungal pigment MCOs (melanin DHN), (E) fungal ferroxidases, (F) fungal and plant ascorbate oxidases, (G) plant LMCOs, (H) CopA (copper resistance), (I) bilirubin oxidases, and (J) CueO (copper efflux). Asterisks in (E) mark the ferroxidases where the corresponding genes are arranged in a mirrored tandem with an iron permease homologue. Note: Cgo_Mco3, Clu_Mco2, Ctr_Mco1, Ctr_Mco2, and Ctr_Mco3 with frame shifts in the genomic sequences. Species codes: Aad, *Arxula adeninivorans*; Aae, *Aquifex aeolicus*; Aau, *Auricularia auricula-judae*; Abi, *Agaricus bisporus*; Afu, *Aspergillus fumigatus*; Aga, *Anopheles gambiae*; Amu, *Acremonium murorum*; Ani, *Emericella nidulans*; Apo, *Auricularia polytricha*; Aps, *Acer pseudoplatanus*; Asp-HI, *Acremonium* sp. HI-25; Ate, *Aspergillus terreus*; Ath, *Arabidopsis thaliana*; Bci, *Botryotinia fuckeliana*; Bha, *Bacillus halodurans*; Bpe, *Bordetella pertussis*; Bsu, *Bacillus subtilis*; Cal, *Candida albicans*; Cci, *Coprinopsis cinerea*; Cco, *Coprinellus congregatus*; Ccr, *Caulobacter crescentus*; Ccv-EN, *Cucurbita* cv. Ebisu Nankin; Cel, *Caenorhabditis elegans*; Cga, *Coriolopsis gallica*; Cgl, *Candida glabrata*; Cgo, *Chaetomium globosum*; Cgu, *Candida guilliermondii*; Cim, *Coccidioides immitis*; Cje, *Campylobacter jejuni*; Cla, *Colletotrichum lagenarium*; Clu, *Candida lusitanae*; Cma, *Cucurbita maxima*; Cme, *Cucumis melo*; Cne, *Filobasidiella neoformans*; Cpa, *Cryphonectria parasitica*; Csa, *Cucumis sativus*; Csu, *Ceriporiopsis subvermispora*; Ctr, *Candida tropicalis*; Dha, *Debaryomyces hansenii*; Dme, *Drosophila melanogaster*; Eco, *Escherichia coli*; Ego, *Ashbya gossypii*; Fgr, *Gibberella zeae*; Ftr, *Funalia trogii*; Fve, *Flammulina velutipes*; Gar, *Gossypium arboreum*; Ggg, *Gaeumannomyces graminis var. graminis*; Ggt, *Gaeumannomyces graminis var. tritici*; Glu, *Ganoderma lucidum*; Gma, *Glycine max*; Kla, *Kluyveromyces lactis*; Led, *Lentinula edodes*; Lpe, *Lolium perenne*; Ltu, *Liriodendron tulipifera*; Mal, *Melanocarpus albomyces*; Mbb, *Mycobacterium bovis* ssp. *bovis*; Mgr, *Magnaporthe grisea*; Mme, *Marinomonas mediterranea*; Mse, *Manduca sexta*; Mtr, *Medicago truncatula*; Mtu, *Mycobacterium tuberculosis*; Mve, *Myrothecium verrucaria*; Ncr, *Neurospora crassa*; Nta, *Nicotiana tabacum*; Oih, *Oceanobacillus iheyensis*; Osa, *Oryza sativa* (*japonica* cultivar-group); Pae, *Pyrobaculum aerophilum*; Pan, *Podospora anserina*; Pbt, *Populus balsamifera* ssp. *trichocarpa*; Pch, *Phanerochaete chrysosporium*; Pci, *Pycnoporus cinnabarinus*; Pcl, *Polyporus ciliatus*; Pco, *Pycnoporus coccineus*; Per, *Pleurotus eryngii*; Phy, *Pimpla hypochondriaca*; PM1, Basidiomycete PM1; Pos, *Pleurotus ostreatus*; Ppu, *Pseudomonas putida*; Pra, *Phlebia radiata*; Pru, *Panus rudis*; Psa, *Pycnoporus sanguineus*; Psc, *Pleurotus sajor-caju*; Psp, *Pleurotus sapidus*; Psy, *Pseudomonas syringae*; Pta, *Pinus taeda*; Rca, *Rhodobacter capsulatus*; Ret, *Rhizobium etli*; Rmi, *Rigidoporus microporus*; Ror, *Rhizopus oryzae*; Rsc, *Ralstonia solanacearum*; Rso, *Thanatephorus cucumeris*; Sce, *Saccharomyces cerevisiae*; Sco, *Schizophyllum commune*; Sla, *Streptomyces lavendulae*; Spo, *Schizosaccharomyces pombe*; Stm, *Salmonella typhimurium*; Sty, *Salmonella typhi*; Thi, *Trametes hirsuta*; Tpu, *Trametes pubescens*; Tsp420, *Trametes* sp. 420; Tsp-AH, *Trametes* sp. AH28-2; Tsp-C30, *Trametes* sp. C30; Tsp-I62, *Trametes* sp. I-62; Tth, *Thermus thermophilus*; Tts, *Trachyderma tsunodae*; Tve, *Trametes versicolor*; Tvi, *Trametes villosa*; Uma, *Ustilago maydis*; Vvo, *Volvariella volvacea*; Xca, *Xanthomonas campestris*; Xfa, *Xylella fastidiosa*; Yli, *Yarrowia lipolytica*; Ype, *Yersinia pestis*.

