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A survey of genes encoding H_2O_2-producing GMC oxidoreductases in 10 Polyporales genomes

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Abstract: The genomes of three representative Polyporales (*Bjerkandera adusta, Phlebia brevispora* and a member of the *Ganoderma lucidum* complex) recently were sequenced to expand our knowledge on the diversity and distribution of genes involved in degradation of plant polymers in this Basidiomycota order, which includes most wood-rotting fungi. Oxidases, including members of the glucose-methanol-choline (GMC) oxidoreductase superfamily, play a central role in the above degradative process because they generate extracellular H_2O_2 acting as the ultimate oxidizer in both white-rot and brown-rot decay. The survey was completed by analyzing the GMC genes in the available genomes of seven more species to cover the four Polyporales clades. First, an in silico search for sequences encoding members of the aryl-alcohol oxidase, glucose oxidase, methanol oxidase, pyranose oxidase, cellobiose dehydrogenase and pyranose dehydrogenase families was performed. The curated sequences were subjected to an analysis of their evolutionary relationships, followed by estimation of gene duplication/reduction history during fungal evolution. Second, the molecular structures of the near one hundred GMC oxidoreductases identified were modeled to gain insight into their structural variation and expected catalytic properties. In contrast to ligninolytic peroxidases, whose genes are present in all white-rot Polyporales genomes and absent from those of brown-rot species, the H_2O_2-generating oxidases are widely distributed in both fungal types. This indicates that the GMC oxidases provide H_2O_2 for both ligninolytic peroxidase activity (in white-rot decay) and Fenton attack on cellulose (in brown-rot decay), after the transition between both decay patterns in Polyporales occurred.

Key words: brown-rot fungi, evolutionary relationships, GMC oxidoreductases, sequenced genome analysis, white-rot fungi

Introduction

Although species from several Basidiomycota (and some Ascomycota) orders contribute to lignocellulose decay, the ability to degrade wood is a typical feature of the order Polyporales. This capability was an essential evolutionary trait acquired by ancestral basidiomycetes in the later Carboniferous period (Floudas et al. 2012), when the amount of carbon fixed by photosynthesis strongly increased due to colonization of land ecosystems by vascular plants. Nowadays fungal decay of wood represents a natural model for the sustainable use of plant resources in lignocellulose biorefineries (Martínez et al. 2009, Ragauskas et al. 2014).

The first basidiomycete genome to be sequenced was that of *Phanerochaete chrysosporium* (= *Phanerodontia chrysosporium*) (Martinez et al. 2004) due to the interest in this white-rot fungus of the order Polyporales as a model lignin-degrading organism (Kersten and Cullen 2007). Wood attack by white-rot fungi is based on their ability to degrade the recalcitrant polymer of lignin in a process that was defined as an enzymatic "combustion" (Kirk and Farrell 1987) and combines extracellular oxidases and peroxidases (Kersten and Cullen 2007, Ruiz-Dueñas and Martínez 2009). With a few exceptions corresponding to poor wood rotters (e.g. species of Jaapiales and Cantharellales), the presence of lignin peroxidase (LiP[3], EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) or versatile peroxidase (VP, EC 1.11.1.16) genes is a constant characteristic of all typical white-rot fungi based on comparative genome analysis (Floudas et al. 2012, 2015; Ruiz-Dueñas et al. 2013). The diversity, distribution and evolutionary relationships of ligninolytic peroxidases in the order Polyporales has been studied (Ruiz-Dueñas et al. 2013).

Brown-rot fungi have developed an alternative strategy, based on Fenton chemistry, to overcome the lignin barrier (Baldrian and Valaskova 2008). H_2O_2 reduction by ferrous iron yields hydroxyl free radical, which is able to access, oxidize and depolymerize wood cellulose with a more or less limited modification of lignin (Kirk 1975, Martínez et al. 2011, Yelle et al. 2011). In 2009 the genome of *Rhodonia placenta* (syn.: *Postia placenta*) was sequenced as the model brown-rot

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fungus to increase our understanding of this type of wood decay (Martinez et al. 2009).

Several oxidases have been related to wood biodegradation as a source of extracellular H_2O_2 (from O_2 reduction), including glucose oxidase (GOX, EC 1.1.3.4) (Kelley and Reddy 1986), methanol oxidase (MOX, EC 1.1.3.13, also known as ethanol/alcohol oxidase) (Nishida and Eriksson 1987, Daniel et al. 2007), aryl-alcohol oxidase (AAO, EC 1.1.3.7) (Guillén et al. 1990), pyranose 2-oxidase (P2O, EC 1.1.3.10) (Daniel et al. 1992) and glyoxal oxidase (GLX, EC 1.1.3.–) (Kersten and Kirk 1987). Although the involvement of some other intracellular oxidases has been suggested (Greene and Gould 1984, Kelley and Reddy 1986), wood decay is an extracellular process and secreted oxidases are more likely involved. Alternative mechanisms for H_2O_2 generation have been suggested, including Mn(III)-mediated oxidation of glyoxylic/oxalic acids (Urzúa et al. 1998).

GLX belongs to the superfamily of copper-radical oxidases (Whittaker et al. 1996) whose distribution in Polyporales genomes has been reported (Kersten and Cullen 2014). In contrast all other oxidases mentioned above are flavooxidases from the GMC oxidoreductase superfamily whose first three members were GOX, MOX and choline dehydrogenase (Cavener 1992). Two additional GMC enzymes, which are inefficient reducing O_2 to H_2O_2, are cellobiose dehydrogenase (CDH, EC 1.1.99.18) and pyranose dehydrogenase (PDH, EC 1.1.99.29) (Zámocký et al. 2006, Kruså et al. 2008, Peterbauer and Volc 2010). All members of the GMC superfamily share similar structural features (Wierenga et al. 1986, Kiess et al. 1998). Recently several GMCs have been classified in the so-called subfamilies AA3_1 (CDH), AA3_2 (AAO/GOX), AA3_3 (MOX) and AA3_4 (P2O) of the CAZy database (Levasseur et al. 2013), but this nomenclature is not used here.

