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On the Diversity of the Laccase Gene: A Phylogenetic Perspective from *Botryosphaeria rhodina* (Ascomycota: Fungi) and Other Related Taxa

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Abstract The present study is the first describing the sequencing of a fragment of the copper-oxidase domain of a laccase gene in the family Botryosphaeriaceae. The aim of this work was to assess the degree of genetic and evolutionary relationships of a laccase gene from *Botryosphaeria rhodina* MAMB-05 with other ascomycete and basidiomycete laccase genes. The 193-amino acid sequences of the copper-oxidase domain from several different fungi, insects, a plant, and a bacterial species were retrieved from GenBank and aligned. Phylogenetic analyses were performed using neighbor-joining, maximum parsimony, and Bayesian inference methods. The organisms studied clustered into five gene clades: fungi (ascomycetes and basidiomycetes), insects, plants, and bacteria. Also, the topologies showed that fungal laccases of the ascomycetes and basidiomycetes are clearly separated into two distinct clusters. This evidence indicated that *B. rhodina* MAMB-05 and other

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closely related ascomycetes are a new biological resource given the biotechnological potential of their laccase genes.

Keywords *Botryosphaeria rhodina* MAMB-05 · Laccase · Ascomycete · Basidiomycete · Phylogenetic analyses · Copper-oxidase domain

Introduction

Laccases (*p*-diphenol:dioxygen oxidoreductases, EC 1.10.3.2) compose one of three polyphenol oxidase groups responsible for the initial fragmentation of the plant cell wall polymer, lignin, resulting in low molecular weight breakdown products (Eriksson et al. 1990; Bernards and Lewis 1998). This enzyme is a member of the multicopper family of oxidases (Messerschmidt 1997) and is typically extracellular with a molecular mass of 50–100 kDa. An important feature is a covalently linked carbohydrate moiety (10–45%), which may contribute to its stability (Claus 2004). Laccases catalyze the one-electron oxidation of a wide variety of reducing substrates (typically mono, di, and polyphenols, but also aromatic amines and nonphenolic compounds) coupled to the four-electron reduction of dioxygen to water via its copper reduction center (Thurston 1994; Piontek et al. 2002; Claus 2004).

Interest in laccases has been fueled not only by their involvement in lignin degradation but also by their potential use in other biotechnological applications (Couto and Herrera 2006), for example: detoxification of environmental pollutants (Duran et al. 2002), prevention of wine decoloration (Minussi et al. 2002), as an aid to bleaching in paper processing (Li et al. 1999), to improve the production of fuel ethanol from renewable raw materials (Larsson et al. 2001), in drug analyses (Bauer et al. 1999), and development of biosensors (Leite et al. 2003).

Laccases are produced by many eukaryotes, e.g., fungi, plants, and insects (Mayer and Staples 2002). The synthesis of laccases by prokaryotes has been reported in Gram-negative and Gram-positive bacteria (Alexandre and Zhulin 2000; Claus 2003). Laccases appear to have different functions in several organisms. For example, laccases play a role in the lignification of xylem tissues in plants (O'Malley et al. 1993; Gavnholt and Knud 2002) and the delignification by fungi (Eriksson et al. 1990), and they appear to be an important factor in plant–pathogen interactions (Edens et al. 1999; Zhu and Williamson 2004). Insect laccases seem to play an important role in the sclerotization of the cuticular exoskeleton (Andersen et al. 1996), and bacterial and fungal laccases are also involved in the melanization process (Langfelder et al. 2003).

Laccase gene sequences have been reported for a number of fungal species, including basidiomycetes and ascomycetes (Germann et al. 1988; Yaver et al. 1996, 1999). The different laccase genes are regulated by factors such as pH, substrate condition, and fungal metabolism (Broda et al. 1995). A multiplicity of laccase genes, differing only slightly in sequence, is not uncommon in fungi (Mansur et al. 1997).

An isolate of *Botryosphaeria rhodina* (MAMB-05) screened for ligninolytic activity (Barbosa et al. 1996) has been extensively studied in our laboratory for the production of laccases (Dekker et al. 2007). This ascomycete produced one kind of polyphenol oxidase, a constitutive laccase, when grown on basal medium (Barbosa et al. 1996). Laccases produced by *Botryosphaeria* isolate MAMB-05 could be induced above constitutive levels when the fungus was grown on basal medium containing the putative inducer and fungal secondary metabolite, veratryl alcohol (Dekker and Barbosa 2001).

