# Evolution of multigene families for lignin degradation in *Pholiota microspora*

**( *Pholiota microspora* でのリグニン分解のための多重遺伝子ファミリーの進化 )**

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## Contents

# Chapter 1

## General introduction

Lignin is a complex aromatic polymers, amorphous and important in the formation of higher plant cell walls. It enables trees to grow taller and compete for sunshine. Wood and other vascular tissues generally are 20-30% lignin and forming a matrix that surrounds the orderly cellulose microfibrils. Lignin physically protects most of the world's cellulose and hemicelluloses from enzymatic hydrolysis. In which fungi play the major role in lignin biodegradation (Kirk and Farrell, 1987). According to differences in chemical and structural change in the plant cell wall during decomposition of wood by fungi. There were three distinguished types of lignin decay; white rot, brown rot and soft rot decay. Typically, white rot fungi simultaneously degrade the three major components of plant cell walls: lignin, hemicellulose and cellulose (Kirk and Highley, 1973). Their ability to metabolize large amounts of lignin is unique among microorganisms. Degradation is usually localized to cells colonized by fungal hyphae and substantial amounts of undecayed wood remains even after advanced decay has occurred. While brown rot and soft rot decay preferentially remove cell wall carbohydrate over lignin. (Kuhar et al., 2007).

The white rot fungi capable of extensive aerobic lignin biodegradation. This property is based on the white rot fungi capability to produce one or more extracellular lignin modifying enzyme (LME). The white rot fungi produce

extracellular enzymes that break down the woody cell wall. The lignin modifying enzymes, such as manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Lcc) are directly involved in the degradation of lignin in their natural lignocellulosic substrates (Wesenberg et al., 2003). Some white rot fungi produce all three LMEs while others produce only one or two of them (Hatakka, 1994).

The wood-rotting basidiomycete *Pholiota microspora* (also known as *P. nameko* or "nameko," the common name in Japanese) (Neda, 2008). Nameko mushroom is one of the most popular edible mushroom and is widely cultivated in Japan. It is sold in local markets for food ingredient in Japan and is widely used as material in fungal research into aspects of sexual reproduction structure and mechanism (Babasaki et al., 2003; Aimi et al., 2005; Yi et al., 2010), DNA-mediated transformation system (Yi et al., 2009), tyrosinase protein structure and function (Kawamura-Konishi et al., 2007; 2011; Moe et al., 2015), inorganic phosphate deficiency (Joh et al., 2001; Tasaki et al., 2002; 2004; 2006), effect of polysaccharide (PNPS-1) of anti-flammatory, anti-tumor and anti-aging (Li et al., 2008; 2010; 2012; 2014; Qian et al., 2015; Zhang et al., 2014; Zheng et al., 2014; 2015), hypersensitivity pneumonitis (Nakazawa and Tochigi, 1989; Ishii et al., 1994; Inage et al., 1996) and production of enzyme lignin peroxidase, and manganese peroxidase (Xu et al., 2013).

In order to understand the lignin degradation system in *P. microspora*, we identify lignin degrading genes that belong to LME. Initially, the deduced amino

acid sequence of known MnP, LiP and laccases of different basidiomycetes such as *Phanerochaete chrysosporium* and *Trametes versicolor* from public databases were subjected to BLASTp searches of the *P. microspora* genome. *LiP* gene was not detected by this inquiry. It suggests that *LiP* absence in *P. microspora*, also, Xu et al. (2013) reported that the enzyme activity of LiP was not detected in their experiment. Therefore, the result obtained, *P. microspora* provides five *MnP* and nine laccase genes in their genome.

Either *MnP* or laccase genes were formed a multigene families that might be caused by gene duplication from ancient time. This phenomenon proposes that *MnP* and laccase genes were redundancy which one or more genes are performing the main function. On the other hand, one or more genes could has little or no effect on the biological phenotype. These suggested that genetic redundancy seems to be wide spread in genomes of higher organisms (Nowak et al., 1997) as well as in basidiomycetous fungi. Moreover, a study into redundancy among nine *MnPs* in *Pleurotus ostreatus* found that the *mnp3* gene was the most strongly expressed; in an *mnp3* knockout mutant, alternative transcription of *mnp4* and *9* were observed on the sampling days, and although total MnP activity was reduced in *Δmnp3* strains, the presence of minor *MnPs* was sufficient for maintaining lignin degradation capacity (Salame et al., 2013). Similar phenomenon was also observed in *Laccaria bicolor*, an ectomycorrhizal basidiomycete. *L. bicolor* has 11 laccase genes and showed the transcription profile by qRT-PCR of laccase genes in different stages of growth. *Lb-*

*lcc3* and *Lb-lcc8* were very abundant in ectomycorrhizas, *Lb-lcc7* was in fruiting bodies, *Lb-lcc9* and *Lb-lcc10* were found in free-living mycelium grown on agar medium (Courty et al., 2009). Therefore, the evolution of multigene families among manganese peroxidase and laccase genes for lignin degradation in *P. microspora* were our major investigation.

## 1-1 Manganese peroxidase gene families in *P. microspora*

Manganese peroxidase (MnP), class II peroxidase, is the most common extracellular lignin-modifying peroxidase produced by lignolytic fungi. This heme-containing glycoprotein was first discovered in *Phanerochete chrysoporium* over 25 years ago and has been studied extensively. Secretory MnP is apparently limited to certain basidiomycetous fungi. Among lignolytic enzymes secreted by white rot fungi, MnP has been reported as the main enzyme involved in lignin depolymerisation (Hofrichter, 2002). Fungal peroxidases have been studied exclusively within the context of wood decomposition and MnP have been suggested to be involved in the degradation of humic compounds. For example, wood decomposers *P. chrysoporium*, *T. versicolor, Phebia radiata* and *Pleurotus* spp. (Hatakka, 1994; Martinez, 2002).

Class II peroxidases belong to the plant peroxidase superfamily (Welinder, 1992), and are secreted as fungal heme peroxidases, including the lignin-modifying peroxidases lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile

peroxidase (VP). There are known to be secreted by several homobasiomycetes (Hatakka, 1994). As mentioned above that *LiP* absence in *P. microspora* and VP was a combination between the catalytic properties of both LiPs and MnPs (Hatakka, 1994; Martinez, 2002; Passardi et al., 2007). Then, only MnP presents in *P. microspora*, MnP oxidized $Mn^{2+}$ to $Mn^{3+}$, which then binds to an appropriate ligand and to polyphenolic substrates, which are oxidized and degraded. Variant *MnP* genes are reported to cluster in number in various fungi, i.e., there are three *mnp* genes in *P. chrysoporium* (Gettemy et al., 1998), two *mnp* genes in *Trametes versicolor* (Johansson et al., 2002), two *mnp* genes in *Dichomitus squalens* (Périé et al., 1996), nine *mnp* genes in *P. ostreatus* (Salame et al., 2013), and two unrelated genetic sequences for *mnp*-encoding genes in *Phlebia radiate* (Hildén et al., 2005). However, it is unclear whether all of these genes function in lignin degradation.

Five MnP nucleotide and deduced amino acid sequences in *P. microspora* will be analysis according to the intron-exon position and phylogenetic analysis. The cluster of *P. microspora* MnPs will be unveiled an evolutionary relationship within organism and among that of comparative basidiomycetous fungi. The duplication event was appeared in MnP family. Based on the evolutionary complexity and the developmental processes observed in higher organisms. The *MnP* gene family in *P. chrysoporium* is actively function (Gettemy et al., 1998), but it was transcribed and expressed dissimilarly. This phenomenon was extensively observed among the *mnp* genes in *P. ostreatus* (Salame et al., 2013). Not only MnPs were unequal

transcription and expression, but different in protein sequences also presence in *P. radiata* (*Pr-mnp2* and *Pr-mnp3*). *Pr-mnp2* and *Pr-mnp3* were different in protein sequences but peroxidase function were similar (Hildén et al., 2005).

## 1-2 Peroxidase gene families

Laccase was first described by Yoshida (1883), and was characterized as a metal containing oxidase that have been reviewed by Mayer and Stables (2002). It is obvious that the laccases are very ancient enzymes from an evolutionary of view. However, fungal laccase were discussed on the role in lignin degradation (Eggert et al., 1996; Youn et al., 1995). Laccases can degrade lignin in the absence of LiP and MnP. But laccase performed browning process in *Lentinula edodes* (Sakamoto et al., 2012). Moreover, laccase activity is strongly regulated and promoted fruiting body development in *Agaricus bisporus* and *Schizophyllum commune* (Wood, 1980; De Vries et al., 1986).

Laccase is belong to phenol oxidases (PO) that are enzymes containing copper atoms in the catalytic centre and are usually called multicopper oxidases. The catalytic activity of these enzymes is oxidation of diphenols to the corresponding quinones (Baldrian, 2006). Not only laccase is PO, but also includes tyrosinases (Durán et al., 2002). Laccases are multicopper enzymes that use molecular oxygen as a terminal electron acceptor and are capable of oxidizing various aromatic compounds such as substituted monophenols and polyphenols, aromatic amines, and

thiol compounds, with subsequent production of radicals (Selinheimo, 2008). Fungi generally produce several laccase isoenzymes encoded by complex multigene families (Giardina et al., 2010). The different transcriptional level can be different regulated mechanism and catalytic properties among isoenzymes. This occurrence suggests that different physiological functions related to fungal life cycle, such as morphogenesis, nutrition, fungal-plant/host interaction, stress defence and lignin degradation (Thurston, 1994). There were nine laccase genes in *P. microspora* according to BLASTp analysis. These genes families conducted us to analysis the possible physiological roles in native *P. microspora* cultivation.

Fungal PO, including tyrosinase, can catalyse melanin formation in fruiting bodies as well as browning of the fruiting body that occurs after harvest, as described in *L. edodes* (Sakamoto et al., 2012), but in *A. bisporus*, in which tyrosinase is responsible for melanogenesis during spore maturation (Hegnauer et al., 1985). Intracellular and extracellular PO are produced in plants and fungi for a variety of purposes. PO from basidiomycetes fungi are connected with melanin production, lignin degradation, and morphogenic processes such as fruiting body formation (Sinsabaugh, 2010). Likewise, *Schizophyllum commune* can secrete high levels of laccase in dikaryotic strains, which are able to form fruiting bodies (De Vries et al., 1986). Furthermore, veratryl alcohol can stimulate laccase production during mycelial growth of *P. ostreatus*, increasing production of fruiting bodies, with fruiting occurring earlier in the medium containing veratryl alcohol than the medium

without (Suguimoto et al., 2001). However, the relationship between the roles of multicopper oxidases and veratryl alcohol in fruiting body development is unclear. Therefore, these phenomena inducted us to study laccase genes in *P. microspora.*

**1-3 The goal of this study**

To estimate physiological roles of MnP and laccase multigene families in *P. microspora* which were five and nine genes, respectively. We conducted quantitative RT-PCR (qRT-PCR) technique to study the transcriptional level of genes in *P. microspora*. Firstly, to understand differential gene expression in different stages during mushroom development. Total RNA were extracted and quantified from mycelia grown in sawdust substrate, primordia and fruiting bodies. Secondly, to understand the substrate specificity for each gene. *P. microspora* was grown in M4 liquid medium containing various aromatic compounds, which were lignin related compounds. Total RNA were extracted and quantified from mycelia to investigate transcriptional level of MnP, laccase and related genes. Thirdly, nucleotide and deduced amino acid sequences were analysis. Number and position of introns were inspected. Phylogenetic trees were generated and analyzed of evolutionary relationship across basidiomycetous fungi.

Therefore, in Chapter 2, we will understand the possible physiological roles of MnPs in lignin degradation, since MnPs is a major lignolytic peroxidase in *P. microspora*. Moreover, the possible roles of laccase among lignin degradation,

pigment synthesis and promote fruiting body development, will be explored in Chapter 3. Finally, the evolution of multigene families among MnPs and laccases for lignin degradation in *P. micrpospora* will be totally concluded and discussed in Chapter 4.

# Chapter 2

## Only one major manganese peroxidase (MnP) is predominantly expressed for mycelial growth of *Pholiota microspora* on sawdust medium

### 2-1 Abstract

In this study, we identified five manganese peroxidase genes (*PnMnPs*) in the *Pholiota microspora* haploid genome. Their amino acid sequences showed high similarity and were used to construct a phylogenetic tree. *PnMnP5, 3, 2* and *4* were clustered tightly, but *PnMnP1* was clustered relatively far from *PnMnP5*. qRT-PCR showed that *PnMnP5* was the only *MnP* gene that was strongly transcribed, showing 15-fold higher expression than other *PnMnPs* in M4 liquid medium, while transcription of *PnMnP5* in sawdust medium was 100 times higher than in M4 liquid medium. These results indicate that *PnMnP5* plays a major role in the ligninolytic peroxidase reaction during mycelial growth in *P. microspora*. Based on a comparison of the position of introns, the phylogenetic relationships among *PnMnPs* and the predominant expression of *PnMnP5*, we believe that all *PnMnPs* are of the same origin and that they were amplified by duplication events in the ancient *P. microspora* genome.