Fungal MCO multigene families

The composition of the MCO arsenal of different fungal taxonomic groups seems to be quite variable. Considering only complete fungal *mco* gene families, i.e. where whole genome sequences are available, half of the basidiomycete and filamentous ascomycete sequences (41 out of 84 total sequences) belong to the laccase *sensu stricto* clusters (Table 2). The other sequences of both basidiomycetes and filamentous ascomycetes are distributed over the fungal pigment MCOs, ferroxidases, and ascorbate oxidases clusters or belong to no cluster. In contrast, MCOs from the ascomycetous yeasts belong almost all to the ferroxidases. According to their grouping in the tree, four of the five MCOs from the zygomycete *R. oryzae* seem to be ascorbate oxidases.

The ferroxidases are the best represented group, being present in 19 of the 22 fungal genomes analyzed here (Table 2). In *S. cerevisiae*, the ferroxidase Fet3p





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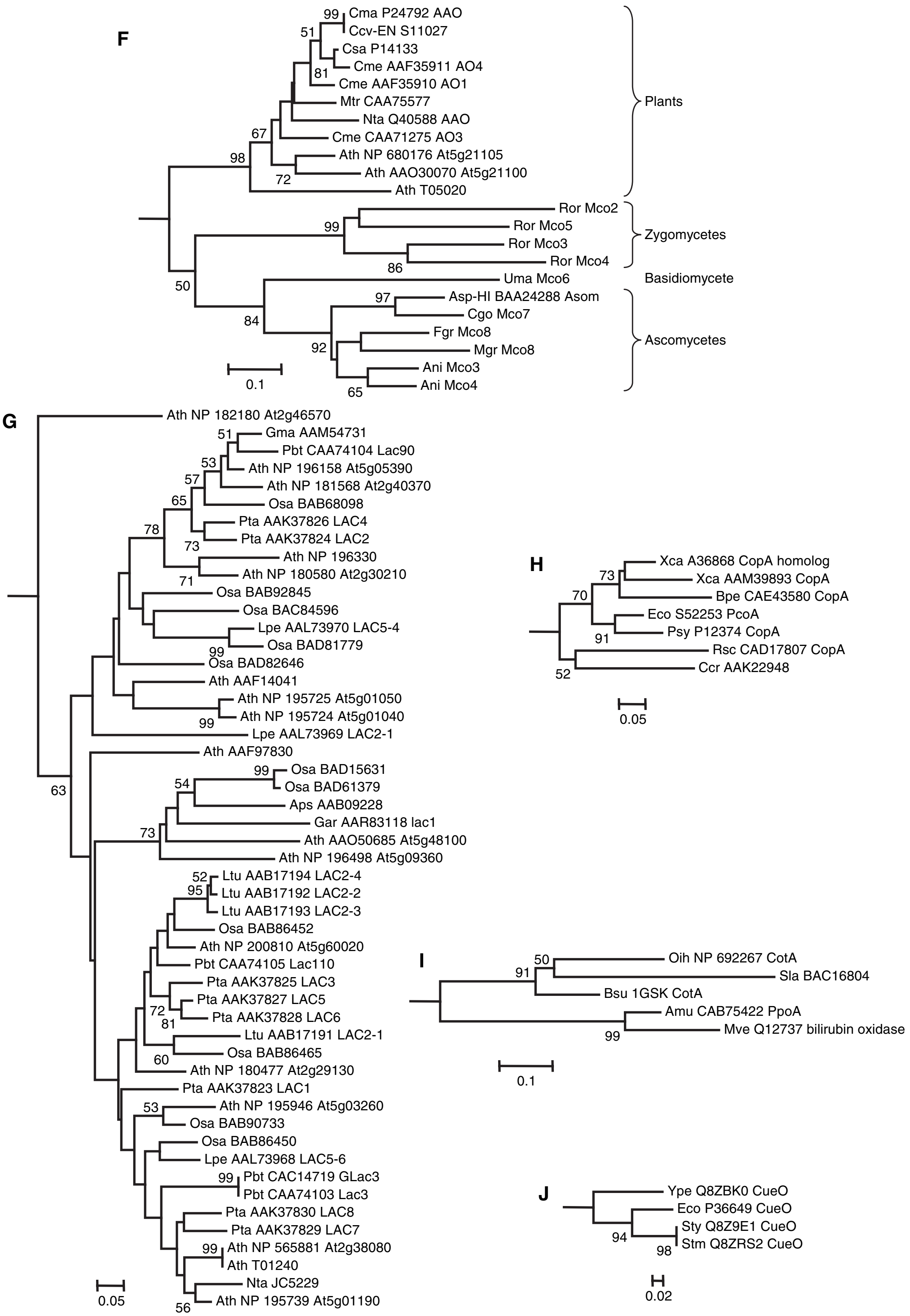


