Supporting Information

Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts

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Materials and methods

Biological material

The fungus-growing ant mutualism has a single origin ca. 50 million years ago (mya) when the ants started to cultivate a basidiomycete fungus (Agaricaceae: Agaricales) in underground gardens as a stable source of food. The clade now comprises more than 230 species divided in 15 genera that vary greatly in colony size, social complexity and fungus culturing behaviour (1). Within this clade, the leaf-cutting ant genera *Atta* and *Acromyrmex* evolved only 8 – 12 mya as a major evolutionary transition that coincided with a significant increase in worker polymorphism, a shift to rearing a single fungal cultivar species (*Leucocoprinus gongylophorus*), an increase in mature colony size (ca. 500-100,000 workers in *Acromyrmex* and several million in *Atta*), and a major change in diet to using fresh leaves as the predominant fungal substrate (1). In addition, leaf-cutting ant queens evolved multiple mating (2) and workers evolved larger metapleural glands (3). These modifications form a remarkable contrast with the other higher attine ant genera (*Trachymyrmex* and *Sericomyrmex*), which have much smaller mature colony sizes (ca. 50-3000), monomorphic workers, singly mated queens, and primarily collect dry leaf litter, insect feces, fallen fruits, flowers and only occasionally leaves. A characteristic synapomorphy for all higher attine ant agricultural systems is that they cultivate fungi with specialized inflated hyphal tips (gongylidia) (1), which implies that the attine symbiosis became obligatorily interdependent for both the ants and the fungi (4). In contrast, the remaining 11 fungus-growing ant genera collectively termed the lower attine ants are characterized by cultivation of a wider spectrum of fungal cultivars from either of two unrelated lepiotaceous clades (clade 1 and 2) (1, 4). Within the lower-attine ants two exceptions to this coevolutionary pattern are known: some species in the genus *Apterostigma* cultivate pterulacaous fungi completely unrelated to the lepiotaceous fungi in clade 1 and 2 and certain species in the genus *Cyphomyrmex* cultivate their lepiotacous cultivars in yeast form, in contrast to all other known fungus-growing ants that cultivate their fungus garden as mycelium (1, 4).

For the present study ant colonies were collected in Parque National Soberanía, Panama (the Gamboa area and forest along Pipeline Road) during 2005-2008, transported to the University of Copenhagen, Denmark and maintained as laboratory colonies at ca. 25 °C and 70% humidity. Colonies were fed a combination of fresh bramble leaves, rice, oatmeal, oak catkins, orange peel, withered flowers, apples and occasionally other fruit items in different proportions adjusted to mimic the natural diet for each species as closely as possible. Laccase activities were measured in the fungus gardens of ant species belonging to four of the five agricultural groups presently recognised in fungus growing ants (see above): (I) Lower attine ants, which cultivate a variety of *Leucocoprinus* (Agaricaceae, Basidiomycota) fungi from two unrelated clades (clade 1 and 2), represented in our study by the ant species *Myrmicocrypta ednaella*, *Mycocepurus smithii*, *Cyphomyrmex costatus*, *C. longiscapus* and *C. muelleri*. (II) Higher attine ants, which cultivate specialized fungi with gongylidia that evolved from clade 1 ancestors, represented here by

Sericomyrmex amabilis, *Trachymyrmex cornetzi*, and *Trachymyrmex* sp. 3 (tentatively also called *isthmicus* or "blackhead"). This *Trachymyrmex* species is an undescribed member of the *T. zeteki/opulentus* species-complex of closely related species that awaits formal revision. (III) Leaf-cutting ants, which are a derived clade of higher attine ants that all cultivate a single species, *L. gongylophorus*, represented here by *Atta colombica*, *At. cephalotes*, *Acromyrmex echinatior*, and *Ac. octospinosus*, and (IV) The *Apterostigma* (lower attine) agricultural group that cultivates a pterulaceous basidiomycete fungus only distantly related to the leucocoprinaceous clade 1 and 2, here represented by *A. collare* and *A. dentigerum*. The fifth category of fungus farming, the *Cyphomyrmex* species that cultivate clade 1 symbionts as yeasts were not included. Fungal clade affiliation (clade 1 or 2) could be inferred from the literature for most lower attine species, except for *Mycocepurus* and *Myrmicocrypta* fungus gardens. In these species, fungal cultivars were genotyped to establish clade identity as previously described (5). Due to mortality of colonies not the exact same *A. echinatior* colonies were available for all experimental and molecular measurements conducted.

Gongylidia abundance in fungus gardens

Fungus garden samples from *A. echinatior* laboratory colonies (Ae331, Ae335, Ae447, Ae490) were collected from five consecutive sections (layers) measured as the distance from the top of the fungus garden (0 – 2.5 cm, 2.5 – 5 cm, 5 – 7.5 cm, 7.5 – 10 cm, and 10 – 12.5 cm). Using a stereomicroscope at 40x magnification a small 3x3mm square ($9mm^2$) was randomly placed on the fungus garden and the number of gongylidia clusters (staphylae) within this area was counted. This was done ten times for each section and the number of staphylae in each section was determined as the average number of all ten measurements. Repeating these measurements for each of the five layers resulted in 50 measurements of each of the four colonies. Numbers were converted to gongylidia clusters (staphylae) per cm^2.

Enzyme extraction

Fungus garden enzymes were extracted from 50 mg fresh fungus garden material from laboratory colonies. Each sample was homogenized and total enzymes extracted in 200 µl of cold 0.1 M Citric acid – sodium citrate buffer (pH = 5.5) followed by centrifugation at 15.000g for 15 min at 4 °C. Supernatants were collected on ice and immediately processed for laccase activity measurements. The four leaf-cutting species were analysed in more detail by collecting and measuring 50 mg fungus garden samples from the three different sections (top, middle, and bottom) corresponding to different stages in substrate degradation. This is because leaf-cutting ant gardens grow at the top while the ants discard the bottom section of the fungus garden where substrate depletion has progressed so far that further maintenance is unproductive (6). Leaf-cutting ant gardens in the field often clearly show the stratification in three distinct sections that reflects the fungal/substrate biomass ratio. Our laboratory rearing procedures where gardens grow under inverted 1 liter plastic beakers accentuate this gradient by constraining the ants to always add substrate at the top and remove depleted fungus garden material at the bottom.

Fecal droplet enzymes were extracted from live *A. echinatior* workers maintained in laboratory colonies on a diet of bramble leaves and fungus garden and from "starved" workers maintained on a diet of bramble leaves and sugar water with no fungus garden for 14 days. Fecal droplets were collected as previously described (7). Briefly, a live worker was held by forceps on a glass plate and with a second pair of forceps pressed down and gently squeezed on the gaster until a fecal droplet (ca. 0.1 - 0.2 µl) was expelled. The droplet was immediately suspended in 4.8 µl ddH_20 to prevent evaporation. Droplets from five individual workers of each dietary scheme were collected and measured separately for three *A. echinatior* colonies.

Laccase activity

Total activity of laccase was measured with Syringaldazine (Sigma-Aldrich Denmark A/S, #S7896). 10 µl of a 16.65 mM Syringaldazine solution (6 mg/mL) in ethanol was mixed on ice with 85 µl 0.1 M Citric acid – sodium citrate buffer (pH = 5.5). Five µl enzyme extract was added yielding a total assay volume of 100 µl and measurements were started immediately by scoring absorbance at 530nm every 10 sec for 10 min on a Versamax plate reader (MDS Analytical Technologies, Sunnyvale, California) at 25 °C. Laccase enzyme from the basidiomycete *Agaricus bisporus* (Sigma-Aldrich Denmark A/S, #40452) was used as a positive control to confirm the assay parameters. Laccase activity was measured as optical density/min during the phase of linear increase in activity as previously reported (8), and unit activity was calculated using the molar absorption coeficient for Syringaldazine of ~65.000 L/mol/cm (9). One unit (U) of activity was defined as the amount of enzyme that oxidizes 1 nmol/min.

Laccase activity analysis

Laccase activities were analysed with ANOVA and means compared with Tukey's correction for multiple comparisons using SAS v. 9.1 for Windows. Fungus garden laccase activity differed significantly between species ($F_{13,39} = 19.48$, $p < 0.0001$, Fig. 2A) and analysis of Tukey's corrected means showed that this was primarily due to the much higher laccase activity in leaf-cutting ant fungus gardens (Fig. 2A, except for *A. cephalotes* gardens, which had enhanced laccase activity but to a lower extent that was not signficantly different from all non-leaf-cutting ants). Tests for serial independence (TFSI) of laccase activity were performed using Phylogenetic Independence v. 2.0 (10) and showed a significant association of fungus garden laccase activity with the phylogenetic branching of both the ants ($C = 0.55$, $p = 0.007$) and the symbiotic fungi (clade 1 only, $C = 0.63$, $p = 0.008$). Therefore the data were analysed using phylogenetically independent contrasts to control for shared traits among related species/genera. We focused on the specific phylogenetic contrasts of leaf-cutting ants versus all other attine ants, both when using the phylogeny of the ants and the phylogeny of the fungal cultivar (Clade 1 only). The most recent phylogenies for fungus-growing ants (1) and the fungal cultivars (4) were used. Species not present in these phylogenies were assumed to be in clades of the closest relatives with branch lengths assumed to be equal to sister species. Categorical variables (leaf-cutting ants versus all other attine ants) were replaced by 0-1 dummy variables to perform the

respective regression analyses. Independent contrast analyses were evaluated using the PDAP (Phenotypic Diversity Analysis Programs) program package for Mesquite ver. 11 (11, 12). The change in fungus garden laccase activity remained significantly correlated with the transition from higher attines to leaf-cuting ants ($F_{1,12}$ = 9.42; p = 0.0097) using the ant phylogeny. However, performing the same analysis using the Clade 1 fungal phylogeny showed no significant correlation between laccase activity and the transition from higher-attines to leaf-cutting ants ($F_{1,7}$ = 0.004; p = 0.953) which was likely due to the longer branch lengths in the fungal phylogeny reducing the power of the regression analysis.

The putative evolutionary trajectory of laccase activity based on the fungal phylogeny for clade 1 (4) was estimated using a maximum likelihood approach as implemented in the BayesTraits program (13). The constant-variance random walk model of evolution was used in the maximum-likelihood analysis, because there was no indication of a directional random walk model giving a better fit to the laccase activity data (likelihood ratio, LR = 0.02, p = 0.9203). The model estimated a punctuated evolutionary trajectory (κ estimate = 0.0) with longer distances (from root to tip) in the fungal phylogeny contributing proportionally more (δ estimate = 2.9) to laccase activity evolution. This would indicate that recent phylogenetic changes (i.e. species-level transitions) may explain most of the evolution of laccase activity independent of overall branch lengths and larger-scale episodes of accelerated evolution. However, none of these estimates were significant (p > 0.50) in likelihood ratio tests of alternative models, so this analysis merely characterized the optimal parameter values in the evolutionary model that described the data best.

