

A peroxidase gene family and gene trees in *Heterobasidion* and related genera

Pekka Maijala

Department of Applied Chemistry and Microbiology,
P.O. Box 56, 00014 University of Helsinki, Finland

Thomas C. Harrington¹

Department of Plant Pathology, 351 Bessey Hall,
Iowa State University, Ames, Iowa 50011, USA

Marjatta Raudaskoski

Department of Biosciences, Division of Plant
Physiology, P.O. Box 56, 00014 University of Helsinki,
Finland

Abstract: Four putative peroxidase-encoding gene fragments, named *mnp1a*, *mnp1b*, *mnp2* and *mnp3*, were amplified with degenerative primers from the white-rot basidiomycete genus *Heterobasidion*. The fragments were cloned and sequenced. Similar fragments were produced and analyzed from the related genera *Amylostereum*, *Bondarzewia* and *Echinodontium*. Each amplified fragment contains three identically positioned introns. According to the predicted amino acid sequence, these fragments are most similar to two Mn peroxidase-encoding genes (*MPGI* and *mnp2*) and gene *pgv* of *Trametes versicolor*. Conserved residues thought to be essential for peroxidase function were identified. All four MnP gene loci of *Heterobasidion* were detected only in *H. parviporum*. Variation occurred in the predicted amino-acid sequences (131–132 amino acids) of all four fragments originating from the 47 *Heterobasidion* isolates tested. Amino acid variation in fragments of *mnp2* and *mnp3* separated European *Heterobasidion parviporum* (“S-type”) and *H. abietinum* (“F-type”), known to have identical rDNA sequences. Asian and western North American isolates from fir, spruce and other hosts had the peroxidase amino acid sequences of European *H. parviporum*. American and European *H. annosum* (“P-type”) isolates had different amino acid sequences and might be cryptic species.

Key words: *Amylostereum*, *Bondarzewia*, *Echinodontium*, manganese peroxidase, phylogeny

INTRODUCTION

The genus *Heterobasidion* (Aphyllphorales: Bondarzewiaceae) comprises several closely related species that cause white rot, primarily in living conifers. *Heterobasidion araucariae* is reported from Australasia, where it inhabits dead conifer wood or causes butt rot of living Araucariaceae. *Heterobasidion insulare* is also a saprophyte or decay fungus in living stems of Pinaceae, distributed in southern and eastern Asia. *Heterobasidion annosum* is a pathogenic species, which forms a complex consisting of three different intersterility groups in Europe: S, F and P. Recently, the European members of the *H. annosum* complex have been separated into three species (Niemelä and Korhonen 1998). In general, *H. parviporum* (= *H. annosum* S group) is a specialized pathogen of Norway spruce, *Picea abies*; *H. abietinum* (= *H. annosum* F group) infects firs (*Abies* spp.); and *H. annosum* (= *H. annosum* P group) occurs primarily on pines (*Pinus* spp.). Groupings are based primarily on interfertility as determined by clamp formation (Korhonen 1978, Capretti et al 1990), but the groups also show differences in physiological and biochemical characteristics (Karlsson and Stenlid 1991, Otrosina et al 1993). The relationships of *H. annosum* populations from Asia and North America to those in Europe are not clear (Harrington et al 1998).

Heterobasidion species are able to degrade lignin, and *H. annosum* is known to secrete laccase (Haars et al 1981, Haars and Hüttermann 1983), but peroxidase activity has been difficult to demonstrate in pure cultures (Haars et al 1981, Boudet et al 1988). In the basidiomycete white-rot fungi, lignin peroxidases (LiPs) and manganese peroxidases (MnPs) are involved in the biodegradation of lignin, and MnP seems to be present in almost all white-rot fungi (Hatakka 1994). Flecks of MnO₂ in wood decayed by *H. annosum* (Blanchette 1984) suggest the presence of MnP activity, and three MnP isozymes have been identified from a homokaryotic isolate of the European *H. annosum* when the fungus was cultivated on spruce wood chips (Maijala 2000).

Knowledge of the molecular genetics of lignin-degrading enzymes has proceeded rapidly during the past decade, especially in the basidiomycete *Phanerochaete chrysosporium* (Cullen 1997, Gold and Alic 1993). More than 50 different fungal peroxidase

TABLE I. Isolate numbers, identified manganese peroxidase genes, location of origin, and hosts for fungal isolates