## 2-2 Introduction

Class II peroxidases belong to the plant peroxidase superfamily (Welinder, 1992), and are secreted as fungal heme peroxidases, including the lignin-modifying peroxidases lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). They show an exceptionally broad substrate spectrum that includes various organic and inorganic compounds (Hammel and Cullen, 2008). To date, MnPs are the most commonly occurring class II peroxidases and no other microorganisms have been reported to express and secrete MnP or contain *mnp* genes, which are limited to certain basidiomycetes families (*Agaricales, Corticiales, Polyporales, Hymenochatales*) (Hofrichter, 2002; Janusz et al., 2013). MnP was first discovered in *Phanerochete chrysoporium* over 25 years ago and has been studied extensively (Hofrichter, 2002; Martínez, 2002). Variant *MnP* genes are reported to cluster in number in various fungi, i.e., there are three *mnp* genes in *P. chrysoporium* (Gettemy et al., 1998), two *mnp* genes in *Trametes versicolor* (Johansson et al., 2002), two *mnp* genes in *Dichomitus squalens* (Périé et al., 1996), nine *mnp* genes in *Pleurotus ostreatus* (Salame et al., 2013), and two unrelated genetic sequences for *mnp*-encoding genes in *Phlebia radiata* (Hildén et al., 2005). However, it is unclear whether all of these genes function in lignin degradation.

The cluster of genes belonging to the MnP family appears to be the result of duplication, based on the evolutionary complexity and the developmental processes observed in higher organisms. The *MnP* gene family in *P. chrysoporium* is actively

functional (Gettemy et al., 1998), but not all of the genes are transcribed and expressed at all times. Differential expression is seen among the *mnp* genes in *P. ostreatus* (Salame et al., 2013) and individual expression levels are affected by culture conditions (Gettemy et al., 1998). There are great dissimilarities in the *mnp* gene family with regard to exon-intron structure, with *Corticiales, Polyporales* and *Hymenochaetales* showing a lower number of introns (from 4 to 7 highly conserved short introns at similar position, except for *Pr-mnp3*) than *Agaricales* (up to 15 in *P. ostreatus*, at positions corresponding to the sites of introns in LiP genes) (Hofrichter, 2002; Hildén et al., 2005).

In this study, we examined *Pholiota microspora* (*P. nameko* or "Nameko", the common name in Japanese) culture under optimal conditions for fruit body formation, as *MnP* gene in this fungus has not previously been studied (Xu et al., 2013). *P. microspora* is traded in local markets for food in Japan and is widely used as a material in fungal research. We identify the five *PnMnP* genes and discuss the phylogenetic relationships among them, and transcription of *PnMnPs* in mycelium cultured in liquid medium containing various aromatic compounds and sawdust medium was investigated by qRT-PCR. Levels of transcription and phylogenetic relationships, including intron position, are also discussed.

## 2-3 Materials and Methods

### 2-3-1 Fungal strains and culture conditions

The monokaryotic strain of *P. microspora* NGW19-6 (*A4*, *pdx1*) with an auxotrophic mutant of pyridoxine, and NGW12-163 (*A3*, *arg4*) with an auxotrophic mutant of arginine (Masuda et al., 1995; Yi et al., 2009) were used. The dikaryotic strain was obtained by crossing NGW19-6 and NGW12-163 and referred to as NGW19-6/12-163 was then used for experiments.

In order to analyze the effects of different aromatic compounds on gene expression, the *P. microspora* NGW19-6/12-163 strain was grown on M4 agar at 25°C for 1 week, and then 10 mycelial agar blocks (3 × 3 mm) were transferred into 20 mL of M4 broth medium (Johansson et al., 2002) (components are presented in terms of g $l^{-1}$: Glucose, 2.20 g; diammonium tartrate, 0.92 g; $KH_2PO_4$, 1.00 g; $NaH_2PO_4 \cdot 2H_2O$, 0.26 g; $MgSO_4 \cdot 7H_2O$, 0.50 g; Thiamine hydrochloride, $1.00 \times 10^{-4}$ g; $CaCl_2 \cdot 2H_2O$ $6.60 \times 10^{-3}$g; $FeSO_4 \cdot 7H_2O$, $5.00 \times 10^{-3}$ g; $MnSO_4 \cdot H_2O$, $3.00 \times 10^{-4}$ g; $ZnSO_4 \cdot 7H_2O$, $5.00 \times 10^{-4}$ g; $CuSO_4$, $6.40 \times 10^{-4}$ g; pH, 5.5; and 15 g agar for solid M4 medium) in a 100-mL Erlenmeyer flask supplemented with aromatic compounds (final concentration): 0.01% lignosulfonate; 0.05 mM 2,5-xylidine; 3 mM veratryl alcohol; 0.1 mM guaiacol; 1 mM ferulic acid; 1 mM veratric acid; and 0.1 mM *O*-anisic acid. *P. microspora* NGW19-6/12-163 was then grown at 25°C for 10 days and the mycelia were harvested by filtration for RNA extraction.

The cultivation of fruit bodies of *P. microspora* was carried out on a sawdust substrate, which was prepared as follows. Beech sawdust was mixed with rice bran at a gravimetric ratio of 5:1 and was adjusted to 65% moisture content using tap water, and this medium was placed into a 100-mL Erlenmeyer flask, followed by autoclaving at 121°C for 60 min. After cooling the media in the air, five mycelial agar blocks (5 × 5 mm) containing NGW19-6/12-163 were inoculated and incubated at 25°C. When the mycelia had colonized the substrate (about 40 days after inoculation), the surface layer was scratched with a spatula, and then 50 mL of sterilized distilled water was poured into the flask. Water was removed after flasks were incubated at 15°C overnight, and then cultivation continued at 15°C until fruiting bodies developed. We defined the day after water removal as day 0. Sample for RNA extraction were taken from triplicate cultures at different stages of the mushroom developmental cycle: 30 days and 90 days.

### 2-3-2. Genomic DNA and total RNA preparation

Genomic DNA extraction from mycelia grown in the liquid medium was performed according to the method of Dellaporta et al. (1983). Harvested mycelia were frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. RNA was extracted using a MagExtractorTM Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. cDNA was synthesized using total RNA as a template with ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (Toyobo, Osaka, Japan). PCR was carried out using Takara Ex Taq® polymerase

(Takara Bio, Japan). The oligonucleotide primers used in this study were listed in Table 2-1. Amplified fragments were subcloned into pMD20 T-vector (Takara Bio, Japan) and sequenced.

### 2-3-3. *P. microspora* genome and retrieved *MnP*s

Whole genomic sequences of monokaryon *P. microspora* NGW 19-6 were determined using the Illumina HiSeq 2000 paired-end technology, with the software (CASAVA ver.1.8.1) provided by Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan), as described by Funo et al. (2014). This sequencing run yielded 30,935,254 high-quality filtered reads with 101-bp paired-end sequencing. The genome was assembled using Velvet assembler (hash length, 85 bp) (Zerbino and Birney, 2008). The final assembly contained 4,770 contigs with a total size 33,400,256 bp, and an $N_{50}$ length of 72,431 bp. According to Higuchi, (2004), the deduced amino acid sequence of known peroxidase genes from public databases [Lip (Q01787) from *Phanerochaete chrysosporium* and MnP (Q6KB19) from *T. versicolor*]. Five peroxidase genes were assigned as *PnMnP1* to *5*, and the coding sequences of intron-exon based on GT-AG rules (Breathnach et al., 1978; Wu and Krainer, 1999)) and open reading frames (ORFs) based on generic rules (start codon, ATG and stop codon TAA, TAG, or TGA) (Brown, 2010) were then predicted. Nucleotide sequences of genomic DNA fragments of *P. microspora MnP* genes were deposited into the DNA Data Bank of Japan (DDBJ) under the following accession

numbers: MnP1 (LC068763); MnP2 (LC068764); MnP3 (LC068765); MnP4 (LC068766); and MnP5 (LC068767).

### 2-3-4 Quantitative RT-PCR (qRT-PCR) assays

The actin gene (*Act1*) was used as the reference gene. Primer pairs for amplification of *PnMnP1-5* and *Act1* cDNAs were designed based on their cDNA sequences using Genetyx software. Amplification of genomic DNA was prevented by designing primers for exon-exon junctions. All primers were tested to ensure that they amplified a single band with no primer-dimers, as shown in Table 2-2. Plasmids inserted into the target gene (*PnMnP1-5*) and the housekeeping gene (*Act1*) were extracted as described by Birnboim (1983). Standard curves were constructed using four ten-fold dilutions of plasmid. Real-time PCR was performed using a KOD SYBR® qPCR Mix kit (Toyobo). Thermocycling was carried out using a PikoReal™ 96 (Thermo Fisher Scientific) with an initial incubation for 1 min at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 1 min. Each run was completed with a melting curve analysis to confirm the specificity of amplification and no primer dimers. Data analysis was performed in accordance with the manufacturer's instructions.

### 2-3-5 Analysis of sequences and phylogenetic tree

Nucleotide and protein sequences data were analyzed using Genetyx ver. 10.0.3 software (Genetyx, Tokyo, Japan). Protein sequence similarity was analyzed using the BLASTp algorithm (Altschul et al., 1997). Peroxidase genes were retrieved

from public domains (NCBI and UniProt). The phylogenetic tree was constructed by MEGA 6.06 software (Tamura et al., 2013) using the minimum evolution method with a bootstrap value of 2,000 replicates. Multiple alignment was performed using ClustalW (Larkin et al., 2007) with the Gonnet distance matrix (Hildén et al., 2005).

### 2-3-6 Statistical analysis

Mean value and standard deviation of the relative results in each treatments were calculated. Comparisons between control and treatment groups were made using Student's *t*-test. Differences were regarded as statistically significant for *P* values under 5% ($P<0.05$).

## 2-4 Results

### 2-4-1 PnMnP sequence analysis and phylogenetic tree

Despite two kind of peroxidase proteins which were LiP and MnP, were subjected to tBLASTn searches of the *P. microspora* genome. Same five peroxidase genes were hit in each tBLASTn searches, and they were assigned as PnMnP1 to 5 from the similarity with MnPs from other fungi. Moreover, LiP activity was not detected in previous study in *P. microspora* (Xu et al., 2013). A phylogenetic tree based on the deduced amino acid sequences of five PnMnPs and other peroxidases from other basidiomycetous mushrooms extracted from the DNA/protein database

is shown in Fig. 2-1. There were four major clusters, assigned as clusters A, B, C and D, respectively. All five PnMnPs were clustered in D. Identity and similarity between *PnMnP5* with *PnMnP1, 2, 3* and *4* was as follows; *PnMnP5* showed 83% identity, 96% similarity to *PnMnP3*; 78% identity and 94% similarity to *PnMnP2*; 78% identity, 95% similarity to *PnMnP4*; and 68% identity, 91% similarity to *PnMnP1*. Therefore, the evolutional distance among *PnMnPs* (1, 2, 3 and 4) to *PnMnP5* was in the rank order of 3, 2, 4 and 1. Moreover, identity and similarity between *PnMnP5* with *PnMnP1* was lower than for the other *PnMnPs* (2, 3 and 4), and the number of introns and their positions in *PnMnP1* showed greater differences than for other PnMnPs. Therefore, to analyze whether the origin of *PnMnP1* differed from that of other *PnMnPs* in the ancient *P. microspora* genome, the position and number of introns were analyzed.

Nucleotide sequences of ORFs of *PnMnP* cDNAs were similar sizes, ranging from 1,049 to 1,074 bp and their genomic DNA sequences contained 5 to 15 introns. *PnMnP* cDNAs were coded from 354 to 372 amino acids proteins and their molecular weights were 36.68 to 39.11 kDa. *PnMnP5* contains 5 introns, which is the lowest number of introns among *PnMnPs*, while *PnMnP1* contains the highest molecular weight and the exons were interrupted by 15 introns, which is the maximum number among *PnMnPs*. Introns among *PnMnPs* were assigned as roman characters from I to XVII (Table 2-3 and Fig. 2-2). During evolution, intron insertion and excision in the genes located on the genome had occurred; therefore,

phylogenetic distance should correspond with the abundance and distribution of introns (Lynch, 2002). The inserted positions of introns I, V, VII, XV and XVII were conserved among *PnMnPs*, thus suggesting that the *PnMnPs* discovered in this study were of the same origin in the ancient genome of *P. microspora*. Moreover, *PnMnP5* had the smallest number of introns, and is therefore believed to be the oldest ancestral gene. If the original *PnMnP* is *PnMnP5*, the hypothetical evolutionary steps would be as follows: i) *PnMnP5* was duplicated as *PnMnP3*, and then intron XII was inserted (intron XII was conserved in *PnMnP1,* 2, 3 and 4); ii) *PnMnP3* was duplicated as *PnMnP2*, and then intron VI was inserted and intron XVII was removed; iii) *PnMnP3* was duplicated as *PnMnP4*, and introns II and III were inserted; iv) finally, *PnMnP2* was duplicated as *PnMnP1*, and then introns II, III, VIII, IX, X, XI, XIII, XIV and XVI were inserted. This series of events is supported by phylogenetic distance based on amino acid sequences.

