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Detecting Manganese Peroxidase (MnP) gene in *Ganoderma* species

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Abstract. – Lignin degradation is achieved by a specific group of enzymes known as Lignin-Modifying Enzymes (LME) where Manganese Peroxidase (MnP) plays a key role. Classified as extracellular enzymes and produced by white-rot fungi (Basidiomycetes, Agaricomycotina, Polyporales), the MnP2 gene was detected in twelve isolates from *Ganoderma australe*, *G. gibbosum*, *G. multiplicatum, G. parvulum* and *G. subamboinense* collected as parasites in legume species, as well as saprophytes in logs from Brazil and Colombia. The presence of this enzyme was barely detected in liquid culture medium, and not at all in solid fermented culture. Analysis based on PCR-RFLP showed a considerable variability in fragment patterns for *G. parvulum* and *G. subamboinense*, however no discrimination was identified for the other species. Sequence analyses from a partial MnP2 gene fragment (~700bp) demonstrated a high degree of similarity in gene structure among species, as well as conserved amino acid residues at the enzyme active sites, in four exons predicted for each isolate. Phylogenetic inference analysis with partial peroxidase sequences from polypore species supports the MnP2 clade for our isolates, although tree topology also indicated the polyphyletic nature of ligninolytic peroxidases, where possible scenarios such as multiple ancestor origin or a single origin with posterior diversification are discussed.

Ganodermataceae / Neotropics / Phylogeny / White-rot fungi

INTRODUCTION

Lignin is the major constituent of plant cell wall responsible for their rigidity and protection (Evert & Einchhorn, 2013). Lignin degradation is a very important factor for carbon recycling in terrestrial environments and is mediated by a group of fungi known as white-rot fungi, classified in the Basidiomycota phylum.

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These organisms are the only ones with the ability to degrade lignin through a process called enzymatic combustion (Levasseur *et al.*, 2014) where enzymes such as Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), Versatile Peroxidase (VP) and Laccase (Lac) are the key factors for lignin mineralization up to water (H_2O) and carbon dioxide (CO_2) (Martínez, 2002; Floudas *et al.*, 2012; Binder *et al.*, 2013). However, all mentioned enzymes, known as Lignin - Modifying Enzymes (LME), possess a differential expression for specific taxa since not all of them are present in certain white-rot fungal species (Hatakka, 1994; Singh & Kumar, 2010). Based on the cooperation of several oxidoreductases in lignin degradation, some applications have been identified to benefit different fields, from the bioconversion of lignocellulosic residues to different added-value products (Milati *et al.*, 2011), degradation of recalcitrant organic pollutants such as polycyclic aromatic hydrocarbons, chlorophenols, industrial dyes and nitro-aromatic compounds (Xu *et al.*, 2017), wood pulp production, bio-bleaching and bioremediation (Janusz *et al.*, 2013).

Ganoderma Karst. species (Polypores, Agaricomycetes) are classified as white-rot fungi and considered serious pathogens in some crop species. However, they are broadly used for medicinal purposes and valued for their ability to produce lignin-degrading extracellular enzymes (Zhou *et al.*, 2013), although varying in the *Ganoderma* genus depending the type of culture used: liquid or solid fermented cultures (Silva *et al.*, 2005; Elissetche *et al.*, 2007). Laccase is perhaps the most commonly detected enzyme within the genus, under various conditions, and different substrates. Manganese Peroxidase has shown little activity in solid fermentation substrates and LiP had only been detected when physical and nutritional conditions enhance its production in solid fermentation processes based on wheat straw and corn residues (D'Souza *et al.*, 1999; Mehboob *et al.*, 2011; Batool *et al.*, 2013).

Manganese Peroxidase [EC 1.11.1.13, Mn (II): hydrogen-peroxide oxidoreductase, MnP] was initially isolated and characterized in *Phanerochaete chrysosporium* Burds (Kuwahara *et al.*, 1984; Paszczynski *et al.*, 1985). Its genome contains 5 MnP genes (MnP 1-5) that codify several isoforms (Martinez *et al.*, 2004), with an average of 370 amino acid residues (Alic *et al.*, 1997), an isoelectric point of 4.5 and 30 – 62.5 kDa of molecular weight (Janusz *et al.*, 2013). Several isoforms have been identified in other species: 11 in *Ceriporiopsis subvermispora* (Pilát) Gilb. & Ryvarden (Lobos *et al.*, 1994), and nine in *Obba rivulosa* (Berk. & M.A. Curtis) Miettinen & Rajchenb., (Miettinen *et al.*, 2016), all of them codified by a cluster of closely related genes (Johansson & Nyman, 1996; Liu *et al.*, 2012). Despite there being no specific number of MnP genes identified in *Ganoderma* species, more than thirty Lignin-Modifying Enzymes (LME) have been isolated, where MnP is the major oxidative enzyme. Additionally, in the *G. lucidum* genome, 7 peroxidase genes have been identified, corresponding the third largest number of peroxidases, thus suggesting strong ligninolytic ability (Xu *et al.*, 2017).