Isolate	Isolate No. ^a	mnp ^b	Location	Host
<i>Heterobasidion abietinum</i>	B1089* ^c	1a, 2, 3	Italy	<i>Abies alba</i>
	B1090*	1a, 2, 3	Italy	<i>Abies alba</i>
	B1162*	1a, 2, 3	Greece	<i>A. cephalonica</i>
	B1165*	3	Bulgaria	<i>Abies alba</i>
	B1166*	1a, 2, 3	Bulgaria	<i>Abies alba</i>
<i>H. annosum</i> -American	B156	1b, 2	California, USA	<i>Pinus jeffreyi</i>
	B163	1b, 3	California, USA	<i>Pinus ponderosa</i>
	B349	1b	California, USA	<i>Pinus lambertiana</i>
	B825	1b, 2, 3	New Hampshire, USA	<i>Picea abies</i>
<i>H. annosum</i> -European	B298*	3	Finland	<i>Picea abies</i>
	B299*	1a	Finland	<i>Pinus sylvestris</i>
	B1169*	1a, 2, 3	Greece	<i>Pinus nigra</i>
	B1256	1a, 2, 3	Scotland	<i>Pinus sylvestris</i>
	B1257	3	Scotland	<i>Pinus sylvestris</i>
	EP0	1a	Finland	<i>Pinus sylvestris</i>
	B1098*	3	Japan	<i>Pinus thunbergiana</i>
<i>H. insulare</i>	B1159*	1a, 2, 3	Japan	<i>Abies firma</i>
	B1279*	1a, 2, 3	China	<i>Pinus koraiensis</i>
	B1281*	1a, 2, 3	China	<i>Abies</i> sp.
	B146	1a, 2, 3	California, USA	<i>Abies concolor</i>
<i>H. parviporum</i>	B227	2, 3	Washington, USA	<i>Abies grandis</i>
	B228	1a, 2, 3	Oregon, USA	<i>Abies concolor</i>
	B304*	2, 3	Sweden	<i>Picea abies?</i>
	B307*	1a, 2, 3	Finland	<i>Picea abies</i>
	B1081	2, 3	Japan	<i>A. sachalinensis</i>
	B1092	1a, 1b, 2, 3	Japan	<i>Abies mariesii</i>
	B1126*	1a, 2, 3	Russia	<i>Picea abies</i>
	B1142	1a, 1b, 2, 3	Mexico	<i>Abies religiosa</i>
	B1180	1a, 1b, 2, 3	Greece	Unknown
	B1181*	1a, 2, 3	Bulgaria	<i>Picea abies</i>
	B1292*	1a, 1b, 2, 3	China	<i>Pinus</i> sp.
	B1295*	1a, 2, 3	China	<i>Abies</i> sp.
	B1300*	1a, 1b, 2, 3	China	<i>Abies</i> or <i>Picea</i> sp.
	B1314*	1a, 2, 3	China	<i>Populus</i> sp.
	B1317	1a, 2, 3	Siberia	<i>Abies siberica</i>
	Fas3*	1a, 1b, 2, 3	Italy	<i>Picea abies</i>
	Fas6*	1a, 2, 3	Italy	<i>Picea abies</i>
<i>H. araucariae</i>	B1080	1a, 2, 3	Papua New Guinea	<i>Araucaria cunninghamii</i>
	B1083	1a, 2, 3	New Zealand	<i>Agathis australis</i>

^a Isolates beginning with the letter B are from the collection of T. C. Harrington. Other reference numbers for these isolates can be found in Harrington et al (1998) and Tabata et al (2000).

^b Indicates identified manganese dependent peroxidase sequences.

^c* Denotes single-basidiospore strain (haploid).

gene sequences are available from 12 different basidiomycete species, which allows comparative studies of the peroxidase gene structure and evolution.

Using degenerative PCR primers designed from previously identified MnPs, Maijala et al (1998a, b) we amplified and cloned a partial sequence of a putative MnP gene from *H. annosum*. Here we report the structure and variation of the amino acid sequences of four putative MnP gene fragments in *H. parviporum*, *H. abietinum*, *H. annosum*, *H. araucariae*

and *H. insulare*, and similar fragments in the closely related genera *Amylostereum*, *Bondarzewia*, and *Echinodontium*. The peroxidase gene sequences have been used to re-evaluate the phylogeny of *Heterobasidion*.

MATERIALS AND METHODS

Fungal strains.—Detailed collection information of the isolates is given in TABLE I. The nomenclature follows that of

Niemelä and Korhonen (1998). Most of the *Heterobasidium* isolates were confirmed to be *H. abietinum*, *H. annosum* or *H. parviporum*, based on dikaryon formation with tester strains. The collection information on *Amylostereum* and *Echinodontium* species can be found in Tabata et al (2000).