Three representative Polyporales—*Bjerkandera adusta, Ganoderma* sp. (*G. lucidum* complex) and *Phlebia brevispora*—were sequenced (Hibbett et al. 2013) and their different GMC gene families are analyzed here. *Bjerkandera adusta* is a strong lignin degrader, which produces AAO (Muheim et al. 1990) together with ligninolytic peroxidases (Kimura et al. 1991, Heinfling et al. 1998). Some species of *Ganoderma* cause extensive wood delignification (González et al. 1986; Martínez et al. 1995, 2011) and little is known about GMC production by these fungi (Peláez et al. 1995, Ralph et al. 1996). Finally, *P. brevispora* was investigated for wood biopulping due to selective lignin removal (Akhtar et al. 1993, Fonseca et al. 2014). Moreover, seven additional sequenced Polyporales genomes were screened and included in the present comparative analysis of GMC-encoding genes. The present study is part of a wider genomic project covering other gene families (Ruiz-Dueñas et al. 2013, Hori et al. 2013, Mgbeahuruike et al. 2013, Syed et al. 2013, Kovalchuk et al. 2013) as an example of genome-enabled mycology to gain insight into the biology and evolution of fungi (Hibbett et al. 2013).

MATERIALS AND METHODS

Genome sequencing.—The genomic sequences of *B. adusta* (HHB-12826-SP), *P. brevispora* (HHB-7030-SS6) and *Ganoderma* sp. (10597-SS1) were obtained at the Joint Genome Institute (JGI), as part of the Saprotrophic Agaricomycotina Project coordinated by D.S. Hibbett (Clark University, USA). The genomes were produced as described by Binder et al. (2013), and the gene prediction is available at http://genome.jgi.doe.gov/Bjead1_1; http://genome.jgi.doe.gov/Gansp1 and http://genome.jgi.doe.gov/Phlbr1, respectively.

Genome screening for GMC gene families in Polyporales.—The above genomes, plus those of *Dichomitus squalens*, *Fomitopsis pinicola*, *Gelatoporia subvermispora* (syn.: *Ceriporiopsis subvermispora*), *P. chrysosporium*, *R. placenta*, *Trametes versicolor* and *Wolfiporia cocos* (= *Wolfiporia extensa*) available at the JGI MycoCosm portal (http://genome.jgi.doe.gov/programs/fungi) (Grigoriev et al. 2012) were screened for genes of the AAO, MOX, GOX, CDH, P2O and PDH families in the GMC superfamily. Among the above genomes, those from the Antrodia clade (*F. pinicola*, *R. placenta*, *W. cocos*) correspond to wood decay by brown-rot species while the other species (*B. adusta*, *D. squalens*, *Ganoderma* sp., *G. subvermispora*, *P. chrysosporium*, *P. brevispora* and *T. versicolor*) cause white-rot decay of wood.

The screening for each of the GMC families was performed by querying an entire set of filtered model proteins for each of the genomes with the following (GenBank) reference sequences: (i) AAO from *Pleurotus eryngii* (AAC72747); (ii) MOX from *Gloeophyllum trabeum, Pichia methanolica* and *Candida boidinii* (ABI14440, AF141329 and Q00922); (iii) GOXs from *Talaromyces flavus*, *Penicillium expasum*, *Penicillium amagasakiense*, *Aspergillus niger* and *Botryotinia fuckeliana* (AAB09442, ABN79922, AAD01493, AAF59929 and CAD88590); (iv) CDHs from *P. chrysosporium, G. subvermispora*, *Coniophora puteana, Pycnoporus cinnabarinus* (syn.: *Trametes cinnabarina*) and *T. versicolor* (CAA61359, ACF60617.1, BAD32781 AAC32197, AAC50004); (v) P2Os from *T. versicolor, Peniophora* sp., *P. chrysosporium*, *Lyophyllum shimeji* and *G. trabeum* (BAA11119, AAO13382, AAS93628, BAD12079 and ACJ54278); (vi) PDHs from *Leucoagaricus meleagris* (syn.: *Agaricus meleagris*), *Agaricus xanthodermus* and *Agaricus bisporus* (AAW82997, AAW92123 and AAW92124).

Sequence analysis.—The genomic sequences with the highest similarities with the reference sequences for the different GMC families first were examined for the automatically annotated introns, searching for consensus 5′–3′ and lariat sequences (Ballance 1986), as well as for the annotation of N- and C-termini. The presence/absence of secretion signal peptides predicted by the JGI automatic annotation pipeline was manually revised to detect possible mistakes (e.g. in neighbor introns) that could result in inaccurate

TABLE I. Inventory of 95 genes from six GMC families in the genomes of 10 Polyporales species (BJEAD, *B. adusta*; PHLBR, *P. brevispora*; PHACH, *P. chrysosporium*; DICSQ, *D. squalens*; GANSP, *Ganoderma* sp., TRAVE, *T. versicolor*; GELSU, *G. subvermispora*; FOMPI, *F. pinicola*; RHOPL, *R. placenta*; and WOLCO, *W. cocos*) from four clades, producing white-rot and brown-rot decay of wood

	Phlebioid			Core polyporoid			Gelato-poria	Antrodia		
Clade	BJEAD	PHLBR	PHACH	DICSQ	GANSP	TRAVE	GELSU	FOMPI	RHOPL	WOLCO
AAO	11	3	3	8	7	3	4	1	2	0
MOX	5	6	3	4	4	4	1	4	4	4
GOX	0	0	1	0	0	0	0	0	2	0
CDH	1	1	1	1	1	1	1	0	0	0
P2O	1	1	1	0	0	1	0	0	0	0
PDH	0	0	0	0	0	0	0	0	0	0
All GMCs	18	11	9	13	12	9	6	5	8	4
Ecology	White rot							Brown rot		

Four allelic variants (SUPPLEMENTARY TABLE I) are excluded from the inventory.

predictions, followed by inspection of the eventually revised sequences with the Signal P 4.0 server (www.cbs.dtu.dk/services/SignalP-4.0) (Petersen et al. 2011). Moreover, other servers as TargetP 1.1 (Emanuelsson et al. 2000), WoLF PSORT (Horton et al. 2007) and TMHMM 2.0 were used to confirm the secreted nature of proteins as well as to predict their putative subcellular locations. Predictions were confirmed by multiple alignment with MUSCLE (Edgar 2004) and by the comparison with reference sequences. Multiple alignments also were used for analysis of motifs conserved in GMC proteins (the ADP-binding domain and, at least, one of the two characteristic Prosite PS00623 and PS00624 sequences) (Cavener 1992). The sequences that lacked these GMC conserved motifs were discarded.