In the present study we describe for the first time the molecular features of a fragment from the copper-oxidase domain of a laccase gene from *B. rhodina* MAMB-05. This information was used to establish a perspective regarding the genetic and evolutionary relationships of laccase genes based on their amino acid sequences using the most critical of phylogenetic methods.

Materials and Methods

Culture Conditions

Botryosphaeria rhodina MAMB-05 used in this work was maintained through periodic transfer on potato-dextrose agar at 4°C. Inoculum preparation and growth of *B. rhodina* MAMB-05 in submerged, liquid shake-cultivation (28°C/180 rpm for 4.5 days) in baffled Erlenmeyer flasks containing basal medium [Vogel minimal salts medium (Vogel 1956) and glucose (10 g/l) as carbon source, pH 6.0] was as previously described by Barbosa et al. (1996).

DNA Isolation

Fungal mycelium was obtained by filtration of the liquid-grown fungal cultures, followed by washing with sterile water, and dried with sterile absorbent paper. Mycelium was ground under liquid nitrogen, and the DNA extracted using the protocol described by Bogo et al. (1996). Pelleted DNA was resuspended in 60 μl of ultrapure water and stored at 4°C. The quality and quantity of extracted DNA samples was monitored routinely by electrophoresis of the material in 1% agarose gels in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA; pH 8.0), stained with ethidium bromide and observed under UV light. Quantification was performed through comparison with lambda-phage DNA.

Amplification of the Copper-Oxidase Domain of a Laccase Gene

PCR amplification was carried out using the degenerate primers (Lac2for 5′GGIACI WIITGGTAYCAYWSICA3′ and Lac3rev 5′CCRTGIWKRTGIAWIGGRTGIGG3′) capable of amplifying a 900 bp laccase gene fragment from ascomycetes and basidiomycetes (Lyons et al. 2003). Ambiguous bases are defined as follows: R, A/G; W, A/T; Y, C/T; S, C/G; K, T/G; I, inosine. Amplifications were developed according to the following conditions: 1 μl DNA sample (5 ng/μl), 2 mM $MgCl_2$; 0.2 mM each

dNTP; 0.6 μM each primer, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 1 U *Taq* DNA polymerase (Invitrogen Life Technologies, Brazil). The amplifications were performed in a Thermocycler (Model T-1 Thermoblock, Biometra) and programmed for an initial denaturation of 3 min at 95°C, followed by 35 cycles each of 1 min at 95°C, 1 min at 45°C, and 3 min at 72°C, followed by a final elongation of 5 min at 72°C. The success of the reaction was confirmed by agarose gel (1%) electrophoresis in TEB buffer (pH 8.0) stained with ethidium bromide and visualized under UV light.

Cloning of the Copper-Oxidase Domain of a Laccase Gene

PCR products were cloned into the vector pCR2.1 using a TOPO-TA cloning kit (Invitrogen Life Technologies, Brazil) as described by the manufacturer. The plasmid DNA containing the PCR product was extracted and purified from 1.5 ml of *Escherichia coli* HB 101 chemically competent cells by the Mini-Prep kit (Invitrogen Life Technologies, Brazil) as described by the manufacturer.

Sequencing of the Copper-Oxidase Domain of a Laccase Gene

Cloned products were sequenced in both directions with M13 reverse (5′ CAGGAAACAGCTATGAC 3′) and M13 forward (5′ GTAAAACGACGGC-CAG 3′) primers by the Sanger modified method using an ABI Prism 377 DNA Sequencing Analyzer (Perkin Elmer). The analysis of sequence quality and construction of the consensus sequence were performed using Sequencer 3.1.1 software. The consensus sequence was compared to sequences in the GenBank DNA database using Blastn, Blastx, and Blastp as search tools (Altschul et al. 1990).

