Molecular characterization of the basidiomycete isolate *Nematoloma frowardii* b19 and its manganese peroxidase places the fungus in the corticioid genus *Phlebia*

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The basidiomycete isolate b19, originally identified by morphological characteristics of the fruiting body as *Nematoloma frowardii*, efficiently produces manganese peroxidase (MNP) and is used for degradation of natural, persistent aromatic polymers (lignin, humic acids and brown coal components). The *N. frowardii* MNP has shown good activity in conversion of xenobiotic compounds such as polycyclic hydrocarbons and trinitrotoluene. However, this biotechnologically promising fungus has not previously been studied at the molecular biology level. We show here that according to the molecular characterization of its main MNP isozyme, *Nf* b19 MNP2, and partial sequencing of its MNP3-, three lignin peroxidase- and two laccase-encoding genes, and the gene encoding the ribosomal SSU 18S RNA, that the fungus has a close phylogenetic relationship to the white-rot basidiomycete *Phlebia radiata* (Fr.). Ribosomal internal transcribed spacer (ITS) sequence (ITS1 + 5.8S + ITS2) phylogeny reclassifies *Nf* b19 as a possible representative of a new species of the genus *Phlebia*, nearest to the *Phlebia acerina* clade. The genus *Phlebia* belongs to a completely different family (Corticaceae) and order (Aphyllphorales) within the phylum Basidiomycota than the genus *Nematoloma*, which is classified in the order Agaricales, family Strophariaceae. Our results thus indicate a need for systematic re-identification of the previously named *N. frowardii* isolate b19.

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INTRODUCTION

Basidiomycetous wood- and litter-degrading white-rot fungi are unique in their ability to decompose the heterogeneous plant polymer lignin by using an array of extracellular lignin-modifying enzymes (LMEs): lignin, manganese and versatile peroxidases (EC 1.11.1.13–16), and laccases (EC 1.10.3.2). Recently, there has been great interest in the potential application of white-rot fungi and their LMEs, in particular manganese peroxidases (MNPs), and laccases in biopulping, biobleaching and

bioremediation of toxic compounds in soil and waters (Breen & Singleton, 1999; Hatakka, 2001; Baldrian, 2006).

The South American isolate *Nematoloma* (*Hypholoma*) *frowardii* b19 (*Nf* b19) is a potent producer of MNP, also on a larger scale for commercial enzyme production, and an efficient degrader of natural aromatic polymers like lignin and humic substances (Hofrichter & Fritsche, 1997; Hofrichter *et al.*, 1999). In addition, the MNP of *Nf* b19 is capable of mineralizing different aromatics directly, such as trinitrotoluene and polycyclic hydrocarbons (PAHs) (Sack *et al.*, 1997; Hofrichter *et al.*, 1998). Similarities between the lignin polymer degradation patterns and LME profiles of *Nf* b19 and other white-rot basidiomycetes were previously observed (Hofrichter & Fritsche, 1997) but extensive genetic studies of the fungus or the molecular characterization of its MNPs, or any other LMEs, have not been carried out.

It has been estimated that about 20 % of the fungal gene sequences deposited in public databases (DDBJ, EMBL, GenBank) are misidentified and even more are poorly annotated (Bridge *et al.*, 2003; Nilsson *et al.*, 2006), usually

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Abbreviations: GAPDH, glyceraldehyde phosphate dehydrogenase; ITS, internal transcribed spacer; LIP, lignin peroxidase; LME, lignin-modifying enzyme; LSU, ribosomal large subunit; MNP, manganese peroxidase; MP, maximum-parsimony; *Nf*, *Nematoloma frowardii*; *Pr*, *Phlebia radiata*; SSU, ribosomal small subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are given in Table 2.

due to incorrect taxonomic identification of the fungus. Taxonomic uncertainty is mostly caused by lack of distinguishable fruiting body, or failures in morphological identification and during the fungal isolation procedure. To avoid more confusion in fungal phylogeny, we believe it is vital to systematically perform comparative functional gene cloning and characterization studies for those fungal strains, such as the *Nf*b19 basidiomycete, that are stored in microbial culture collections and reported for use in biotechnological applications like enzyme production.