Finally, molecular models of 94 out of the 95 GMC sequences (references in SUPPLEMENTARY TABLE I) could be generated at the Swiss-Model server (www.swissmodel.expasy.org), which selected the most adequate templates (Bordoli et al. 2009). For AAO, MOX, GOX, CDH and P2O sequences, the crystallographic structures of *P. eryngii* AAO (PDB 3FIM), *Arthrobacter globiformis* choline oxidase (PDB 3LJP, note that no MOX crystal structure is available), *A. niger* GOX (PDB 1CF3), *P. chrysosporium* CDH (PDB 1KDG) and *Aspergillus oryzae* P2O (PDB 1TTO), respectively, were used as templates. Strictly conserved histidine and histidine/asparagine residues at the active site (Hernández-Ortega et al. 2012c, Wongnate et al. 2014) were searched for in all the models, and sequences lacking these residues were discarded.

GMC evolutionary history.—The evolutionary history of the (95) GMC sequences obtained was estimated with RaxML 7.7.1 (Stamatakis et al. 2008) from the multiple alignment obtained with MEGA 5 (Tamura et al. 2011) (alignment in SUPPLEMENTARY FIG. 1). For evolutionary tree construction, a maximal likelihood with clustering method was used, with the WAG model of amino acid substitutions, and the gaps treated as deletions (a 100-iteration bootstrap was performed). Identity degrees among all the above sequences were obtained after pairwise alignment with Clustal W2.

Reconciliation analyses.—The histories of gene duplication and losses for total GMCs (and the individual families) were inferred with Notung 2.6 (Durand et al. 2006). The gene tree was used as input and combined with a Polyporales phylogenetic tree (Binder et al. 2013) from TreeBASE (www.treebase.org, tree ID Tr67497). The estimated numbers of gene duplications and deletions on each branch were used to hypothesize the number of sequences at the ancestral nodes. Two different threshold levels (30% and 90%) were used to assess the significance of the predictions obtained.

RESULTS

GMC gene families in three recently sequenced and other Polyporales genomes.—A total of 41 GMC genes—21 AAO, 15 MOX, 3 CDH and 2 P2O genes (TABLE I)—were identified in the recently sequenced genomes of *B. adusta*, *Ganoderma* sp. and *P. brevispora*. Family classification was completed by inspection of the enzyme molecular models described below for characteristic flavin environment and catalytic residues (Gadda 2008, Hernández-Ortega et al. 2012a, Wongnate and Chaiyen 2013, Romero and Gadda 2014). The genome of *B. adusta* has the highest number of GMC genes (a total of 18), while similar numbers (11–12 genes) were found in the two other genomes (TABLE I). No GOX or PDH genes were found in any case and P2O genes also were absent from the *Ganoderma* sp. genome. AAO genes are the most abundant GMC genes in *B. adusta* and *Ganoderma* sp. (11 and 7, respectively) while MOX genes are the most abundant in *P. brevispora* (six genes). None of the 41 GMC genes identified in the three genomes had been cloned and deposited in databases (TABLE I).

Annotated genomes from seven more species of Polyporales were included for a wider comparison. The resulting 10 genomes include representatives of the Phlebioid (*B. adusta, P. brevispora, P. chrysosporium*),