Genomic studies of *Ganoderma* species and other polypores have uncovered the existence of a large number of genes that codify for peroxidases from different subfamilies, especially for MnP. Evolutionary reconstruction analyzes have shown that the MnP gene could be the precursor of all class-II ligninolytic peroxidases (Ruiz-Dueñas *et al.*, 2013) since this enzyme is the most widespread ligninolytic peroxidase (Morgenstern *et al.*, 2008; Ruiz-Dueñas *et al.*, 2009). This interesting scenario demonstrates the great diversity and complexity among these enzymes: not only a remarkable ability for lignin degradation but also of all its biotechnological applications. In the present study, the detection and sequencing of a partial region of the MnP2 gene was carried out in twelve white-rot fungal *Ganoderma* isolates

comprising five species that include *G. australe* (Fr.) Pat., *G. gibbosum* (Blume & T. Nees) Pat., *G. multiplicatum* (Mont.) Pat., *G. parvulum* Murrill and *G. subamboinense* (Henn.) Bazzalo & Wright.

MATERIAL AND METHODS

Fungal specimens and culture

Ganoderma australe, *G. gibbosum, G. multiplicatum*, *G. parvulum*, and *G. subamboinense* basiodomes were collected from leguminous trees in urban and protected areas in Brazil and Colombia (Table 1). The specimens were prepared for taxonomic studies and cultured in malt extract (MEA) at 2%. Twenty-five mycelium disks of 5-mm from each strain were retrieved from MEA culture, inoculated in 100 mL of malt extract and incubated at 30°C for one week. Afterwards, they were filtered, washed with sterile water, and dried out for molecular analysis.

To identify MnP activity in all isolates, a solid fermentation substrate based on sibipiruna wood (*Caesalpinia peltophoroides* Benth.) was developed and treated in a solution of water and malt extract.

Table 1. *Ganoderma* specimens evaluated with their respective Herbarium code, GenBank accession number for MnP gene and host information.

Species	*Origin*	*Herbarium*	*MnP GenBank accession*	*Host*
G. australe	Brazil	SP417784	KX458229	Dead stump
		SP417796	KX458230	Dead stump
		SP417793	KX458231	Dead stump
G. gibbosum	Brazil	SP417774	KX458232	Inga vera Kunth
		SP417798	KX458234	Caesalpinia peltophoroides Benth.
		SP417788	KX458233	Dead stump
G. multiplicatum	Brazil	SP417797	KX458226	Caesalpinia peltophoroides Benth.
		SP417780	KX458227	Dead stump
G. parvulum	Colombia	CUVC60084	KX458223	Leucaena leucocephala L.
		CUVC60081	KX458224	Cassia fistula L
		CUVC60087	KX458225	Phitecelobium dulce (Roxb.) Benth.
G. subamboinense	Brazil	SP417773	KX458228	Caesalpinia ferrea Mart. ex Tul

DNA isolation and PCR amplification

Fifty milligrams of fungal mycelium culture were ground in liquid nitrogen for DNA extraction under conditions proposed by Raeder & Broda (1985). For the amplification of a partial segment of MnP gene, primers E2FB (GAC CTS CAG AAG AAC CTG TTC SA) and E8R (CGG AGY TGS GTC TCG ATG AAG) (Binder & Hibbett, 2003) were used. The final volume of 25 µL mix was as follows: 2.5 µL PCR buffer 10X $(NH_4)_2SO_4$, 2.5 µL $MgCl_2$ 25mM, 4.8 µL dNTP's 5mM, 1 µL of

each primer 10mM, 0.5 µL Bovine Serum Albumin (BSA) 5X, 1 µL Threhalose 10%, 2 µL DNA stock, and 9.6 µL ultrapure water. The PCR thermal profile was followed as described by (Vilgalys & Hester, 1990). The amplified products were visualized in 1.5% agarose gel.

Cloning and sequencing

A partial fragment amplified (~700) for each specimen was cloned using the cloning vector system pGEM-T-easy Promega® according to the manufacturer's instructions and inserted in *Escherichia coli* DH5α. After transformation, solid LB media was prepared for bacterial growth in the presence of ampicillin 100mg/mL and X-gal 20mg/mL and incubated for 16 hours at 37◦C. White colonies were picked and inoculated in 15mL of liquid SOC culture media overnight. Plasmids were retrieved using the Plasmid Miniprep System Promega® Kit according to the manufacturer's instructions. To verify the incorporation of the recombinant DNA, a PCR reaction was performed using primers T7 and SP6 flanking promoter regions of the cloning vector. This reaction was carried out as follows: 95ºC for 2min, followed by 36 cycles at 94ºC for 30sec, 50ºC for 1min and 72ºC for 1 min. The final extension was at 72ºC for 5min. To identify polymorphisms between samples, the amplified PCR products were placed under restriction reactions with *HinfI* endonuclease enzyme (Vilgalys & Hester, 1990), identified in 2.5% agarose gel and, then, corroborated by *in silico* analysis performed in Geneious R10®. (Kearse *et al.*, 2012), using sequences obtained directly from the inserts flanked by the cloning vector promoters T7 and SP6.