Fig. 2. (Continued).

Table 1. Biochemically characterized basidiomycete laccases with corresponding sequences in the detailed tree in Fig. 3

No.[b]	Species	Acc. no.	Protein	p*I* value	Optimal pH[a] ABTS	Optimal pH[a] SGZ	Optimal pH[a] Other substrates	Redox potential	Kinetics[a,c]	Ref.
1	*Trametes* sp. AH28-2	AAW28933	LacA	4.2			GUA 4.5		ABTS K_m 25, k_{cat} 692 (27.7), GUA K_m 420, k_{cat} 69 (0.16), DMP K_m 25.5, k_{cat} 81 (3.2)	88
2	*Trametes pubescens*	AAM18407	Lap2	2.6	3	4.5	GUA 3, DMP 3, *p*-anisidine 4.5, catechol 3.5, hydroquinone 3.5, ferrocyanide 3.0		ABTS K_m 14, k_{cat} 690 (48), GUA K_m 360, k_{cat} 180 (0.51), DMP K_m 72, k_{cat} 400 (5.6)	89
3	*Trametes versicolor*	AAL07440	Lac1	2.75–3.23					ABTS K_m 60, k_{cat} 220 (3.7), 2HF K_m 230, k_{cat} 32 (0.14), 2HF-4 CL K_m 380, k_{cat} 140 (0.37), 2HF-5 CL K_m 240, k_{cat} 63 (0.26), 4HF K_m 600, k_{cat} 47 (0.08), 4HF-5 CL K_m 220, k_{cat} 97 (0.44)	90
4	*Trametes villosa*	Q99044	LCC1	3.5	≤ 2.7	5–5.5				78
5	*Pycnoporus cinnabarinus*	AAG13724	Lac1	< 3.5						91
6	*Pycnoporus cinnabarinus*	AAC39469	Lcc3–1	3.7			GUA 4			92
7	*Trametes* sp. C30	AAF06967	LAC1	3.6		4.5–5		0.73 V	SGZ K_m 1.8, k_{cat} 30 (16.7), GUA K_m 71, k_{cat} 38.3 (0.5), ABTS K_m 10.7, k_{cat} 55.8 (5.2)	77, 93
8	Basidiomycete PM1	CAA78144	Laccase	3.6			GUA 4.5			94, 95
9	*Trametes villosa*	Q99046	LCC2	6.2–6.8	6	5–5.5				78
10	*Trametes* sp. C30	AAM66349	Lac2	3.2		5.5–6		0.56 V	SGZ K_m 6.8, k_{cat} 1093.3 (160.8) GUA K_m 1006, k_{cat} 1261.3 (1.3), ABTS K_m 536, k_{cat} 683.3 (1.3)	77
11	*Ceriporiopsis subvermispora*	AAC97074	Lcs1	Approx. 3.6						96
12	*Lentinula edodes*	BAB83131	LeLcc1	3.0	4		GUA 4.0, DMP 4.0, *p*-phenylenediamine 5.0, pyrogallol 4.0, ferrulic acid 5.0, catechol 4.0		ABTS K_m 108, GUA K_m 917, DMP K_m 557, catechol K_m 22400, pyrogallol K_m 417, *p*-phenylenediamine K_m 256, ferrulic acid K_m 2860	97

Table 1. (Continued).