Laccase protein sequencing and analysis

To obtain fungal laccases that are active and transferred via ant fecal droplets, the fecal droplet proteome was separated on a SDS-PAGE gel and the individual bands cut out and sequenced using MALDI TOF and tandem mass-spectrometry. The methodology is described in detail in (7). Briefly, 50 fecal droplets from *A. echinatior* workers (colony Ae263) were collected and combined in 2 x SDS-PAGE loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and separated on a 12.5% polyacrylamide gel. The gel was stained (0.25% Coomassie Brilliant Blue R250, 0.44% ethanol, 9.2% acetic acid) and visible bands were excised individually. Proteins in gel bands were subjected to in-gel protein digestion using trypsin following previously described protocols (7*,* 14). The resulting peptide mixtures were desalted on custom-made reverse-phase microcolumns, prepared with R2 resin (Perseptive Biosystems Inc., Framingham, MA, USA) and were eluted with 0.8 µL of matrix solution (5 µg/µL of a-cyano-4-hydrocynnamic acid in 70% acetonitrile and 0.1% TFA) directly onto the matrix assisted laser desorption ionization (MALDI) target plate. Peptide mass spectra were obtained in positive reflector mode on a 4800 Plus MALDI-TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA, USA) using 20 kV of acceleration voltage. Each spectrum was externally calibrated with peptides derived by tryptic digestion of β-lactoglobulin

and tandem mass spectra were acquired using the same instrument in MS/MS-positive mode. Because no protein identification could be obtained by searching protein sequence databases at the National Center for Biotechnology (NCBI Prot., April 2008) manual *de novo* sequencing was performed on the basis of the presence of b and y peptide fragment ions in MS/MS spectra (15). To facilitate the process, samples were derivatised by adding 7 μL of 10 μg/μL 4-sulfophenyl isothiocyanate dissolved in 50 mM NaHCO3, pH 8.6. The reaction was allowed to proceed for 30 min. at 50°C and terminated using 1 μL of 1% TFA. The mixture was then desalted and eluted directly on the target and analyzed using 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in MS/MS mode. Derived peptides (showing a mass difference of 215 Da compared to the original MS spectra) were sequenced using the same instrument in MS/MS-positive mode. The obtained MS/MS spectra from underivatized and derivatized samples were analyzed manually supported by the AminoCalc program (Protana A/S, Odense, Denmark) to find the distance between fragment ions and to obtain amino acid sequences. The observed similarity between peptide mass spectra and cDNA sequences (96%, 2 out of 51 aa, Fig. 3*A*) was not 100% because of some variation between samples used for fecal droplet proteome and cDNA analysis.

RNA isolation

Using a stereo microscope, gongylidial clusters (staphylae) and mycelium were collected from fungus garden fragments in the middle section of fungus gardens and put immediately in liquid nitrogen. The two different tissue types were sampled right next to each other to minimize variation in age and kind of substrate supporting these different types of hyphae. RNA was isolated from the fungal symbiont *L. gongylophorus* from laboratory *A. echinatior* maintained fungus gardens using a modified protocol of the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany, # 74104). The fungal material was disrupted with a FastPrep® sample preparation machine (Qbiogene, MP Biomedicals, Santa Ana, California) at 4.5m/sec for 45 sec in 500 μL RLC lysis buffer, 5 μL β-mercaptoethanol, and glassbeads. The RNA was extracted twice with an equal amount of phenol/chloroform/isoamyl alcohol (25:24:1), pH 8, followed by one extraction with chloroform/isoamyl alcohol (24:1). The final extract was then purified using the RNeasy Plant Mini Kit including an on-column DNase I treatment step as described in the protocol (Qiagen, Hilden, Germany, # 79254). cDNA was generated with standard oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA, #18080-051) following the manufacturers descriptions and used as template for subsequent PCR reactions unless otherwise stated.

Gene finding

The obtained laccase amino acid sequences were used to design two laccase specific degenerate primers Sp1-F1 and Sp3-R1 (Table S1), which were used in combination with a RACE strategy to sequence the full length gene product of *LgLcc1*. 3′- and 5′ - RACE libraries were made from approximately 1 μg of the purified RNA with the SMART RACE cDNA kit (Clontech,

Mountain View, CA, USA), and gene sequences were PCR amplified from these libraries using specific primers designed from the fungal genome sequence along with the primers enclosed in the SMART RACE cDNA kit (Table S1-3). The following PCR scheme was used to amplify the genes: one cycle of 95°C for 4 min, 10 cycles of 94°C for 20 sec, 72°C for 30 sec (with a decrease in temperature of 0.5°C in every cycle) and 72°C for 2 min, followed by 35 cycles of 94°C for 20 sec, 67°C for 30 sec and 72°C for 2 min, and ending with one cycle of 72°C for 7 min. All PCR products were cloned into pCR4-TOPO using the TOPO TA cloning method (Invitrogen, Carlsbad, CA, USA) before sequencing.

To search for further putative laccase-coding genes present in the *L. gongylophorus* genome, the four conserved laccase signature regions L1 – L4 (16) were identified in *LgLcc1* and used as query sequences for a psi-tblastn search strategy. The first psi-tblastn iteration was searched against the non-redundant protein sequence (nr) database in January 2010 and the position specific scoring matrix (PSSM) retrieved. The PSSM was then used to search the raw and unassembled draft genomic sequence information of the fungus *L. gongylophorus* cultivated by another, sympatric colony of *A. echinatior* (Ae322). This sequence set was generated using two full 454-sequencing runs and represents 700 Mb of sequence data with an estimated 7x coverage (based on actual observed coverage of known fungal sequences) of the heterokaryotic genome and is part of a genome sequencing project of *L. gongylophorus*, coordinated by Jacobus J. Boomsma, Centre for Social Evolution, Copenhagen University, 2012 (The raw sequence data of the fungal genome have been deposited in the European Nucleotide Archive (ENA) at the EMBL-EBI database under the accession number ERP001825). This yielded 167 unique raw reads that were assembled into 24 contigs using CAP (17). Manual trimming reduced the number of contigs to 14 for which full gene lengths (~2500-3000 bp) were obtained using further local BLAST against the unassembled genome sequence set. Gene prediction was performed with AUGUSTUS (18) using hidden-markov models trained on the agaric basidiomycete *Laccaria bicolor* and gave a final set of 9 putative laccase-coding genes. The high sequence divergence of the identified putative laccase sequences (Figures S3-S5), including a ferroxidase (*LgLcc9*), implies that our search strategy was exhaustive and all putative laccase coding genes should have been identified in the sequence data. To confirm gene prediction all putative laccase-coding genes were sequenced using a combination of specific primers and RACE PCR as described above (for primer sequences see Table S1-3). Potential signal peptide sequences were estimated with SignalP (19) and the subcellular location predicted with TargetP (20, 21).

Expression analysis

Using a stereomicroscope, undifferentiated mycelium and clusters of gongylidia (staphylae) were collected with a fine forceps from fragments of fungus garden belonging to four colonies of *Acromyrmex echinatior* (Ae263, Ae280, Ae322, and Ae335) and three colonies of *Trachymyremx cornetzi* (Tra.sp1.C#004_270408, Tra.sp1.C#005_270408Tsp-5, and Tra.sp1.C#010_010508) that were analyzed separately as biological replicates. The fungus

material was immediately put into liquid nitrogen before RNA extraction (see RNA isolation section above). Expression levels of putative laccase-coding genes in gongylidia and gongylidia-free mycelium were determined using qPCR. cDNA was generated from either 200 ng or 1,000 ng of total RNA from undifferentiated mycelium or staphylae using Superscript III reverse transcriptase (Invitrogen) and an oligo(dT) primer and subsequently diluted with water 8 or 40 times depending on the amount of starting material. 0.5 µL template cDNA was used in a 20-µL qPCR reaction with 10 µL of 2× SYBR Premix Ex Taq (TaKaRa Bio Inc., Otsu, Japan) and 0.4 µl of each primer (10 µM) and run on a Mx3000P QPCR system (Agilent, Santa Clara, CA, USA). PCR conditions consisted of one cycle of 95°C for 2 min, then 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Finally a melting curve was included after each run. All primers used for qPCR were positioned in the 3′ end of genes and amplified a DNA fragment of ~150-250 bps. Primers were designed using Primer3 (22) to prevent amplification of genomic DNA by letting at least one primer in each primer pair span an intron (Table S1-3). Transcript levels were normalized using three reference genes: Elongation factor 1-α, ubiquitin and glyceraldehyde 3-phosphate dehydrogenase and the efficiency of each qPCR reaction measured with a dilution series of templates at four different concentrations. For analysis, the mean Ct values of triplicate qPCR reactions were used. The relative gene expression levels between mycelium and gongylidia were determined using the software program REST (23), which uses a pairwise fixed reallocation test with 10,000 random reallocations to assess the significance of obtained values. Because the expression levels were measured as ratios they are non-normally distributed with more variability in the upper standard errors (23). Absolute quantification was achieved by measuring cDNA content in the template used to generate the dilution series standard curve with Quant-It^{tm} PicoGreen$^{®}$ (Invitrogen, CA, USA, Carlsbad) following the manufacturers recommendations. The absolute number of transcripts was calculated using the formula:

$$Number\ of\ transcripts = \frac{ng * 6.022 * 10^{23}}{length * 10^{9} * 650}$$

where $6.022*10^{23}$ molecules / mol represents Avogadro's number, and assuming that the average weight of a basepair is 650 Dalton. The absolute number of transcripts was standardized to the total amount of RNA and not corrected using the reference genes, which increased variation between samples slightly (Fig. 3*D*).

Recombinant expression of LgLcc1

The laccase *LgLcc1* cDNA sequence was heterologously expressed in *Saccharomyces cerevisiae* yeast to determine whether the fungal laccase was functionally active and to confirm the polyphenol oxidative potential of the enzyme. The coding sequence of *LgLcc1* was amplified with the primers Cont0_F_yea_BamHI and Cont0_R_EcoRI containing a BamHI and EcoRI restriction site tail, respectively, using cDNA as template (Table S1-3). PCR conditions were one

cycle of 94°C for 4 min, then 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec, followed by 20 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and ending with one cycle of 72°C for 10 min. The amplified cDNA PCR product was inserted into the yeast expression vector *pYES2* (Invitrogen) under the control of a galactose inducible promoter. To ensure that no PCR errors had been incorporated into the construct the amplified cDNA was sequenced. The yeast strain INVSc1 (*S. cerevisiae*: MAT**a/α** *his3Δ1 leu2 trp1-289 ura3-52* (Invitrogen)) was transformed with either the pYES2 vector containing the laccase gene (*pYES2 – LgLcc1*) or an empty *pYES2* vector (*pYES2-*) using the LiOAc/poly-ethylene glycol (PEG) method (22). Positive transformants (*Ura+*) were selected after incubation at 25°C for 3 days on uracil depleted yeast medium consisting of: 2% glucose, 2 % bacto-agar, 0.7 % (wt/vol) yeast nitrogen base (Invitrogen) and 0.2 % yeast synthetic drop-out media supplement without uracil (Sigma- Aldrich). Successfully transformed INVSc1 yeast colonies are able to synthesize uracil from the *ura3-52* gene located on the plasmid and six *Ura+* colonies were selected and transferred to an induction medium containing: 2% galactose, 2 % bacto-agar, 0.7 % (wt/vol) yeast nitrogen base, 0.2 % yeast synthetic drop-out media supplement without uracil, 0.5 mM $CuSO_4$ and 0.5 % Gallic acid (Sigma-Aldrich Denmark A/S, #241105). After incubation for five days at 25°C a dark colouration became visible in the transformed yeast colonies, which indicated a positive polyphenol oxidase reaction when gallic acid was present in the medium.