In this study, we describe the ORF of the gene (*Nf mnp2*) encoding the main MNP isozyme (MNP2) of isolate *Nf* b19. Molecular characterization of the *Nf* b19 MNP2, and fragments of its MNP3, three lignin peroxidase (LIP) and two laccase encoding genes, as well as of the ribosomal SSU 18S RNA and internal transcribed spacer ITS1+5.8S+ITS2 sequences used as phylogenetic markers, suggests that isolate *Nf* b19 in fact belongs to the white-rot corticioid genus *Phlebia*. According to the ITS phylogeny, *Nf* b19 is positioned close to the clade of the species *Phlebia acerina*, which is next to the *P. radiata* clade.

Our data imply a need for systematic reidentification of isolate *Nf* b19, in view of its evident molecular relatedness to *Phlebia* spp., which are classified in the family Corticiaceae (order Aphyllophorales, class Homobasidiomycetes) (Hibbett & Thorn, 2001) rather than in the previously proposed genus *Nematoloma* (*Hypholoma*) belonging to the family Strophariaceae (order Agaricales, class Homobasidiomycetes).

METHODS

Fungal strains and cultivation. *Nematoloma frowardii* b19 (DSM 11239) and *Phlebia radiata* 79 (ATCC 64658) were maintained on 2 %

malt extract (ME), 2 % agar slants. For LME production and isolation of DNA, the fungi were cultivated on LN-AS-glucose and ME broth media (Hildén *et al.*, 2005; Mäkelä *et al.*, 2006).

Amplification and cloning of LME-encoding genes. A genomic-PCR-based strategy was first used to clone and identify the *Nf mnp2* gene. The previously described degenerate primer pairs that were targeted at conserved *mnp* codon regions (Table 1) were used to amplify a 669 bp genomic fragment of *mnp2*. According to the nucleotide sequence, gene-specific primers were then designed (Table 1) to amplify the lacking 5' and 3' ends by genome walking using the Universal Genome Walker kit (Clontech). Cloning of the 1576 bp fragment on pCR2.1 in *Escherichia coli* TOPO (Invitrogen) yielded the full-length *mnp2* ORF. The same PCR-aided subcloning strategy was used to isolate partial fragments of *Nf mnp3*, *lip1*, *lip3*, *lip4*, *lac1*, *lac2* (laccase) and *gapdh* (glyceraldehyde phosphate dehydrogenase, GAPDH) genes with degenerate primer pairs (Hildén *et al.*, 2006; Mäkelä *et al.*, 2006). Cloning and sequencing were performed as previously described (Hildén *et al.*, 2005).

Sequence analyses. For primary sequence analyses, the software tools on the EBI-EMBL (<http://www.ebi.ac.uk>) and NCBI (<http://www.nih.ncbi.gov>) servers were used. The ITS and 18S sequences with highest identity were obtained by nucleotide BLAST searches (BLASTN, <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Protein and nucleotide phylogeny analyses were carried out using the MEGA 4.0 software (<http://www.megasoftware.net>) (Tamura *et al.*, 2007). For protein and nucleotide sequence phylogenies, both minimum evolution-neighbour joining (ME-NJ) and maximum-parsimony (MP) methods were adopted. Gonnet250 matrix was used for protein and DNA weight matrix for nucleotide sequences, respectively, in the multiple alignments (CLUSTAL W). Bootstrapping (1000 replicates) was conducted to test branching of the minimum-evolution trees. The MP trees were obtained using the close-neighbour-interchange algorithm with search level 4, in which the initial trees were obtained with the random addition of sequences (500 replicates). All alignment gaps were treated as missing data. The alignments were manually trimmed (overhangs were removed and gaps were corrected) prior to phylogenetic calculations.