PCR and cloning.—Genomic template DNA for the PCR of *B. montana* was kindly provided by David Hibbett (Clark University, Worcester, Massachusetts, USA). Genomic DNA of *Heterobasidium* species was extracted using the method of DeScenzo and Harrington (1994). Degenerative forward and reverse PCR primers were designed from multiple fungal peroxidase sequence alignments, revealing consensus areas in the putative Ca²⁺-binding motifs in the enzyme (Poulos et al 1993). The respective forward and reverse primers were DP1 (5'-GG(A/C/T)GGTGCCGATGG(C/G)TC-3') and DP2 (5'-GG(A/G)GTGGAGTC(A/G)AACGG-3'). More specific primers used for each of the three peroxidases in *Heterobasidium* were PX11 (*mnp1*-specific, forward, 5'-GATGGGTCCATCATCGTA-3'), PX12 (*mnp1*-specific, reverse, 5'-GAGTTCCTGGGATCGTCAC-3'), PX21 (*mnp2*-specific, forward, 5'-TGCCGATGGGTC(A/G/T)ATATC-3'), and PX31 (*mnp3*-specific, forward 5'-GATGGGTCCCT(C/T)AT(C/T)GTG-3'). These primers amplify a fragment of about 570 bp. In general, PCR included 50 pmol of each primer, 50–100 ng of genomic DNA, 2 mM dNTPs, 1.5 mM MgCl₂, and 2.5 units of DNA-polymerase (Promega) per 100 µL reaction. Cycling conditions typically included an initial denaturation at 94 C for 95 s and 34 cycles of 94 C for 35 s, 52 C for 1 min, and 72 C for 2 min, with a final elongation of 10 min at 72 C. Slow ramping (1 C for every 5 s) was used from annealing to extension temperatures. Resulting products were cut from the agarose gel and cloned into the pGEM-easy vector (Promega). For sequencing, cloned products were cycle-sequenced with an automated ABI sequencer in both directions with the T7 and SP6 universal primers that flank the cloning site.

Representative DNA sequences and inferred amino-acid sequences of the amplified fragments were deposited in GenBank (AJ507469–AJ507485). The *mnpA* fragments from *Amylostereum* and *Echinodontium* species were deposited in GenBank with accession numbers AF218404 (*A. areolatum*), AF218405 (*A. ferreum*), AF218408 (*A. laevigatum*), AF218410 (*E. tinctorium*), and *B. montana* peroxidase fragments by AF218413 and AF218414 (Tabata et al 2000). Other available amino acid sequences were downloaded from various sources: *Trametes versicolor* *pgv* = X77154, *mnp2* = AF102515, *mnp1* = Z30668, *ulg2* = M91818, *lpgIII* = Z30666, *ulg1* = M55294, *lpgIV* = Z31011, *lpgII* = Z75655, *npr* = AF008585; *Phlebia radiata* *lgp3* = 126290; *Phanerochaete chrysosporium* *lipB* = X54257, *lipG* = AF140063, *lipI* = O282 (Schalch et al 1989), *lipE* = L08963, *lipA* = M27884, *lipC* = X55343, *lipF* = M77508, *lipH* = M24082, *lipJ* = AF140062, *lipD* = M18743; *Bjerkandera adusta* *lpo1* = 444058; *Trametes hirsuta* *lip* = E07702; *Ceriporiopsis subvermispora* *mnp1* = AF013257, *mnp3* = AF161585, *mnp2a* = AF161078, *mnp2b* = AF161584; fungus IZU-154, (Matsubara et al 1996); *Dichomitus squalens* *mnp1* = 157474, *mnp2* = 157475; *Pleurotus ostreatus* *mnp1* = U21878, *mnp2*

TABLE II. Levels of similarity^a between four different MnP gene fragments at the nucleotide/amino acid level identified in European *H. parviporum*-isolate B1292

Gene	<i>per1b</i>	<i>per2</i>	<i>per3</i>
<i>per1a</i>	75.3/92.9	70.3/85.5	71.2/88.6
<i>per1b</i>	—	68.2/86.3	67.4/87.0
<i>per2</i>		—	67.8/85.9

^a Calculated by using the program BESTFIT of Wisconsin Package, version 10, Genetics Computer Group, Madison, Wisc., USA.

= AJ243977; *Pleurotus eryngii* *mnp1* = AF007224; and *Coprinus cinereus* *cip1* = X70789.

Putative amino-acid sequences were analyzed with parsimony (PAUP 4.0b3a, Swofford 1998) after manually aligning the sequences by inserting gaps. A total of 136 characters, including gaps, were in the aligned data set. Gaps were treated as missing data. Maximum-parsimony heuristic searches were performed with all characters having equal weight and with tree-bisection-reconnection. The robustness of the internal branches of the tree was evaluated by 100 bootstrap replications using heuristic searches.