Gene annotations and bioinformatic analysis

All raw sequences were assembled and edited independently using Geneious R10® (Kearse *et al.*, 2012). After contig assembly, ~150 pb belonging to the cloning vector (T7 and SP6) were removed from each isolate, obtaining a sequence of ~700bp for MnP gene. Primers E2FB and E8R were mapped for each sequence. For intron – exon identification, several MnP gene sequences were retrieved from both Coding DNA Sequence (CDS) and whole MnP gene from the National Center for Biotechnology Information (NCBI) and from the Joint Genome Institute (JGI) databases. *Ganoderma australe* (DQ267753), *Trametes versicolor* (D86493, X77154, Z30668), and *Ganoderma sp*. (10597 SS1) were included. Gene annotations considered intron – exon prediction. Afterwards, a multiple sequence alignment was performed between the references and our isolates using muscle alignment algorithm in Geneious R10® software. However, *T. versicolor* (Z30668) showed the highest similarity (77%) with our isolates and was employed for the intron – exon prediction of our sequences. Additionally, amino acid residues were obtained from both the reference and our isolated sequences. For the identification of amino acid residues involved in Heme, Substrate, Mn+2 and Ca+2 Binding Sites, a *Ganoderma lucidum* (ACA48488) reference sequence was used for this purpose. Finally, for the identification of conserved regions of the molecule, a multiple sequence alignment was performed using amino acid sequences from *G. australe* (DQ267753), *G. lucidum* (ACA48488) and *T. versicolor* (D86493 and Z30668), and the 12 *Ganoderma* isolates of the present study.

Nucleotide analysis

Network analysis was carried out using Network Software v. 5.0 Fluxus Engineering (Bandelt *et al.*, 1999) using nucleotide and amino acid sequences. The median – joining algorithm was performed using weighted transition / transversion at a 1:3 ratio, generating the most parsimonious network. DNAsp v. 5.0 (Librado & Rozas, 2009) was employed for Tajima's D test and for all parameters related to nucleotide patterns. Mega v. 7.0 (Tamura *et al.*, 2007) was used to identify genetic distances between and within species following the Kimura 2 parameter model under 1000 bootstrap replicates.

Phylogenetic analysis

Forty-three MnP gene sequences were analyzed, 31 of them retrieved from Morgenstern *et al.* (2008, 2010); Saroj *et al.* (2013); Zhou *et al.* (2014), and 12 from our isolates. The phylogenetic background reconstruction was inferred by the maximum likelihood method using PAUP v. 4.0b10 (Swofford, 2002) software, and a heuristic search with a hundred replicates. The TBR algorithm (Tree-Bisection-Reconnection) was used to adjust the topology (Branch-swapping). The Akaike criterion selected the model of nucleotide substitution in Model Test v. 2.1.3 (Darriba *et al.*, 2012). All node branches were supported through Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) analysis models. MP was performed with 10,000 bootstrap re-sampling, although it was 100 in the case of ML. Bayesian inference analysis was carried out using Mr. Bayes software v. 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsendbeck, 2003) under Markov-Chain Monte Carlo (MCMC) algorithm, with 10 million generations and tree sampling every 100th generations. A consensus tree was constructed using the last 50,000 trees (50% of the most representative trees).

RESULTS AND DISCUSSION

Twelve *Ganoderma* isolates, belonging to five species, generated a partial fragment of ~700bp for the MnP gene, indicating the presence of this enzyme. The single, low activity detection identified was for *G. parvulum* in liquid culture medium (data not shown). As demonstrated by Morgenstern *et al.* (2010) and Xu *et al.* (2017), this variability in MnP gene expression is significant at intra and interspecific level where certain conditions, such as the stability of mRNA transcription (Morgenstern *et al.*, 2010), Metal Responsive Elements interactions (MREs) and Heat Shock Elements (HSEs) in the gene sponsor region (Gold & Alic, 1993; Tello *et al.*, 2000), as well as availability of Carbon and Nitrogen (Pease & Tien, 1992), affect gene expression.

Three subfamilies of MnP enzymes have been identified from filtered culture of *Polyporus strigoso-zonata*, *Fomitoporia mediterranea* and *Trametes versicolor* (Floudas *et al.*, 2012). The first subfamily is characterized by a short sequence of amino acids; the second by an extra-large sequence with a terminal carbon for metal binding that provide enzyme stability (Sundaramoorthy *et al.*, 1994); and the third is a version of MnP with two residual acids (Floudas *et al.*,

2012). Since we did not cover the whole gene sequence for MnP in our isolates, it is hard to classify them into a specific subfamily.

Restriction analysis in the 12 *Ganoderma* isolates showed a differential genetic profile for *G. parvulum* (KX458223, KX458224, KX458225) and *G. subamboinense* (KX458228) with respect to all other isolates. For *G. parvulum,* a specific fragment pattern was identified, sharing only a fragment of 52bp common for all isolates. This fragment pattern indicates considerable nucleotide variation, not only in *G. parvulum*, but also in *G. subamboinense*. Nevertheless, this method was not sensitive for polymorphism identification in *G. australe* (accession numbers KX458229, KX458230 and KX458231), *G. gibbosum* (KX458232, KX458233 and KX458234) and *G. multiplicatum* (KX458226 and KX458227).

Manganese Peroxidase gene structure

Our isolates showed the greatest similarity in nucleotide composition (77%) in two sequences retrieved from NCBI databases (*Trametes versicolor* Z30668 and D86493) reported as MnP2 by Johansson *et al.* (1993) and MnP by Iimura (1996) respectively, where the first was used as reference for gene structure configuration. Size and position of introns and exons were conserved among our isolates and the reference, where our sequences merged with exons 2, 3 and 4, as well as the introns between (2 and 3).