Table 1. Oligonucleotides used as primers for gene amplification

Gene and primer type	5' primer (5'→3')	3' primer (5'→3')	Amplicon length (bp)
<i>Nf mnp2</i> , degenerate	gghggtgccgatggstc	tcractggagsgcgcac	669
<i>Nf mnp2</i> , full length	atggccttcaacttgctgc	ctacgagctgtctgcaccac	1576
<i>Nf mnp2</i> , 5'GW		gataccaagggttcggaagaagg	Arbitrary*
<i>Nf mnp2</i> , 5'GW nested		ggttcgaccgttaggaagatgag	Arbitrary*
<i>Nf mnp2</i> , 3'GW	ttcaaggacgctggtaacttcag		Arbitrary*
<i>Nf mnp2</i> , 3'GW nested	gtattaccgctgctgatcacgtt		Arbitrary*
<i>Nf mnp3</i>	accaggcgaggttgagt	gctctcggagtgaccgagg	289
<i>Nf lip1</i>	ccccactcactaccacact	cgttcggaaccggagaact	256
<i>Nf lip3</i>	tcgtctgactttccacgatg	gagcgatttctgtgacaga	961
<i>Nf lip4</i>	gga/t/cggtgccgatggg/ctc	ggccagaccttatactcagtcg	1324
<i>Nf lac1</i> , A	atgcacactttccttcgct	a/gtgg/actg/atgg/atacca/gaangt	556
<i>Nf lac1</i> , B	ggtgacaacttcagatc	cagcttgatcgggcacct	243
<i>Nf lac2</i>	atgcgctttacagcccttg	cgtgttcaaggccgtatctt	297
<i>Nf gapdh</i>	gagtcaccgggtgtcttcac	aagtgctgtggtgaccttg	215
<i>Nf</i> ITS/ <i>Pr</i> ITS	tccgtaggtgaacctgcgg (ITS1†)	tcctccgcttattgatgc (ITS4†)	642/637
<i>Nf</i> 18S/ <i>Pr</i> 18S	gtagtcatatgtctgtctc (NS1†)	gcatacagacctgttatgcctc (NS6†)	1424/1424

*Genome walking PCR with variable product size.

†<http://plantbio.berkeley.edu/~bruns/tour/primers.html>.

RESULTS AND DISCUSSION

Here we describe the complete ORF of the MNP2-encoding gene (*mnp2*), and deduced amino acid sequences of genomic fragments of another MNP-encoding gene (*mnp3*), as well as three LIP-, two laccase- and one GAPDH-encoding gene of the basidiomycete isolate *Nf* b19.

The degenerate primer pairs that were targeted at conserved codon regions, and the genome-walking approach, aided in obtaining the genomic ORF clone of *Nf* b19 *mnp2*. The nucleotide sequence with description of intron–exon structure is deposited in GenBank (accession EF491855, Table 2). The beginning of the predicted mature MNP2 was identical to the experimentally determined N-terminal peptide sequence of the *Nf* b19 MNP2 enzyme (M. Hofrichter and others, unpublished results) excluding the 23 aa leader peptide. Comparison of the primary structure of *Nf* b19 MNP2 reveals 96 % amino acid identity to the MNP2 of *Phlebia radiata* 79 (gene *Pr mnp2*, Table 2, Uniprot accession Q70LM3) with differences within 15 amino acids (Fig. 1) that are apparently not involved in enzyme catalysis or haem coordination.

The splicing positions of the seven introns were also identical to those detected within *Pr mnp2* (Hildén *et al.*, 2005). According to the gene and primary protein structure, *Nf* b19 *mnp2* belongs to the classical long MNPs within group B of fungal haem peroxidases (Hildén *et al.*, 2005; Martínez, 2002), where the so far cloned MNPs of the white-rot basidiomycetes *Ceriporiopsis subvermispora* (four *mnp* genes), *Dichomitus squalens* (two genes), *Phanerochaete chrysosporium* (four genes, five in the genome) and *Phanerochaete sordida* (three genes), and the long MNPs from *Physisporinus* (*Ceriporiopsis*) *rivulosus* (gene *mnpA*) (Hakala *et al.*, 2006), *Phlebia* sp. MG60 (two genes) and *Phlebia radiata* 79 (gene *mnp2*) (Hildén *et al.*, 2005) also belong (Fig. 2). These fungal *mnp* genes contain six or seven highly conserved short introns at similar positions (Hildén *et al.*, 2005; Hakala *et al.*, 2006).