RESULTS

Characterization of the fragment sequences.—Putative peroxidase encoding gene fragments ranging in size from 560–570 bp were successfully amplified in all *Heterobasidium* species. Sequencing of the cloned PCR products revealed several related genes, with similarity levels from 50 to more than 80% to known LiP and MnP sequences at the inferred amino-acid level. Four distinct products, arbitrarily designated *mnp1a*, *mnp1b*, *mnp2* and *mnp3*, were detected in *Heterobasidium parviporum*, including several haploid isolates (TABLE I), clearly indicating the existence of at least four distinct peroxidase loci in *H. parviporum*. The four gene sequences of *H. parviporum* share 67–75% and 85–93% similarities at the nucleotide level and the putative amino-acid levels, respectively (TABLE II). Three introns tentatively were identified in all fragments (FIG. 1). Examination of the 106 DNA sequences produced from 47 *Heterobasidium* isolates showed that most of the variation in each of the peroxidase genes was in the intron sequences, but variability existed also at the inferred amino acid level.

The predicted 131–132 amino acid residues of different species of *Amylostereum*, *B. montana*, *Heterobasidium* and *Echinodontium* are shown in FIG. 2. Sequences are aligned with *Coprinus cinereus* peroxidase *cip1*, *Trametes versicolor* genes *pgv*, *MPGI* and *lpgIII*, *Phanerochaete chrysosporium* LiP-gene *lipA* and MnP-gene *mnp1*, and *Pleurotus eryngii* peroxidase *mnp1*. The closest similarity to peroxidase fragments

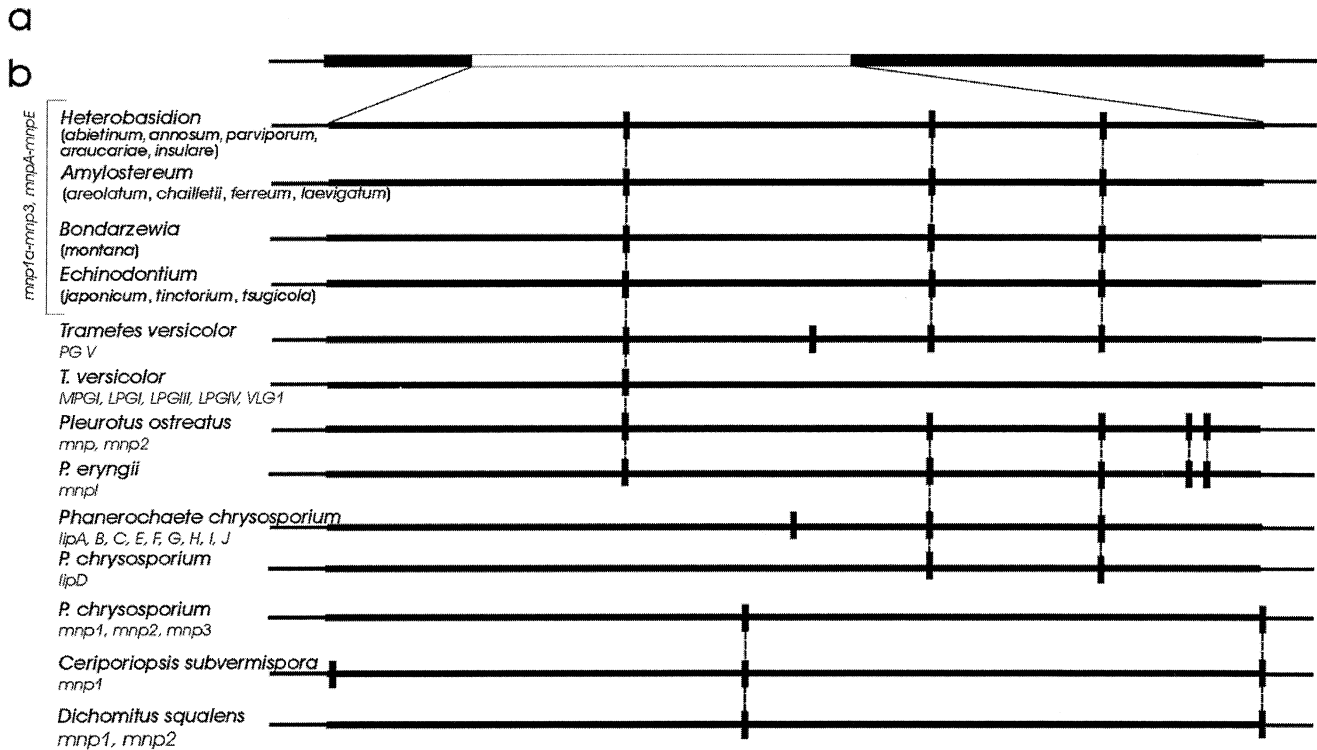


FIG. 1. Exon-intron organization in peroxidases of selected white-rot fungi. **a.** Schematic presentation of a fungal peroxidase gene, showing the amplified area within the gene as a white bar. **b.** Horizontal lines representing open reading frames of the amplified region of the genomic DNA of LiPs and MnPs in selected fungi. The first four groups have been investigated in this study. *P. chrysosporium* LiP-genes are classified according to Stewart and Cullen (1999). Vertical bars indicate intron positions. Intron sites sharing common positions are connected with dashed lines.

of *Heterobasidion* is with *T. versicolor* MnP genes (85.5%), whereas *T. versicolor* LiP genes share less than 70% similarity.