### 2-4-2 *PnMnP5* gene expression in the presence of aromatic compound used for substrate for MnP

In order to investigate differences in the roles of *PnMnPs*, expression of *PnMnPs* in mycelia grown in M4 liquid medium supplemented with aromatic compound used for substrate for MnP was studied by quantitative RT-PCR analysis. *PnMnP1, 2, 3* and *4* transcripts were detected at very low levels, and no effect for supplemented aromatic compound was observed (Fig. 2-3). Surprisingly, however,

quantity of *PnMnP5* mRNA in 10-day cultures with and without aromatic compound used for substrate for MnP were 10- to 15-fold larger than those of the other *PnMnPs* mRNA. In particular, *PnMnP5* was not affected with 0.01% lignosulfonate, 0.05 mM 2,5-xylidine and 3 mM veratryl alcohol. These results suggest that: i) transcription of *PnMnP5* is constitutive in M4 liquid medium, but the presence of aromatic compounds did not enhance *PnMnP5* transcription; and ii) transcription of *PnMnP1*, *2*, *3* and *4* is very low, suggesting that only *PnMnP5* functions in lignin degradation in *P. microspora*.

### 2-4-3 *PnMnP5* is the major manganese peroxidase in *P. microspora*

Transcription of *PnMnPs* in mycelia cultured on sawdust medium was then investigated. Mycelia were harvested from sawdust culture at 1 month after inoculation and at 2 months after *kinkaki*, which was performed at 1 month after inoculation, and total RNAs were extracted. Figure 2-4 shows expression levels of *PnMnPs* in the sawdust medium; and 100-fold higher transcription of *PnMnP5*, as compared with *PnMnP1, 2, 3* and *4,* was detected in total RNA from mycelia cultured for 1 and 3 months. Transcription of *PnMnP5* in sawdust medium was also 100-fold higher than that of *PnMnP5* in liquid M4 medium supplemented with aromatic compound used for substrate for MnP. Therefore, transcript of *PnMnP5* with aromatic compounds such as veratryl alcohol was much lower than with sawdust. It is possible that aromatic compounds are not suitable for research into

lignin degradation by *P. microspora*, as they do not accurately reflect the response of mycelia to lignin. Moreover, transcription of *PnMnP1, 2, 3* and *4* was also low in the sawdust medium. We thus concluded that *PnMnP5* plays a major role in lignin degradation by *P. microspora* during mycelial growth on sawdust substrate.

## 2-5 Discussion

It has been well documented that nitrogen levels influence *MnP* production by white rot fungi (Johansson et al., 2002; Hamman et al., 1997). The results of this study show that *PnMnP5* transcription in *P. microspora* is much higher than the other *MnP* genes presents in the genome. In addition, expression levels of *PnMnP5* were not encouraged by various aromatic compound that used for substrate for MnP in modified liquid medium. But, when *PnMnP5* transcription in M4 medium was compared with that in sawdust medium, we must consider that M4 liquid basal medium might not be suitable for MnP expression of *P. microspora*. Because contents of basal medium was not investigate in this study and aeration were much different between liquid culture and solid culture such as sawdust medium (Elisashvili et al., 2008; Camassola et al., 2013). The relatively poor production of the four *PnMnPs* in our experiments raised the issue of why transcription in the presence of lignin was unequal. In this respect, our results suggest the need for further consideration of assessing intragenic noncoding DNA in the context of evolutionary processes.

Our results showed that a well-used aromatic compound, veratryl alcohol, was unable to effectively induce the gene expression of *PnMnP5* in *P. microspora*. This suggests that enhancement of *MnP* and *LiP* transcription in response to various aromatic compounds does not occur in white rot fungus as common feature (Cancel et al. 1993). *PnMnP5* is expressed natively and is important for the ligninolytic system. A study into redundancy among nine *MnPs* in *P. ostreatus* found that the *mnp3* gene was the most strongly expressed; in an *mnp3* knockout mutant, alternative transcription of *mnp4* and *9* were observed on the sampling days, and although total MnP activity was reduced in *Δmnp3* strains, the presence of minor *MnPs* was sufficient for maintaining lignin degradation capacity (Salame et al., 2013). The accumulation of transcripts of *mnp1, 2* and *3* genes in mycelia of *P. chrysosporium* cultured in Mn and nitrogen-limited medium indicated that *mnp2* and *mnp1* transcriptions are respectively predominant, but *mnp3* transcription does not appear to be significantly regulated. These phenomena suggest that *MnP* genes are differentially regulated at the transcriptional level and respond to dissimilar environmental conditions (Gettemy et al., 1998). Moreover, the white rot basidiomycete *Ganodema lucidum* is grown and has the highest *MnP* activity on 10-day culture with poplar wood, but not in cultures grown with pine wood (D'Souza et al., 1999). Similar results have been reported for *T. versicolor MnP*. In addition, the transcription of *P. chrysosporium* genes was studied, and an important role for putative metal response elements (MREs) in transcriptional regulation was suggested, but they do not appear to regulate *MnP* in *T. versicolor* (Johansson et al.,

2002). Based on these phenomena, the differences in lignin degradation activity among white rot fungal species is the result of differential expression and transcription *MnPs*, not the number of genes in their genome.

With regard to genetic variability of gene families, *MnP* belongs to the class II peroxidases, which include lignin-modifying peroxidase, as well as *LiP* and *VP*. *LiP* is catalytically the most powerful class II fungal peroxidase, having the ability to directly oxidize dimeric lignin model and nonphenolic substrates. *LiP* and *MnP* have been extensively studied with regard to substrate oxidation capacity, and *LiP* shares similarities with *VP* (Hofrichter, 2002). Our results suggest that *PnMnP* is an important gene for lignin degradation by *P. microspora*, as the *LiP* gene is absent from its genome. In addition, the lignin degradation capacity of *P. microspora* is relativity lower when compared with white rot basidiomyceteous fungi such as *P. chrysosporium*, *P. radiata* and *T. versicolor* (Hatakka, 1994), which possess the multi-peroxidase gene family. Because of the high homology of amino acid sequences among the five *PnMnPs*, the genes appear to be part of the same family that was amplified by a duplication event during evolution. Therefore, *P. microspora* has only one type of enzyme.

Expression levels of *PnMnP5* were highest; therefore, we believe it to be the original *MnP* in the *P. microspora* genome, as intragenic noncoding DNA interrupting the exons in the five *PnMnPs* is unequally present. The fewest introns are present in the most actively transcribed *PnMnP5* gene. Intron excision and

insertion events promote mutation of *PnMnPs*, while duplication events promote disruption of promoter regions in *PnMnPs*. This may be why only *PnMnP5* is strongly expressed and transcription of other *PnMnPs* is very low.

In this study, we examine the role of *PnMnPs* in *P. microspora* at the transcriptional level, and found that the number of genes is unimportant for lignin degradation. To clarify the relationship between lignin degradation capacity and related multi-gene families, such as laccase genes, in the fungal genome, we are planning further qRT-PCR studies.

**Table 2-1.** Primer sets for cDNA and plasmid construction.

| Genes | Primers | 5'→ 3' |
|---|---|---|
| ***Act1*** | PnActin1_F1 | CGAAATTTCAGCTCTCGTCGT |
| | PnActin1_R1 | CTGGAGCACGGAATCGCT |
| ***PnMnP1*** | PnMnP1_F1 | GCTAATCGTGGCCACAAAT |
| | PnMnP1_R1 | GGACTTGGGAATAACATCGG |
| ***PnMnP2*** | PnMnP2_F1 | GCTCTCAGCGCTACAGTCAAT |
| | PnMnP2_R1 | GCATCCTAGTCTGGTCGTTGAT |
| ***PnMnP3*** | PnMnP3_F1 | CAACGCTGCATGTTCTCTGTT |
| | PnMnP3_R1 | CTCCTGGTATCTTGACCGAGAA |
| ***PnMnP4*** | PnMnP4_F1 | CTCTCACCTCGCCTCCATC |
| | PnMnP4_R1 | GAGTCGTGTGGCAAGCAAT |
| ***PnMnP5*** | PnMnP5_F1 | GATCAATGCTGCCCTCACC |
| | PnMnP5_R1 | CAGTTCGGGGCAAGCAAGT |

**Table 2-2.** Primer sets for qPCR.

| **Genes** | **Primers** | **5'→ 3'** |
|---|---|---|
| ***Act1*** | PnActin1_CF1 | GCTATGCTATGTCGCGCTTGAT |
| | PnActin1_R1 | CTGGAGCACGGAATCGCT |
| ***PnMnP1*** | PnMnP1_CF1 | CTTCGCGCTCTCTCAAGACC |
| | PnMnP1_R1 | GGACTTGGGAATAACATCGG |
| ***PnMnP2*** | PnMnP2_CF1 | CTGTTCCCCGGAAATGGATC |
| | PnMnP2_R1 | GCATCCTAGTCTGGTCGTTGAT |
| ***PnMnP3*** | PnMnP3_CF1 | GCGATGGTCAACGACCAA |
| | PnMnP3_R1 | CTCCTGGTATCTTGACCGAGAA |
| ***PnMnP4*** | PnMnP3_CF1 | GGCAAGCCATGATCAACGA |
| | PnMnP4_R1 | GAGTCGTGTGGCAAGCAAT |
| ***PnMnP5*** | PnMnP5_CF1 | GTGGCAAGCTATGGTTAACGA |
| | PnMnP5_R1 | CAGTTCGGGGCAAGCAAGT |

**Table 2-3.** Intron position according to the highest number of introns (*PnMnP1*). Intron position is indicated by roman characters I to XVII from the N-terminal to the C-terminal. (+) indicates introns that are shared, while (-) indicates no intron in that position.

| ***Intron position*** | **I** | **II** | **III** | **IV** | **V** | **VI** | **VII** | **VIII** | **IX** | **X** | **XI** | **XII** | **XIII** | **XIV** | **XV** | **XVI** | **XVII** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***PnMnP5*** | + | - | - | - | + | - | + | - | - | - | - | - | - | - | + | - | + |
| ***PnMnP3*** | + | - | - | - | + | - | + | - | - | - | - | + | - | - | + | - | + |
| ***PnMnP2*** | + | - | - | - | + | + | + | - | - | - | - | + | - | - | + | - | - |
| ***PnMnP4*** | + | + | + | - | + | - | + | - | - | - | - | + | - | - | + | - | + |
| ***PnMnP1*** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - |

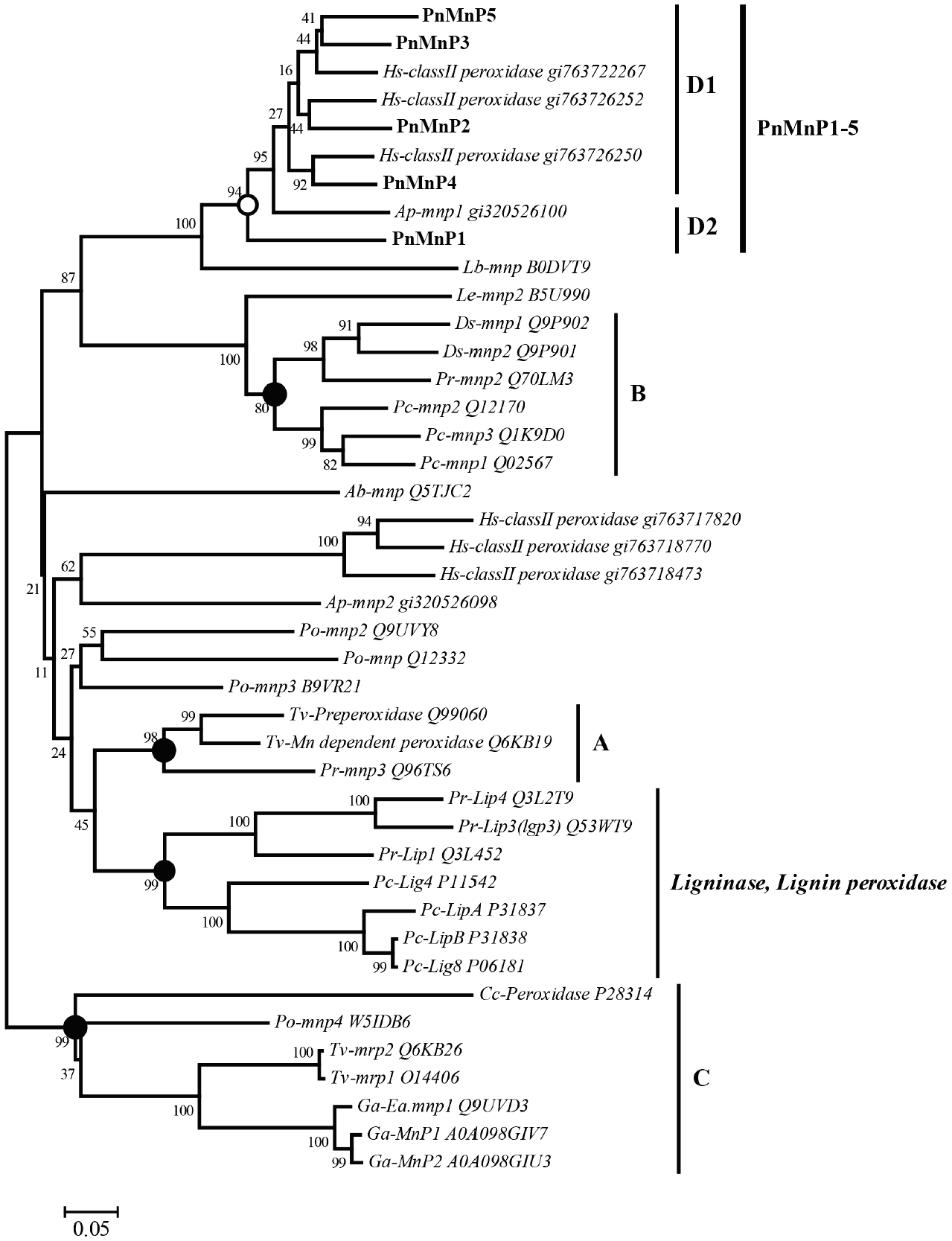
PnMnP5
PnMnP3
Hs-classII peroxidase gi763722267
Hs-classII peroxidase gi763726252
PnMnP2
Hs-classII peroxidase gi763726250
PnMnP4
Ap-mnp1 gi320526100
PnMnP1
Lb-mnp B0DVT9
Le-mnp2 B5U990
Ds-mnp1 Q9P902
Ds-mnp2 Q9P901
Pr-mnp2 Q70LM3
Pc-mnp2 Q12170
Pc-mnp3 Q1K9D0
Pc-mnp1 Q02567
Ab-mnp Q5TJC2
Hs-classII peroxidase gi763717820
Hs-classII peroxidase gi763718770
Hs-classII peroxidase gi763718473
Ap-mnp2 gi320526098
Po-mnp2 Q9UVY8
Po-mnp Q12332
Po-mnp3 B9VR21
Tv-Preperoxidase Q99060
Tv-Mn dependent peroxidase Q6KB19
Pr-mnp3 Q96TS6
Pr-Lip4 Q3L2T9
Pr-Lip3(lgp3) Q53WT9
Pr-Lip1 Q3L452
Pc-Lig4 P11542
Pc-LipA P31837
Pc-LipB P31838
Pc-Lig8 P06181
Cc-Peroxidase P28314
Po-mnp4 W5IDB6
Tv-mrp2 Q6KB26
Tv-mrp1 O14406
Ga-Ea.mnp1 Q9UVD3
Ga-MnP1 A0A098GIV7
Ga-MnP2 A0A098GIU3
D1
D2
PnMnP1-5
B
A
Ligninase, Lignin peroxidase
C
0.05