Phlebia radiata 79 (*Pr* 79, ATCC 64658) was isolated in Finland as an efficient lignin-degrading white-rot fungus,

and was shown to produce three MNP and three LIP isoforms (Moilanen *et al.*, 1996; T. K. Lundell and others, unpublished results). Recently, we have cloned and characterized the *Pr* 79 *mnp2*, *mnp3*, *lip1*, *lip3* and *lip4* genes (Hildén *et al.*, 2005, 2006). The five lignin-modifying-peroxidase-encoding genes as well as two laccases (*Pr lac1* and *lac2*) are concomitantly expressed when the fungus is grown on its natural substrates, either hardwood or softwood (Hildén *et al.*, 2006; Mäkelä *et al.*, 2006).

In view of the high level of sequence identity between *Nf* b19 *mnp2* and *Pr mnp2*, we looked for the presence of other peroxidase- and laccase-encoding genes in isolate *Nf* b19 as previously found in *Pr* 79. This approach led to partial cloning of *mnp3*, *lip1*, *lip3*, *lip4*, *lac1*, *lac2* and *gapdh* gene fragments using PCR primer pairs designed for amplification of the respective genes from *Pr* 79. Within all these *Nf* b19 gene fragments (Table 2), introns were at identical positions and similar in length as in the respective *Pr* 79 genes. Moreover, nucleotide sequence similarity was over 80 % between the *Nf* b19 gene fragment and the corresponding region in the *Pr* 79 gene (Table 2).

Translated ORF regions of genomic *Nf* b19 *mnp3*, *lip1*, *lip3*, *lip4* and *gapdh* fragments showed 98 % amino acid identity to the predicted *Pr* 79 peroxidases and GAPDH (Fig. 3). The translated 51 aa of the *lac1* fragment was 100 % identical to the respective region in *Pr* Lac1 (Uniprot accession Q01679) whereas the 68 aa sequence of the *Nf* b19 *lac2* fragment was 89 % identical to *Pr* Lac2 (Uniprot accession Q0KHD1).

To more thoroughly investigate the phylogenetic relationship between these two seemingly unrelated basidiomycetous isolates, according to their original fruiting body morphology and taxonomic classification to two distinct orders and families under the class Homobasidiomycetes (Hibbett & Thorn, 2001), the nuclear ribosomal SSU 18S rDNA and more variable ITS (includes ITS1, 5.8S and ITS2) regions were amplified from *Nf* b19 and *Pr* 79 with the universal fungal primers NS1 and NS6, and ITS1 and ITS4 (Table 1), respectively, and cloned and sequenced.

Table 2. Nucleotide similarity between protein encoding genes of isolate *Nf* b19 and *Phlebia radiata* 79 (*Pr* 79)

Cloned genomic fragment	Nucleotide sequence accession (<i>Nf</i> b19/ <i>Pr</i> 79*)	Length (<i>Nf</i> b19/ <i>Pr</i> 79) (bp)	No. of introns†	Nucleotide similarity (%)
<i>mnp2</i>	EF491855/AJ315701	1576/1574	7 (7)	89
<i>mnp3</i>	EF491856/AJ310930	252/247	2 (11)	82
<i>lip1</i>	EF491857/AY743218	217/218	2 (11)	82
<i>lip3</i>	EF491858/AY749105	923/934	7 (11)	83
<i>lip4</i>	EF491859/AY745250	1275/1269	9 (11)	83
<i>lac1</i>	EF491860/X52134	206/208	1 (9)	80
<i>lac2</i>	EF491861/AJ884685	297/297	1 (12)	88
<i>gapdh</i>	EF491862/EF491863	231/231	1	93

*Accessions for *Pr* 79 are for complete protein-coding gene sequences except for *gapdh*.

†Number of introns within the cloned genomic fragment; the known number of introns in the completely cloned *Pr* 79 gene is given in parentheses.