Ura+ INVSc1 colonies were also grown in liquid induction medium consisting of: 2% galactose, 0.7 % (wt/vol) yeast nitrogen base, 0.2 % yeast synthetic drop-out media supplement without uracil, and 0.5 mM $CuSO_4$ - or in non-inducible selective medium by galactose substituted with glucose and later transferred to the induction medium. 5 ml yeast cultures were harvested by centrifugation and 300 µl 0.1 M Citric acid – sodium citrate buffer (pH = 5.5) and glassbeads were added before cells were disrupted with a FastPrep® lysis machine as previously described. Extracts were centrifuged at 15.000g for 20 min at 4°C before the supernatants were mixed on ice with a set of protease inhibitors. Laccase activity was measured as previously described and general polyphenol oxidase activity was spectrophotometrically measured using 20mM Gallic acid in 0.1 M Citric acid – sodium citrate buffer (pH = 5.5) as substrate. However, because of unknown yeast inhibitors also previously reported for gal-induced heterologous expression of fungal laccase enzymes in yeast (25-27), this method produced distinctly positive but very low and variable amounts of laccase enzyme activity after extended incubation periods at varying temperatures.

Positive selection analysis

In order to estimate laccase *LgLcc1* sequence evolution the homologous enzymes were sequenced from closely related ant-cultivated fungi within Clade 1 (4). Fungus garden RNA was extracted from *Trachymyrmex cornetzi* (Tra.sp1.C#004_270408) representing the higher-attines and *Cyphomyrmex longiscapus* (Cyp.lon.C#012_180507) representing the lower-attines and cDNA constructed as described above. A 141bp region of the laccase gene, between the two

highly conserved copper binding regions I and II was amplified using the primers LccF1 and LccR1 (28) in combination with the specific degenerate primers LccF1-1 and LccR1-1 (Table S1-3) placed internal to the primers LccF1/R1. The full lengths of these partial laccase gene fragments were obtained with RACE-PCR (except for the final 5' end (ca. 300bp) of the laccase gene *ClLcc1* of the *C. longiscapus* fungus garden which could not be amplified). BLASTx searches of the NCBI GenBank database were performed to confirm the sequence similarity and presence of the laccase consensus sequence signature of the cloned laccase gene fragments (16). Genbank accession numbers are JQ307232 and JQ307233.

Tests for significantly positive natural selection were performed with the branch model and branch-site model test and statistical significance was evaluated with likelihood-ratio tests of the estimated models using codeml as implemented in PAML 4.4 (29). Maximum likelihood estimates of the dN/dS (ω) ratio at sites along a protein indicate positive Darwinian selection if ω > 1 and thus allows for the detection of lineages and sites subject to divergent selection. Tests were performed on a small subtree with five laccase sequences: *LgLcc1*, *TcLcc1*, *ClLcc1*, *Lac* from *Cyathus bulleri* (EU195884), and *Lac2* from *Coprinus comatus* (JQ228449) based on sequence similarity in a neighbour-joining analysis (Fig. S4). We believe that this number of taxa represents an optimal compromise between having sufficient outgroup comparison and avoiding position change saturation that would follow from including more distantly related sequences. The alignment was trimmed until the first conserved region by, respectively, 26 codons from the start and 9 codons from the end, to avoid artefacts resulting from misalignment in the variable end regions. The Branch-site test allows for varying dN/dS ratios (ω) both between particular branches and between particular amino-acid positions. A specific branch is selected *a priori* dividing the tree into background and foreground branches. The model then divides the sites at the background lineages into two classes: conserved sites with $0 < \omega_0 < 1$ and neutral sites with $\omega_1 = 1$. The foreground lineages contain the same site classes, but with a proportion $p = 1 - p_0 - p_1$ of sites in a third site class with $\omega_2 > 1$. The model parameters are estimated with maximum likelihood and compared with a log-likelihood ratio test to the null model where $\omega_2 = 1$ across all sites in foreground lineages. Following a significant branch-site model test, a Bayes empirical Bayes analysis was used to estimate the specific codons with a significant probability of positive selection ($\omega > 1$) as described in (30). Each model was run three times with varying initial values of ω to avoid local maximum likelihood estimates. The validity of this analysis was verified by performing the same analysis using two other outgroups with only laccase sequences from the family Agaricaeae to which the attine fungi belong (Table S9). These additional analyses showed the same result, although the inclusion of more distantly placed (Fig. S4) and likely paralogous laccase sequences indicated positive selection also on the branch leading to *TcLcc1* cultivated by *T. cornetzi* (Table S9).

Protein model

The structural properties of *LgLcc1* were analysed with homology modelling of the secondary protein structure using Swissmodels (31-33), Cphmodels (34), and 3D-Jigsaw (35, 36). The different models were evaluated with the Qmean score (37) and the final model evaluated with a Ramachandran plot using Procheck (38). The crystal structure of a laccase enzyme from the free-living basidiomycete *Trametes trogii* (NCBI-Structure: 2hrgA) was used as template for the homology model (Blast E-value = 7.66e-165, Sequence identity = 60.8%) based on iterated blast searches. The final model predicted the position of 486 amino acids (98% of the total AA sequence) and manual inspection of the Ramachandran plot for this model indicated normal molecule tension in the 3D structure (98.3% residues in core + allowed region). The molecular mass of 54 kDa and the isoelectric point of 4.7 were calculated using WinPep (39) and H++ respectively (40, 41). An isoelectric point of 4.7 is lower than the observed pI of 6.3 – 6.5 obtained by isoelectric focusing of the laccase from leaf-cutting fecal droplets in (42), a difference that likely reflects secondary enzyme modifications that were not accounted for in our protein model.

Additional analysis

Laccase enzyme activity of fungal cultivars grown in-vitro

Laccase activity of isolated fungal cultures was studied with liquid cultures grown in standard potato dextrose media and thus grown with no specific induction of laccase activity. Fungal cultures were isolated by placing a small tuft of mycelium from fungus gardens on Potato Dextrose Agar media (Sigma-Aldrich Denmark A/S, #P2182) containing streptomycin and successful isolates were subcultured and maintained on PDA media. Liquid cultures were prepared by placing a small agar plug (0.5 cm^3) from each fungus culture in 10 mL Potato Dextrose Broth medium (Sigma-Aldrich Denmark A/S, #P6685). All fungus cultures were grown without movement at 25 °C in the dark. Liquid cultures were harvested by washing the fungal cultures on filterpaper with cold ddH_20 to remove growth media after ca. 30 days when the fungal cultures were still actively growing and there was excess media left. The washed cultures were placed on a pre-cooled ceramic tray with a recess (ca. 2 cm diam.) standing on a cooling element. 30 mg sea sand (Merck, 1.07712.1000, grain size 0.1-0.3) and 100 µl 0.1 M Citric acid – sodium citrate buffer (pH = 5.5) was added before cultures were homogenized using the bottom of a glass tube, which together with the sand particles grinded the fungus cultures into a homogenous solution that was transferred to an eppendorf tube. 1000 µl 0.1 M Citric acid – sodium citrate buffer (pH = 5.5) was added and the solution vortexed.

The homogenized fungal cultures were standardized to ensure that possible differences in enzyme activity were not due to differences in biomass. 100 µl of homogenized culture were measured in triplicate by spectrophotometry and the optical density at 650nm was used as a measure of fungal biomass in the sample. The leftover culture solution was subsequently aliquoted as 150 µl samples in 5 separate eppendorf tubes and diluted with 0.1 M Citric acid –

sodium citrate buffer (pH = 5.5) so all culture solutions had the same optical density (OD = 0.8). Enzymes were then extracted by centrifugation at 15.000g for 15 min at 4 °C, and the laccase activity measured as previously described. Due to some mortality of colonies over the course of this research and contamination of some fungal cultures not the exact same fungal isolates were available for these experiments as in the assays measuring laccase activity of intact fungus gardens. Laccase activity in liquid cultures did not differ significantly between species ($F_{13,39}$ = 2.02, p = 0.0620) but showed the same trend of higher laccase activity in fungal cultures from leaf-cutting ants except for isolated cultivars of *A. cephalotes* (Fig S1).

References

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Tables

Table S1. Primers used in this study for the *Acromyrmex echinatior* cultivar: *Leucocoprinus gongylophorus.*

Gene	Primer function	name	Strand	Sequence (5' - 3'), (X = Inosin)	Ref.
Fungal laccases	Fungal laccase primer	LccF1	F	CAYTGGCAYGGNTTYTTYCA	(*52*)
	Attine cultivar laccase primer	LccF1-1	F	AGTTCCGTCKGCCCAASY	This study
	Fungal laccase primer	LccR1	R	RTGRCTRTGRTACCARAANGT	(*52*)
	Attine cultivar laccase primer	LccR1-1	R	CAGAAGGTACCRGCTTGG	This study
LgLcc1	Protein seq. deg. primer	Sp1-F1	F	GCTGTCMTXCARGTNGARCA	This study
	Protein seq. deg. primer	LccF2	F	ACNTTYTGGTAYCAYAGYCA	This study
	qPCR	LccF3	F	CCTACAACTACGACAATCC	This study
	Protein seq. deg. primer	LccF4	F	GTNCARYTNTTYACXGGXCA	This study
	Expression cloning	Cont0_F_yea _BamHI	F	GGATCCACACAATGAAGACGGCT TTAGGTCT	This study
	Protein seq. deg. primer	Sp3-R1	R	TGACCRGCRAAXAKYTGNAC	This study
	Protein seq. deg. primer	LccR2	R	AGDATYTGNAGNAGNACXGG	This study
	Protein seq. deg. primer	LccR3	R	TGNCCRTGNAGRTGRAAXGG	This study
	Protein seq. deg. primer	LccR4	R	TGNCCNGTRAAXARYTGXAC	This study
	Protein seq. deg. primer	LccR5	R	TTNARRTTRTCRTAXGTXGT	This study
	Protein seq. deg. primer	LccR6	R	ACATTGACCACCCCTATATCAG	This study
	qPCR	LccR7	R	CATAAGTCACTCCAAGCATCG	This study
	Expression cloning	Cont0_R_Eco RI	R	GAATTCTCAGGTTCCCGAATCTTG ATCT	This study
LgLcc2	RACE and gene sequencing	Cont1_for1	F	CCAACCAAGCCATATACTGT	This study
	RACE and gene sequencing	Cont1_for2	F	GGACCGGTAATTACAATGAG	This study
	RACE and gene sequencing	Cont1_for3	F	ATTTCGAAGTCAATGGTCAC	This study
	qPCR	Cont1_for4	F	TCGTCAGAGACACTGTCAAC	This study
	RACE and gene sequencing	Cont1_for5	F	ATGCACTTTTCTTTCTTATCTACTC	This study
	RACE and gene sequencing	Cont1_3race1	F	CCTGGACCGTGGATCTTGCAC	This study
	RACE and gene sequencing	Cont1_rev1	R	ACAAAGATCGGTCCAAGAA	This study
	RACE and gene sequencing	Cont1_rev2	R	CGTTGACTGGAGTAGATTCG	This study
	RACE and gene sequencing	Cont1_rev3	R	GCGTAGATTTGAATCGTGTT	This study
	qPCR	Cont1_rev4	R	TCAATGTGACAGTGCAAGAT	This study
	RACE and gene sequencing	Cont1_rev5	R	CTGACCGGCGTAGATTTG	This study
LgLcc3	RACE and gene sequencing	Cont2_for1	F	CCCTTGCTTACACCATTG	This study
	RACE and gene sequencing	Cont2_for2	F	TGATCCCAACTTCGTATTCT	This study
	RACE and gene sequencing	Cont2_for3	F	AACTCAATCTTCCCTCTGGT	This study
	RACE and gene sequencing	Cont2_for4	F	AGATACCGTTGGCGATTTTG	This study
	qPCR	Cont2_for5	F	ATTGCCATATTGATTTCCAC	This study
	RACE and gene sequencing	Cont2_for6	F	ATGTTACCTAATCTTCAATTTGC	This study
	RACE and gene sequencing	Cont2_3race1	F	CTTTGCTGAAGCCCCGGAAGATA	This study