**Fig. 2-1.** Minimum evolution tree with 2000 bootstrap replications by Mega 6.06 based on ClustalW multiple sequence alignment using the Gonnet distance matrix and ignoring gaps in distance correlation, according to the method of Hildén et al.2005. PnMnP1-5 were clustered into the same ancestry as *Hs-class II peroxidase, Ap-mnp1* and *Lb-mnp*, and was distant from groups A, B and C. (**A**) *Pr-mnp3* was clustered with ligninase, lignin peroxidase (LiP) of *Tv-Mn dependent peroxidase* and *Tv-Pre-peroxidase, Po-mnp* and *Po-mnp3*; (**B**) *Pr-mnp2* was clustered with *Ds-mnp1-2*, *Pc-mnp1-3* and *Le-mnp2*; and (**C**) no *Pr-mnp*s were clustered in the clade with *Ga-Ea-mnp1, Ga-MnP1, Ga-MnP2*, *Tv-mrp1,2* and *Cc-peroxidase*. Initials refer to fungal species with gene name and protein ID; Ab (*Agaricus bisporus*), Ap (*Agrocybe praecox*), Cc (*Coprinopsis cinerea*), Ds (*Dichomitus squalens*), Ga (*Ganoderma applanatum*), Hs (*Hypholoma sublateritium*), Lb (*Laccaria bicolor*), Le (*Lentinula edodes*), Pc (*Phanerochaete chrysosporium*), Pn (*Pholiota nameko*), Po (*Pleurotus ostreatus*), Pr (*Phlebia radiata*) and Tv (*Trametes versicolor*).

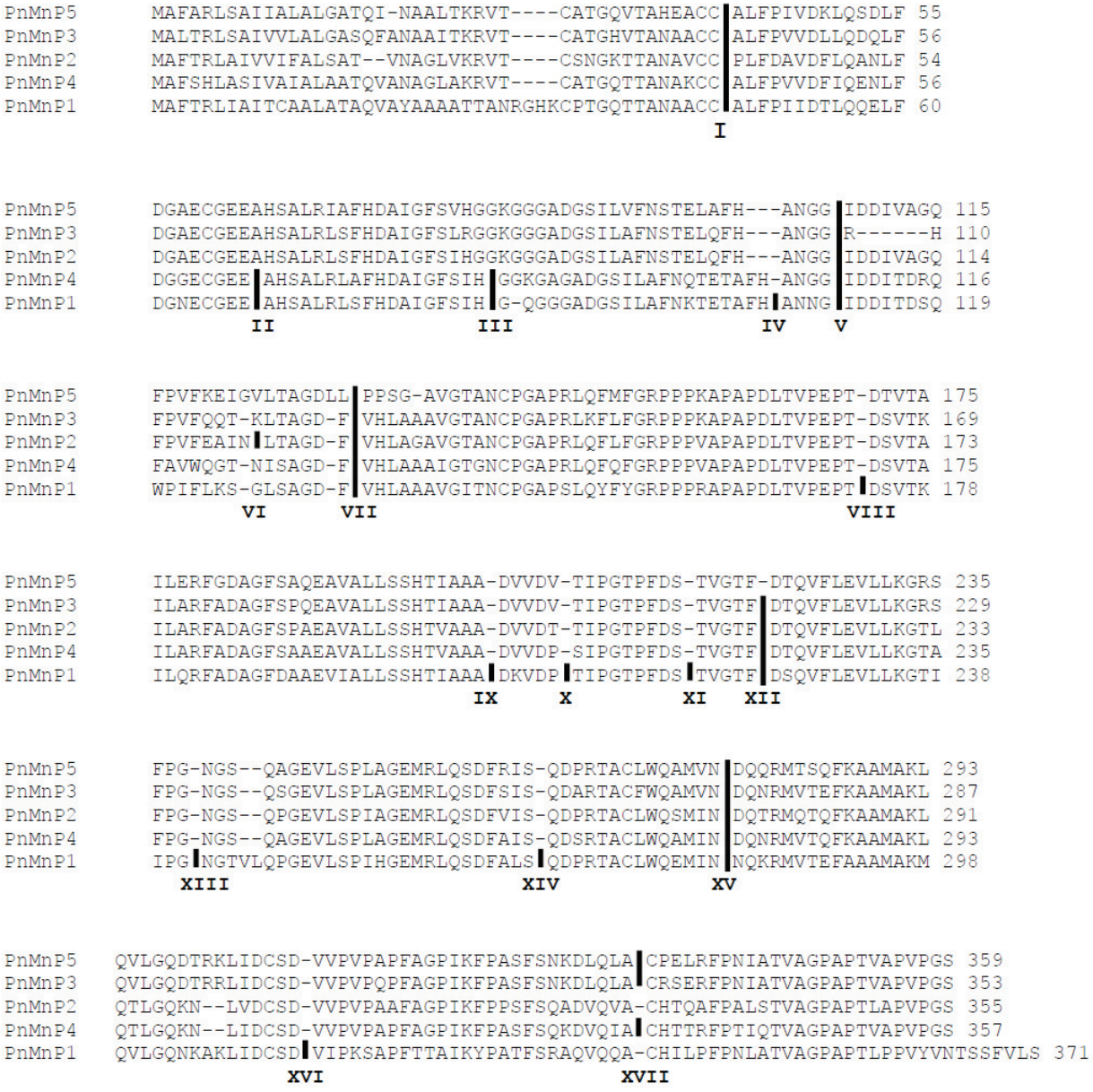


**Fig. 2-2.** Alignment of deduced amino acid sequences of *PnMnP1-5* show intron-exon junctions. Sequences labeled from top to bottom are *PnMnP5, 3, 2, 4* and *1*, according to the phylogenetic tree (Fig. 2-1). Roman characters I to XVII refer to intron positions. Black vertical bars refer to interrupted intron positions and (-) indicates the gap.

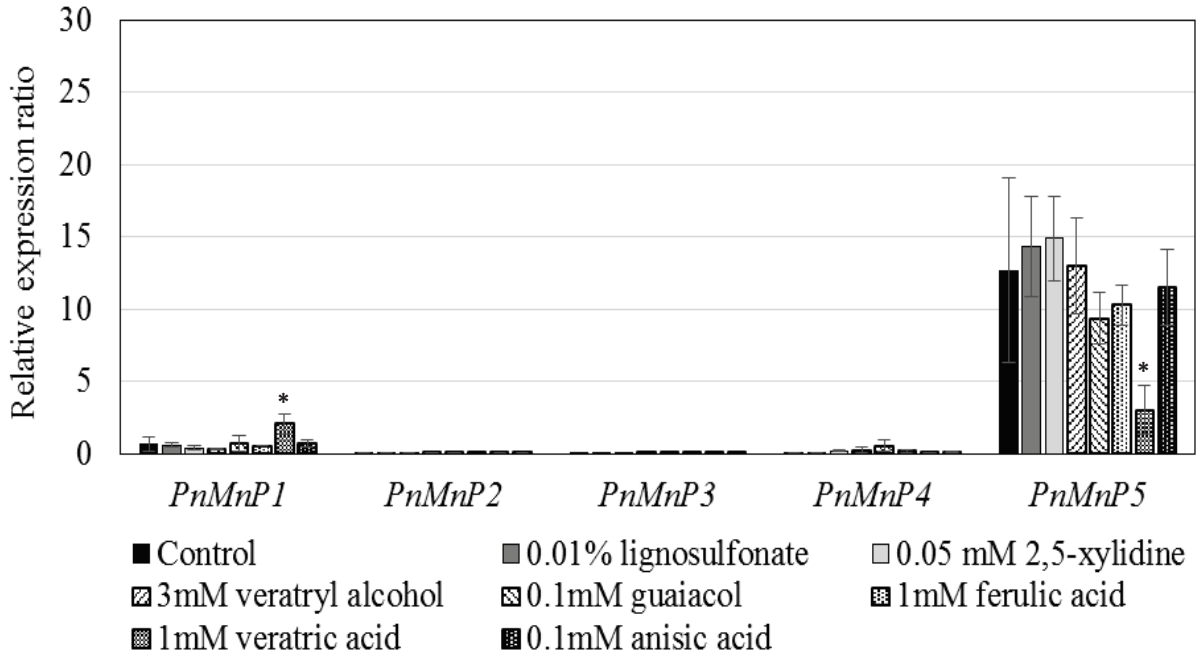


**Fig. 2-3.** Relative expression ratio of *PnMnP1-5* genes in *P. microspora* mycelial culture in amended M4 medium with aromatic compound used for substrate for MnP. Total RNA was observed on day 10 by qRT-PCR in triplicate of samples, which are denoted by standard error bars. Asterisks indicate that the difference in expression level is significant between control basal medium and supplemented with aromatic compound used for substrate for MnP (*t*-test, $p < 0.05$).

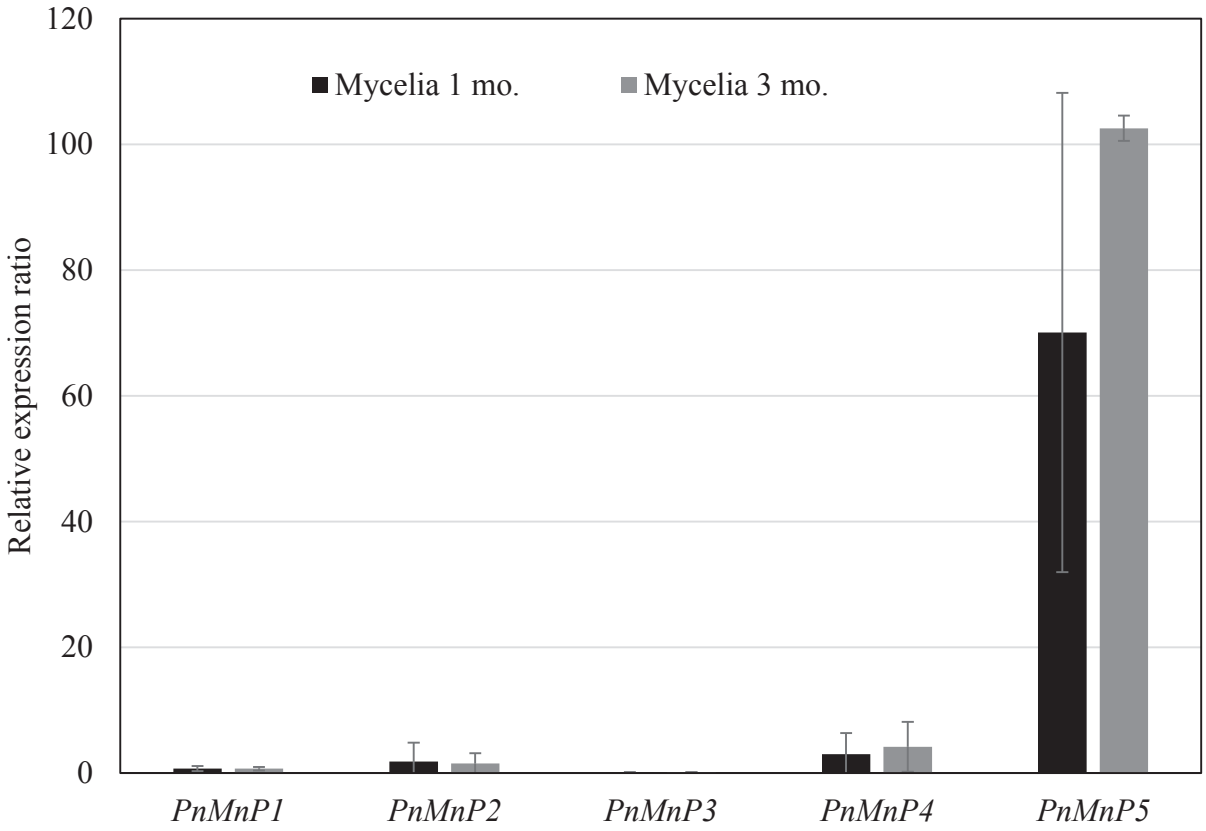


**Fig. 2-4** Relative expression ratio of *PnMnP1-5* genes expression from mycelial growth of *P. microspora* in sawdust medium. Total RNA acquired by mycelial stages at 1 and 3 months. All samples were done in triplicates to denote in standard error bars.