No.[b]	Species	Acc. no.	Protein	p*I* value	Optimal pH[a] ABTS	SGZ	Other substrates	Redox potential	Kinetics[a,c]	Ref.
13	*Pleurotus ostreatus*	Q12739	POX2	3.3	2.5		DMP 3.5	0.74 V	ABTS K_m 39, k_{cat} 1866 (47.8), DMP K_m 7.6, k_{cat} 1150 (151.3)	98
14	*Pleurotus ostreatus*	CAA06292	PoxA1b	6.9	3	6	DMP 4.5	0.65 V	ABTS K_m 370, k_{cat} 1500 (4.1), SGZ K_m 220, k_{cat} 333.3 (1.5), DMP K_m 260, k_{cat} 6000 (23.1)	98, 99
15	*Volvariella volvacea*	AAO72981	lac1	3.7	3	5.6	DMP 4.6		ABTS K_m 30, SGZ K_m 10, DMP K_m 570	100
16	*Coprinopsis cinerea*	AY464531	Lcc1	3.7 and 4	4	6.5				101
17	*Pleurotus ostreatus*	CAC69853	Poxa3	POXA3a 4.3, 4.1 POXA3b	3.6	6.2	DMP 5.5		POXA3a ABTS K_m 70, k_{cat} 73333 (1047.6), SGZ K_m 36, k_{cat} 2833.3 (78.7), DMP K_m 14000, k_{cat} 23333.3 (1.7) ABTS K_m 74, k_{cat} 158333.3 (2139.6), SGZ K_m 79, k_{cat} 11666.6 (147.7), DMP K_m 8800, k_{cat} 20000 (2272.2)	102
18	*Thanatephorus cucumeris*	S68120	Laccase 4	7.5	≤ 2.7	7				103

[a]ABTS, 2,2′-azinobis (3-ethylbenzo-6-thiazolinesulfonic acid); SGZ, syringaldazine; DMP, 2,6-dimethoxyphenol; GUA, guaiacol; 2HF, *N′*,*N′*-dimethyl-*N*-(2-hydroxyphenyl)urea; 2HF-4 CL, *N′*,*N′*-dimethyl-*N*-(4-chloro-2-hydroxyphenyl)urea; 2HF-5 CL, *N′*,*N′*-dimethyl-*N*-(5-chloro-2-hydroxyphenyl)urea; 4HF, *N′*,*N′*-dimethyl-*N*-(4-hydroxyphenyl)urea; 4HF-5 CL, *N′*,*N′*-dimethyl-*N*-(5-chloro-4-hydroxyphenyl)urea. [b]No. refers to numbers in circles in Fig. 3. [c]K_m in μM, k_{cat} in s^{-1}, ratio k_{cat}/K_m given in brackets in $\mu M^{-1} \cdot s^{-1}$.

Table 2. Number of sequences from complete fungal *mco* multigene families in the different clusters and presence of homologues of representative genes of the high affinity iron uptake pathways.

	Basidiomycetes				Filamentous ascomycetes						Ascomycetous yeasts											Zygomycete
Cluster[a]	Cci	Cne	Pch	Uma	Ani	Ncr	Fgr	Mgr	Cgo	Cim	Ego	Sce	Cal	Cgl	Cgu	Clu	Ctr	Kla	Dha	Yli	Spo	Ror
Total MCOs	17	6	5	6	7[b]	10	13	11	7	2	3	3	5	3	3	3	3	3	3	3	1	5
Basidiomycete laccases	17																					
Ascomycete laccases					2	8	5	4	4	1												
Fungal pigment MCOs (melanin DHN)				2	3		3	2							1	1			1			
Fungal ferroxidases		4	1	1		2	2	2	2		2	2	5	2	2	2	3	2	2	3	1	1
Fungal and plant ascorbate oxidases				1	2		1	1	1													4
Not in any cluster		2	4	2			2	2		1	1	1		1				1				
Genes of high affinity iron uptake pathways																						
ftr1 homologue(s)	−	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
ftr1 homologue(s) clustered with MCOs[c]	−	2	1	1	−	1	2	1	1	−	−	−	−	−	−	−	−	−	−	1	1	1
sid1/sidA homologues	+	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	−[d]