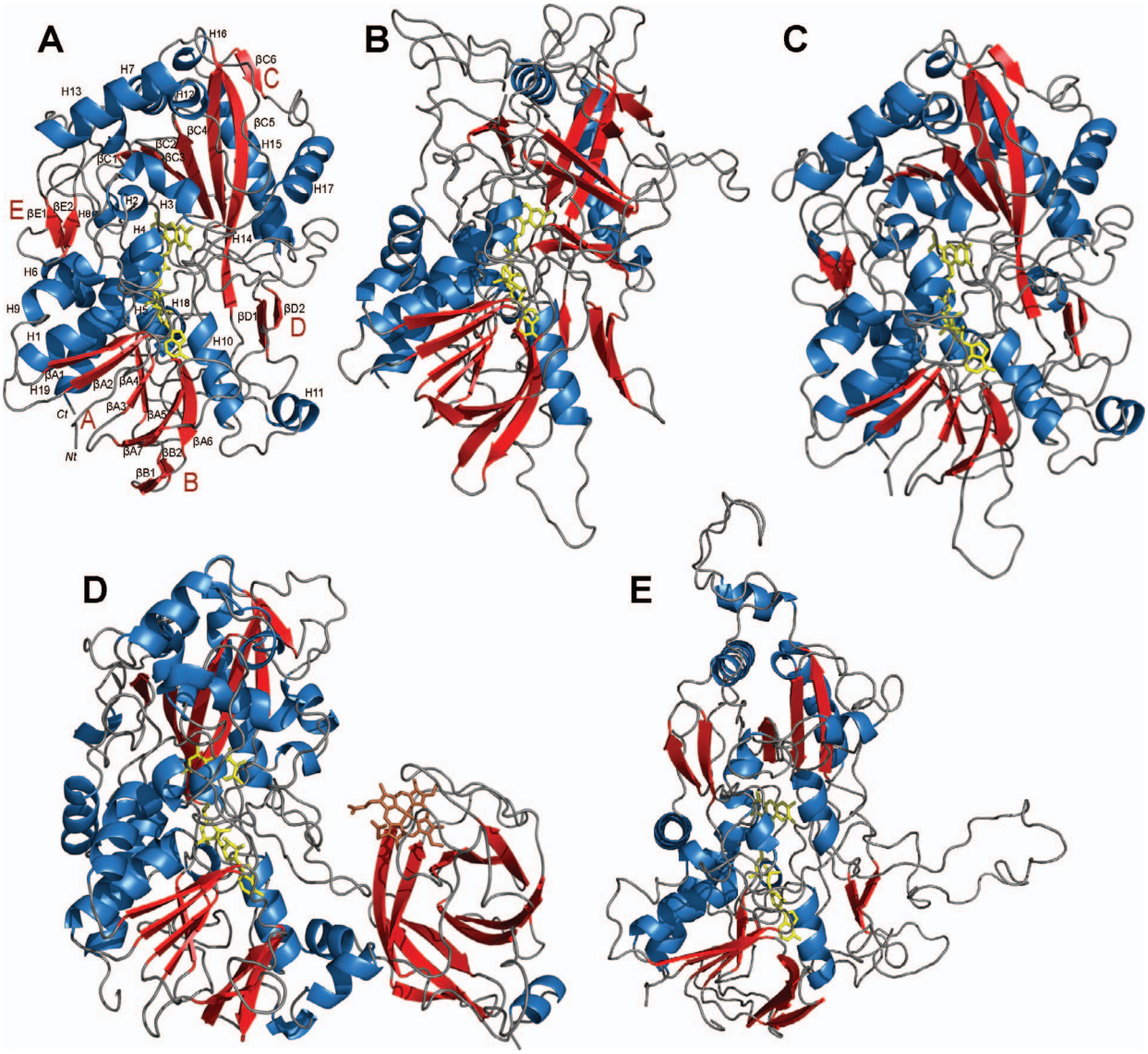

FIG. 1. Ribbon models for the molecular structures of representative members of the five GMC oxidoreductase families found in 10 Polyporales genomes (flavin and heme cofactors are shown as sticks). A. AAO of *B. adusta* (JGI protein ID 245059) indicating the position of four β-sheets, individual β-strands and 19 α-helices. B. MOX (monomer) of *F. pinicola* (JGI protein ID 156775). C. GOX (monomer) of *P. chrysosporium* (JGI protein ID 131961). D. CDH of *G. subvermispora* (JGI protein ID 84792) (flavin domain in the left and heme domain in the right. E. P2O (monomer) of *B. adusta* (JGI protein ID 34622). The molecular models were built with crystal structures of related proteins as templates.

core Polyporoid (*D. squalens*, *Ganoderma* sp., *T. versicolor*) Gelatoporia (*G. subvermispora*) and Antrodia (*F. pinicola*, *R. placenta*, *W. cocos*) clades (Binder et al. 2005). The number of genes of the different GMC families in each of the genomes is included herein (TABLE I), up to a total of 95 (JGI protein ID references are included [SUPPLEMENTARY TABLE I], as well as the existence of alleles and recognized signal peptides; and the complete sequences are provided in the alignment [SUPPLEMENTARY FIG. 1]). MOX genes are equally present in the white-rot and brown-rot genomes (average 4.0–4.4 genes/genome) while those of AAOs are nearly sixfold more abundant in the genomes of white-rot (av. 5.7 genes/genome) than brown-rot (av. 1.0 gene/genome) species. Moreover, CDH genes were present in all the white-rot genomes (one copy per genome) but absent from the brown-rot genomes. Finally, P2O genes also were absent from the brown-rot genomes and no PDH genes were found in any of the genomes.

Structural modeling of GMC oxidoreductases from Polyporales genomes.—Most of the predicted GMC sequences (94 of 95) were modeled with related crystal structures as templates. Five representative structures (FIG. 1)

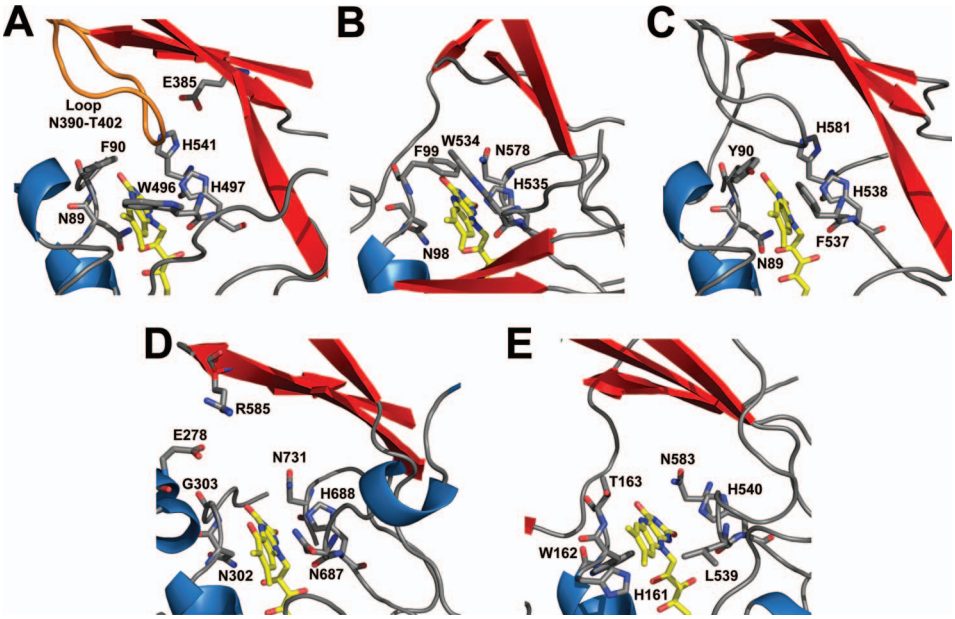

FIG. 2. Detail of active-site residues in the molecular models for the five Polyporales GMCs (FIG. 1). A. *B. adusta* AAO. B. *F. pinicola* MOX. C) *P. chrysosporium* GOX. D. *G. subvermispora* CDH. E. *B. adusta* P2O. Residue numbering corresponds to the putative mature proteins. FAD and the selected residues are shown as sticks. The N390-T402 loop of AAO is shown in A.