Sequence Alignment and Phylogenetic Analysis

Thirty-eight amino acid sequences of the copper-oxidase domain of laccase, including ascomycetes, basidiomycetes, insects, plants, and bacteria, were retrieved from GenBank and aligned within the fragment sequenced in this work (Table 1). The criterion used to select these sequences was the best score verified by the comparison using the Blastx tool for the *B. rhodina* MAMB-05 sequence with those deposited in the database. Alignments were performed using BioEdit version 5.0.9 (Hall 1999) and Clustal X (Thompson et al. 1997). Additionally, the alignment was visually inspected at each nucleotide position.

The aligned sequences were exported as a Nexus file, and maximum parsimony (MP/equal weights; Fitch 1977) and neighbor-joining (Saitou and Nei 1987) were conducted with Paup version 4.0b10 (Swofford 2000) using the PaupUp graphic interface (Calendinni and Martini 2005). MrBayes version 3.1.1, as described by Huelsen et al. (2001), was used to conduct the Bayesian inference analysis. *Mycobacterium bovis* was the outgroup for the whole analysis. Heuristic searches were performed by stepwise taxon addition (100 replicates), combined with tree-bisection-reconnection as the branch swapping algorithm. Parsimony analysis was also carried out with the characters treated as unordered and their states optimized

Table 1 Data from amino acid sequences retrieved from GenBank and used for alignment and phylogenetic analyses

Species	Phylum	GenBank acc. no.
Fusarium proliferatum	Ascomycota	AAK72900
Botryotinia fuckeliana	Ascomycota	AAK77953
Gaeumannomyces graminis	Ascomycota	CAD24841
Stagnospora sp.	Ascomycota	AAN17288
Phaeosphaeria spartinicola	Ascomycota	AAN17290
Cryphonectria parasítica	Ascomycota	AAA09235
Mycosphaerella sp.	Ascomycota	AAN17293
Chaetomium thermophilum	Ascomycota	AAT84322
Phaeosphaeria halima	Ascomycota	AAN17286
Colletotrichum lagenarium	Ascomycota	BAB32575
Melanocarpus albomyces	Ascomycota	CAE00180
Chaetomium globosum	Ascomycota	EAQ90355
Marine ascomycete	Ascomycota	AAN17287
Podospora anserine	Ascomycota	CAA70061
Ganoderma sp.	Basidiomycota	AAR04342
Rigidoporus microporus	Basidiomycota	CAE81289
Polyporus ciliatus	Basidiomycota	AAG09230
Trametes sp.	Basidiomycota	AAQ12269
Pycnoporus cinnabarinus	Basidiomycota	CAD12769
Spongipellis sp.	Basidiomycota	BAE79811
Coriolopsis gallica	Basidiomycota	AAK02068
Lentinus tigrinus	Basidiomycota	AAX07469
Panus rudis	Basidiomycota	AAR13230
Flammulina velutipes	Basidiomycota	AAR21096
Coriolus versicolor	Basidiomycota	BAA23284
Phlebia radiate	Basidiomycota	CAA36379
Ceriporiopsis subvermispora	Basidiomycota	AAC97074
Flammulina velutipes	Basidiomycota	BAE80732
Schizophyllum commune	Basidiomycota	BAA31217
Coprinus cinereus	Basidiomycota	AAD30966
Coprinellus congregatus	Basidiomycota	CAD62686
Pleurotus pulmonarius	Basidiomycota	AAY41066
Termitomyces sp.	Basidiomycota	BAE53769
Auricula polytricha	Basidiomycota	AAT73204
Populus trichocarpa	Streptophita	CAC14719
Mycobacterium bovis	Actinobacteria	CAD93731
Manduca sexta	Insecta	AAN17507
Anopheles gambiae	Insecta	AAX49502

for both Acctran and Deltran criteria. Heuristic searches were conducted on a maximum number (MaxTrees) of 10,000 trees. Majority rule consensus was also computed. Bootstrap and jackknife analyses were performed to assess the support of

the resulting topology and were based on 1000 replicates of the heuristic search as described above. Bayesian inference was performed with 1,000,000 generations of 4 Markov chains, and the posterior probabilities of branches were obtained computing the whole set of generations.