Exon/intron arrangement.—The amplified fragments of the four *Heterobasidion* genes contain three relatively short introns that range in size from 49–62 bp. The overall exon-intron structure (FIG. 1) of the peroxidase-encoding fragments in this study appears to be unique among the known fungal peroxidases. In other fungi, LiP and MnP genes contain short, 50–80 nucleotide introns, positions of which are moderately conserved in different species. Gene *pgv* encoding a peroxidase with currently unknown function in *T. versicolor* (Jönsson et al 1994) and peroxidase-encoding genes in *Pleurotus* species share the three intron positions with the fragments investigated but have additional intron(s) within the analyzed region (FIG. 1). All peroxidase fragments of *Heterobasidion*, as well as the peroxidase fragments of *B. montana*, *Amylostereum*, and *Echinodontium* species, possess the predicted consensus splice sites GT(a/g)NG(c/t) for the 5' end and (c/t)N(c/t)AG for the 3' ends of eukaryotic introns (Ballance 1986).

In all *Heterobasidion*, *Amylostereum*, *Bondarzewia*

and *Echinodontium* fragments, the position of intron I matches exactly the position of the third intron in *T. versicolor* MnP encoding gene *MPGI* (Johansson and Nyman 1996) and LiP encoding genes *LPGI*, *LPGIII*, *LPGIV* (Jönsson and Nyman 1994, Johansson and Nyman 1996) and *VLG1* (Black and Reddy 1991). This intron position also is shared with *Pleurotus ostreatus* *mnp*-genes (Asada et al 1995, unpubl) and with *P. eryngii* versatile peroxidase encoding gene *mnpl* (Ruiz-Dueñas et al 1999). The positions of introns II and III match those in all *P. chrysosporium* LiP genes (Gold and Alic 1993) and *mnp*-genes of the *Pleurotus* species. MnP genes from *Ceriporiopsis subvermispora* (Lobos et al 1998, Tello et al 2000), *Dichomitus squalens* (Li et al 1999) and *P. chrysosporium* have no intron positions in common with *Heterobasidion* (FIG. 1).

Structural and functional domains.—The amplified region includes several structural and functional domains that are conserved in all fungal secretory peroxidases (TABLE III), such as the invariant proximal His177 ligand to the heme (based on *T. versicolor* *MPGI*-gene numbering; Johansson and Nyman 1996) and calcium-binding regions including residues

TABLE III. Important amino acid residues for protein structure and function within the investigated region of ligninolytic peroxidases

Residue	Function	Reference
Gly66, Asp68, Ser70	Distal Ca ²⁺ -binding	Sundaramoorthy et al. 1994
Thr/Ser176, Asp 193, Thr195, Thr198	Proximal Ca ²⁺ -binding	Sundaramoorthy et al. 1994
His82, Ala83, Asn84, Trp171, Leu172	LRET pathway	Schoemaker et al. 1994
His177	Heme ligand	Sundaramoorthy et al. 1994
(Arg181)*, Asp183	Mn ²⁺ -binding	Sundaramoorthy et al. 1997
Phe194	Stability	Kishi et al. 1997
Glu79	Heme pocket; e ⁻ -transfer	Poulos et al. 1993; Banci, 1997
Phe142	Substrate binding	Veitch et al. 1995
Cys121	Structural rigidity	Sundaramoorthy et al. 1994
Asn103, Asn131, Asn135, Asn160	N-Glycosylation site	

* Residues described in *P. chrysosporium* peroxidases are shown in parentheses.

Gly67, Asp69, Ser71, Thr/Ser178, Asp195, and Thr197 (Sundaramoorthy et al 1994). In the three-dimensional structure of LiP and MnP, residues Phe82, His83, Pro/Ala84, and Asn85 are located at the surface of the protein, and they form one putative, long-range electron transfer (LRET) pathway (Schoemaker et al 1994, Camarero et al 1999). In *mnp3* and *mnpA*, position 82 is occupied by a redox-active Tyr-residue, so far only found in one of the *P. chrysosporium* LiP H2 isozyme-encoding genes, *lipD* (de Boer et al 1987) and in a manganese-repressible peroxidase of *T. versicolor* (Collins et al 1999). The presence of two glycine residues at the site that represents the heme-opening channel in the three-dimensional structure (Poulos et al 1993, Sundaramoorthy et al 1994) is another distinct feature of the *mnp3* fragments of different *Heterobasidion* species and *A. areolatum mnpC*. Cysteine residues in fungal secretory peroxidases are all conserved, and the cysteine occupying Position 121 is invariant in the analyzed fragments, except in one *H. parviporum* fragment (FIG. 2). An atypical Cys is found in *A. areolatum mnpC* at Position 109 and in *B. montana mnpB* at position 132.