	qPCR	Cont2_rev1	R	CCAAGTCTGACAAAGCTGAT	This study
	RACE and gene sequencing	Cont2_rev2	R	GATATTTGAATCAGGGGTCA	This study
	RACE and gene sequencing	Cont2_rev3	R	GTTTCAATGATGGTGAGGTC	This study
	RACE and gene sequencing	Cont2_rev4	R	ACTCAGCAGGTGGGGTCTTA	This study
	RACE and gene sequencing	Cont2_rev5	R	AGTAACGCTGACCTGCTAAG	This study
LgLcc4	RACE and gene sequencing	Cont3_for1	F	GCTCACTGTAGAAAGAGACAAA	This study
	RACE and gene sequencing	Cont3_for2	F	TGGAGACTCATTCCTCTACAA	This study
	RACE and gene sequencing	Cont3_for3	F	CCTGAGGAGTGCTCACTGTA	This study
	qPCR	Cont3_for4	F	CTTGATGTTGGCTTTGCTAT	This study
	RACE and gene sequencing	Cont3_for5	F	CAATATCACACCAACTTTATGG	This study
	RACE and gene sequencing	Cont3_for6	F	GGTATCAATCAGTGCCCTATC	This study
	RACE and gene sequencing	Cont3_for7	F	ATGGCCCGAAGCTCATATT	This study
	RACE and gene sequencing	Cont3_for8	F	GTTCGACTCAAAGAAGATGG	This study
	RACE and gene sequencing	Cont3_for9	F	CCCGAAGCTCATATTTTGT	This study
	RACE and gene sequencing	Cont3_3race1	F	TTCGCAGAGGCACCAGAACAAAC	This study
	qPCR	Cont3_rev1	R	GTTGCGTTAGGATGAGTTTC	This study
	RACE and gene sequencing	Cont3_rev2	R	TGAAGATGGAATGGGTGAG	This study
	RACE and gene sequencing	Cont3_rev3	R	ATAGCTCTTCCCATTGCTTT	This study
	RACE and gene sequencing	Cont3_rev4	R	ACTCCTCAGGGGACAGTAG	This study
	RACE and gene sequencing	Cont3_rev5	R	AAATTATGCCCGTGAAGAT	This study
	RACE and gene sequencing	Cont3_rev6	R	ATATTGCACCCCGAAATG	This study
LgLcc5	RACE and gene sequencing	Cont4_for1	F	CGACATTAACGGTATTTCCT	This study
	RACE and gene sequencing	Cont4_for2	F	GTGCTTCTTGCTGGTCTTT	This study
	RACE and gene sequencing	Cont4_for3	F	GCGACGTGTATCCTATCAAT	This study
	qPCR	Cont4_for4	F	CAATTTCAAGAATCCTCTACG	This study
	RACE and gene sequencing	Cont4_for5	F	ATTCATCTCAGCGCTTGC	This study
	RACE and gene sequencing	Cont4_3race1	F	GATTGGCACCTGGAAGCTGGTCT	This study
	RACE and gene sequencing	Cont4_rev1	R	AGTGGGTGAAATGTAGGAAA	This study
	qPCR	Cont4_rev2	R	GCAGAGATCTTTCCAATCCT	This study
	RACE and gene sequencing	Cont4_rev3	R	TGCCAATCAATATGACAGTG	This study
	RACE and gene sequencing	Cont4_5race1	R	AAAAAGCGAGTCGCAGCGAAGAC	This study
	RACE and gene sequencing	Cont4_5race2	R	CGCTGGAAAGACCAGCAAGAAGC	This study
LgLcc6	RACE and gene sequencing	Cont5_for1	F	ACATTCCAGCACCTACAATC	This study
	RACE and gene sequencing	Cont5_for2	F	TTCAATGTTGTCAGAAGTGC	This study
	RACE and gene sequencing	Cont5_for3	F	ACAATAGCGAACAAGGACAT	This study
	RACE and gene sequencing	Cont5_for4	F	CCCCATCCTTTCCACTTACA	This study
	qPCR	Cont5_for5	F	TCCCTGGATCTTACATTGTC	This study
	RACE and gene sequencing	Cont5_for6	F	AGATGCTGCTCGTCACTC	This study
	RACE and gene sequencing	Cont5_3race1	F	GTGCCCTACCTGGAATGCGTTTT	This study
	RACE and gene sequencing	Cont5_rev1	R	GTAGGGCACAGTTGATTGTAA	This study
	RACE and gene sequencing	Cont5_rev2	R	CTGAACGTTGACAACTGCTA	This study
	qPCR	Cont5_rev3	R	TTGTAATTGTCTGAGAGAGGAGGA	This study

	RACE and gene sequencing	Cont5_5race1	R	TCGAGAAAACCCGTCAGGGGATA	This study
	RACE and gene sequencing	Cont5_5race2	R	GCAACGACAACAGCAGCCAAAGT	This study
LgLcc7	RACE and gene sequencing	Cont8_for1	F	ATCTTCACGGGCATACGTT	This study
	RACE and gene sequencing	Cont8_for2	F	AGTCAATTCAGCCAACAACT	This study
	RACE and gene sequencing	Cont8_for3	F	CACTGTAGGCCCTGTTATCT	This study
	qPCR	Cont8_for4	F	GGATTCTCCACTGTCACATTA	This study
	RACE and gene sequencing	Cont8_3race1	F	GATCTTGGCTTTGCGGTTGTGTTC	This study
	RACE and gene sequencing	Cont8_for5a	F	ATGCGCCTAGCTGATGCT	This study
	RACE and gene sequencing	Cont8_for5b	F	ATGCTGGTTTGTCAAGCA	This study
	qPCR	Cont8_rev1	R	GAGTTCGGATGTGTTGTTTT	This study
	RACE and gene sequencing	Cont8_rev2	R	CAGAGCTGATCCCAATTTTT	This study
	RACE and gene sequencing	Cont8_rev3	R	AGTTGTTGGCTGAATTGACT	This study
	RACE and gene sequencing	Cont8_rev4	R	TTAAGTTGTTCGCCCTTCT	This study
	RACE and gene sequencing	Cont8_5race1	R	TGGTTACCCTTATTTGACCTGGGT CTG	This study
	RACE and gene sequencing	Cont8_5race2	R	CGCCCTTCTTTGCCCAGATAACA	This study
LgLcc8	RACE and gene sequencing	Cont10_for1	F	ACCACTCCTATGACTTCTGG	This study
	RACE and gene sequencing	Cont10_for2	F	GAAATACCTTGCTTTCCTTG	This study
	qPCR	Cont10_for3	F	AGCACTGGACCTTTCATGT	This study
	RACE and gene sequencing	Cont10_for_4	F	TATGAAATACCTTGCTTTCCTT	This study
	RACE and gene sequencing	Cont10_3race	F	GTTGACCCTCAAGGCACTCGTGA	This study
	RACE and gene sequencing	Cont10_rev1	R	GCAGGAAGAGCATCGTAA	This study
	qPCR	Cont10_rev2	R	ATCGTAAGCTGGGCAAAGAG	This study
	RACE and gene sequencing	Cont10_rev3	R	GCCAGAAGTCATAGGAGTGG	This study
	RACE and gene sequencing	Cont10_rev4	R	CGAGAAAGAGTAGCATTTTGA	This study
LgLcc9	RACE and gene sequencing	Cont6_for1	F	TCCTGCTATTCTCTCTGCTC	This study
	RACE and gene sequencing	Cont6_for2	F	GTACCGACGTCGAAGAGTC	This study
	RACE and gene sequencing	Cont6_for3	F	CTGCCTCCATGTTCCTCT	This study
	qPCR	Cont6_for4	F	AGCTCAAGGACAACTGTGAA	This study
	RACE and gene sequencing	Cont6_3race1	F	GAAGCGCTTGGAATGCCTGTTTC	This study
	qPCR	Cont6_rev1	R	AAAACACATCCAAACATTGC	This study
	RACE and gene sequencing	Cont6_rev2	R	AGTCCATAGAACCGACCTCT	This study
	RACE and gene sequencing	Cont6_rev3	R	TTTCCAGCATCAGAGTTCTT	This study
	RACE and gene sequencing	Cont6_rev4	R	GGTACCGTCGACTTCAATAA	This study
	RACE and gene sequencing	Cont6_5race1	R	TGACACGACGCTCAAAGAGACCA	This study
	RACE and gene sequencing	Cont6_5race2	R	GGTCAGGTCCCACCAGAGTTCGT	This study
Ubiquitin	qPCR		F	AACGATAATGGGACCCGGTG	(*13*)
	qPCR		R	GATTGGGATCAGTCAGCATTG	(*13*)
GADPH	qPCR		F	TCAACGGCAAGCTCACTGGT	(*13*)
	qPCR		R	ACAAAATTCCCGTTCAAAGGAAT C	(*13*)
Ef1a	qPCR		F	TTGGAGGAATCTCCCAACATG	(*13*)
	qPCR		R	AACGGACTTGACTTCAGTAGTC	(*13*)

Table S2. Primers used in this study for the *Trachymyrmex cornetzi* cultivar: *Leucocoprinus* sp.