Results

The use of degenerate primers described above in PCR reactions consistently produced a single amplification product of approximately 900 bp. It was possible that on sequencing, a 615 bp fragment (GenBank accession no. EF690301) was translated into 193 coded amino acids. Nucleotide sequence analysis with the Blastn tool (nucleotide/nucleotide comparison) did not retrieve any similarity. In spite of this, Blastx (nucleotide/translated sequence comparison) retrieved more than 100 high-scored amino acid sequences. Blastp (amino acid/amino acid comparison) confirmed that our sequence corresponded to the copper-oxidase domain of a laccase gene.

Aligned sequences totaled 192 amino acids in length, of which 15 characters were constant, 23 were parsimony uninformative, and 154 were parsimony informative. NJ distances ranged from 11 to 89.5% and revealed three different genetic units (Fig. 1). Twelve ascomycete laccases grouped together. *Botryotinia fuckeliana*, *Stagnospora* sp., and *Phaeosphaeria spartinicola* arose as a distinct genetic group apart from the main ascomycete grouping. In the basidiomycetes, 19 species grouped together at the same phenetic unit (Fig. 1).

The laccase variant from *Auricularia politricha* was the single gene variation ungrouped with the main cluster as aforementioned. Laccases from *Flammulina velutipes* arose as two unrelated variations, but both are included in the main basidiomycete grouping. Laccase sequences from *Populus trichocarpa*, *Anopheles gambiae*, and *Manduca sexta* formed the third additional genetic group.

Parsimony analysis revealed two monophyletic lineages (ascomycete and basidiomycete) except *Auricularia polytricha* (basidiomycete) that arose as the sister laccase to ascomycete (Fig. 2). In the ascomycetes, laccases from *B. rhodina* MAMB-05 and *Fusarium proliferatum* are closely related, and they arose as the sister group of the remaining ascomycete. The laccase from a marine ascomycete is interstitially located within the second main evolutionary lineage. In the basidiomycetes, three distinct lineages were observed. The laccases from *Termitomyces* sp. and *Schizophyllum commune* are evolutionarily closely related, and they arose as the sister group to the two remaining groups. Likewise, an additional lineage was evidenced comprising *Populus trichocarpa*, *Anopheles gambiae*, and *Manduca sexta*. The bootstrap and jackknife values at the node that separates *B. rhodina* MAMB-05 and *F. proliferatum* from the ascomycetes were 71 and 75, which give support to this separation (Fig. 2).

Bayesian inference (Fig. 3) results were similar to those mentioned above, with satisfactory posterior probabilities from the Monte Carlo–Markov chains. In general, previous relationships were reinforced, especially those from parsimony analysis. The fungi were equally separated and the intrinsic relationships revisited.