In the three-dimensional structure of the MnP from *P. chrysosporium*, three carboxylate ligands, provided by conserved Glu37, Glu41 and Asp183, are important for proper Mn²⁺-binding (Sundaramoorthy et al 1997). Asp183 residue is present in all the cloned and sequenced fragments, except in the *mnpB* fragment from *B. montana*, in which Asp183 is replaced by a glutamine. In the other MnP-fragment from *B. montana* (*mnp2*), the correct Asp183 residue needed for Mn²⁺ binding is present. Potential N-glycosylation sites (Asn-X-Thr/Ser) (Kornfeld and Kornfeld 1985) are not markedly conserved among the genes or species analyzed in this study (FIG. 2).

Phylogenetic analyses of putative amino acid sequences. Examination of the aligned amino acid sequences (FIG. 2) suggests that the fragments amplified from *Heterobasidion*, *Bondarzewia*, *Amylostereum* and *Echinodontium* are similar and distinct from those of the amino-acid sequences of peroxidases identified in other white-rot fungi. In the results of the parsimony analysis shown in FIG. 3, the tree is rooted to *Coprinus cinereus* peroxidase, which belongs to a separate class of secretory fungal peroxidases (Baunsgaard et al 1993). There is no bootstrap support for the branch containing the representative sequences of the fragments of *Heterobasidion*, *Bondarzewia*, *Amylostereum* and *Echinodontium*, but this clade was inferred in all six most-parsimonious trees. The amino acid sequences of *T. versicolor* MnP encoding genes *mnp2* (unpubl), *MPGI* (Johansson and Nyman 1996) and a gene *pgv* (Jönsson et al 1994) appear to be most similar to those of *Heterobasidion* and related genera (FIG. 3).

The tree in FIG. 3 suggests phylogenetic relationships among the various peroxidase genes, although bootstrap analysis shows little support for most of the tree. The topology of the six most-parsimonious trees was identical except for the branches for *Pc-lipB*, *Pc-lipG*, *Pc-lipI*, and *Pc-lipE*. There is bootstrap support (83%) for grouping lignin peroxidase genes of *T. versicolor*, *T. hirsuta*, *Bjerkandera adusta*, *Phlebia radiata* and *Phanerochaete chrysosporium*, and there was strong support (100%) for grouping the MnP sequences of *Ceriporiopsis subvermispora*, *P. chrysosporium*, fungus IZU-154 and *Dichomitus squalens*. The *T. versicolor* gene *Tv-npr* did not group with the other MnP and lignin peroxidase genes (FIG. 3).

Using the *T. versicolor* gene *Tv-pgv* as an outgroup, there was bootstrap support (74%) for grouping the amino-acid sequences of the fragments from *Heterobasidion*, *Bondarzewia*, *Amylostereum*, and *Echinodon-*

structural differences are manifested as differences in substrate specificity and catalytic mechanism. Asp183 residue needed for Mn²⁺-binding (Kishi et al 1996, Sundaramoorthy et al 1997) is present in all cloned and sequenced fragments, except in the *mnpB* fragment from *B. montana*. Position 172 in most of the analyzed peroxidase fragments is Ala, whereas, in *E. tsugicola mnpE*, it is Ser. Trp at position 172 that has been postulated to be involved in binding of veratryl alcohol (VA) (Doyle et al 1998, Timofeevski et al 1999) and thus in the VA-oxidizing activity of these enzymes. The variable amino acids present at position 172 in our amplified fragments, and the presence of Asp-residue at position 183, required for Mn-ion binding in MnPs, support the idea that the cloned fragments are from genes encoding MnP without VA-oxidizing activity.