Intron – exon boundaries were identified following the reference sequence intron – exon structure. These splicing junctions considered the GT – AG rule of Eukaryotic genes (Janusz *et al.*, 2013) found at the beginning (GT) and at the end (AG) of our intron sequences. However, exons 4 and 5 from our sequences merged into exon 4 of the reference. Specific introns varied slightly in size among our isolates (i.e. *G. australe* showed 69bp, *G. gibbosum* 68bp, *G. multiplicatum* 72bp, *G. parvulum* 72bp and *G. subamboinense* 69bp).

Finally, 4 exons and 3 introns were obtained for each isolate. The gene structure pattern found at intron – exon configuration is related, as proposed by Levasseur *et al.*, (2014), to the multiple numbers of genes present in the white-rot fungi that codify MnP. These genes have also been reported as the most heterogeneous in *Pycnoporus cinnabarinus* fungus as compared to LiP and VP genes. Additionally, in this species, exons 2 and 3 of the *mnp2/mnp3* pair merge into a single exon in *mnp1*; likewise exons 3, 4 and 5 of the *mnp1/mnp2* correspond to a single exon in *mnp3* (Levasseur *et al.*, 2014). This differential distribution has also been observed by Alic *et al.* (1997) who proposes that the ancestry of the *mn2* gene in *P. chrysosporium* is due to the presence of an extra intron in the sequence.

Several studies have demonstrated multiple MnP isoforms. For instance, in *Obba rivulosa,* nine MnP genes were identified (Miettinen *et al.*, 2016) whereas Liu *et al.* (2012) identified 7 peroxidase genes in the *Ganoderma lucidum* genome. These duplicated genes seem to generate a cluster of genes that facilitate their expression and organization as previously identified by Johansson & Nyman (1996), Passardi *et al.* (2007) and Levasseur *et al.* (2014) which indicate that these genes are linked and can be transferred together.

Some class II peroxidase genes are grouped in the same scaffold (*mnp3*, *lip1*, *lip2* and *lip3*) separated by 2kb in the same transcriptional direction, suggesting a recent gene duplication event (Passardi *et al.*, 2007). Furthermore, Levasseur *et al.* (2014) identified 9 ligninolytic peroxidases in *P. cinnabarinus* genome, three of them belonged to MnP class II enzymes, suggesting the exploitation of several

strategies for ligninolysis including class II mediator peroxidases requiring hydrogen peroxide, or Laccases in the presence of redox mediators.

Manganese Peroxidase gene richness is considerable and is related to the complexity of filamentous fungal genomes, their hyphal morphology and elongation and penetration of complex substrata, thus generating a range of nonspecific highly reactive radicals where multiple peroxidase isozymes are required, and providing specificities essential for the effective hydrolysis of wood complex polymers (Martinez *et al.*, 2004). Moreover, these duplication events are essential factors contributing to novel functions giving rise to evolutionary adaptability such as sub-functionalization or neo-functionalization (Levasseur *et al.*, 2007, 2014; Levasseur & Pontarotti, 2011). Passardi *et al.* (2007) proposed a coevolution between ligninolytic activity and the diversity found in plant cell wall composition, leading to duplication events in ligninolytic fungi accumulating several copies of peroxidases of either the same or a different gene family.

Nucleotide composition

After comparison of our sequences with the reference (Z30668), reported by Johansson *et al.* (1993), is composed of 2.125bp and structured with six exons and five introns that cover 1.397bp of the gene (Fig. 1), we obtained an average fragment of 544bp (excluding introns) and between 180 - 182 amino acid residues (Table 2).

Most of the CDS genes reported for white-rot fungi MnP possess relatively similar length (993 – 1098bp) (Martinez *et al.*, 2004) and intron – exon number (6 – 7) (Martínez, 2002; Levasseur *et al.*, 2014). However, some MnP genes reported showed a considerable number of exons: for instance, *C. cinereus* possess 14 exons, *T. versicolor* 12 (Martínez, 2002), *Ganoderma sp*. 13 (Binder *et al.*, 2013) and *G. australe* 11 (Lay & Hseu, 2005). In our case, four exons and three introns were identified in approximately 700bp for the 12 isolate sequences, with intron sizes ranging from 58bp in *G. multiplicatum* to 72bp in *G. parvulum*, similar to the intron size identified for fungal peroxidases that vary between 43bp and 85bp. For the reference sequence, the average size of exons was 183 nucleotides, exon 4, 443 nucleotides long, being the largest and the exon 6, 13 nucleotides long, the smallest. On the other hand, intron size ranges from 55 (intron 5) to 63 nucleotides (intron 1) with an average of 60 nucleotides.