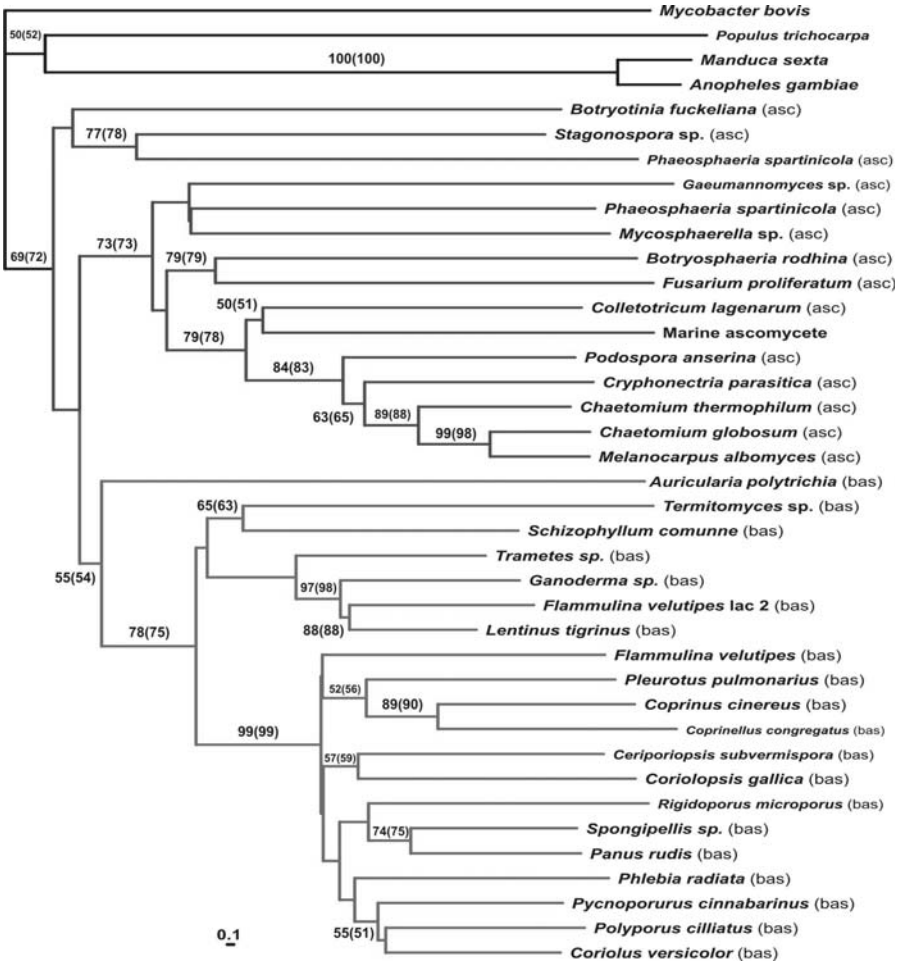

Fig. 1 Neighbor-joining tree of laccase amino acid sequences. Numbers on the branches are bootstrap values (jackknife values in parentheses) obtained for 1000 pseudoreplications. (asc), ascomycete fungi; (bas), basidiomycete fungi

Moreover, the Bayesian inference also presented evidence that the alleles from *Populus trichocarpa*, *Anopheles gambiae*, and *Manduca sexta* were distinct evolutionary lineages.

Discussion

Molecular phylogenetics has been increasingly dominant in several biological approaches such as organismal and molecular evolution (Hillis et al. 1996). Thus, the whole topologies obtained showed that the evolution of laccase divided the dataset into four distinct genetic and evolutionary groups: ascomycetes,