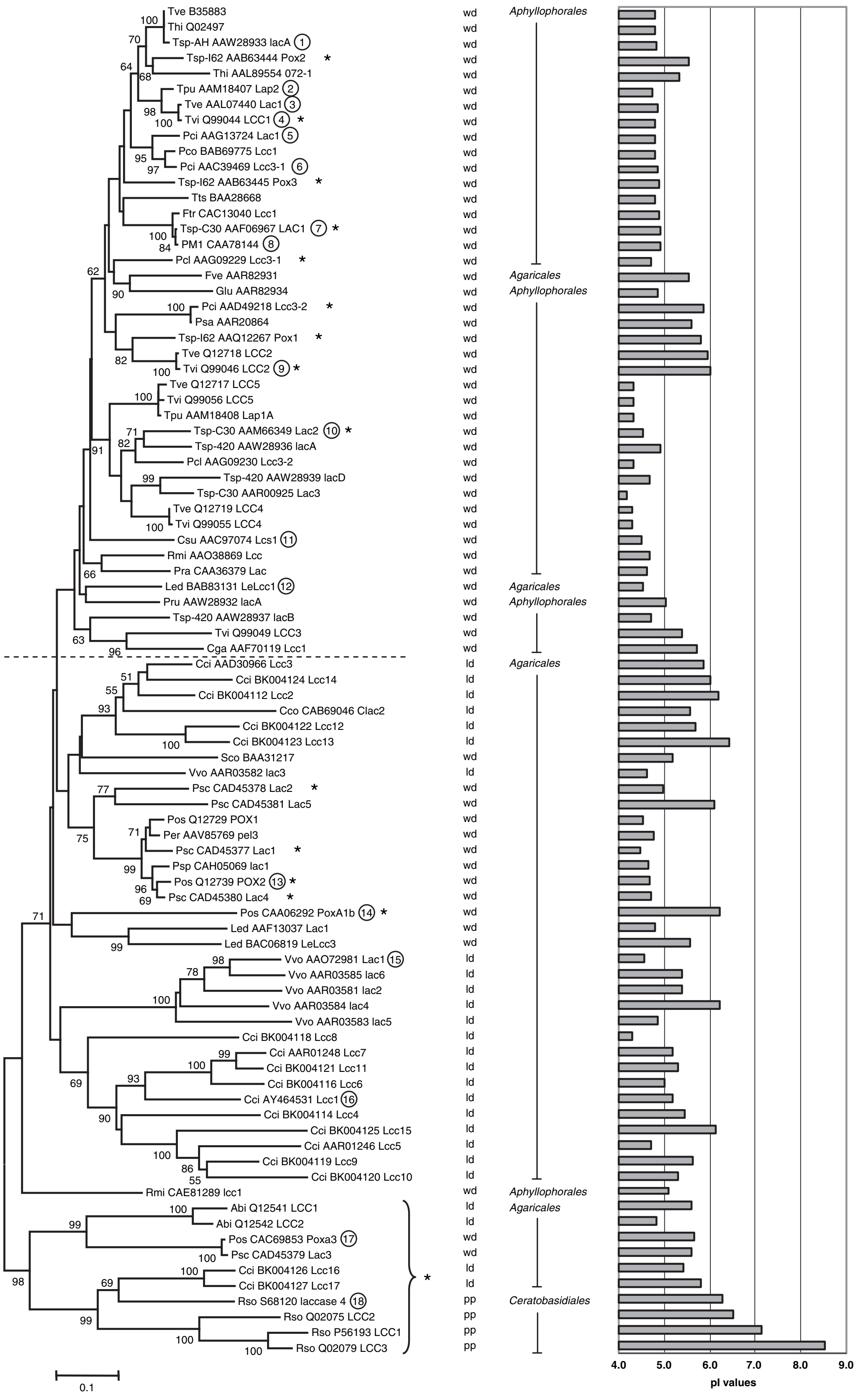
[a]Cluster according to phylogenetic tree in Fig. 1. For abbreviations, see Fig. 2. [b]Not including one MCO lacking the L1 signature sequence in the predicted sequence. [c]See Fig. 2E. [d]Instead of the hydroxamate siderophores typical for fungi, zygomycetes produce siderophores of the carboxylate group [75].