correspond to *B. adusta* AAO (A), *F. pinicola* MOX (B), *R. placenta* GOX (C), *G. subvermispora* CDH (D) and *B. adusta* P2O (E) mature proteins. All these GMCs show a common folding with the lower domain harboring the FAD cofactor. Specific features are present in AAO, which possesses a loop partially covering the entrance to the active site (FIG. 2A, left); and CDH, which has a heme domain connected by an unstructured linker (FIG. 1D). Of interest, AAOs and CDHs are known as monomeric proteins while GOXs, P2Os and MOXs form oligomers (Romero and Gadda 2014). One large β-sheet is present in both the FAD-binding (sheet A) and the substrate-binding (sheet C) domains, the former being accompanied by two small sheets (B, D) and the latter by only one (sheet E) (FIG. 1A). Similar numbers of α-helices exist in the FAD-binding and the substrate-binding domains (9–10 in AAO), some of them (e.g. AAO helices 1, 4, 10) conserved in most GMCs. All the predicted models present the ADP-binding $\beta\alpha\beta$ motif near their N-termini (SUPPLEMENTARY FIG. 2A) and the GMC signatures 1 and 2 (Prosite PS00623 and PS00624, respectively; SUPPLEMENTARY FIG. 2B, C), with the only exception of P2O that lacks signature 1.

The FAD flavin ring enters the GMC upper domain, where several residues form a substrate-binding site at the *re*-side of the isoalloxazine ring (FIG. 2). They include a histidine strictly conserved in the superfamily (SUPPLEMENTARY FIG. 1 multiple alignment), corresponding to *B. adusta* AAO His497 (FIG 2A), *F. pinicola* MOX His535 (FIG. 2B), *P. chrysosporium* GOX His538 (FIG. 2C), *G. subvermispora* CDH His688 (FIG. 2D) and *B. adusta* P2O His540 (FIG. 2E). A second conserved histidine in AAO and GOX (His541 and His581 in FIG. 2A, C, respectively) is replaced by an asparagine in MOX, CDH and P2O proteins (Asn 578, Asn731, Asn583; FIG. 2B, D, E, respectively). An aromatic residue often precedes the fully conserved histidine, being a tryptophan in AAO (Trp496) and MOX (Trp534) and a phenylalanine in GOX (Phe537), while a leucine (Leu539) and an asparagine (Asn687) occupy this position in the P2Os and CDHs, respectively (FIG. 2). At the opposite (*si*) side of the isoalloxazine ring another aromatic residue, which points toward the active site, is conserved, being a phenylalanine in AAO (Phe90) and a tyrosine in MOX (Tyr99) and GOX (Tyr90) (FIG. 2A–C). An asparagine preceding the latter position is conserved in all the Polyporales GMCs (Asn89, Asn98, Asn89, Asn322 in FIG. 2 AAO, GOX, MOX, CDH, respectively) with the exception of P2Os. This asparagine residue, also conserved in other GMCs, is involved in flavin bent conformation (Kiess et al. 1998).

Evolutionary history of GMC oxidoreductases in the Polyporales genomes.—The evolutionary history of the 95 GMCs identified in the 10 Polyporales genomes (five allelic variants, SUPPLEMENTARY TABLE I, excluded) was inferred by comparing their predicted amino-acid sequences (mature proteins). It is worth

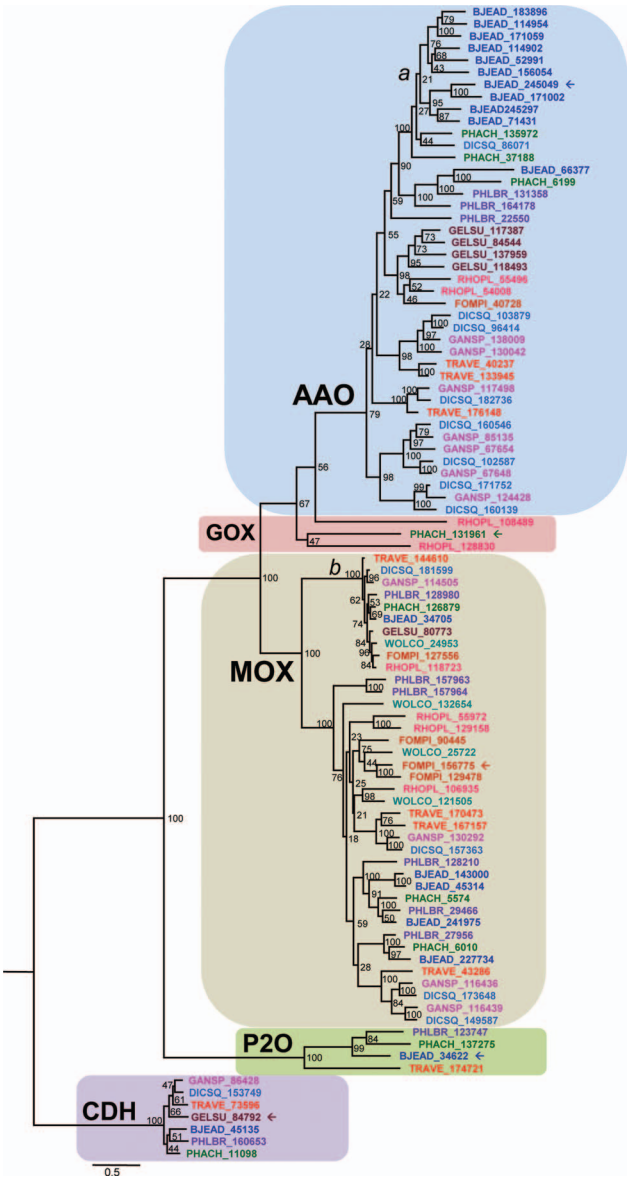

FIG. 3. Maximal likelihood evolutionary tree of the 95 GMC sequences (five allelic variants listed in SUPPLEMENTARY TABLE I excluded) from 10 Polyporales genomes (different color labels), prepared with RaxML (with gaps treated as deletions). The AAO, MOX, P2O and CDH groups (and the *a* and *b* subgroups mentioned in the text) are shown, together with a few GOX sequences related to AAOs. Numbers at nodes indicate bootstrap values. Those modeled sequences (FIGS. 1, 2) are indicated by arrows. Abbreviations of the fungal species are provided (TABLE I) as are complete amino-acid sequences (SUPPLEMENTARY FIG. 1).