Several introns that maintain the same position in several groups have been identified, not only in MnP genes (Godfrey *et al.*, 1990) but also in LiP genes (Stewart *et al.*, 1992; Gold & Alic, 1993; Levasseur *et al.*, 2014), indicating a common ancestry of class II peroxidases and subsequent diversification as a possible mechanism of adaptation to lignin polymer diversity in plant cell walls (Passardi *et al.*, 2007). On the other hand, Morgenstern *et al.* (2008, 2010) suggests that LiPs, VPs and the "classical" MnPs are derived from enzymes with a manganese-dependent activity. This homology has been used to define subfamilies of genes encoding ligninolytic enzymes (Godfrey *et al.*, 1990; Gold & Alic, 1993; Stewart *et al.*, 1992). A high degree of protein conservation has also been identified in core fungal genomes through genomic analyzes, suggesting a considerably number of orthologue genes in these taxa (Levasseur *et al.*, 2014; Riley *et al.*, 2014). Moreover, Passardi *et al.* (2007) proposed that class II peroxidases could have evolved from existing class I genes (Cytochrome *c* peroxidases) through evolutionary processes in response to very high redox potentials of lignin polymers. In this case, some key amino acid

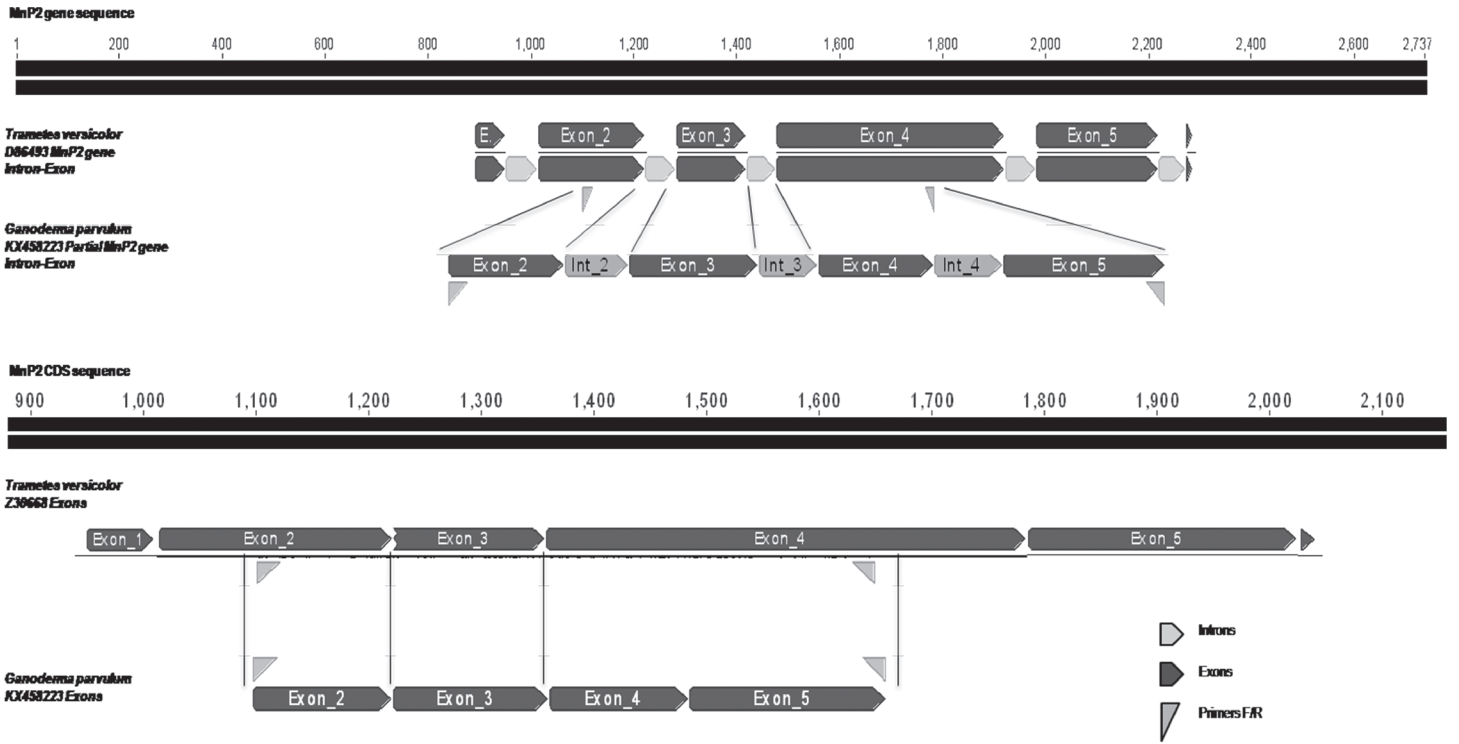

Fig. 1. Schematic of Manganese Peroxidase gene structure where intron – exon positions are shown, also primers binding sites are represented. Upper side: Reference sequence *T. versicolor* (Z30668) and isolate *G. parvulum* (KX458223) (~700bp) representing intron – exon configuration. The coverage sequence obtained includes the region from exon two to exon four respect to the reference. Lower side: coding DNA sequence (CDS) for both, reference and *G. parvulum* isolate (KX458223); primer binding positions and sequence coverage are shown.

Table 2. MnPII gene partial sequence information. Intron and exon sizes in bp is shown for every species, as well as synonymous and non-synonymous substitutions at nucleotide level are indicated.