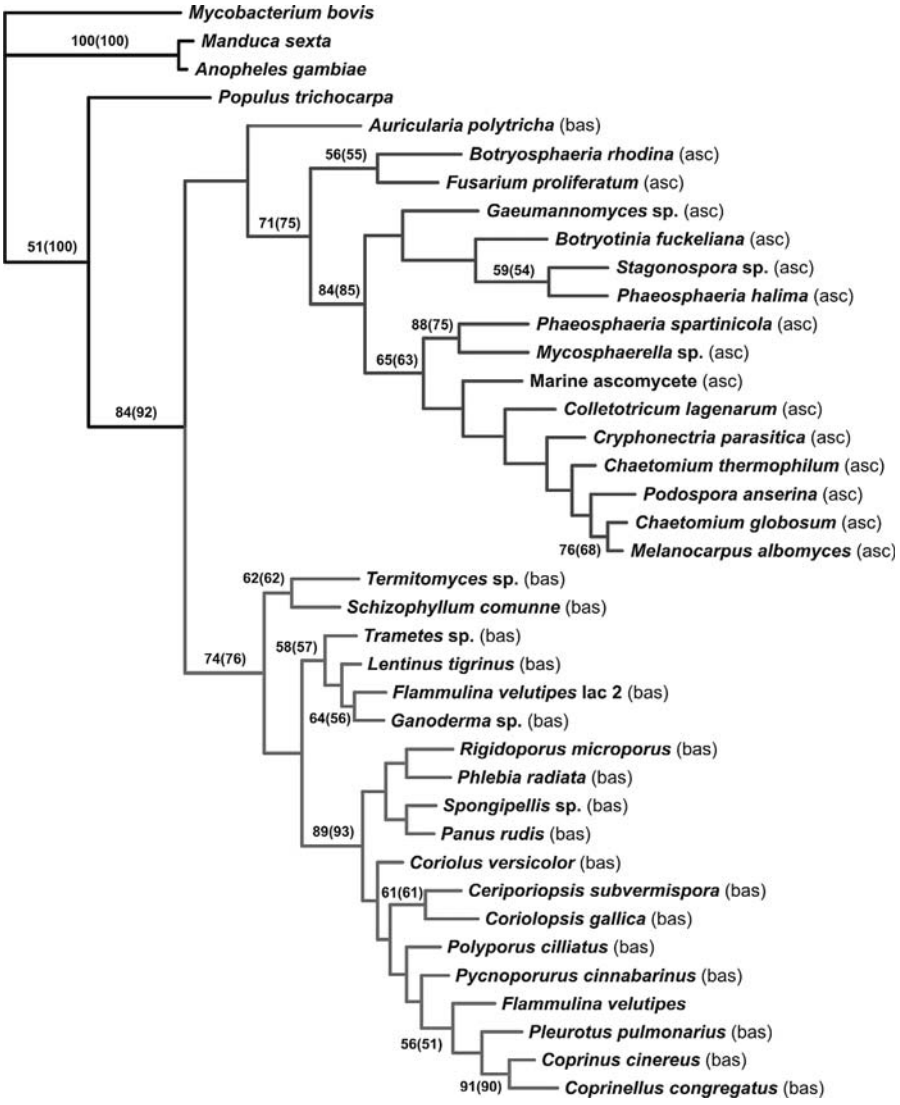

Fig. 2 Maximum parsimony phylogeny (majority rule consensus) from laccase amino acid sequences. Numbers on the branches are bootstrap values (jackknife values in parentheses) obtained for 1000 pseudoreplications

basidiomycetes, insects, and plants. This evidence might suggest that laccase could be useful as a robust molecular marker for organismal evolution on macroevolutionary time scales. Support for this hypothesis may be gathered from the different metabolic biases of the laccase sequences studied here. Laccases in plants and insects are involved in different functions. Plant laccases have a role in the lignification of differentiating xylem tissues (O'Malley et al. 1993; Gavnholt and Knud 2002), and in insects laccases play an important role in cuticular sclerotization