noting that all the sequences from each of the GMC families cluster together in the maximal likelihood tree (FIG. 3). The two main groups correspond to the 39 MOXs and the 42 AAOs (100% and 79% bootstrap, respectively), with the only 3 GOXs distantly associated to the AAOs. Of interest, 10 of the 11 *B. adusta* AAOs are included in a 13-member subgroup (*a*, 100% bootstrap), suggesting recent duplication. In contrast MOXs include a subgroup (*b*, 100% bootstrap) of 10 sequences, each from one of the genomes. These 10 sequences share an insertion and a slightly longer C-terminus (SUPPLEMENTARY FIG. 1) involved in oligomerization and/or secretion of the enzymes through a unique secretory pathway (Danneel et al. 1994), suggesting a common origin of these genes. At the basal nodes the well supported (100% bootstrap) P2O (four sequences) and CDH (seven sequences) families appear unrelated between them and with the rest of the GMCs. The distant position of the latter families and the relatedness between AAOs, GOXs and MOXs agree with the pairwise identity values across and within gene families (SUPPLEMENTARY FIG. 3). In fact the average pairwise (interfamily) identity between P2O and CDH sequences is 8% and, among them and the rest of the families, range between 11% and 14%. These values are significantly lower than those between AAO and MOX (25% interfamily average), GOX and MOX (24% interfamily average) and AAO and GOX sequences (31% interfamily average). On the other hand the pairwise (intrafamily) identities within the CDH and P2O families are higher, 73% and 51%, respectively; whereas AAOs, GOXs and MOXs show values of 46%, 30% and 57%, respectively.

GMC gene duplication and loss during diversification of Polyporales.—The expansion or reduction in the number of GMC genes upon evolution of Polyporales was investigated by reconciliation of the evolutionary tree of the 95 GMC genes (FIG. 3) and the phylogenetic tree of the 10 species of Polyporales (from TreeBASE) using Notung. The results (using two different threshold levels) suggest that the ancestors of Polyporales had a high number of GMC genes, more than found in any of the extant species or the predicted intermediate ancestors (FIG. 4). Therefore during GMC evolution 14 contraction events and two expansions (from nodes *d* to node *g* and from node *e* to node *h*) were predicted. A similar tendency was observed for each of the individual GMC families (SUPPLEMENTARY FIG. 4A–E) with a total of 39 contractions and seven expansions. In this case expansions resulted in higher AAO (in node *g* and in *B. adusta*; SUPPLEMENTARY FIG. 4A), GOX (in *R. placenta*; SUPPLEMENTARY FIG. 4C) and P2O (in node *c*; SUPPLEMENTARY FIG. 4E) gene numbers (often after previous contractions) than predicted for the initial Polyporales ancestor. The stronger contraction of GMC gene numbers was evident in the Antrodia clade, resulting in only 4–5 genes in *W. cocos* and *F. pinicola*, and the largest expansion was observed

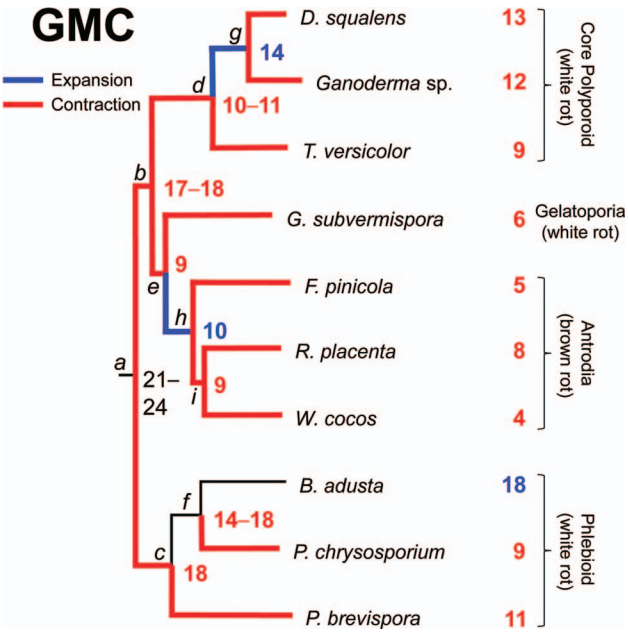

FIG. 4. Estimated range of GMC gene copies at the ancestral nodes (and extant species) of the represented phylogeny of Polyporales taken from Binder et al. (2013) after reconciliation with the gene evolutionary history (FIG. 3) using Notung (Durand et al. 2006). Branches and numbers after gene expansion and contraction are in black and gray, respectively (for reconciliation of the individual GMC families, see SUPPLEMENTARY FIG. 4).

in *B. adusta* (Phlebioid clade) with 18 GMC genes, including 11 AAOs (FIG. 4). Of interest, most of the remaining GMC genes in the Antrodia clade correspond to the MOX family (4/5 in *F. pinicola*, 4/8 in *R. placenta* and 4/4 in *W. cocos*).

DISCUSSION

The global reaction in initial wood decay by white-rot and brown-rot basidiomycetes is iron-catalyzed oxidation of lignin or polysaccharides, respectively, by H_2O_2 generated by oxidases (from the GMC and/or the copper-protein radical superfamilies). In white-rot decay this reaction is catalyzed by Fe^{3+} in the heme cofactor of ligninolytic peroxidases, while in brown-rot decay free Fe^{2+} reduces H_2O_2 forming the highly reactive hydroxyl radical (Martínez et al. 2005; Kersten and Cullen 2007; Baldrian and Valaskova 2008, 2009). The information available on the presence and relevance of GMC families in Polyporales species is discussed below.