Species	*Intron size (bp) 2-3-4**	*Exon size (bp) 2-3-4-5**	*CDS (bp)*	*# Amino acid residues*	*NS*	*Exon Position of NS*	*Codon change*	*Amino acid Change*	*AA biochemical property*
G. australe	65 - 62 - 69	122 - 135 - 122 - 168	546	182	89	1	TTC - TCC	Phe - Ser	Nonpolar - Polar
					101	1	ATG - ACG	Met - Thr	Nonpolar - Polar
					482	4	CTG - CCG	Leu - Pro	Nonpolar - Nonpolar
					503	4	CGC - CCC	Arg - Pro	(+) Charged - Nonpolar
					521	4	CGC - CCC	Arg - Pro	(+) Charged - Nonpolar
G. gibbosum	64 - 61 - 68	122 - 135 - 122 - 168	546	182	223	2	ATT - TTT	Ile - Phe	Nonpolar - Polar
					236	2	CGC - CAC	Arg - His	(+) Charged - (+) Charged
					446	4	CCA - CGA - CGG	Pro - Arg - Arg	Nonpolar - (+) Charged - (+) Charged
G. multiplicatum	62 - 58 - 72	116 - 135 - 122 - 168	541	180	26	1	AAC - AGC	Asn - Ser	Polar - Polar
					169	2	CTC - TTC	Leu - Phe	Nonpolar - Nonpolar
					515	4	CTC - CCC	Leu - Pro	Nonpolar - Nonpolar
G. parvulum	69 - 63 - 72	122 - 135 - 122 - 168	547	182	509	4	AAC - AGC	Asn - Ser	Polar - Polar
G. subamboinense	61 - 62 - 69	122 - 135 - 122 - 168	547	182	—	—	—	—	—

*Intron and exon number position; **NS** = nucleotide substitution position

residues changed thus enabling the enzyme to oxidize new targets, consequently taking advantage of new niches.

Nucleotide diversity in the MnP gene

Nucleotide diversity in the MnP gene found in the 12 *Ganoderma* isolates was 0.061. However, when analysis was carried out within species, diversity varied from 0.006 for *G. australe*, *G. gibbosum* and *G. parvulum* to 0.020 for *G. multiplicatum*, indicating that this last species is the most diverse. The total number of mutations for the 12 strains was 98, where 90 were polymorphic and segregating sites. On the other hand, 450 nucleotides were monomorphic. The most common nucleotide substitutions were transitions (A/G 10.8%; G/A 16.8%; T/C 9.3%; and C/T 14.1%), than transversions (A/T - A/C 5.02%; T/A - T/G 4.5%; C/A - C/G 6.9%; and G/T – G/C 7.7%). KX458224 and KX458225 strains of *G. parvulum* showed no variation in nucleotide composition, nevertheless this species showed 5-point mutations respect to the KX458223 isolate. The other isolates showed no identical sequences, and point mutations varied from 5 (*G. australe*, *G. gibbosum*, and *G. parvulum*) to 11 (*G. multiplicatum*).

A network analysis was constructed to visualize the number of nucleotide and amino acid substitutions (through median-joining algorithm [MJ]) (Bandelt *et al.*, 1999), showing that almost all of them were synonymous (data not shown). *G. subamboinense* showed the greatest differentiation in nucleotide composition (33 nucleotide substitutions), *G. gibbosum* was close to *G. australe* (22 nucleotide substitutions), and *G. multiplicatum* to *G. parvulum* (18 nucleotide substitutions). Moreover, when nucleotide and amino acid networks were compared, complexity decreases for the amino acid network, indicating that the greatest nucleotide variation is found at the third codon position generating no amino acid changes in most of the mutations.

Genetic distance analysis showed that *G. multiplicatum* and *G. parvulum* were the least distant (0.003), followed by *G. australe* and *G. gibbosum* (0.013), and, finally, *G. subamboinense* was the most distant with values of 0.037, 0.034, 0.023, 0.036, respectively.

Amino acid sequence comparisons within species showed some variations. For instance, *G. australe* presented 5 substitutions, *G. gibbosum* 3, *G. multiplicatum* 3, and *G. parvulum* 2. However, for *G. parvulum* and *G. multiplicatum,* changes in amino acid residues were between those with similar biochemical properties, but not for *G. australe* and *G. gibbosum* (Table 2). Nevertheless, comparison of amino acid sequences across all *Ganoderma* strands showed 93% conserved positions. Additional analysis was carried out to identify binding site positions (amino acid residues involved in the molecule active sites) using *Ganoderma lucidum* protein as a reference sequence (Genbank accession number ACA48488) (Huang *et al.*, 2009). All amino acid residues involved in the substrate (Gly, Phe, His), heme (His, Glu, Leu, Arg, Phe), Mn^{2+} (Glu, Glu) and Ca^{2+} (Asp, Gly, Asp, Ser) binding sites at the 5' end region of the sequences were well conserved (Fig. 2). Additional conserved regions have been reported by Levasseur *et al.* (2014) at the internal heme propionate region (at the 5' end of the sequence) for MnP I, II and III, and Xu *et al.* (2017) in aromatic substrate oxidation, heme pocket binding sites and Ca^{+2} binding sites, not only in *Ganoderma lucidum* MnP, but also from various other fungi and plant peroxidases.