# Chapter 3

## Relationship between fruiting body development and phenol oxidase gene expression in *Pholiota microspora*

### 3-1 Abstract

We analysed nucleotide sequences of phenol oxidase genes in *Pholiota microspora* and identified three types of phenol oxidase: laccase (*Lcc1-Lcc8*), ferroxidase (*Lcc9*), and tyrosinase (*Tyr*). The expression of *Lcc1* to *Lcc9* and *Tyr* genes in *P. microspora* was examined by qRT-PCR. We quantified transcripts of these ten genes in mycelia, primordia, and fruiting bodies grown on sawdust substrate and in mycelia grown in M4 liquid medium supplemented with aromatic compounds. All *Lcc* genes were expressed at a very low level in mycelia grown on sawdust medium, but *Lcc1* was transcribed at a level 8-fold higher in M4 liquid medium when supplemented with 3 mM veratryl alcohol. On the other hand, *Lcc9* and tyrosinase were highly expressed in primordia and fruiting bodies. These results suggest that the content of melanin and related pigments in the fruiting body might be determined by complementary activity of two types of phenol oxidase, such as *Lcc* and *Tyr*, in *P. microspora*.

## 3-1 Introduction

Phenol oxidases (PO) are enzymes containing copper atoms in the catalytic centre and are usually called multicopper oxidases. The catalytic activity of these enzymes is oxidation of diphenols to the corresponding quinones (Baldrian, 2006). PO includes tyrosinases and laccases (Durán et al., 2002). Tyrosinases oxidize *p*-monophenols and *o*-diphenols to the corresponding quinones, whereas laccases are capable of oxidizing various aromatic compounds such as substituted monophenols and polyphenols, aromatic amines, and thiol compounds, with subsequent production of radicals (Selinheimo, 2008). Intracellular and extracellular PO are produced in plants and fungi for a variety of purposes. PO from basidiomycetes fungi are connected with melanin production, lignin degradation, and morphogenic processes such as fruiting body formation (Sinsabaugh, 2010).

In fruiting bodies, fungal PO can catalyse melanin formation, as well as browning of the fruiting body that occurs after harvest, as described in *Lentinula edodes* (Sakamoto et al., 2012). In general, melanin involved in gill browning is considered to be synthesized from β-(3,4-dihydroxyphenyl)alanine (DOPA), derived from tyrosine. Oxidation of tyrosine is commonly catalysed by tyrosinase. The mechanisms of mushroom browning have been investigated extensively in *Agaricus bisporus*, in which tyrosinase is responsible for melanogenesis during spore maturation (Hegnauer et al., 1985); non-reproductive tissues have higher tyrosinase levels than fresh fruiting bodies (Burton, 1988). Pigmentation of mushrooms is

largely mediated by tyrosinase (Jolivet et al., 1998). Moreover, a correlation between melanin synthesis and intracellular laccase in *Cryptococcus neoformans* has been reported (Ikeda et al., 2002). Laccase activity also increases in fruiting bodies of *L. edodes* after harvest. Laccase purified from *L. edodes* fruiting bodies after harvest can oxidize DOPA (Nagai et al., 2003). Therefore, PO are involved in melanin synthesis in fruiting bodies.

On the other hand, white-rot basidiomycetes produce extracellular PO to degrade lignin, and laccases are believed to be important for lignin degradation (Youn et al., 1995; Eggert et al., 1996). Almost all species of white-rot fungi reportedly produce laccase (Hatakka, 2001). In *Pycnoporus cinnabarinus*, which is capable of lignin degradation, laccase was described as the sole ligninolytic enzyme produced by this species (Eggert et al., 1996). Moreover, fruiting body development might be dependent on laccase activity in some fungi. In *Schizophyllum commune*, dikaryotic strains that are able to form fruiting bodies can secrete high levels of laccases, but monokaryotic strains do not produce any (De Vries et al., 1986). In *A. bisporus*, laccase activity is strongly regulated during senescence of fruiting bodies; therefore, fruiting bodies rapidly brown after harvest (Wood, 1980). These phenomena indicate that laccase may play an important role in the morphogenesis of mushrooms. Furthermore, veratryl alcohol can stimulate laccase production during mycelial growth of *Pleurotus ostreatus*, increasing production of fruiting bodies, with fruiting occurring earlier in the medium containing veratryl alcohol than the

medium without (Suguimoto et al., 2001). However, the relationship between the roles of multicopper oxidases and veratryl alcohol in fruiting body development is unclear.

The wood-rotting basidiomycete *Pholiota microspora* (=*Pholiota nameko*, also known as “nameko” in Japan) is sold in local markets as food in Japan and is widely used as material in fungal research into aspects of sexual reproduction (Aimi et al., 2005; Yi et al., 2010) and DNA-mediated transformation (Yi et al., 2009). To understand the role of laccase, we identified nine *Lcc* genes and evaluated their phylogenetic relationships, and then investigated the relationship between pigment production and lignin degradation.

## 3-3 Materials and Methods

### 3-3-1 Fungal strains and culture conditions

The monokaryotic strains *P. microspora* NGW19-6 (*A4*, *pdx1*), a pyridoxine auxotrophic mutant, and NGW12-163 (*A3*, *arg4*), an arginine auxotrophic mutant (Masuda et al., 1995; Yi et al., 2009) were used. A dikaryotic strain was obtained by crossing NGW19-6 and NGW12-163, referred to as NGW19-6/12-163, and used for experiments.

In order to analyse the effects of different aromatic compounds on gene expression, the *P. microspora* NGW19-6/12-163 strain was grown on M4 agar at

25°C for 1 week, and then 10 mycelial agar blocks (3 × 3 mm) were transferred into 20 mL of M4 broth medium (Johansson et al., 2002) (components are presented in terms of g $l^{-1}$: glucose, 2.20 g; diammonium tartrate, 0.92 g; $KH_2PO_4$, 1.00 g; $NaH_2PO_4$•$2H_2O$, 0.26 g; $MgSO_4$•$7H_2O$, 0.50 g; thiamine hydrochloride, $1.00 \times 10^{-4}$ g; $CaCl_2$•$2H_2O$ $6.60 \times 10^{-3}$g; $FeSO_4$•$7H_2O$, $5.00 \times 10^{-3}$ g; $MnSO_4$•$H_2O$, $3.00 \times 10^{-4}$ g; $ZnSO_4$•$7H_2O$, $5.00 \times 10^{-4}$ g; $CuSO_4$, $6.40 \times 10^{-4}$ g; pH, 5.5; for solid M4 medium, 15 g agar was added) in a 100-mL Erlenmeyer flask supplemented with aromatic compounds (final concentration): 0.01% lignosulfonate; 0.05 mM 2,5-xylidine; 3 mM veratryl alcohol; 0.1 mM guaiacol; 1 mM ferulic acid; 1 mM veratric acid; 0.1 mM anisic acid; 1 mM gallic acid and 1 mM L-DOPA. *P. microspora* NGW19-6/12-163 was then grown at 25°C for 20 days and the mycelia were harvested by filtration for RNA extraction.

The cultivation of fruiting bodies of *P. microspora* was carried out on a sawdust substrate, which was prepared as follows. Beech sawdust was mixed with rice bran at a gravimetric ratio of 5:1 and adjusted to 65% moisture content using tap water, and this medium was placed into a 100-mL Erlenmeyer flask, followed by autoclaving at 121°C for 60 min. After cooling the medium in the air, five mycelial agar blocks (5 × 5 mm) containing NGW19-6/12-163 were inoculated and incubated at 25°C. When the mycelia had colonized the substrate (about 40 days after inoculation), the surface layer was scratched with a spatula, and then 50 mL of sterilized distilled water was poured into the flask. Water was removed after flasks

were incubated at 15°C overnight, and then cultivation continued at 15°C until fruiting bodies developed. We defined the day after water removal as day 0. Samples for RNA extraction were taken from triplicate cultures at mycelial stages of the mushroom developmental cycle (30, 90 days), primordia, and fruiting body (1 cm).

### 3-3-2 Genomic DNA and total RNA preparation

Genomic DNA was extracted according to the method of Dellaporta et al. (1983). RNA was extracted using a MagExtractor™ Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. cDNA was synthesized using total RNA as a template with ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (Toyobo, Osaka, Japan). PCR was carried out using a Takara Ex Taq® polymerase (Takara Bio, Japan). The oligonucleotide primers listed in Table 3-1. Amplified fragments were subcloned into pMD20 T-vector (Takara Bio, Japan) and sequenced.

### 3-3-3 *P. microspora* genome and retrieved genes

Whole genomic sequences of monokaryon *P. microspora* NGW 19-6 were determined using Illumina HiSeq 2000 paired-end technology with the software (CASAVA ver.1.8.1) provided by Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan), as described by Funo et al. (2014). This sequencing run yielded 30,935,254 high-quality filtered reads with 101-bp paired-end sequencing. The genome was assembled using Velvet assembler (hash length, 85 bp) (Zerbino and Birney, 2008). The final assembly contained 4,770 contigs with a total size

33,400,256 bp and an $N_{50}$ length of 72,431 bp. The deduced amino acid sequences of known laccase and tyrosinase genes from public databases were searched against the *P. microspora* genome using the BLASTp algorithm. The nine laccase genes and tyrosinase gene identified were named *Lcc1-9* and *Tyr*. The coding sequences of intron-exon junctions were based on GT-AG rules (Breathach et al., 1978; Wu and Krainer, 1999) and homology of amino acid sequences to laccase proteins in the DNA Data Bank of Japan (DDBJ). The nucleotide sequences of genomic DNA fragments of *P. microspora* laccase genes were deposited in the DDBJ under the following accession numbers: *Lcc1* (LC093451); *Lcc2* (LC093452); *Lcc3* (LC093453); *Lcc4* (LC093454); *Lcc5* (LC093455); *Lcc6* (LC093456); *Lcc7* (LC093457); *Lcc8* (LC093458); and *Lcc9* (LC093459). Subcellular localization was predicted using the PSORTII (Nakai and Kanehisa, 1992) (http://psort.hgc.jp/form2.html), and SOSUI (Hirokawa et al., 1998) (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) online tools.

### 3-3-4 Quantitative RT-PCR (qRT-PCR) assays

The actin gene (*Act1*) was used as a reference gene. Primer pairs for amplification of *Lcc1-9, Tyr*, and *Act1* cDNAs were designed based on their cDNA sequences using Genetyx software. Amplification of genomic DNA was prevented by designing primers for exon-exon junctions. All primers were tested to ensure that they amplified a single band with no primer-dimers, as shown in Table 3-2. Plasmids with inserted of the target gene (*Lcc1-9, Tyr*) and the housekeeping gene (*Act1*) were

extracted as described by Birnboim (1983). Standard curves were constructed using four ten-fold dilutions of plasmid. Real-time PCR was performed using a KOD SYBR® qPCR Mix kit (Toyobo). Thermocycling was carried out using a PikoReal™ 96 system (Thermo Fisher Scientific) with an initial incubation for 1 min at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 1 min. Each run was completed with a melting curve analysis to confirm the specificity of amplification and absence of primer-dimers. Data analysis was performed in accordance with the manufacturer's instructions.

### 3-3-5 Analysis of sequences and phylogenetic tree

Nucleotide and protein sequence data were analysed using Genetyx ver. 10.0.3 software (Genetyx, Tokyo, Japan). Protein sequence similarity was analysed using the BLASTp algorithm (Altschul et al., 1997). Laccase genes were retrieved from public domains (NCBI and UniProt). A phylogenetic tree was constructed by MEGA 6.06 software (Tamura et al., 2013) using the neighbour joining method with a bootstrap value of 1,000 replicates. Multiple alignment was performed using ClustalW (Larkin et al., 2007).