and the iron permease Ftr1p physically interact with each other to form a multicomponent system for high affinity iron uptake [71]. Interestingly, the three species that do not have a ferroxidase (*C. cinerea*, *A. nidulans*, and *Coccidioides immitis*) also lack homologues of the iron permease gene *ftr1*, whereas the other 19 species have at least one gene coding for a putative Ftr1p as determined by Blast searches (Table 2). Furthermore, in the filamentous species and the yeasts *Yarrowia lipolytica* and *Schizosaccharomyces pombe*, at least one of the *fet3* homologues is arranged in a mirrored tandem with the *ftr1* homologue (Table 2, Fig. 2E), i.e. the putative start codons are less than 5 kb apart and they are divergently transcribed. Such an arrangement could indicate a common regulation and function of the genes in iron metabolism as it was proposed for the *fet3/ftr1* homologues of *S. pombe* or *P. chrysosporium* [72,73]. Thus, the presence of *ftr1* homologues only in the fungal genomes that also have a ferroxidase suggest that at least one of the ferroxidases in each of those species may play a similar role as in *S. cerevisiae*. In addition to the reductive pathway involving Fet3p/Ftr1p, many fungi also developed another high affinity mechanism to accumulate iron, namely the siderophore-dependent pathway [74]. The presence of homologues of the *sid1* or *sidA* genes (Table 2), encoding a L-ornithine-N^5-monooxygenase catalyzing the first step in hydroxamate siderophore biosynthesis in *U. maydis* and *A. nidulans* [75], respectively, suggests that the species lacking ferroxidases use only this alternative pathway for their (high affinity) iron uptake.

Evolution of basidiomycetous laccases

In order to understand more about the evolution of the basidiomycetous laccases, we subjected all sequences from the basidiomycete laccase cluster to a more stringent analysis (see Experimental procedures). The clustering of the sequences in the NJ tree does not strictly follow taxonomical relationships of the species they were derived from (Fig. 3). Similar subclusters as in the NJ tree were observed in the tree generated by the maximum likelihood method (not shown). The arrangement of the sequences suggests that clustering

Fig. 3. Neighbour joining tree of basidiomycete laccases based on realigned sequences. Putative allelic sequences were omitted. Bootstrap values are from 500 replications; only values ≥ 50% are shown. Wd, wood-decaying (including uncharacterized *Trametes* sp. C30 (formerly misidentified as *Marasmius quercophilus* [104]), *Trametes* sp. 420, and basidiomycete PM1 isolated from wastewater but shown to be ligninolytic [94]); ld, litter-decomposing; pp, plant pathogen. Circled numbers refer to characterized laccases in Table 1, asterisks indicate characterized laccases mentioned in the discussion. Dashed line indicates border of upper and lower part of the tree as discussed in the text. The bar diagram shows calculated p*I* values.


Aphyllophorales
Agaricales
Ceratobasidiales
wd
ld
pp
pI values
4.0
5.0
6.0
7.0
8.0
9.0
0.1

is at least in part according to the function of the respective enzymes. The laccases in the upper part of the tree beginning with *Coriolopsis gallica* Lcc1 are all from typical wood decay species and this group may be specific to wood degradation. In the lower part of the tree, some sequences from the same species were found among different subclusters. This could be explained by, within the same species, the possible variability in demands on oxidative enzymes causing the paralogous laccase copies to diversify. The phylogenetic analysis clearly supports the presence of multiple laccases in the ancestors of these species that have been maintained during the speciation and diversification of the Homobasidiomycete fungi.

Evidence for different functions of the various laccases is provided by expression studies and biochemical characterizations of different members from laccase multigene families. Unfortunately, the sequence-enzyme link has been established only for a few laccases so far (Table 1). Most information is available on laccases from typical white rot fungi by which the enzymes are thought to be involved in lignin degradation. Laccase LAC1 and LAC2 from *Trametes* sp. C30 are well separated from each other on the NJ tree (Fig. 3). Whereas LAC1 is constitutively produced in liquid malt extract medium, LAC2 synthesis is induced by the addition of copper and *p*-hydroxybenzoic acid [76]. Further, LAC2 has a redox potential of 0.56 V compared to 0.73 V of LAC1 [77]. Due to the differences in expression pattern and biochemical properties of the enzymes, Klonowska *et al.* [77] suggested different physiological roles for these two enzymes. Expression of the *lcc1* gene from *Trametes villosa* could be induced 17-fold by addition of 2,5-xylidine to the liquid culture, whereas *lcc2* was not induced but present at a constitutive level [78]. Lcc1 has a p*I* value of 3.5, an optimal pH for ABTS of 2.7 and for syringaldazine of 5–5.5. The properties for Lcc2 are quite different with a p*I* value of 6.2–6.8, optimal pH for ABTS of 6 and for syringaldazine of 5–5.5 [78]. Lcc2 clustered with a group of five laccases with predicted p*I* values of 5.6–6 (Fig. 3), all higher than the average for all basidiomycete laccases at 5.2. It was suggested that the surface charge (directly correlated to p*I* values) on laccases might affect catalytic activity towards phenolic substrates whose oxidation accompanies proton release [67]. In fact, *T. villosa* Lcc2 activity dropped down to 15% of its optimal activity at pH 4 whereas Lcc1 still retained 50–60% [78]. Because of its differences in expression and enzymatic properties, it is likely that Lcc2 functions under different physiological or environmental conditions than Lcc1. Interestingly, Lcc2 is the only enzyme among all MCOs analyzed here, except for some more heterogeneous bacterial enzymes, which is lacking a highly conserved aspartate residue at the 13th position of the L1 signature sequence as defined by Kumar *et al.* [46]. Instead of the aspartate, Lcc2 has a glutamate residue. It was shown recently that the Asp serves as a proton donor in *M. verrucaria* bilirubin oxidase [79]. Point mutations at this site showed that the presence of a carboxyl group is required, although the enzymatic activity of the Glu-mutant of bilirubin oxidase was reduced to 46% [79]. In the case of *T. villosa* Lcc2, the Glu may be an adaptation to higher pH environments as its carboxyl group shows different proton dissociation properties compared to the one from Asp.