The synonymous substitutions found in our sequences indicates neutral mutations with no negative effect on the fitness of the molecule (Kimura, 1991; Takahata, 1996; Laurent, 2008). Other substitutions with changes in amino acids occur among those with the same biochemical properties. To verify this, a neutrality test (Tajima's D test) was carried out in the 12 isolates, indicating that all nucleotide substitutions correspond to neutral mutations ($D = 0.1713$, $P > 0.10$). However, it is necessary to consider the fact that the comparison was made among 5 *Ganoderma* species, using the same MnP *Locus* (MnP2). The diversity found in the MnP2 isozyme in *Ganoderma* isolates is possibly related to a neutral process which has permitted diversification and, as reported by Levasseur & Pontarotti (2011), these evolutionary mechanisms are positively selected for adaptation to a particular environment. This kind of mutations could lead to specialization of catalytic activity of all oxidative substrates and their capability to depolymerize complex wood polymers to CO_2 and H_2O (Martinez *et al.*, 2004; Levasseur *et al.*, 2014; Riley *et al.*, 2014).

Additionally, a sub-functionalization process can be associated with gene duplication, leading to refinement by natural selection to become more efficient for lignin degradation. Also, worldwide distribution of white-rot fungi, including *Ganoderma* spp. (Xu *et al.*, 2017), could be associated with local adaptations with imprints in genetic regions with high nucleotide diversity. Here, all variations of phenolic compounds and multiple lignin substrata found in gymnosperms and angiosperms have possibly triggered adaptive conditions in order for white-rot fungi to obtain carbon and energy (Passardi *et al.*, 2007).

Phylogenetic analysis in *Ganoderma spp.*

Phylogenetic analysis has provided a picture of relationships among *Ganoderma* species and the polyphyletic nature of ligninolytic enzymes in white-rot fungi (Morgenstern *et al.*, 2008, 2010; Zhou *et al.*, 2014). A total of 877 characters

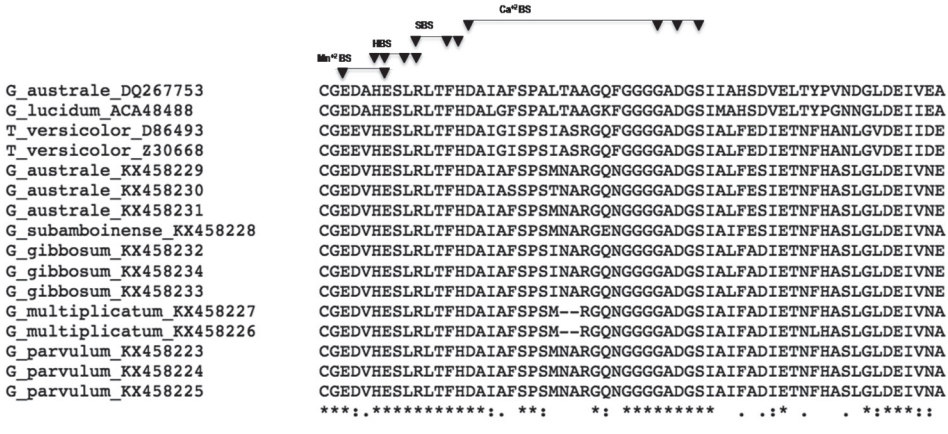

Fig. 2. Amino acid residues alignment for Manganese Peroxidase gene at 5' end of the molecule. The inversed triangles indicate the amino acid residues involved in: Manganese Binding Site (Mn+2 BS); Heme Binding Site (HBS), Substrate Binding Site (SBS) and Calcium Binding Site (Ca+2 BS). The alignment includes sequences from NCBI databases and the twelve isolates analyzed in the present study. For *G. multiplicatum* (KX458226 and KX458227) there are two gaps indicating the absence of amino acid residues on that positions.

per nucleotide sequence were obtained, 93 of them being constant, 699 parsimoniously informative and 85 parsimoniously non-informative. The most parsimonious tree had a length of 4.873, a consistency index of 0.3614 and a retention index of 0.2265. (Fig. 3). Nucleotide frequencies were T= 0.2533, C= 0.3270, A= 0.1812 and G= 0.2384. Three clusters were clearly identified in the phylogenetic inference (MnP1, MnP2 and LiP) where all our isolates clustered together in the MnP2 clade, *Trametes versicolor* being the closest taxa.

All nodes were well supported by MP, ML and BI analysis with values over 95% and 1.0 for the main nodes, respectively.

In the MnP2 cluster, *G. parvulum* and *G. multiplicatum* were close to each other, as well as *G. australe* and *G. gibbosum*; and *G. subamboinense* was located between the mentioned clusters. This grouping is in agreement with the network analysis indicating the closeness among these species, as well as with the phylogenetic inference topology proposed by Zhou *et al.* (2014). The polyphyletic topology identified here with respect to MnP1 and MnP2 reflects either the possibility of an origin from different ancestors or dramatic changes in amino acid sequences. Nevertheless, there are other plausible perspectives such as the wide distribution of MnPs in terms of taxonomic occurrence and phylogenetic distribution (Morgenstern *et al.*, 2010; Zhou *et al.*, 2014), gene duplication events with posterior refinement by natural selection, and early occurrence and diversification of MnPs before the splitting of the major linages of Agaricomycetes (Morgenstern *et al.*, 2008).