Aryl-alcohol oxidase.—AAO first was isolated from *Pleurotus* species (Agaricales) (Bourbonnais and Paice 1988; Guillén et al. 1990; Sannia et al. 1991, 1992) where it generates H_2O_2 by redox-cycling of anisaldehyde (Guillén and Evans 1994), an extracellular fungal metabolite (Gutiérrez et al. 1994). Subsequent studies focused on the *Pleurotus eryngii* enzyme, which was cloned and sequenced (Varela et al. 1999), heterologously expressed (Varela et al. 2001, Ruiz-Dueñas et al. 2006), crystallized (Fernández et al. 2009) and its reaction mechanisms investigated by a variety of techniques (Ferreira et al. 2005, 2006, 2009, 2010, 2015; Hernández-Ortega et al. 2011a, b, 2012b, c). Then a Polyporales AAO was isolated from *B. adusta* (Muheim et al. 1990). Although the above enzymes are known as secreted proteins (Hernández-Ortega et al. 2012a), recognized signal peptides are missing from four of the 42 sequences from the 10 Polyporales genomes, including one sequence from *B. adusta* and two from *D. squalens* and *P. chrysosporium*. The latter is in agreement with the description of an intracellular AAO in this fungus (Asada et al. 1995).

AAO activity has been detected in cultures of a few other Polyporales species (Peláez et al. 1995), although a Southern blot (using a *P. eryngii* probe) did not detect the corresponding gene in many of these (Varela et al. 2000), suggesting gene variability among different fungi. AAO activity in *B. adusta* (Romero et al. 2010), whose sequence corresponds to BJEAD_171002 from the JGI genome, has been characterized largely showing higher activity on *p*-hydroxy and chlorinated benzyl alcohols than *Pleurotus* AAO (Romero et al. 2009). *p*-Hydroxybenzyl alcohols are the typical substrates of vanillyl alcohol oxidase, a flavoenzyme from a different superfamily (Leferink et al. 2008), but they are not efficiently oxidized by *Pleurotus* AAO, whose best substrates are *p*-methoxylated benzyl alcohols (Guillén et al. 1992, Ferreira et al. 2005). Therefore the best characterized Polyporales AAO shows catalytic properties intermediate between Agaricales AAO and vanillyl-alcohol oxidase. The higher activity of *B. adusta* AAO on chlorinated benzyl alcohols, which was noticed first by de Jong et al. (1994), is related to the ability of this species to synthesize 3-chloro-*p*-methoxybenzaldehyde (de Jong et al. 1992, de Jong and Field 1997). Redox cycling of this and related chlorinated compounds provides a continuous source of H_2O_2 to *B. adusta* peroxidases (de Jong et al. 1994), similar to the *Pleurotus* anisaldehyde redox cycling. Chloroaromatics also could help wood colonization due to their antibiotic properties.

Glucose oxidase.—In contrast to AAO, which has been reported rarely in ascomycetes (Goetghebeur et al. 1992), GOX has been largely studied in *A. niger* (Frederick et al. 1990) and other ascomycetous fungi but rarely in basidiomycetes (Danneel et al. 1993). This is the protein with the largest sequence identity with

AAO, as shown in the gene tree, both sharing the general folding and active-site residues (Hecht et al. 1993, Wohlfahrt et al. 1999, Witt et al. 2000).

GOX is widely used in biosensors and other biotechnological applications (Bankar et al. 2009), but its involvement in lignocellulose degradation was discarded because the best known representatives are confirmed intracellular enzymes. However, two of the only three GOX sequences identified in the Polyporales genomes include a typical signal peptide, suggesting participation in the extracellular attack on lignocellulose.

Pyranose 2-oxidase.—P2O, which differs from GOX in glucose oxidation at the C2 (instead of the C1) position, is known as a secreted enzyme (Daniel et al. 1994) involved in lignocellulose degradation (Nyanhongo et al. 2007). This oxidoreductase first was investigated in *P. chrysosporium* (Artolozaga et al. 1997), and these studies suggested that P2O rather than GOX is secreted during wood decay (Volc et al. 1996). However, none of the four genes found in the Polyporales genomes have a recognized signal peptide, in agreement with the sequence obtained by Koker et al. (2004) for the cloned P2O gene from *P. chrysosporium.* Therefore if secreted this would be by an alternative mechanism, as suggested for MOX (see below).

P2O is produced by other Polyporales, including *Trametes multicolor* (= *Trametes ochracea*) (Leitner et al. 2001), and most recent P2O research focuses on this enzyme, whose reaction mechanisms have been elucidated in a variety of crystallographic, spectroscopic, directed mutagenesis, isotope labeling and kinetic studies (Hallberg et al. 2004; Sucharitakul et al. 2008; Prongjit et al. 2009, 2010; Pitsawong et al. 2010; Wongnate et al. 2011, 2014).

Methanol oxidase.—MOX is mostly known as a peroxysomal enzyme in methylotrophic ascomycetous yeasts, such as *Pichia pastoris* or *C. boidinii* (Ozimek et al. 2005). The first basidiomycete MOX was purified and characterized from *P. chrysosporium* (Nishida and Eriksson 1987) and it is also known from *Phlebiopsis gigantea* (Danneel et al. 1994). MOX was proposed as the main oxidase in brown-rot decay based on biochemical characterization and expression analyses in *Gloeophyllum trabeum* (Daniel et al. 2007). The corresponding gene is present in the genome of *R. placenta* (Martinez et al. 2009) and was overexpressed in wood-containing cultures of this brown-rot fungus and also in those of the white-rot *P. chrysosporium* (Vanden Wymelenberg et al. 2010).

The MOX gene of *G. trabeum* and other basidiomycetes does not include a recognized signal peptide. However, the extracellular location of MOX has been demonstrated and operation of an alternative secretion mechanism was proposed (Daniel et al. 2007). The rationale for MOX involvement in brown-rot decay is that demethoxylation, resulting in methanol release, was reported first by Kirk (1975) and confirmed by 2D-NMR analyses (Martínez et al. 2011) as the main lignin modification in brown-rot decay.