Gene	Primer function	name	Strand	Sequence (5' - 3')	Reference
TcLcc1	RACE and gene sequencing	Tsp1-3_Lcc_F2	F	CCGGCACCTTCTGGTATCATTC	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F3	F	TCATTCCCATCGTTCTACGCAGT	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F4	F	CCTGCGCGGTGCATTAGTTATT	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F5	F	ACGATGAGGACGGCTTTGAGTC	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F6	F	ATGGGCACGCTTTCGATGTAAT	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F7	F	TGTAATCCGGAGTGCGGGTAGT	This study
	qPCR	Tsp1-3_Lcc_F8	F	CATATAACTACGTCAATCC	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F9	F	CGCAGGGACACTGTATCGT	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F10	F	GTATCGTTGGGAGAGACAGG	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F11	F	GTTGGGAGAGACAGGGGATA	This study
	RACE and gene sequencing	Tsp1-3_Lcc_F12	F	ACGAATTTACTCGCCGAGACCA	This study
	RACE and gene sequencing	Tsp1-3_Lcc_R2	R	CGCAGGCCATCACAATACTGC	This study
	RACE and gene sequencing	Tsp1-3_Lcc_R3	R	TATCCCCTGTCTCTCCCAACGA	This study
	RACE and gene sequencing	Tsp1-3_Lcc_R4	R	TGGTCTCGGCGAGTAAATTCGT	This study
	qPCR	Tsp1-3_Lcc_R5	R	CATAGATCACTCCAGGAATCA	This study
	RACE and gene sequencing	Tsp1-3_Lcc_R6	R	TCTTGGTCGGCGTATGTTGAA	This study
	RACE and gene sequencing	Tsp1-3_Lcc_R7	R	CCGCTATCTTGGTCGGCGTA	This study
	RACE and gene sequencing	Tsp1-3_Lcc_R8	R	TCACTCCAGGAATCAGTGACA	This study
	RACE and gene sequencing	Tsp1-3_Lcc_R9	R	CCAGGAATCAGTGACAGCTAA	This study
	qPCR	Tsp1_510_LccF8	F	CCTATAACTACGACAACCC	This study
	qPCR	Tsp1_510_LccR5	R	CATAGGTCACTCCAGGAATCG	This study
Ef1a	qPCR (Tsp1-4)	EF1a-F1-Tsp1A	F	TTGGAGGAGTCTGAAAACATG	This study
	qPCR (Tsp1-5 & Tsp1-10)	EF1a-F1-Tsp1B	F	TTGGAAGAGTCTGAGAATATG	This study
	qPCR (Tsp1-4)	EF1a-R1-Tsp1B	R	CCGATACCACCAATTTTGTA	This study
	qPCR (Tsp1-5 & Tsp1-10)	EF1a-R1-Tsp1A	R	CCGATACCACCGATCTTGTA	This study
Ubc	qPCR (Tsp1-4)	Tsp14_Ubc_F4	F	AACGATAATGGGACCTGGTG	This study
	qPCR (Tsp1-5 & Tsp1-10)	Tsp1510_Ubc_F4	F	GACGATAATGGGACCTGGTG	This study
	qPCR	Tsp1_Ubc_R4	R	GGTTGGGATCGGTCAGCATTG	This study
GADPH	qPCR	Tsp1_GADPH_F4	F	TCAACGGCAAGCTCACTGGC	This study
	qPCR (Tsp1-4)	Tsp14_GADPH_R4	R	ACAAAATTCCCATTCAGCGATATC	This study
	qPCR (Tsp1-5 & Tsp1-10)	Tsp1510_GADPH_R4	R	ACAAAATTCCCATTCAACATAATC	This study

Table S3. Primers used in this study for the *Cyphomyrmex longiscapus* clade 1 cultivar *Leucocoprinus* sp.

Gene	Primer function	name	Strand	Sequence (5' - 3')	Reference
ClLcc1	RACE and gene sequencing	Clon12_Lcc_F1	F	ACCGTTGGAGTTACGCAATG	This study
	RACE and gene sequencing	Clon12_Lcc_F2	F	ATGCCCAATATCTCCTGGAA	This study
	RACE and gene sequencing	Clon12_Lcc_F3	F	GAGCGTTTACACTCTTCCTCGTA	This study
	RACE and gene sequencing	Clon12_Lcc_F4	F	CAGCACTTACAACTACGTCAATCC	This study
	RACE and gene sequencing	Clon12_Lcc_R1	R	TCGCCTCGAACTGATAAAGG	This study
	RACE and gene sequencing	Clon12_Lcc_R2	R	CCAGGAGATATTGGGCATTG	This study
	RACE and gene sequencing	Clon12_Lcc_R3	R	CAACGGTTCCATCGGCCCAAC	This study
	RACE and gene sequencing	Clon12_Lcc_R4	R	GGCCAGTCGCCTCGAACTGATAAAG	This study
	RACE and gene sequencing	Clon12_Lcc_R5	R	CGCAGGCCATCACAGTATTGCGTAG	This study

Table S4 (Next page). Alignment of laccase consensus sequences of *L. gongylophorus* with example fungal laccase sequences for comparison presented as in (43). **A.** Laccase sequences and **B.** multi-copper oxidase sequences with ferroxidase activity. Laccase and ferroxidase consensus sequences are from Kumar et al. (16) and Larrondo et al. (44), respectively.

Table S4

A

Organism	Gene	Acc. Code	L1	L2	L3	L4
L. gongylophorus	LgLcc1	JQ307223	HWHGMFQNGTA-WADGTSGVTQCPI	GTFWYHSHRSTQYCDGLRGAL	HPFHLHGH	GPWILHCHIDWHLDVGLAVVM
L. gongylophorus	LgLcc2	JQ307224	HWHGIFQHRTS-FMDGTEGVTQCPI	GTFWYHSHFGEQYCDGIRGGL	HPFHLHGH	GPWILHCHIDFHLKEGLALVF
L. gongylophorus	LgLcc3	JQ307225	HWHGMFQTRTN-FMDGTDGVTQCPI	GTYWYHSHFGVQYCDGVRGGF	HPFHLHGH	GPWILHCHIDFHLVTGLAIVF
L. gongylophorus	LgLcc4	JQ307226	HWHGILQYHTN-FMDGAAGINQCPI	GTYWYHSHFGVQYCDGVRGAL	HPFHLHGH	GPWILHCHIDFHLDVGFAIVF
L. gongylophorus	LgLcc6	JQ307228	HWHGILQKGTS-PMDGTAGVTQCPI	GTYWYHSHLGNQYCDGLRGAL	HPFHLHGH	GPWILHCHIDFHLVNGLALVI
L. gongylophorus	LgLcc7	JQ307229	HWHGILQRGTN-FMDGTAGVTQCPI	GTYWYHSHIGLQYCDGVRGAL	HPMHLHGH	GPWILHCHINPHLDLGFAVVF
L. gongylophorus	LgLcc5	JQ307227	HWHGLFQARTS-DMDGPAFVNQCPI	GNFWYHSHLSTQYCDGLRGAI	HPFHLHGH	GAWFLHCHIDWHLEAGLAVVF
L. gongylophorus	LgLcc8	JQ307230	NLDGIFFNSNNIFEEAEPFVNACPI	GSFWYHSELSVQYVDGFRGPL	HPFHLHGM	GPFMFHCHIFWHKFAGLASVQ
L. bicolor	Lcc1	FJ432084	HWHDMFQHGSS-WADGPVGVTQCPI	GTFWYHSHHSTQYCDGLRGAL	HPIHLHGT	GPWILHCHIDWHLNLGLAVVF
C. cinerea	Lcc1	AY338756	HWHGLFQRGTN-WADGADGVNQCPI	GTFWYHSHFGTQYCDGLRGPM	HPFHLHGH	GPWFFHCHIEFHLMNGLAIVF
Cy.bulleri	laccase	ABW75771	HWHGLFQHGTT-WADGPVGVNQCPI	GTFWYHSHHSTQYCDGLRGPL	HPFHLHGH	GPWILHCHIDWHLELGLAIVF
P.nameko	laccase	ABR24264	HWHGFFQRGSS-WADGPVGVTQCPI	GTFWYHSHHSTQYCDGLRGAM	HPIHLHGH	GPWIMHCHIDWHFEIGLSVVF
L.edodes	laccase1	BAB83131	HWHGLFQKTTN-WADGPAFVNQCPI	GTFWYHSHLSTQYCDGLRGPL	HPFHLHGH	GPWFLHCHIDWHLEAGFAIVF
T.versicolor	laccase	1KYA	HWHGFFQKGTN-WADGPAFINQCPI	GTFWYHSHLSTQYCDGLRGPF	HPFHLHGH	GPWFLHCHIDFHLEAGFAVVF
M.albomyces	laccase	1GW0	HWHGIHQKDTN-LHDGANGVTECPI	GTSWYHSHFSAQYGNGVVGTI	HPMHLHGH	GAWLFHCHIAWHVSGGLSVDF
Laccase consensus			HWHxxxxxxxx-xxDGxxxxxQCPI	GTxWYHSHxxxQYCDGLxGxF L I M	HPxHLHGH	GPWxLHCHIDxHxxxGLxxxL A F A F F V E M M

B

Organism	Gene	Genbank	L1	L2	L3	L4
L. gongylophorus	LgLcc9 (fer)	JQ307231	HHHGMFFNSTS-YFDGAQGVSECGI	GTYWVHAHASGHYVDGLRAPL	HPFHLHGH	GVWFFHCHIEWHLEAGLAIQL
L. bicolor	Lcc10	FJ432093	HHHGMFFNTTS-WMDGAQGISECGI	GTYWVHGHSSGQYVDGLRAPL	HPFHLHGH	GVWFLHCHIEWHLEVGLSVQF
P. chrysosporium	Mco1	AY225437	HWHGIPQNGTA-YYDGTAGITECGI	GTTWWHSHYDTQYTDGVTGAL	HPFHLHGY	GAWTLHCHISWHMSAGLLMQF
Fet3 consensus			HWHGxxxxxxx-xMDGxxxxTQCPI	GTYWYHSHxxxQYxDGxRxxF A V L	HPFHLHGH	GVWLFHCHIEWHLxxGLxxxL VA FI D F M

Table S5. Gene information for *L. gongylophorus* laccase sequences. Cellular localisation was estimated with TargetP (21, 22).

Gene	Cellular localisation	Introns	DNA (bp)	mRNA (bp)	Protein (aa)	Accession codes
LgLcc1	secretory	13	2209	1557	519	JQ307223
LgLcc2	secretory	12	2208	1587	529	JQ307224
LgLcc3	secretory	12	2259	1587	529	JQ307225
LgLcc4	secretory	12	2235	1584	528	JQ307226
LgLcc5	secretory	14	2327	1560	520	JQ307227
LgLcc6	secretory	12	2238	1578	526	JQ307228
LgLcc7	unknown	13	2330	1599	533	JQ307229
LgLcc8	secretory	12	2221	1569	523	JQ307230
LgLcc9	secretory	8	2426	1911	637	JQ307231

Table S6. Maximum likelihood estimated dN/dS ratios (ω) for each branch in the tree presented in Figure 3*E*. *LgLcc1*: *L. gongylophorus, TcLcc1*: *Leucocoprinus sp.* cultivated by *T. cornetzi*, *ClLcc1*: *Leucocoprinus* sp. cultivated by *C. longiscapus, C. bulleri*: *Lac* from *Cyathus bulleri* (EU195884), *C. comatus*: *Lac2* from *Coprinus comatus* (JQ228449).