<i>Nf</i> -b19	MNP2	MAFNFAAILAFVSLAAVTSAA PS GTTC	66
<i>Pr</i> -79	MNP2	MAFNFAAILAFVSLAAVTSAA PS KTTCS	66
<i>Nf</i> -b19	MNP2	LIFHDAIAISQSMGPSAGGGADG S MLIFPTVEPAFFPNLGIADSVNNLIPFLSQFPTISAGDLV Q F	132
<i>Pr</i> -79	MNP2	LIFHDAIAISQSMGPSAGGGADG S MLIFPTVEPAFFPNLGIADSVNNLIPFLSQFPTISAGDLV H F	132
<i>Nf</i> -b19	MNP2	AGAVAISNCPGAPQLEFLAG R SNATAPAI D GLIPEPQDDVT K ILAR F ADAGN F T P DEVVALLASH S	198
<i>Pr</i> -79	MNP2	AGAVAISNCPGAPQLEFLAG R PNATAPAI D GLIPEPQDDVT K ILAR F ADAGN F S P AEVVALLASH S	198
<i>Nf</i> -b19	MNP2	IARADHVDPTLDAAFPDSTPFD F DTQVFLEVLLKG T GF P GLD N NTGEVSSPLPVT D GT D VGELRL Q	264
<i>Pr</i> -79	MNP2	IARADHVDPTLDAAFPDSTPFD F DTQVFLEVLLKG V GF P GL A NTGEVSSPLPVT D GT D VGELRL Q	264
<i>Nf</i> -b19	MNP2	SDFV L ARDERTACAWQSFVNE Q QAMADAF K EAVKKLAVLGH S SDLVDCSAVVPVKPATGT P AT F	330
<i>Pr</i> -79	MNP2	SDF A LARDERTACAWQSFVNE Q EAMATA F K N AVKKLAVLGH N RNDLVDCSAVVPVKPATGT P AT F	330
<i>Nf</i> -b19	MNP2	PASTG P QDLELTCTT V PFPTLSTAPGAQQT L I P HCS D GT M TCNSVQFDGPATNFGGAD S	390
<i>Pr</i> -79	MNP2	PASTG P QDLELTCTT E PFPTLSTAPGAQQT L I P HCS D GT M TCNSVQFDGPATNFGGAD S	390

Fig. 1. CLUSTAL W (<http://www.ebi.ac.uk/ClustalW>) alignment of predicted MNP2 amino acid sequences of isolate *Nf* b19 (this work) and *Pr* 79 (Uniprot accession Q70LM3). Dissimilar amino acids are indicated in bold, and predicted N-terminal leader peptides are highlighted in grey.

Fig. 2. Maximum-parsimony (MP) subtree of typical long manganese peroxidases (MNPs) of group B of fungal class II secreted haem peroxidases (Hildén *et al.*, 2005). The translated ORF amino acid sequence of the *Nf* b19 *mnp2*, here named as *Nf*-b19 *Phlebia* sp. MNP2 (in-frames), and other predicted basidiomycetous MNPs were retrieved via UniProt (<http://beta.uniprot.org>) search. Only complete ORF amino acid sequences were included in the multiple alignment. The subtree of a MP consensus tree inferred from 19 most parsimonious trees, generated with 92 taxa, is shown. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. The MP tree was obtained using the close-neighbour-interchange algorithm (Tamura *et al.*, 2007) with search level 2, in which the initial trees were obtained with the random addition of sequences (500 replicates). Fungal species names are followed by translated protein name and sequence accession (dbj, DDBJ; emb, EMBL; gb, GenBank).