Evolution of MnP genes in Heterobasidium and related genera.—The identical intron positioning in the MnP fragments and the high similarity of the genomic MnP sequences indicate close relatedness of *Amylostereum*, *Bondarzewia*, *Echinodontium* and *Heterobasidium*, consistent with the previous grouping of these genera, referred to as “Group 2” based on nuclear rDNA and mitochondrial rDNA sequences (Hibbett and Donoghue 1995, Hibbett et al 1997). The similarity of the amino-acid sequences of *Heterobasidium mnp2* and one of the fragments of *B. montana*, in particular, suggests a close relationship between these two genera and supports the placement of *Heterobasidium* in the *Bondarzewiaceae*. The MnP genes (*Tv-mnp2*, *Tv-mpg1*, and *Tv-pgv*) of the polypored fungus *Trametes versicolor* show the closest relationship to amino-acid sequences of the MnP genes of *Heterobasidium* and relatives. Our interpretation of these data is that the so-called “Group 2” genera split long ago from *Trametes* and that there has been considerable MnP gene duplication since that split.

Previous DNA sequencing of the internal transcribed (ITS) and intergenic spacer regions (IGS) of rDNA (Harrington et al 1998, Harrington and Rizzo 1999) have not differentiated European S-type and F-type groups, and these together were grouped with Asian and North American isolates as a “fir” lineage within *H. annosum* (Harrington et al 1998). However, differences in RAPD (La Porta et al 1997) and isozyme markers (Karlsson and Stenlid 1991, Otrosina et al 1993) indicated that S- and F-type isolates in

Europe were distinct. Mating type tests also have shown that European S and F types were infertile only partially (Korhonen et al 1992, 1997). Although these populations form an unresolved polytomy in *mnp1a* gene sequences, S and F types from Europe differed in the inferred amino-acid sequences of the other peroxidase genes. The Asian and North American isolates of the F-type had the same inferred amino-acid sequences of the S type, now recognized as *H. parviporum*. To date, all F-type (now *H. abietinum*) isolates have been from southern Europe. Failure of rDNA spacer sequences to separate closely related species that are sympatric has been noted in *Heterobasidium* and *Ceratocystis* (Harrington and Rizzo 1999, Witthuhn et al 2000). The identical rDNA spacer regions in European *H. parviporum* and in *H. abietinum* might have resulted from rare hybridization events in Europe, and along with the concerted evolution and homogenization of the nuclear ribosomal genes, only one of the parental rDNA types has emerged.

Asia appears to be the center of diversity for the genus *Heterobasidium*, and it has been speculated (Harrington et al 1998) that Asia or Australasia is the center of origin. *Heterobasidium parviporum* is the only species found in Europe, Asia and North America and shows the greatest diversity in rDNA and peroxidase sequences. Thus far, we have been able to amplify both *mnp1a* and *mnp1b* sequences only in *H. parviporum*. One of many interpretations of these data is that the *Heterobasidium* ancestor had both *mnp1a* and *mnp1b*, and lineage sorting has resulted in the other derived species having either of the two genes. Most of the sequence diversity in peroxidase amino-acid sequences and in rDNA is found in *H. parviporum* populations from Asia and North America, and either of these two continents might have served as the origin of *H. parviporum* (Harrington et al 1998).

Because of the limited variation at the amino-acid level and the relatively small number of isolates studied, it is difficult to infer phylogenetic relationships among the *Heterobasidium* species. The presence of *mnp1a* and *mnp1b* in *H. parviporum*, but only *mnp1a* in *H. abietinum*, might be explained by an early splitting of *H. abietinum* from *H. parviporum* (Korhonen et al 1997, Harrington et al 1998), with the loss of the *mnp1b* paralog in *H. abietinum*. This split might

←

uninformative. The trees were 255 steps in length, with a consistency index = 0.6863, a retention index of 0.9214, and a rescaled consistency index of 0.6323. Bootstrap values greater than 50 are indicated above the branches, and branches with 80% or greater bootstrap support are in bold.