## 3-4 Results

### 3-4-1 Role of *Lcc1* and *Lcc9*

Transcription of the nine laccase genes (*Lcc1-9*) in mycelia and fruiting bodies grown on sawdust medium was investigated. Mycelia were harvested from sawdust medium 1 month after inoculation and 2 months after *kinkaki*. Primordia and fruiting bodies were also harvested and total RNA was extracted from the tissue and the mycelium. Fig. 3-1(A) shows expression levels of the *Lcc*s *(1-9)*. Significant expression was detected in only *Lcc1* and *Lcc9*. On the other hand, no or only slight expression of the other *Lcc*s *(2-8)* was detected. *Lcc9* showed the highest expression of all *Lcc*s in the fruiting body. *Lcc9* expression in primordia was also relatively high. On sawdust medium, the expression of *Lcc9* in primordia was 10-fold higher and in fruiting bodies 15-fold higher than in mycelia.

In order to investigate the roles of *Lcc1* and *Lcc9*, expression of *Lcc1* and *Lcc9* in mycelia grown in M4 liquid medium supplemented with aromatic compounds was studied by quantitative RT-PCR [Fig. 3-1(B)]. Maximum expression of *Lcc1* was observed in mycelium grown in M4 liquid medium supplemented with 3 mM veratryl alcohol, and expression was 8-fold higher than that of *Lcc1* in mycelia grown in M4 liquid basal medium. Other tested aromatic compounds did not affect *Lcc1* expression. In contrast, *Lcc9*, which was transcribed at the highest level in fruiting bodies, did not show any significant response in the presence of aromatic compounds.

Therefore, the role of *Lcc1* may be lignin degradation, and *Lcc9* may be related to morphogenesis involving colouration of the fruiting body.

### 3-4-2 Expression of *Tyr* also involved in fruiting body

Laccase and tyrosinase are PO and are closely related to production of melanin pigment in the fruiting body. Thus, in order to confirm the relationship between PO expression and fruiting body development, transcription of *Tyr* in mycelia, primordia, and fruiting bodies that were cultured on sawdust medium were investigated. Expression of *Tyr* in primordia was 250-fold higher and in fruiting bodies 300-fold higher than that of *Tyr* in mycelia (Fig. 3-2). These results indicated that tyrosinase is required for processes during fruiting body development in *P. microspora* such as pigment production.

### 3-4-3. Phylogenetic relationship among Lccs and origin of the *Lcc* genes

From a phylogenetic tree based on the deduced amino acid sequences of *P. microspora* Lccs and laccases from other basidiomycetous mushrooms extracted from the DNA and protein databases, Lcc1-7, Lcc8, and Lcc9 fit into three major clusters (Fig. 3-3). The first cluster, containing Lcc1-7, fell into the same clade. Based on their phylogenetic relationships and the structure of the genes including intron position, *Lcc2-7* may have been amplified from *Lcc1*. Therefore, the origin of

the *Lcc1* group was *Lcc1*. These laccases are clustered tightly with other fungi in the same family, *Hypholoma sublateritium* and *Stropharia aeruginosa*, whereas laccase gene families of other basidiomycetes are tightly clustered within the same genus. The second cluster, containing Lcc8, included laccase protein sequences from the anamorphic fungi *Thanatephorus cucumeris* (*Rhizoctonia solani*) and *Flammulina velutipes*. Transcription of *Lcc8* was not detected in mycelia, primordia, or fruiting bodies grown on sawdust substrate or in mycelia grown in M4 liquid medium supplemented with aromatic compounds. These results suggested that *Lcc8* has a different function than lignin degradation or morphogenesis or has lost its function, although its origin differed from *Lcc1* and *Lcc9*. The third cluster, Lcc9, was grouped with ferroxidase. The role of ferroxidase in iron uptake has been analysed extensively in *Saccharomyces cerevisiae* (De Silva et al., 1995), but remains unclear in basidiomycetes, although it has strong activity to iron (Larrondo et al., 2003). Thus, three types of laccases might have originally been present in the *P. microspora* genome. The PSORTII and SOSUI program estimated that the Lcc1, 3, 4, 5 and 8 were extracellular proteins, Lcc2 and 7 were cytoplasmic proteins, and Lcc6 and 9 were transmembrane proteins. Moreover, Tyrosinase was estimated as cytoplasmic protein. Therefore, development of the colour might be occur in cytoplasm or periplasmic spaces of the cell.

### 3-4-4 Intron structure and origin of *Lcc*s

To analyse whether the origin of *Lcc1* differed from that of *Lcc8* and *Lcc9* in the ancient *P. microspora* genome, the position and number of introns were analysed. Nucleotide sequence of *Lccs* ranged from 2118 bp to 2643 bp. ORFs of *Lcc8* and *9* were the largest. The nucleotide sequence of *Lcc*s belonging to the *Lcc1* group (*Lcc1-7*) ranged from 1551 to 1575 bp. Fig. 3-4 shows the structure of the *Lcc* genes. There were 18 introns in *Lcc8*; 13 in *Lcc1, 2*, *5,* and *7*; 12 in *Lcc3 and Lcc6*; 10 in *Lcc4*; and 9 in *Lcc9*. Introns I to XIII were inserted in the same positions for *Lcc1-7*, which were clustered in the same group in the phylogenetic tree, with the exception of introns VII, VIII, and XII, which were absent in *Lcc4* and intron XIII, which was absent in *Lcc3*. Based on these results, the position of the introns corresponded with phylogenetic and evolutionary relationships; therefore, the ancient *P. microspora* genome originally had three genes, a *Lcc* gene belonging to the *Lcc1* group*, Lcc8*, and *Lcc9*.

All *P. microspora* laccase coding sequences were used as search queries against the DDBJ using the Blastx algorithm. The percentage of protein sequence identity of *Lcc1* to *Lcc9* was 83% to multicopper oxidase in *H. sublateritium* (KJA22755.1), 80% and 81% to two laccases in *S. aeruginosa* (AFE48786.2 and AFE48786.2), 61% to multicopper oxidase in *H. sublateritium* (KJA22010.1), 74% to a laccase-like protein in *Galerina marginata* (KDR80952.1), 79% to multicopper oxidase in *H. sublateritium* (KJA22017.), 71% to multiple oxidase in *H.*

*sublateritium* (KJA22017.1), 66% to multicopper oxidase in *Sphaerobolus stellatus* (KIJ32231.1), and 83% to multicopper oxidase in *H. sublateritium* (KJA26904.1). Consensus motifs (L1, L2, L3, and L4) among laccases (Larrondo et al., 2003; Kumar et al., 2003) were conserved in all *P. microspora Lcc*s. Therefore, *Lcc1-8* were identified as laccases and *Lcc9* as a homologue of ferroxidase.

## 3-5 Discussion

In this study, high *Lcc9* and *Tyr* expression in primordia and fruiting bodies were shown, so the colour of the fruiting body in *P. microspora* may be determined by the combined activity of these two enzymes because they are closely related to oxidation of phenolic compounds and melanin production. *Lcc9* is a ferroxidase that can reduce $Fe^{3+}$ to $Fe^{2+}$, and the substrate specificity of ferroxidase for $Fe^{3+}$ was higher than for phenolic compounds in *Phanerochaete chrysosporium* (Larrondo et al., 2003). In order to minimize production of active oxygen in redox cycling in fungi, the coupling of $Fe^{3+}$ reduction with a reductant is required (De Luca and Wood, 2000; Kosman, 2003). Therefore, this highly suggests that *Lcc9* and *Tyr* are related to not only colouring, but also active oxygen scavenging. Furthermore, in *L. edodes,* laccases are believed to have a role in morphogenesis of fruiting bodies (Zhao and Kwan, 1999). Likewise, in *P. ostreatus*, the presence of phenolic compounds such as veratryl alcohol in the culture medium promotes fruiting body formation and shortens the culture period[16]. These observations support our results that metabolism

of phenolic compound by PO was connected with fruiting body formation. Moreover, small lignin degradation products might be one of the initiation signals for fruiting body development.

Our results showed that expression of all *Lcc*s was poor in mycelia grown on sawdust substrate. Therefore, laccases do not directly degrade lignin during growth on sawdust substrate. However, *P. microspora* contains manganese peroxidase, which is highly expressed in mycelia on sawdust medium, for degrading lignin (Sutthikhampa et al., 2015). *Lcc1* was induced by aromatic compounds and *Lcc2-7* were expressed at a basal level during mycelial growth on sawdust substrate. Based on the evolutionary distance of the first cluster, position of the introns, and expression profile, *Lcc1-7* might have been generated by repeated duplication from a single *Lcc*, and *Lcc1* might be the origin of *Lcc2-7*. Moreover, enzyme activities in *P. microspora* are affected by their level of expression rather than the number of genes, because only two of the nine genes were actively transcribed.

In this study, we investigated phenol oxidase expression in *P. microspora*, finding that *Lcc9* and *Tyr* are closely related to fruiting body formation, though their role remains unresolved. In subsequent experiments, therefore, we will further study their role by knockout of *Lcc9* to determine the relationship between this gene and fruiting body formation, including pigment production, in *P. microspora*.

**Table 3-1.** Primer sets for cDNA and plasmid construction.

| **Genes** | **Primers** | **5'→ 3'** |
|---|---|---|
| *Act1* | PnActin1_F1 | CGAAATTTCAGCTCTCGTCGT |
| | PnActin1_R1 | CTGGAGCACGGAATCGCT |
| *Lcc1* | PnLcc1-F1 | GACGAGCACCAGCATTCATT |
| | PnLcc1-R1 | CTGATTCGTGTTGAGCACGAA |
| *Lcc2* | PnLcc2_F3 | CTGGCATGGCCTGTTCCAA |
| | PnLcc2-R1 | GACGACGGCAAGACCAACAT |
| *Lcc3* | PnLcc3-F1 | GTTGACGAGCACCAGTATTCATT |
| | PnLcc3-R2 | CAGGGTCTTCGATAGTCGCATT |
| *Lcc4* | PnLcc4-F1 | GCTTACTGGCGTCAAGGGA |
| | PnLcc4-R1 | CGTATGACGGGATTGTGGTAATT |
| *Lcc5* | PnLcc5-F1 | CGGGCCTATTGCCAATCTTT |
| | PnLcc5-R1 | GACGGAGAATGCGTGAGGA |
| *Lcc6* | PnLcc6-F1 | CCTGAGCGTCTTCGGTTT |
| | PnLcc6-R1 | CGACTTCGATGATCGTCATGA |
| *Lcc7* | PnLcc7-F1 | GTTGGTCAGCACGAGTATACATT |
| | PnLcc7-R1 | GCCAGTACTATGGCTAGACCAA |
| *Lcc8* | PnLcc8-F1 | GACTAATTCTGAGGACGGACCT |
| | PnLcc8-R1 | GGCAATAATGTTGAGCAGGGTA |
| *Lcc9* | PnLcc9-F1 | CATCGAAGTCGATGGTACCGA |
| | PnLcc9-R1 | GGTAAGCACGCATCCGAA |
| *Tyr* | PnTyr_F1 | CCTACGTTCTTCTCTACGAGCA |
| | PnTyr_R1 | GAGAACGGGGTCAGAGGAGT |

**Table 3-2.** Primer sets for qPCR.

| Genes | Primers | 5'→ 3' |
|---|---|---|
| *Act1* | PnActin1_CF1 | GCTATGCTATGTCGCGCTTGAT |
| | PnActin1_R1 | CTGGAGCACGGAATCGCT |
| *Lcc1* | PnLcc1-F1 | GACGAGCACCAGCATTCATT |
| | PnLcc1-CR1 | CCGTCACAGTATTGCGTCGAAT |
| *Lcc2* | PnLcc2_CF1 | CTATTCACTTGCACGGCCACA |
| | PnLcc2-R1 | GACGACGGCAAGACCAACAT |
| *Lcc3* | PnLcc3-F1 | GTTGACGAGCACCAGTATTCATT |
| | PnLcc3_CR1 | GAGACCGTCGCAATACTGTGTAGA |
| *Lcc4* | PnLcc4-F1 | GCTTACTGGCGTCAAGGGA |
| | PnLcc4-CR1 | CCGTCACAATACTGAGTGGAAT |
| *Lcc5* | PnLcc5-F1 | CGGGCCTATTGCCAATCTTT |
| | PnLcc5-CR1 | GCCAATGAATGCTCGTGCT |
| *Lcc6* | PnLcc6-F1 | CCTGAGCGTCTTCGGTTT |
| | PnLcc6-CR1 | CATTGAGATTGAAGGTATCGCCCT |
| *Lcc7* | PnLcc7-CF1 | GATCACCTGGTTTCCCGCA |
| | PnLcc7-R1 | GCCAGTACTATGGCTAGACCAA |
| *Lcc8* | PnLcc8-F1 | GACTAATTCTGAGGACGGACCT |
| | PnLcc8-CR1 | GGGTCAACGGGGTCATAAACA |
| *Lcc9* | PnLcc9-CF1 | GTCTGGTTCCTTCATTGCCA |
| | PnLcc9-R1 | GGTAAGCACGCATCCGAA |
| *Tyr* | PnTyr_CF1 | GATCCTGCTGTCGCTGCTT |
| | PnTyr_R1 | GAGAACGGGGTCAGAGGAGT |