Branch	**dN/dS**	**dN**	**dS**
LgLcc1 + TcLcc1	0.1490	0.0653	0.4382
LgLcc1	0.1392	0.0245	0.1763
TcLcc1	0.1327	0.0432	0.3254
ClLcc1	4.2038	0.0253	0.0060
LgLcc1+TcLcc1+ClLcc1	0.0013	0.0802	59.9155
C. bulleri	0.6010	0.1091	0.1815
C. comatus	0.0118	0.1001	8.4757

Table S7. Branch-site test of positive selection on specific codon positions among the attine cultivar branches in the tree (Figure 3*E*) using the outgroup sequences: *Lac2 C. comatus* (JQ228449) and *Lcc C. bulleri* (EU195884) (see table S9). For each branch, likelihood-ratio tests were used to estimate the probability that positive selection has occurred on individual sites on the foreground branch/branches using model A with ω_2 fixed ($\omega_2 = 1$) or variable ($\omega_2 > 1$) as described in (30). Because several branches in the phylogenetic tree were tested, statistical significance (p) was controlled with a Bonferroni correction for multiple comparisons.

Branch	**ln*L* Branch-site model A, with $\omega_2 = 1$**	**ln*L* Branch-site model A, with $\omega_2 > 1$**	**$-2\Delta\ln L$**	***p***	**Parameter estimates with significant selection model ($\omega_2 > 1$)**
LgLcc1	-5591.87	-5591.84	0.07	1.000	
TcLcc1	-5590.36	-5588.70	3.31	0.275	
(*LgLcc1*+ *TcLcc1*)	-5584.99	-5581.27	**7.44**	**0.025**	$p_0 = 0.795$, $p_1 = 0.143$, $p_2 + p_3 = 0.062$, $\omega_0 = 0.039$, $\omega_2 = 6.090$
ClLcc1	-5592.08	-5592.06	0.04	1.000	

Table S8. Codon positions in *LgLcc1* predicted to have a dN/dS > 1 (p > 90%) and details for the significant positions obtained following a branch-site test for positive selection using bayes-empirical-bayes estimation (30). The placement of positively selected codons in the protein structure could not be related to any particular protein region.

Position	Codon
109	S Serine
378	E Glutamic acid
410	S Serine
512	S Serine

Table S9. Branch-site tests of positive selection on specific codon positions among the attine cultivar branches with three different outgroups showing consistent positive selection on the branch subtending the clade of *LgLcc1* and *TcLcc1*. Gene id numbers are given for sequences obtained from the *Agaricus bisporus* genomes: *A. bisporus* var *bisporus* (H97) v2.0 and *A. bisporus* var *burnettii* JB137-S8 available at http://genome.jgi-psf.org. Statistical significance (*p*) was adjusted with a Bonferroni correction for multiple comparisons.

Outgroup	***LgLcc1***	***TcLcc1***	**(*LgLcc1*+ *TcLcc1*)**	***ClLcc1***
Lac2 C. comatus (JQ228449) *Lcc C. bulleri* (EU195884)	2ΔlnL=0.07 p=1.000	2ΔlnL=3.31 p=0.275	2ΔlnL=7.44, p=0.025	2ΔlnL=0.04 p=1.000
Lac2 *C. comatus* (JQ228449) *A. b.* var *bisporus* (184993) *A. b.* var *burnettii* (131729)	2ΔlnL=1.37 p=0.967	2ΔlnL=2.91 p=0.352	2ΔlnL=7.42 p=0.026	2ΔlnL=0.01 p=1.000
Lac2 C. comatus (JQ228449) *Lac1 C. comatus* (JQ228450) *Lcc1d A. b. var. eurotetrasporus* (FJ898370) *Lcc1 A. bisporus* (L10664) *Lcc2d A. b. var. eurotetrasporus* (FJ907540) *Lcc2 A. b. var. bisporus* (FJ838791) *Lcc2t A. b. var. eurotetrasporus* (JN039361) *Lcc1 A. bisporus* (L10663)	2ΔlnL=1. 62 p=0.814	2ΔlnL=6.15 p=0.052	2ΔlnL=9.70 p=0.007	2ΔlnL=0.00 p=1.000

Figures
Figure S1

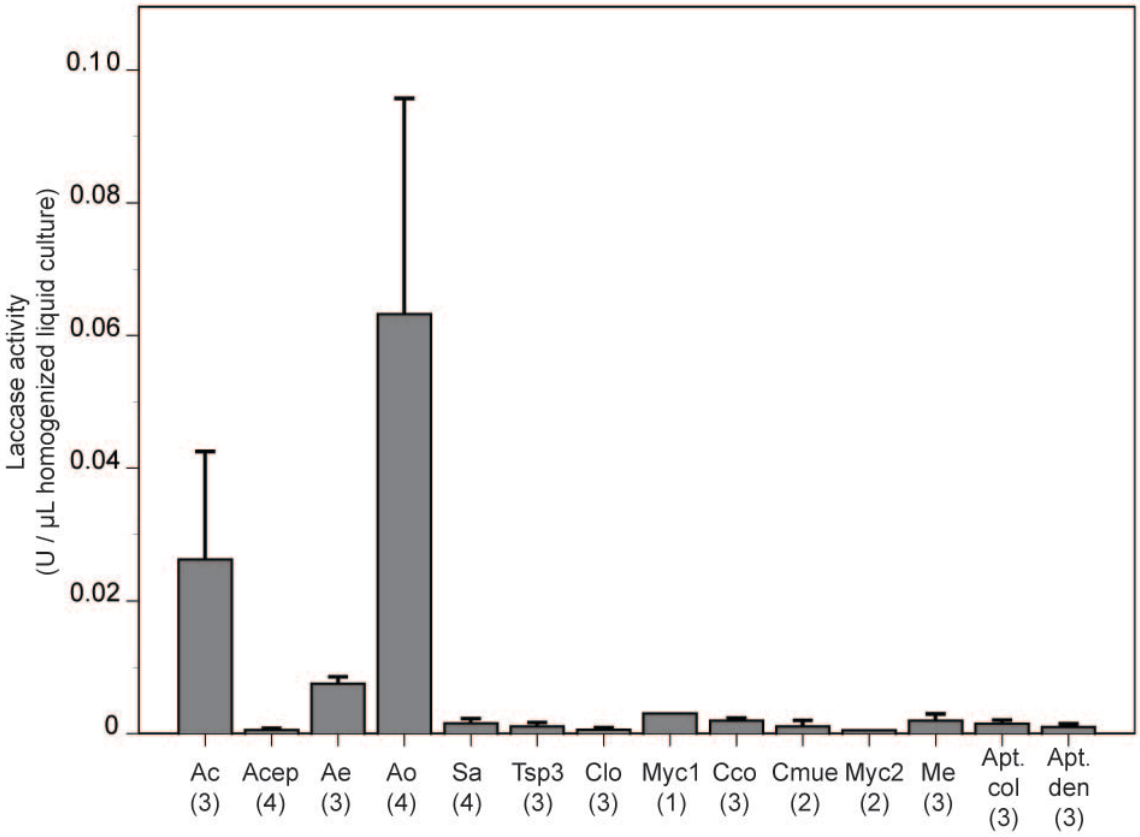


Figure S1. Laccase activity (+SE of n>2) of isolated fungal cultivars grown *in-vitro* in liquid potato dextrose broth cultures from 13 species of fungus-growing ants showing high laccase activity in the leaf-cutting ant cultivars except for *A. cephalotes*. Abbreviations used are: Ac=*Atta colombica*, Acep=*A. cephalotes*, Ae=*Acromyrmex echinatior*, Ao=*A. octospinosus*, Sa=*Sericomyrmex amabilis*, Tsp1=*Trachymyrmex* sp. 1 cf. *cornetzi*, Tsp3=*T.* sp. 3 "blackhead", Clo=*Cyphomyrmex longiscapus*, Myc1=*Mycocepurus smithii* (clade 1 cultivar), Cco=*C. costatus*, Cmue=*C. muelleri*, Myc2=*M. smithii* (clade 2 cultivar), Me=*Myrmicocrypta ednaella* (clade 2 cultivar), Apt.col=*Apterostigma collare*, Apt.den=*A. dentigerum*. The numbers of fungal isolates assayed are given in parentheses.

Figure S2

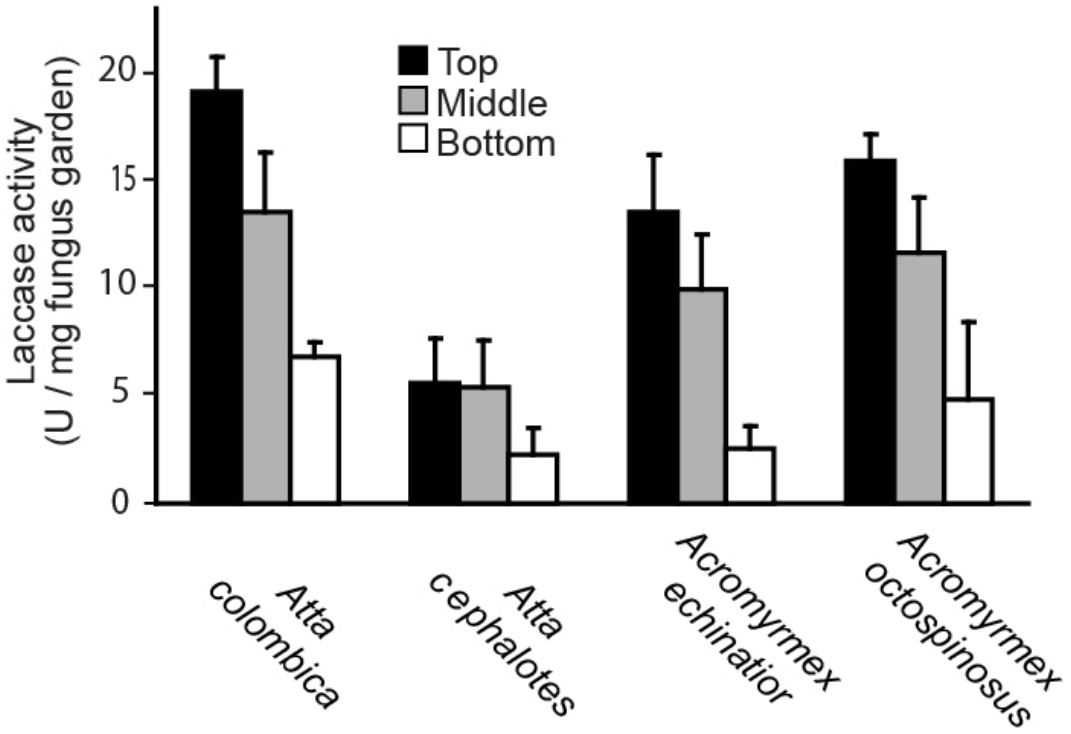


Figure S2. Laccase activity (U/mg±SE) in the top, middle and bottom sections of three laboratory fungus gardens of four common Panamanian leaf-cutting ant species showing that laccase activity decreases from top to bottom (ANOVA: $F_{2,24} = 19.23$, $p < 0.0001$). *A. cephalotes* gardens have a lower overall laccase activity than the other three species tested, which resulted in a significant ANOVA interaction term between fungus garden sections and leaf-cutting ant species (ANOVA: $F_{9,24} = 3.50$, $p = 0.0067$).