Complex lignin-like compounds such as coal-derived humic acids increased *P. cinnabarinus lcc3–1* but not *lcc3–2* transcript levels [80]. *pox1* and *pox2* transcription in *Trametes* sp. I-62 was induced at different growth stages by the lignin degradation product veratryl alcohol, whereas *pox3* transcripts remained constant. On the other hand, the latter gene seemed to be carbon catabolite repressed [81]. These examples suggest different roles for the members of the laccase families during the lifecycle of the organism.

Further evidence that the clustering at least partially reflects the function was obtained by a phylogenetic analysis using partial laccase sequences from the ascomycetes *Xylaria* sp. and *Hypoxylon* sp. [82]. The sequences from the xylariaceous ascomycetes were clustering among those from wood-decaying basidiomycetes (data not shown). Compared to most other ascomycetes, xylariaceous fungi seem to be capable of lignin mineralization [82–84]. Therefore, the close similarities of the laccases may be based on the same presumed function as for those in the wood-decaying basidiomycetes.

Next to lignin degradation, other biological roles for laccases have been described (e.g. involvement in different developmental processes, see above) and the close similarity of laccases from fungi occupying different niches may be due to a shared function independent of the ecological niche. This may be the case for the cluster involving the laccases from the litter-decomposing *A. bisporus* (LCC1 and LCC2) and *C. cinerea* (Lcc16 and Lcc17), the wood-decaying *P. ostreatus* (POXA3) and *Pleurotus sajor-caju* (Lac3), and the herbaceous plant pathogen *R. solani* (LCC1 to LCC4). Compared to other members of the *P. sajor-caju* laccase gene family (*lac1*, *lac2*, and *lac4*), *lac3* is constitutively expressed and not inducible by nutrient nitrogen and carbon, copper, manganese, and several different aromatic compounds [85]. *P. ostreatus* POXA3 is differentially regulated at the protein level. The protease

PoS1 is involved in the activation of POXA3, whereas POXA1b was degraded in presence of PoS1 and POXC was not affected [86]. Furthermore, considerable differences in their enzyme kinetics suggest different substrate specificities (Table 1). Neither expression data nor enzyme properties are yet available for the *C. cinerea* Lcc16 and Lcc17, making up their own subfamily among the 17-member multigene family of the species (Kilaru *et al.*, unpublished results). As the only sequences in the basidiomycete cluster, Lcc16 and Lcc17 have a glutamate residue (E191 and E192, respectively) which otherwise is only present among sequences from the ferroxidase cluster and the ferroxidase/laccase grade and four sequences outside of the main clusters. This Glu is conserved in yeast ferroxidases and was shown to be essential for activity of Fet3p from *S. cerevisiae* [87]. As *C. cinerea* does not have a *ftr1* homologue required for a high affinity iron uptake (see above), Lcc16 and Lcc17 may play a cytoprotective role as suggested by Stoj and Kosman [42].

Conclusion

The classification of enzymes from the MCO family according to enzymatic activities in many cases is a challenging task due to the wide and overlapping substrate specificities of most members. The present phylogenetic analysis of amino acid sequences of over 350 MCOs provides a valuable additional means to categorize enzymes in this family. The detailed analysis of basidiomycetous laccases suggested that clustering of the sequences was at least partially according to the function of the respective enzymes. Therefore, we conclude that these analyses will be helpful in evaluating the function of yet uncharacterized enzymes. Nevertheless, detailed and comparable biochemical characterizations of more MCOs are now needed in order to refine potential predictions based on our classification.