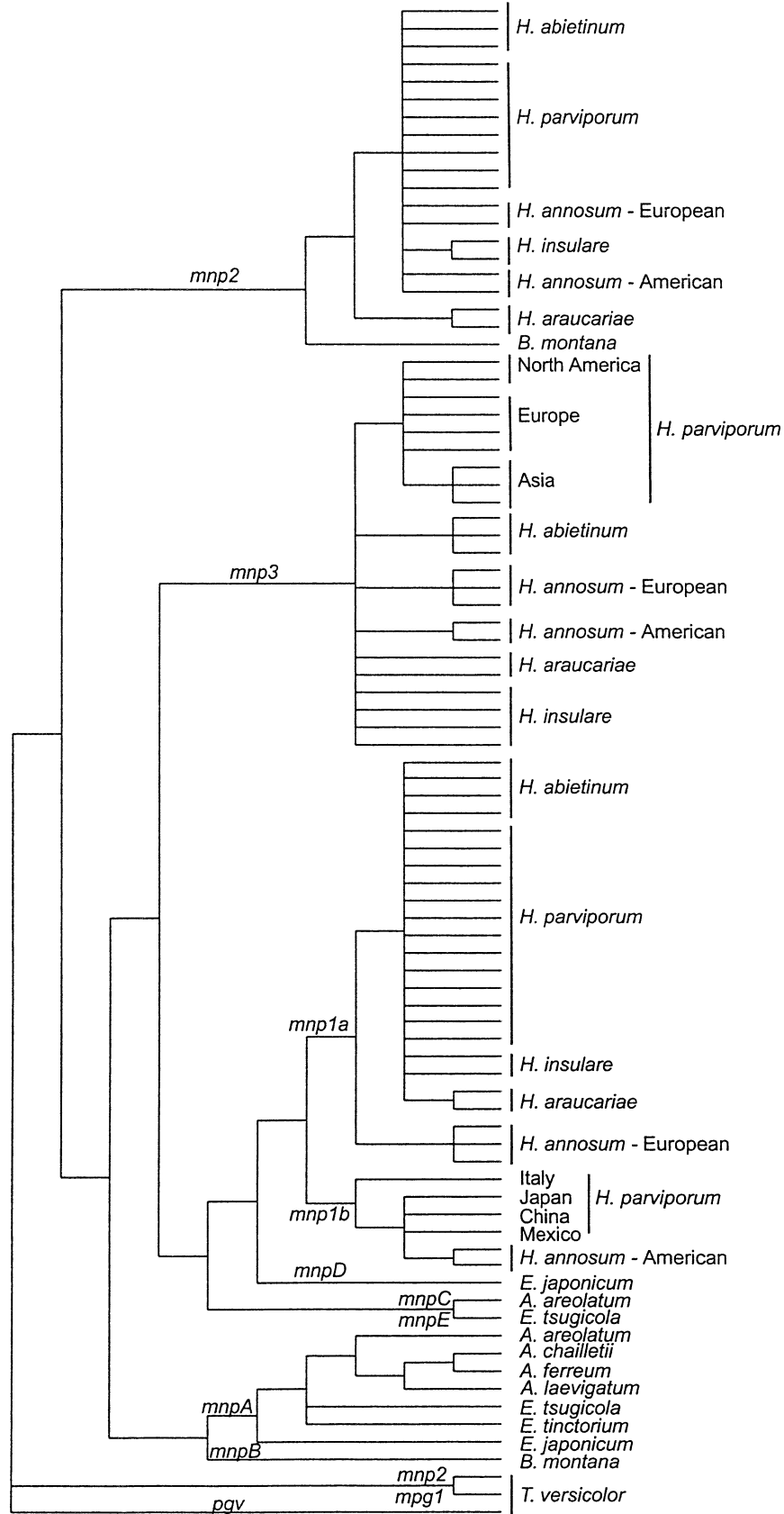


FIG. 5. A strict consensus tree of the 60 most-parsimonious trees of the partial amino acid sequences of the manganese peroxidase genes in *Heterobasidion*, *Amylostereum*, *Echinodontium*, *Bondarzewia*, and *Trametes*. The various peroxidase genes are indicated on the branches. One of the most-parsimonious trees is shown in FIG. 4.

have occurred in eastern Asia, with *H. abietinum* following a southerly route on *Abies* hosts and *H. parviporum* migrating later from Asia to Europe along the more northerly *Picea abies/Abies sibirica* host route to Europe (Korhonen et al 1997). Although *H. abietinum* is known only in Europe, analysis of peroxidase amino-acid data and the data from the rDNA spacer regions (Harrington et al 1998, Harrington and Rizzo 1999) is consistent with an Asian origin of the species (Korhonen et al 1997).

The identification of only *mnp1a* in the European form of *H. annosum* and only *mnp1b* in the American form of *H. annosum* further questions whether these two "pine" lineages are one species (Harrington et al 1998). Both ITS and IGS rDNA analyses fail to group these two geographically isolated populations (Harrington et al 1998, Harrington and Rizzo 1999). The low level of interfertility between European and American *H. annosum* isolates (Harrington et al 1989) further suggests that they represent distinct species.

The DNA and amino-acid sequences of ecologically important genes, such as the genes coding for lignin-degrading enzymes in white-rot fungi, present tremendous opportunity for inferring phylogenies. Such approaches, however, are hindered by the complexities of gene duplications, variation in sequences among alleles and lineage sorting. With the genus *Heterobasidion*, we have a relatively small group of well-characterized species with a well-known ecology and biogeography. Using degenerative primers, a unique class of MnP genes was identified in this genus and in related genera, and the amino acid sequences of these genes, although highly conserved, have proven superior to sequences of the rDNA-spacer regions in separating the recently diverged *H. parviporum* and *H. abietinum*. Analyses of the DNA sequences of these individual peroxidase genes with a larger sample of isolates should allow further insight into the evolutionary history of this genus.

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