Figure S3

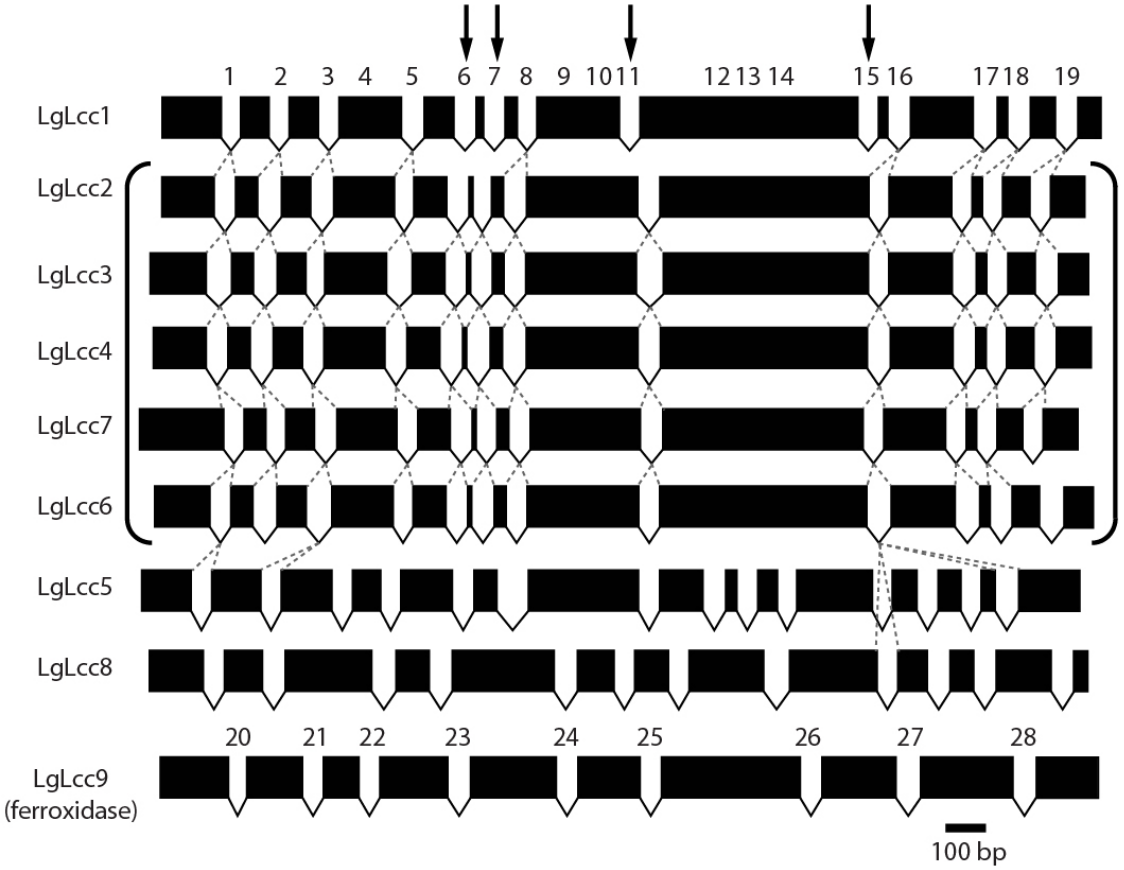


Figure S3. Intron-exon structure of the 9 laccase-coding genes. Intron-exon boundaries that occur at similar base pair positions between gene copies are connected with dotted lines. The four arrows indicate the four introns where *LgLcc1* is different compared to the five *LgLcc* sequences (*LgLcc2,3,4,6,*7) that have nearly identical intron-exon structure (marked in brackets).

Figure S4

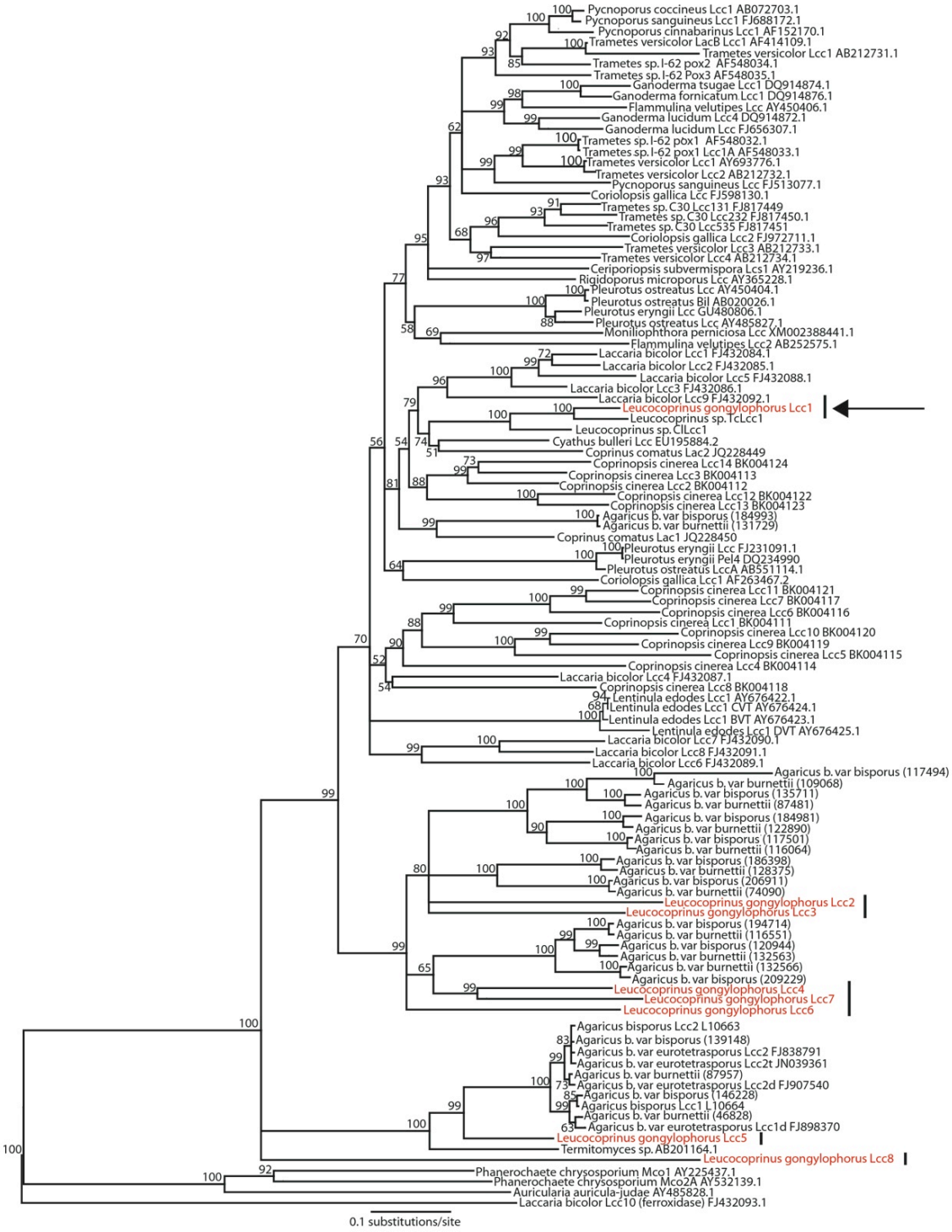


Figure S4. Neighbour Joining tree of Basidiomycete laccase amino-acid sequences. The eight *L. gongylophorus* laccase sequences are marked in red and the arrow indicates *LgLcc1*. Branch support was estimated by 10.000 bootstrap replications and the tree rooted with the ferroxidase *Lcc10* from *L. bicolor*. Gene id numbers are given for sequences obtained from the *Agaricus bisporus* genomes: *A. bisporus* var *bisporus* (H97) v2.0 and *A. bisporus* var *burnettii* JB137-S8.

Figure S5

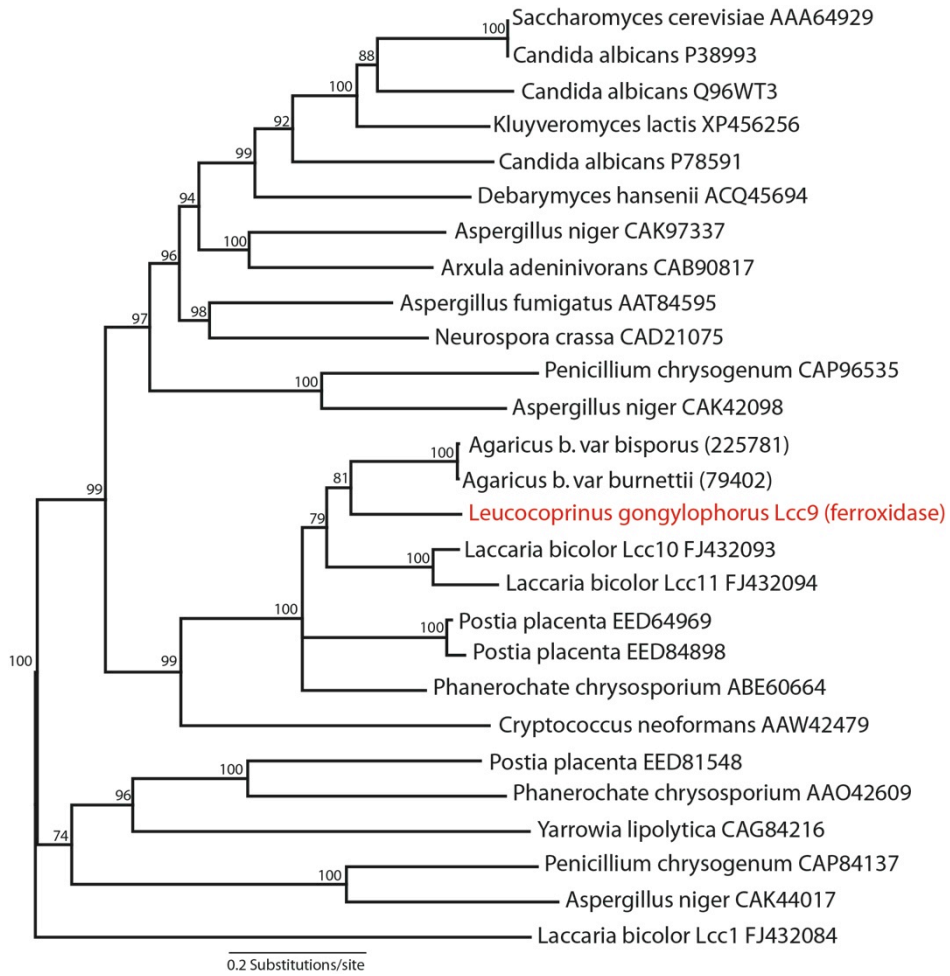


Figure S5. Neighbour Joining tree of Basidiomycete ferroxidase amino-acid sequences. The *L. gongylophorus* ferroxidase sequence (*LgLcc9*) is marked in red. Branch support was estimated by 10.000 bootstrap replications and the tree was rooted with the laccase *Lcc1* from *L. bicolor*.