Experimental procedures

The NCBI GenBank database was mined by BlastP searches with different multicopper oxidase sequences (*P. ostreatus* Q12739, *Trametes versicolor* A35883, *P. cinnabarinus* AAG09231, *Lentinus edodes* BAB83132, *R. solani* S68120, *C. neoformans* A36962, *N. crassa* KSNCLT, *C. albicans* CAA70509, *Glycine max* AAM89257). More sequences were obtained by using the BLink option from GenBank in entries identified from published reports. In addition to the GenBank sequences, we deduced further sequences from the publicly available genome sequences of *P. chrysosporium* (http://www.jgi.doe.gov/whiterot/), *A. nidulans*, *Candida guilliermondii*, *Candida lusitanae*, *Candida tropicalis*, *Chaetomium globosum*, *C. immitis*, *C. cinerea*, *C. neoformans* Serotype A, *Fusarium graminearum*, *Magnaporthe grisea*, *N. crassa*, *R. oryzae*, and *U. maydis* (all from http://www.broad.mit.edu/annotation/) by tblastn searching and annotating by hand. Sequences were selected for the presence of the four conserved Cu-oxidase consensus patterns typical for the MCOs (see above). Only complete sequences were kept for further analyses. Proteins that could not be aligned over extended regions (e.g. MnxG from Bacillus SG-1) or lacking considerable stretches of sequence (e.g. EpoA from *Streptomyces coelicolor* and SLAC from *Streptomyces griseus* defined as two-domain multicopper blue proteins by Nakamura and Go [47]) were excluded. When such sequences were included initially, the alignment had to be restricted to the most conserved parts of the sequences because of ambiguity in the alignment. This restriction, however, also caused a reduction of the resolution of our phylogenetic analysis (not shown). Redundant sequences, i.e. sequences from the same species with 100% identity were also removed. Because of the lack of available information, we could not differentiate between allelic and nonallelic sequences and therefore kept all sequences with identities smaller than 100%.

For phylogenetic analysis of all MCOs, an alignment was created with CLUSTALX Version 1.81 (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) using the default settings for multiple sequence alignments. The obtained alignment was adjusted manually with GENEDOC Version 2.6.002 (http://www.psc.edu/biomed/genedoc/). Based on this alignment we constructed phylogenetic trees with MEGA Version 3.1 (http://www.megasoftware.net/) by the neighbour joining method using three different distance estimation models (p-distances, Dayhoff or PAM, Jones-Taylor-Thornton or JTT). Bootstrapping was carried out with 500 replications. The large dataset prevented the reasonable application of other phylogenetic inference methods (e.g. maximum likelihood based).

For the more detailed phylogenetic analysis of the basidiomycete laccases, a new alignment only including the sequences from the basidiomycete cluster from the MCOs tree was created. After manual adjustments, only conserved regions, i.e. where the assignment of positional homology was possible, were used for tree construction, all other regions were masked (excluded). Groups of very similar sequences (p-distances $< 5\%$) were reduced to one representative sequence for better visualization. A NJ tree was constructed using the JTT substitution-rate matrix in MEGA. Bootstrapping was performed with 500 replications. For further evaluation of the tree, the maximum likelihood method was used to generate another tree using the PROML program from the PHYLIP package Version 3.63 (http://evolution.genetics.washington.edu/phylip.html). The JTT model for amino acid substitution was chosen and *N. crassa* laccase KSNCLO was used as an outgroup. Tree topology was visualized using TREEVIEWX Version 0.5.0 (http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/index.html).

Analysis with the partial sequences from *Xylaria* sp. and *Hypoxylon* sp. [81]. was performed by creating an alignment using only the corresponding region from all MCO sequences spanning the segment from the L1 (HWHG…) to the middle of the L2 signature sequence (…WYHSH) according to Kumar *et al.* [46]. A NJ tree (p-distances) based on this alignment was constructed with MEGA.

Fungal genomes were searched for the presence of homologues of representative genes of the high affinity iron uptake pathways in the NCBI GenBank Genome database using the tblastn option. Protein query sequences were *S. cerevisiae* Ftr1p (Acc. No. NP_011072) and Arn1p (NP_011823), *U. maydis* Sid1 (P56584), and *A. nidulans* SidA (AAP56238).

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