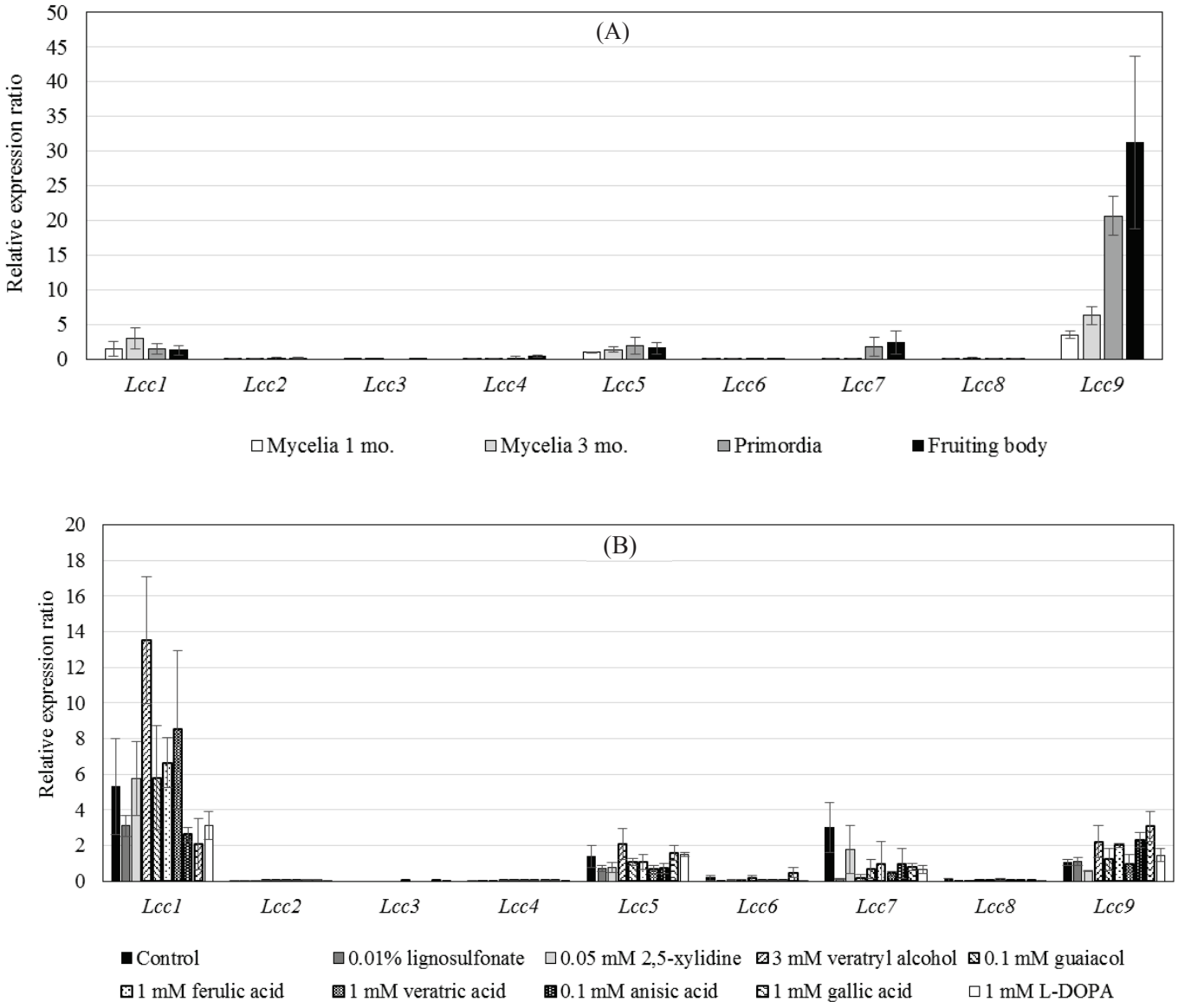


**Fig. 3-1** Relative expression ratio of *Lcc1-9* genes during development of *P. microspora* in sawdust medium. Total RNA was extracted from mycelia cultivated for 1 and 3 months (mo.), primordia, and fruiting bodies (**A**). Mycelia cultured in M4 medium with aromatic compounds (**B**) were observed by qRT-PCR. All samples were assessed in triplicate, with variation denoted by standard error bars.

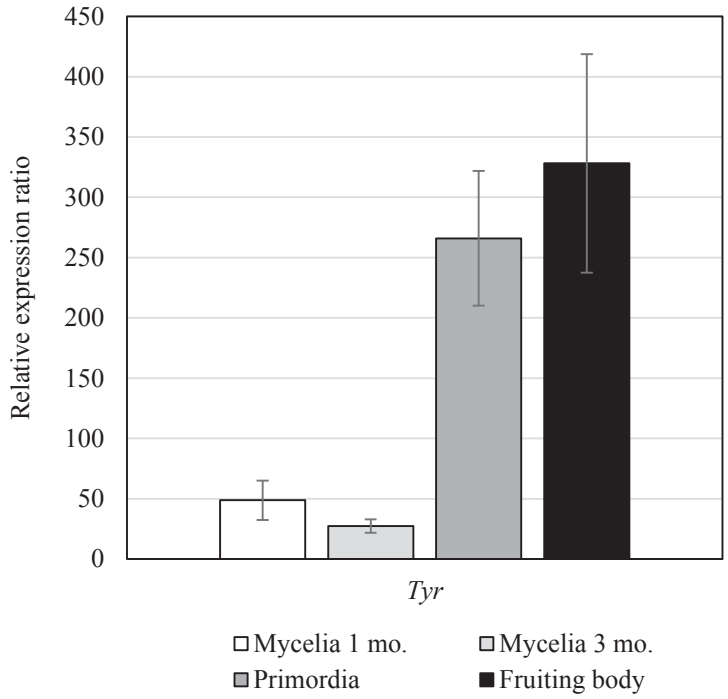


**Fig. 3-2** Relative expression ratio of *Tyr* gene during development of *P. microspora* in sawdust medium. Total RNA was extracted from mycelia cultivated for 1 and 3 months (mo.), primordia and fruiting bodies. All samples were assessed in triplicate, with variation denoted by standard error bars.

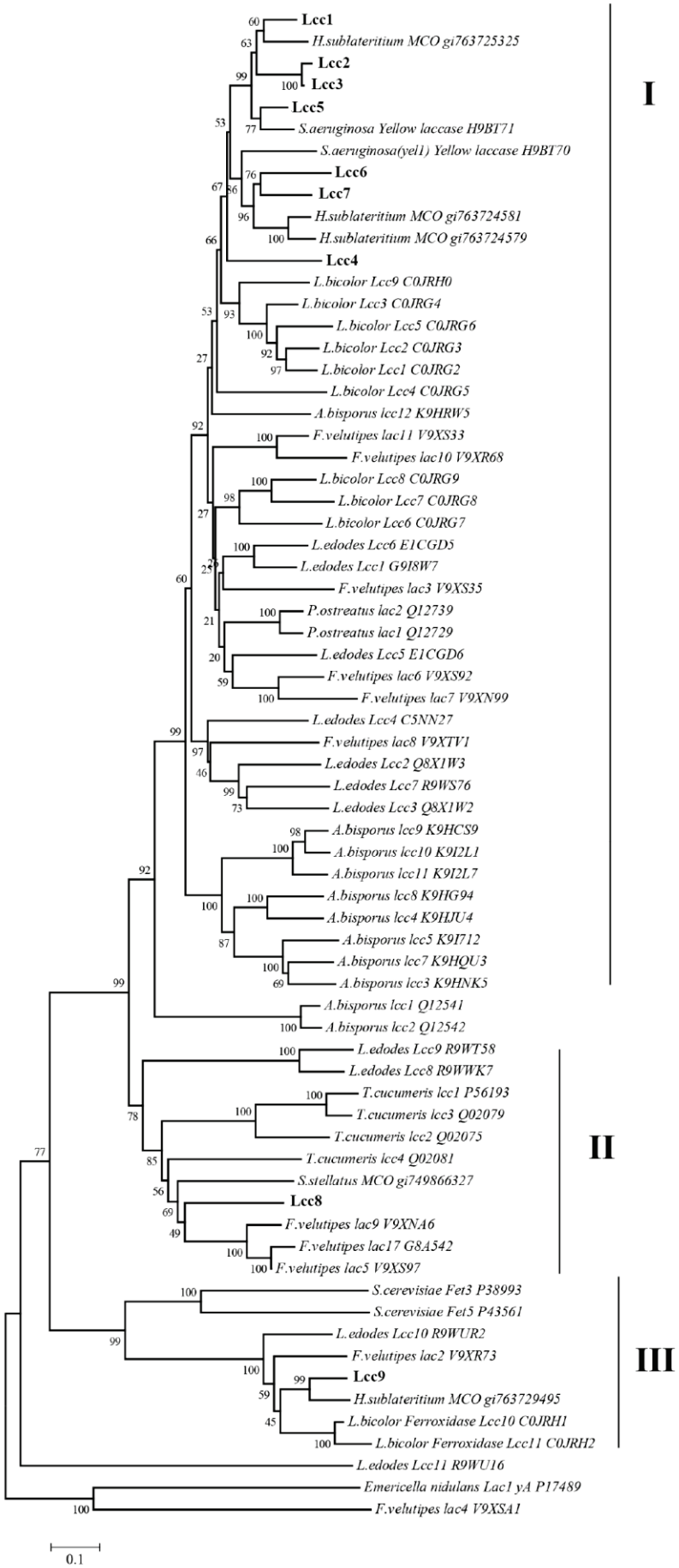
Lcc1
H.sublateritium MCO gi763725325
Lcc2
Lcc3
Lcc5
S.aeruginosa Yellow laccase H9BT71
S.aeruginosa(yel1) Yellow laccase H9BT70
Lcc6
Lcc7
H.sublateritium MCO gi763724581
H.sublateritium MCO gi763724579
Lcc4
L.bicolor Lcc9 C0JRH0
L.bicolor Lcc3 C0JRG4
L.bicolor Lcc5 C0JRG6
L.bicolor Lcc2 C0JRG3
L.bicolor Lcc1 C0JRG2
L.bicolor Lcc4 C0JRG5
A.bisporus lcc12 K9HRW5
F.velutipes lac11 V9XS33
F.velutipes lac10 V9XR68
L.bicolor Lcc8 C0JRG9
L.bicolor Lcc7 C0JRG8
L.bicolor Lcc6 C0JRG7
L.edodes Lcc6 E1CGD5
L.edodes Lcc1 G9I8W7
F.velutipes lac3 V9XS35
P.ostreatus lac2 Q12739
P.ostreatus lac1 Q12729
L.edodes Lcc5 E1CGD6
F.velutipes lac6 V9XS92
F.velutipes lac7 V9XN99
L.edodes Lcc4 C5NN27
F.velutipes lac8 V9XTV1
L.edodes Lcc2 Q8X1W3
L.edodes Lcc7 R9WS76
L.edodes Lcc3 Q8X1W2
A.bisporus lcc9 K9HCS9
A.bisporus lcc10 K9I2L1
A.bisporus lcc11 K9I2L7
A.bisporus lcc8 K9HG94
A.bisporus lcc4 K9HJU4
A.bisporus lcc5 K9I712
A.bisporus lcc7 K9HQU3
A.bisporus lcc3 K9HNK5
A.bisporus lcc1 Q12541
A.bisporus lcc2 Q12542
L.edodes Lcc9 R9WT58
L.edodes Lcc8 R9WWK7
T.cucumeris lcc1 P56193
T.cucumeris lcc3 Q02079
T.cucumeris lcc2 Q02075
T.cucumeris lcc4 Q02081
S.stellatus MCO gi749866327
Lcc8
F.velutipes lac9 V9XNA6
F.velutipes lac17 G8A542
F.velutipes lac5 V9XS97
S.cerevisiae Fet3 P38993
S.cerevisiae Fet5 P43561
L.edodes Lcc10 R9WUR2
F.velutipes lac2 V9XR73
Lcc9
H.sublateritium MCO gi763729495
L.bicolor Ferroxidase Lcc10 C0JRH1
L.bicolor Ferroxidase Lcc11 C0JRH2
L.edodes Lcc11 R9WU16
Emericella nidulans Lac1 yA P17489
F.velutipes lac4 V9XSA1
I
II
III
0.1

**Fig. 3-3** Phylogenetic tree of laccases. The tree was constructed by the neighbour joining method with 1000 bootstrap replications of Lcc1-9 and laccases from public databases according to gene family: *Emericella nidulans, Flammulina velutipes, Hypholoma sublateritium, Laccaria bicolor, Lentinula edodes, Pleurotus ostreatus, Saccharomyces cerevisiae, Sphaerobolus stellatus, Stropharia aeruginosa*, and *Thanatephorus cucumeris*. Taxa contain organism and gene name, then protein ID.