Pyranose and cellobiose dehydrogenases.—PDH and CDH use electron acceptors different from O_2 and therefore do not contribute to H_2O_2 supply. However, they oxidize plant carbohydrates and participate in electron transfer to other lignocellulose-degrading oxidoreductases.

PDH catalyzes the same oxidations of P2O but uses quinones as electron acceptors, being an enzyme of interest in biotechnology (Peterbauer and Volc 2010). The first PDH was isolated from *Agaricus bisporus* (Volc et al. 1997) and also found in related species (Kujawa et al. 2007, Kittl et al. 2008) including *L. meleagris* where it was thoroughly investigated (Tan et al. 2013; Krondorfer et al. 2014a, b). Screening for PDH revealed its exclusive presence in the above and other litter-degrading Agaricales (Volc et al. 2001), an observation that is consistent with its absence from all the (wood-rotting) Polyporales genomes analyzed.

CDH includes both flavin and heme domains, the former being able to oxidize cellobiose to cellobiolactone by transferring the electrons to Fe^{3+} via the heme domain (Henriksson et al. 2000, Zámocký et al. 2006). CDH first was described in *P. chrysosporium* (whose conidial state was referred as *Sporotrichum pulverulentum* in some of these studies) (Ayers et al. 1978, Bao et al. 1993). The ancestral fusion between the two CDH domains and the subsequent evolution in different fungi has been discussed (Zámocký et al. 2004). One CDH gene was present in the genomes of the seven white-rot Polyporales analyzed and absent from the three brown-rot Polyporales genomes, in agreement with Hori et al. (2013), in which CDH was found only in white-rot genomes. However, this GMC seems to be present in other brown-rot fungi, as revealed by its early description in *C. puteana* (order Boletales) (Schmidhalter and Canevascini 1993) and its detection in the genomes of brown-rot fungi from other Agaricomycotina orders (Floudas et al. 2012).

Its ability to generate hydroxyl radical by simultaneous Fe^{3+} and O_2 reduction has been suggested (Kremer and Wood 1992), but O_2 reduction by CDH is inefficient and only takes place in the absence of Fe^{3+}. However, recent studies showed that CDH increases the cellulolysis yield and contributes to the action of lytic polysaccharide monooxygenase (Langston et al. 2011).

CDH from *P. chrysosporium* experiences proteolytic cleavage in cultures releasing the flavin domain (Wood

and Wood 1992), which was described as a different enzyme, cellobiose-quinone oxidoreductase (Westermark and Eriksson 1974). However, the physiological significance of such cleavage and the role of cellobiose-quinone oxidoreductase under natural conditions is unknown (Raices et al. 2002).

GMC oxidoreductases in Polyporales: final evolutionary/ecological remarks.—The total number of GMC genes cloned to date from species of the order Polyporales is fewer than 10: from *P. chrysosporium*, *P. cinnabarinus*, *Pycnoporus sanguineus* (syn.: *Trametes sanguinea*), *T. ochracea* and *T. versicolor* (Leitner et al. 1998, Raices et al. 1995, Dumonceaux et al. 1998, Moukha et al. 1999, Vecerek et al. 2004, de Koker et al. 2004, Sulej et al. 2013). However, the present survey of GMC genes from a broader sampling including 10 Polyporales genomes (from different clades and survival strategies) reveals nearly 100 GMC genes representing five of the six best-known families (no PDH genes present).

The GMC superfamily is thought to have evolved from an old common ancestor, which very likely exhibited broad substrate specificity and poor kinetic parameters and gave rise to more specialized and efficient enzymes as evolution proceeded (Cavener 1992). The present study suggests that this diversification took place at a more ancestral stage of fungal evolution, with predominant gene loss among members of the Polyporales. This resulted in two main GMC types (groups) corresponding to AAO and MOX, with an average of ~ 4 gene copies per genome, and three small groups corresponding to P2O, CDH and GOX (neighbor to the AAO group) with 0–1 copies per genome, in agreement with Zámocký et al. (2004) and Kittl et al. (2008).

While ligninolytic peroxidases (from the LiP, MnP and VP families) were absent from the brown-rot fungal genomes but present in all the white-rot fungal genomes (Ruiz-Dueñas et al. 2013), H_2O_2-producing GMCs were present in genomes of both white-rot and brown-rot species. Floudas et al. (2012) showed that the first wood-rotting fungus appeared by incorporation of secreted high redox-potential (ligninolytic) peroxidase genes in the genome of an ancestral basidiomycete. This was most likely accompanied by the evolution of several extracellular H_2O_2-producing oxidases, some of them with different evolutionary origin. These included copper-radical oxidases and several families of GMCs derived from related enzymes involved in intracellular metabolism.

White-rot decay was likely the ancestral survival strategy in wood-decay basidiomycetes (Floudas et al. 2012, Ruiz-Dueñas et al. 2013) and brown-rot evolved several times among Polyporales and other Agaricomycotina orders. The white-rot to brown-rot transition in Polyporales included loss of the ligninolytic peroxidase genes, which are not required because lignin remained polymeric in brown-rotted wood. However, extracellular H_2O_2, used as peroxidase-activating substrate in white-rot decay, also plays an important role in brown-rot decay as the precursor of the hydroxyl radical formed by Fenton reaction. Therefore it seems that the same H_2O_2-generating oxidase types present in white-rot fungi remained in the derived brown-rot species. During evolution some differences in the frequency of the individual GMC families appeared. In this way MOX genes are the most abundant GMC genes in the brown-rot Polyporales while AAO genes are the most abundant in the white-rot species (up to 11 copies in *B. adusta*). Finally, the number of CDH genes predicted in the ancestor of Polyporales diminished, but all the white-rot species maintain one CDH gene, which contributes to polysaccharide degradation by these fungi. However, CDH genes disappeared in brown-rot fungi, where Fenton chemistry is the main mechanism for polysaccharide attack.

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