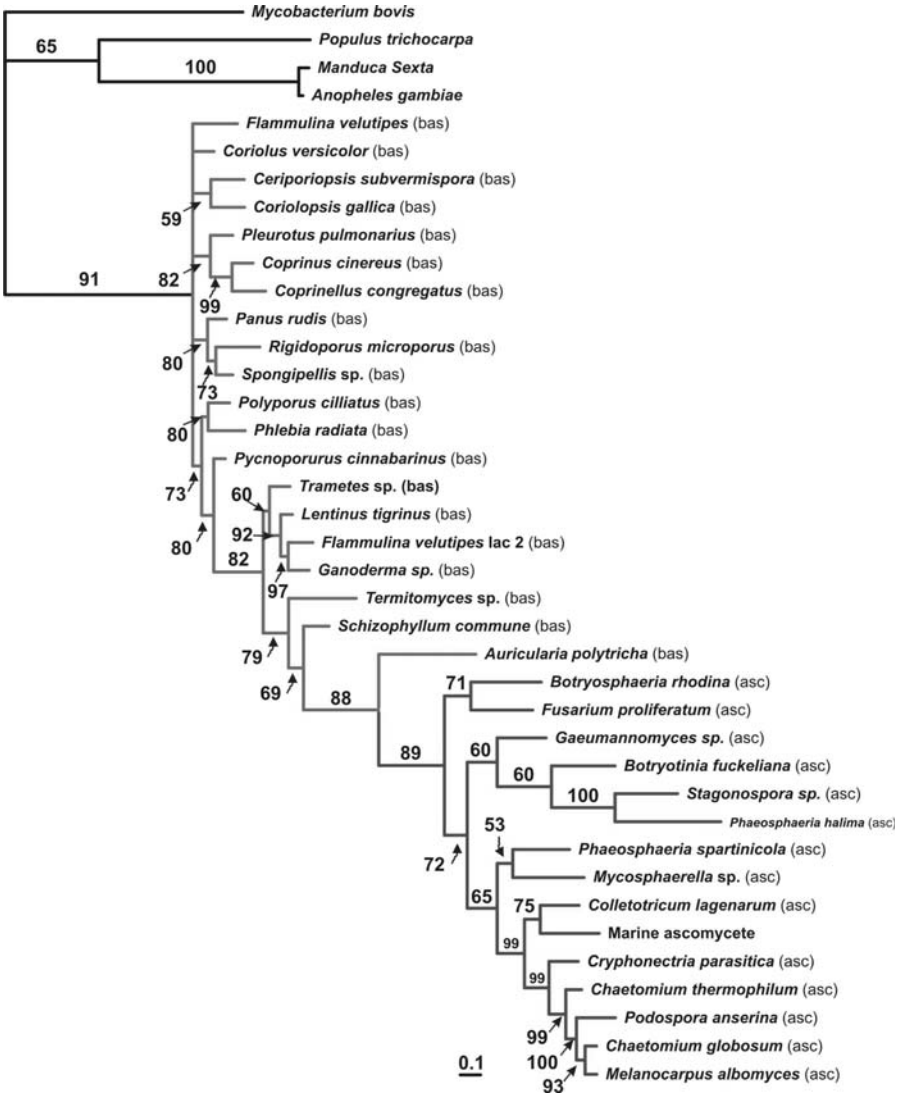

Fig. 3 Bayesian phylogeny from laccase amino acid sequences. Numbers on the branches are posterior probabilities obtained for 1,000,000 generations of MCM chains

(Andersen et al. 1996). Similar phylogenetic results were previously reported (Hoegger et al. 2006).

Regarding the genetic and phylogenetic division observed between the fungal lineages (Figs. 1–3), an interesting association may be highlighted. The laccase of the white rot fungi (basidiomycetes) appears to be a complex enzyme, given that it is involved in both the biodegradation of lignin and mineralization of lignin, whereas in brown rot fungi (ascomycetes), the laccases tend to be less efficient in the decomposition of lignin (D'Souza et al. 1996). This functional diversity of

fungal laccases could be associated with the genetic and phylogenetic division observed in the topologies (Figs. 1–3). The diversity of the laccase sequences among salt marsh fungi of the southeastern United States has also been reported (Lyons et al. 2003).

Multiple laccase genes can be found in many organisms; e.g., *Trametes villosa* has 5 (Yaver et al. 1996), *Pleurotus ostreatus* 4 (Palmieri et al. 1997), and *Coprinus cinereus* up to 17 (Kilaru et al. 2006). In this way, the whole set of analyses performed showed that the laccase from *Flammulina velutipes* was phenetically and evolutionarily distinct. This evidence reinforces the laccase diversity and suggests intraspecific variations occurring in this genetic pool.

One of the most important criteria in phylogenetic systematics is the issue of homology (for details see Wiley et al. 1991; Henning et al. 1999). In terms of molecular data, three types of homology are defined: (i) orthology, when the sequences have a single and the same ancestor; (ii) paralogy, when they originate from a gene duplication event, and (iii) xenology, when they originate by horizontal gene transfer (Nei and Kumar 2000). Thus, the tree topologies presented here suggest that laccase is possibly a paralogue-based enzyme. The two or more laccase genes in different organisms could be the result of duplication events, after which the enzymes evolved to perform similar biochemical processes.

Regarding the laccase from *Auricularia polytricha*, both phylogenetic topologies (Figs. 2 and 3) showed the sequence grouped out of the basidiomycetes cluster. The laccase gene fragment sequenced from *B. rhodina* MAMB-05 grouped inside the main ascomycete fungi cluster, which corroborates the hypothesis that the laccase amino acid sequence from this species seems to be most genetically, and phylogenetically, similar to the laccase sequences from ascomycetes rather than those of the basidiomycetes.

Interesting data gathered here using systematic techniques for understanding molecular evolution are those grouping *B. rhodina* MAMB-05 and *Fusarium proliferatum* laccases (Figs. 1–3). Recently, laccases from *F. proliferatum* were demonstrated to have potential application for upgrading industrial lignins from paper-pulp mill effluents arising from Kraft-anthraquinone processing (Hernández Fernaud et al. 2006). This evidence suggests that similar useful enzymes for biotechnological process applications may also exist for *B. rhodina* MAMB-05 laccases. Thus, the present analyses reinforce the use of molecular systematic methodologies for understanding the historical processes of molecular evolution as well as to indicate and select elements of biodiversity for new biotechnological advances.

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