Figure S6

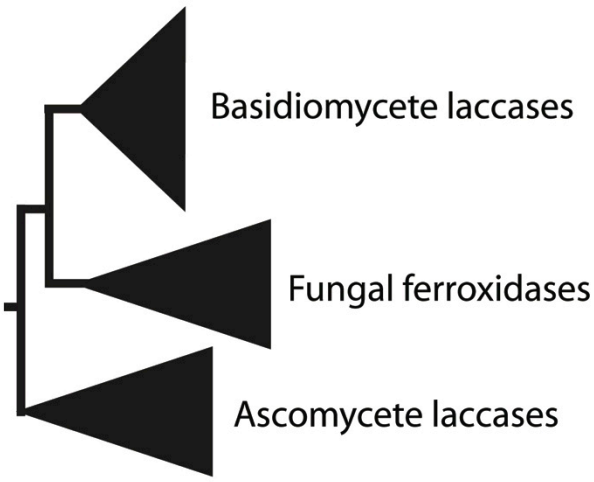


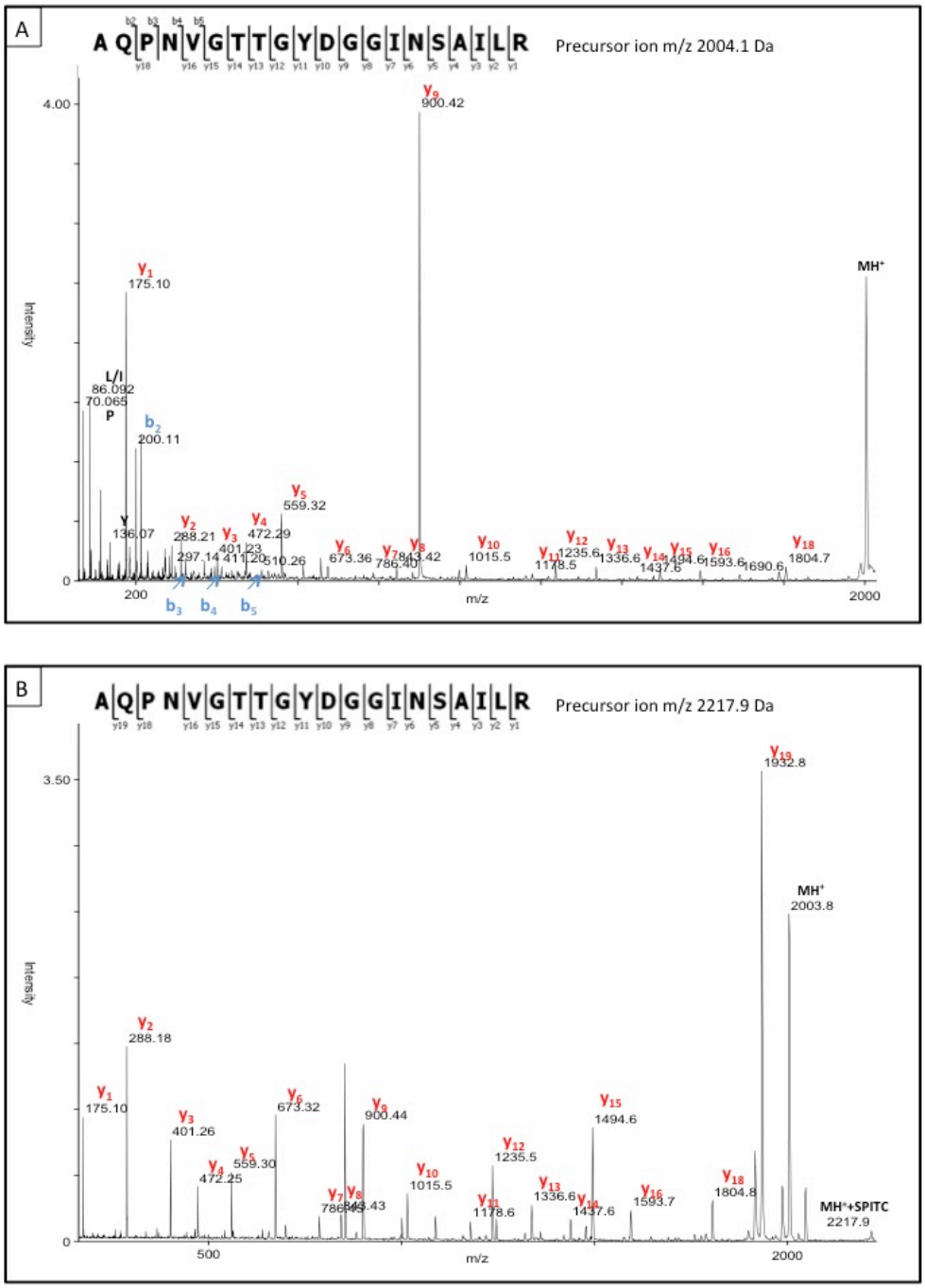
Figure S6. Schematic tree showing the relationship between fungal laccases and ferroxidases after Courty et al. (43).

Figure S7 (Next page). Tertiary structure of the putative substrate binding region of *LgLcc1*. **A.** Protein model of *LgLcc1* with the four substrate-binding regions (loops I-IV) highlighted in blue, green, red and yellow. **B.** Partial protein alignment of *LgLcc1* (numbers refer to amino acid positions) with the higher and lower attine fungal homologs (*TcLcc1* and *ClLcc1*) and similar laccase sequences from: 1. The non-symbiotic free-living basidiomycetes: *Cyathus bulleri* (*C.b.*) (ABW75771) and *Coprinus comatus* (*C.c.*) (JQ228449), and 2. The free-living basidiomycete *Trametes versicolor* (*T.v.*) (PDB acc. code: 1KYA) and ascomycete *Melanocarpus albomyces* (*M.a.*) (PDB acc. code: 1GW0) for which secondary structures are known so substrate binding regions can be predicted. Conserved amino acids in direct contact with the substrate, as determined by x-ray crystallography to construct 1KYA and 1GW0 are highlighted in bold-face print with grey shading.

Figure S7

A
Loop I
Loop III
Loop IV
Loop II
B
Loop I
Loop II
Loop III
Loop IV
Species and ecology
B1-B2
B4-B5
B7-B8
C1-C2
C4-C5
C7-C8
Gongylidia-bearing symbiont (LgLcc1)
Gongylidia-bearing symbiont (TcLcc1)
Non-gongylidia-bearing symbiont (CfLcc1)
Saprophytic free-living fungus (C.h.)
Saprophytic free-living fungus (C.c.)
Saprophytic free-living fungus (T.v.)
Saprophytic free-living fungus (M.a.)

Figure S8


A
A Q P N V G T T G Y D G G I N S A I L R
Precursor ion m/z 2004.1 Da
Intensity
m/z
MH+
B
A Q P N V G T T G Y D G G I N S A I L R
Precursor ion m/z 2217.9 Da
MH+
2003.8
MH+SPITC
2217.9

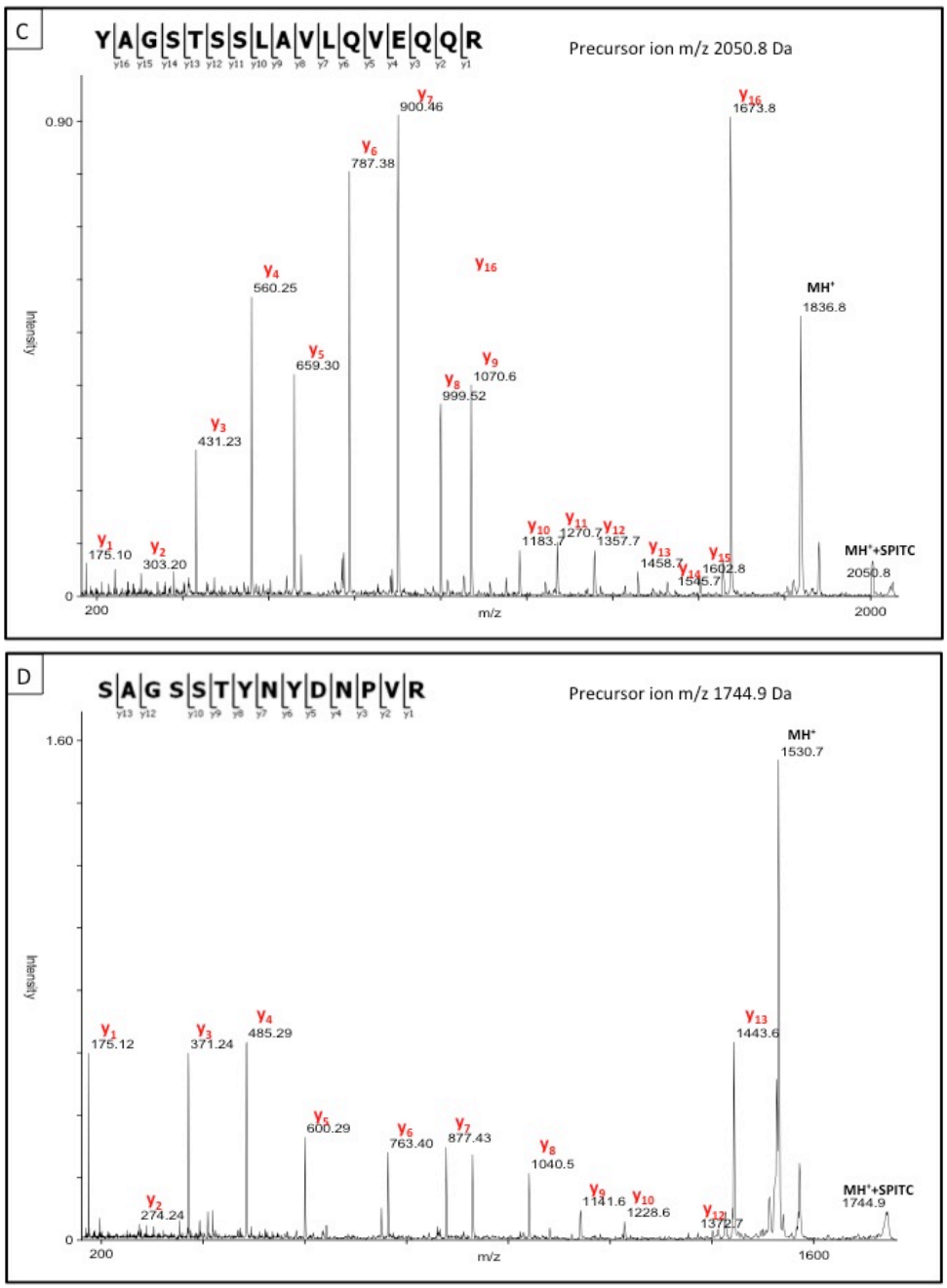


Figure S8. Mass spectrometry based *de novo* sequencing of Laccase peptides. The annotated MALDI MS/MS fragmentation spectra of the precursor ions: **A.** m/z 2004.1 Da; **B.** m/z 2217.9 Da, derivatised with SPITC; **C.** m/z 2050.8 Da, derivatised with SPITC; **D.** m/z 1744.9 Da, derivatised with SPITC. Precursor ions MH+, fragment ions of types y and b, and immonium ions are marked in the spectrum, and the deduced sequence and fragment ions used for the calculations are given for each precursor ion. The mass spectrometry analysis does not allow distinction between I and L and between Q and K. The assignment of correct amino acid residues has therefore been based on the results obtained from DNA sequencing.