The occurrence of ligninolytic enzymes are derived from a manganese-dependent activity, but there is still not enough resolution to understand these transitions (Morgenstern *et al.*, 2008). Nevertheless, some sequences of the MnP gene for *Ganoderma* species are confusing, due to the fact that they have been reported indifferently as either MnP or LiP. For instance in Saroj *et al.* (2013) enzymes from *Ganoderma australe* (DQ267753), *G. formosanum* (DQ267752) and *G. applanatum* (AB035734) are reported as MnP however clustering apart, indicating the high divergence of these enzymes among different taxonomic groups. In Morgenstern *et al.* (2010) there is no enzyme assignation for those accessions that are clustered apart from both MnP1 and MnP2 clades making them difficult to classify, despite being supported with bootstrap values of 99%. In contrast, Zhou *et al.* (2014) reported them as LiP and clustered in the LiP clade with bootstrap values of 100%. However, this clade is reported as both LiP and VP clade, their group assignation still uncertain.

These conflicting results are probably due to the high level of similarity among ligninolytic enzymes as reported by Janusz *et al.* (2013) who identified 40% homology among them, although Saroj *et al.* (2013) reported a similarity as high as 50% among ligninolytic peroxidases. In the present study, the phylogenetic topology coincided with the results obtained by Zhou *et al.* (2014) with respect to the LiP clade showing high support values on the main nodes.

It is important to consider the very close structural similarity between MnPs, LiPs and VPs active sites residing basically in the mode of hydrogen peroxide activation. The location of residues potentially able to bind Mn+2 suggests a very similar three-dimensional fold. Additionally, superposition analysis, depicting 10 Cysteine residues generating 5 disulfide bonds in MnP and 8 Cysteine residues for four disulfide bonds in LiP and VP, showed that the initial four disulfide bonds from MnP are the same as observed in LiP and VP (Saroj *et al.*, 2013). An interesting perspective from Zhou *et al.* (2014) indicates that the topology obtained using several ligninolytic enzymes, where all of them often clustered in the same group with high bootstrap values, can be caused by the overall information of amino acid

sequences rather the key residues involved in substrate oxidation. In that sense, the topologic structure reflects a phylogenetic relationship.

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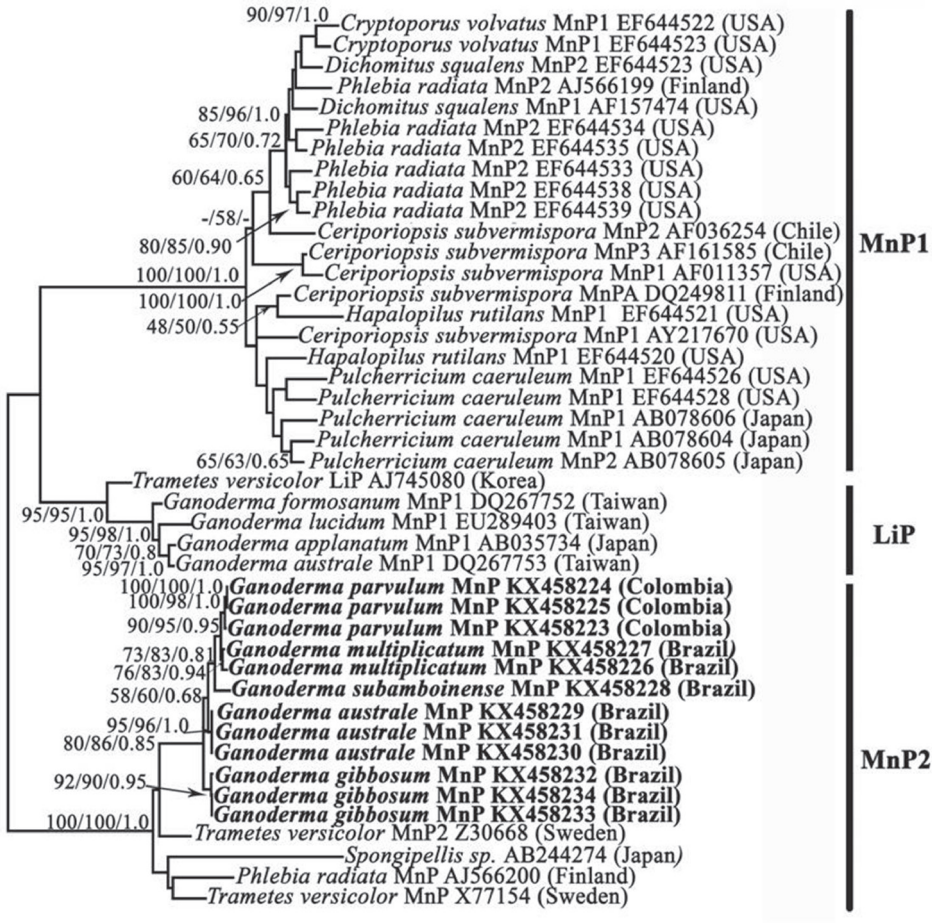

Fig. 3. Phylogenetic analysis for Manganese Peroxidases (MnP1 and MnP2) and Lignin Peroxidase (LiP) considering several Polyporales species and twelve *Ganoderma* isolates obtained in the present study (in bold). Maximum Parsimony, Maximum Likelihood and Bayesian Analysis support each node respectively. Arrows indicate nodes support.

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