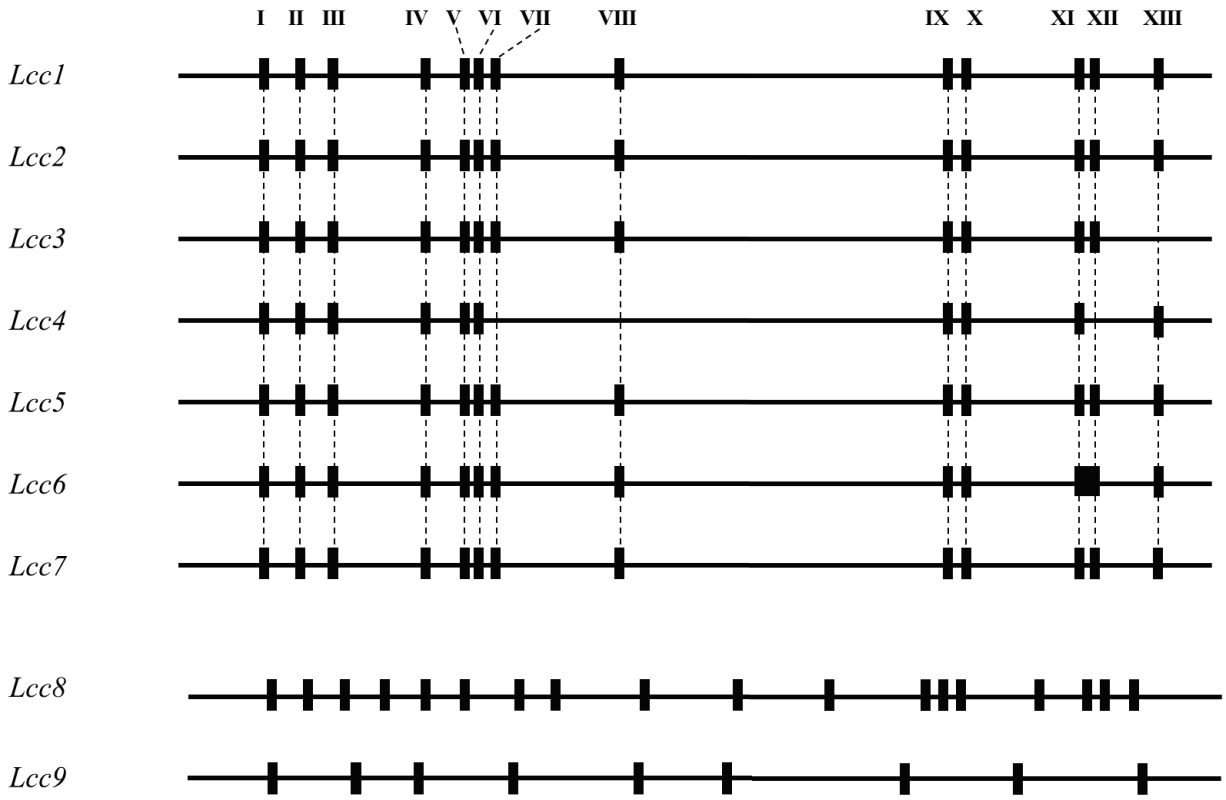


**Fig. 3-4** Intron positions within laccase genes *Lcc1* to *Lcc9* of *P. microspora* define three gene types. Horizontal lines indicate the laccase genes, and vertical bars indicate intron positions. Dotted lines indicate introns that interrupt the coding sequence of the different genes at exactly the same codon position for *Lcc1-7*. The Roman characters I to XIII indicate intron positions. *Lcc8* and *Lcc9* do not have introns at the same positions.

# Chapter 4

## Conclusions and discussions

From the evolutionary perspective, genetic redundancy of *MnP* and *laccase* gene families might be occurred by gene duplication since ancient time. From the results suggested that among five *MnP*, only one *MnP* predominant transcript in mycelia grown on sawdust substrate and relatively constitutive transcription even grown in liquid medium with or without aromatic compounds. Moreover, due to nucleotide and protein sequences analysis, we believe that all *P. microspora MnPs* are of the same origin and that they were amplified by duplication events in the ancient *P. microspora* genome. Furthermore, analysis of nine laccases in *P. microspora*, the result obtained that *Lcc1-9* were low level of expression in mycelia grown on sawdust medium, also in liquid medium containing aromatic compounds. It suggested that *P. microspora* laccases were not involved in lignin degradation. But *Lcc9* and tyrosinase was highly expressed in primordia and fruiting bodies. The results suggest that the content of melanin and related pigment in the fruiting body might be decided by harmony of the two kinds of phenol oxidase activity such as *Lcc* and *Tyr* in *P. microspora*.

In the Chapter 2, *P. microspora MnP5* transcription in *P. microspora* is much higher than the other *MnP* genes presents in the genome. This result may occurred by the concentration of nitrogen that presence in M4 liquid medium. Since, the

nitrogen levels can influence MnP production, which has been well documented (Johansson et al., 2002; Hamman et al., 1997). In contrast, the relatively low transcription of the four *P. microspora MnPs* in this investigation also raised the issue of why transcription in the presence of lignin was unequal. In this regard, evolutionary relationship of nucleotide and protein sequences analysis were then consideration. In addition, the transcription of *P. chrysosporium* genes was suggested the role of putative metal response elements (MREs) in transcriptional regulation but absence in *T. versicolor* (Johansson et al., 2002). With regard to genetic variability of gene families, *P. microspora* presents only *MnPs* but *LiP* gene is absent from its genome. The lignin degradation capacity of *P. microspora* is relativity lower when compared with white rot basidiomyceteous fungi such as *P. chrysosporium*, *P. radiata* and *T. versicolor* (Hatakka, 1994). However, in conclusion, *P. microspora* has only one type of enzyme and the differences in lignin degradation activity among white rot fungal species is the result of differential expression and transcription *MnPs*, not the number of genes in their genome.

In the Chapter 3, from the results of nucleotide and protein sequences analysis was showed that *P. microspora* exists three types of laccases. However, all *P. microspora* laccases was poor expression in mycelia grown on sawdust substrate. Therefore, laccases do not directly degrade lignin during growth on sawdust substrate. Also, the relatively low expression level in mycelia grown in liquid medium containing aromatic compound, except *Lcc1* which suggests to be sole

origin of *Lcc2-7*. Nonetheless, *P. microspora* contains MnP, which is highly expressed in mycelia on sawdust medium, for degrading lignin (Sutthikhampa et al., 2015). Furthermore, the high expression level of *Lcc9* and *Tyr* in primordia and fruiting bodies were revealed, thus the color of the fruiting body in *P. microspora* may be determined by the combined activity of these two enzymes because they are closely related to oxidation of phenolic compounds and melanin production.

Final conclusion, we investigated the possible physiological roles of manganese peroxidase and phenol oxidase expression in *P. microspora* at the transcriptional level. Manganese peroxidase is required for lignin degradation in mycelia during growth on sawdust medium, but phenol oxidase including laccase and tyrosinase are required for related pigment synthesis in the fruiting body of *P. microspora*.

## Abstract

### Evolution of multigene families for lignin degradation in *Pholiota microspora*

The wood-rotting basidiomycete *Pholiota microspora* (or "nameko" in Japanese). Nameko mushroom is one of the most popular edible mushroom and is widely cultivated in Japan. Lignin degradation is based on the white rot fungi capability to produce extracellular lignin modifying enzyme. In this study, we identified ligninolytic gene families in *P. microspora*. There were five manganese peroxidase (*MnP*) and nine laccase (*Lcc*) genes, but not lignin peroxidase that present in *P. microspora*.

Firstly, to analyse lignin degrading genes. Five *MnP* genes were identified. Nucleotide and amino acid sequences were analyzed intron-exon position and phylogenetic relationship, respectively. *PnMnP5*, *3, 2* and *4* were clustered tightly, but *PnMnP1* was clustered relatively far from *MnP5*. Moreover, qRT-PCR unveiled that *PnMnP5* gene only that was strongly transcribed, 15-fold higher expression than other *MnPs* in M4 liquid medium. While transcription of *PnMnP5* in sawdust medium was 100 times higher than in M4 liquid medium. Therefore, the results indicate that *PnMnP5* plays a major role in the ligninolytic peroxidase reaction during mycelial growth in *P. microspora*. Based on a comparison of the position of introns, the phylogenetic relationships among *PnMnPs* and the predominant

expression of *PnMnP5*, we believe that all *PnMnPs* are of the same origin and that they were amplified by duplication events in the ancient *P. microspora* genome.

Secondly, to estimate the physiological role of phenol oxidase. We analyzed nucleotide sequences of phenol oxidase genes; nine laccases and a tyrosinase. The expression of *Lcc1* to *Lcc9* and *Tyr* genes in *P. microspora* was examined by qRT-PCR. We quantified transcripts of these ten genes in mycelia, primordia, and fruiting bodies grown on sawdust substrate and in mycelia grown in M4 liquid medium supplemented with aromatic compounds. All *Lcc* genes were expressed at a very low level in mycelia grown on sawdust medium, but *Lcc1* was transcribed at a level 8-fold higher in M4 liquid medium when supplemented with 3 mM veratryl alcohol. On the other hand, *Lcc9* and tyrosinase were highly expressed in primordia and fruiting bodies. These results suggest that the content of melanin and related pigments in the fruiting body might be determined by complementary activity of two types of phenol oxidase, such as *Lcc* and *Tyr*, in *P. microspora*.

Final conclusion, we investigated the possible physiological role of manganese peroxidase and phenol oxidase expression in *P. microspora* at the transcriptional level. Manganese peroxidase is required for lignin degradation in mycelia during growth on sawdust medium, but phenol oxidase including laccase and tyrosinse are required for related pigment synthesis in the fruiting body of *P. microspora*.

# 和文摘要

## *Pholiota microspora* でのリグニン分解のための多重遺伝子ファミリーの進化

木材腐朽担子菌、*Pholiota microspora*（なめこ）は、最も人気がある食用キノコの一つであり、日本において広く栽培されている。木材の難分解性リグニン分解は、細胞外リグニン分解酵素を生産する白色腐朽菌能力に基づいている。本研究では、*P. microspora* におけるリグニン遺伝子ファミリーを同定した。*P. microspora* には、5 個のマンガンペルオキシダーゼ（*MnP*）と 9 個のラッカーゼ（*Lcc*）の遺伝子があったが、リグニンペルオキシダーゼは存在しなかった。

まず初めに、リグニン分解遺伝子を分析し、5 個の *MnPs* の遺伝子を同定した。ヌクレオチドおよびアミノ酸配列についてそれぞれ、イントロン - エクソン位置と系統発生関係を解析した。*PnMnP5*、*3*、*2* と *4* が緊密にクラスターを形成していたが、*PnMnP1* は *PnMnP5* から比較的遠い系統関係にあった。また、qRT-PCR の結果からは、*PnMnP5* 遺伝子のみが強く転写されており、M4 液体媒体中の他の *MnP* よりも、15 倍高く発現した。おがくず培地の *PnMnP5* は M4 液体培地のものよりも 100 倍の転写量があった。これらより、*PnMnP5* は、*P. microspora* の菌糸成長において、リグニンペルオキシダーゼ反応で重要な役割を果たしていることが示された。

イントロンの位置の比較による *P. microspora* の *MnPs* と *PnMnP5* の優勢発現の系統関係により、すべての *P. microspora* の *MnPs* は同じ起源のものであり、それらは古代 *P. microspora* の遺伝子中で複製することで増幅されていると考えられた。

次に、フェノールオキシダーゼの生理学的役割を評価するために、フェノールオキシダーゼ遺伝子である 9 個のラッカーゼおよびチロシナーゼの塩基配列を分析した。*P. microspora* における *Lcc1*～*Lcc9* と *Tyr* 遺伝子の発現を qRT-PCR によって解析した。おがくず培地上に成長した菌糸体、原基、子実体、及び芳香族化合物を添加した M4 液体培地中で増殖させた菌糸体において、これらの 10 個の遺伝子の転写産物を定量した。すべての *Lcc* 遺伝子は、おがくず培地上で増殖させた菌糸体においては非常に低い水準で発現したが、*Lcc1* はベラトリルアルコール 3 mM を添加した M4 液体培地中で M4 液体培地の発現水準よりも 8 倍高かった。一方、*Lcc9* とチロシナーゼは原基および子実体で非常に多く発現した。これらの結果より、子実体中のメラニンと関連色素の含有量は、*P. microspora* における *Lcc* および *Tyr* などのフェノールオキシダーゼの 2 種類の相補的活性によって決定される可能性が示唆された。

以上の結果から、転写レベルでの *P. microspora* のマンガンペルオキシダーゼとフェノールオキシダーゼの発現における生理的役割は、マンガ

ンペルオキシダーゼは、おがくず培地上で成長する菌糸にとってリグニン分解のために必要であること、ラッカーゼとチロシナーゼ含むフェノールオキシダーゼは、*P. microspora* の子実体における関連色素の合成に必要であること、と結論できた。

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## List of publications

Surasit Sutthikhampa, Yoshiko Kawai, Mirai Hayashi, Sophon Boonlue, Norihiro Shimomura, Takeshi Yamaguchi and Tadanori Aimi: Only one major manganese peroxidase (MnP) is predominantly expressed for mycelial growth of *Pholiota microspora* on sawdust medium. Mushroom Science and Biotechnology, **23**,159-165 (2016) (The corresponding content is in chapter 2)

Surasit Sutthikhampa, Yoshiko Kawai, Mirai Hayashi, Sophon Boonlue, Norihiro Shimomura, Takeshi Yamaguchi and Tadanori Aimi: Relationship between fruiting body development and phenol oxidase gene expression in *Pholiota microspora*. Mushroom Science and Biotechnology, **23**, 151-158 (2016) (The corresponding content is in chapter 3)