<i>Nf</i> -b19	MNP3	PLHGEIRLQSDSELARDSRTACEWQSFVNNQAKIQSAF R AAFRKMT	46
<i>Pr</i> -79	MNP3	PLHGEIRLQSDSELARDSRTACEWQSFVNNQAKIQSAF K AAFRKMT	46
<i>Nf</i> -b19	LIP1	PLTTTPHFPAKG S MADVEQACAETFPFALRSDPGPQTAVPAVPSSPVRN	35
<i>Pr</i> -79	LIP1	PLTTTPHFPAKG L ADVEQACAETFPFALRSDPGPQTAVPAVPSSPVRN	35
<i>Nf</i> -b19	LIP3	IAISPAMEATGQFGGGGADGSIMIFSDIET A FHPNIGLDEVVESFRPFQQRSGMGVADFIQFSGAV	66
<i>Pr</i> -79	LIP3	IAISPAMEATGQFGGGGADGSIMIFSDIET K FHPNIGLDEVVESFRPFQQRSGMGVADFIQFSGAV	66
<i>Nf</i> -b19	LIP3	GTSNCPGAPT L NAFIGRKDATQAAPDGLVPEPFHDVNTILARFNDAGDFDELET V WFLIAHSVAAQ	132
<i>Pr</i> -79	LIP3	GTSNCPGAPT L NAFIGRKDATQAAPDGLVPEPFHDVNTILARFNDAGDFDELET V WFLIAHSVAAQ	132
<i>Nf</i> -b19	LIP3	NDIDPTVSHAPFDSTPSVMDGQFFIETQLRG I EFIGTGGIEGVAESPVKGEFRLM	187
<i>Pr</i> -79	LIP3	NDIDPAVSHAPFDSTPSVMDGQFFIETQLRG V EFIGSGGIEGVAESPVKGEFRLM	187
<i>Nf</i> -b19	LIP4	IMIFSKTETA F HHPNIGLDEVVESFRPFQERSGMGVADFIQFSGAVGTSNCPGAPVLNVSIGRVDAT	66
<i>Pr</i> -79	LIP4	IMIFSKTETA F HHPNIGLDEVVESFRPFQERSGMGVADFIQFSGAVGTSNCPGAPVLNVSIGRVDAT	66
<i>Nf</i> -b19	LIP4	R AAPDGLVPEPFHDVNTILARFADAGEFDELET V WFLIAHSVAAQNDIDPTVSHAPFDSTPEIMDG	132
<i>Pr</i> -79	LIP4	R PAPDGLVPEPFHDVNTILARFADAGEFDELET V WFLIAHSVAAQNDIDPTVSHAPFDSTPEIMDG	132
<i>Nf</i> -b19	LIP4	QFFIETQLRGEGFIGK S GIEGVAKSPVRGEFRLQSD E LLARDNRTACEWQSFQTDQAKLQNR F QFI	198
<i>Pr</i> -79	LIP4	QFFIETQLRGEGFIGK A GIEGVAKSPVRGEFRLQSD E LLARDNRTACEWQSFQTDQAKLQNR F QFI	198
<i>Nf</i> -b19	LIP4	FQAMGELGTDPTTLIDCSDVLP T PPDLTTVPHPAGV R INDVQAACAETFPF T LPTDPGPATAV A AVP	264
<i>Pr</i> -79	LIP4	FQAMGELGTDPTTLIDCSDVLP T PPDLTTVPHPAGV S INDVQAACAETFPF T LPTDPGPATAV A PVP	264
<i>Nf</i> -b19	LAC1	NVIDELTNATMLKTTT I HWHGFFQHGTNWADGPAF I NCPIASGDSFLYN F	51
<i>Pr</i> -79	LAC1	NVIDELTNATMLKTTT I HWHGFFQHGTNWADGPAF I NCPIASGDSFLYN F	51
<i>Nf</i> -b19	LAC2	AALL P SLLSEV H ATN N GIGPVTDL H ISNAVVSPDGF S RD A VVVQGRF S PLIKGNKG D R F LLNVIN Q L	68
<i>Pr</i> -79	LAC2	ATLL S SLLSEV Y ATN S GIGPVTDL R ISNAVVSPDGF S RD A VVVQGF S PLIKGNKG D M F KLNVIN Q L	68
<i>Nf</i> -b19	GAPDH	K IETAS A HLAGGAKKV I ISAPSADAPMFVVGVN L DKYDSSYKV I SNASCTTN C LAP L	57
<i>Pr</i> -79	GAPDH	T VETAS A HLAGGAKKV I ISAPSADAPMFVVGVN L EKYDSSYKV I SNASCTTN C LAP L	57

Fig. 3. CLUSTAL W (<http://www.ebi.ac.uk/ClustalW>) alignment of the translated partial LME (MNP, LIP, LAC) and GAPDH amino acid sequences from isolate *Nf* b19 and *Pr* 79.

The SSU and LSU rRNA-encoding genes are highly conserved and universally present in all living organisms, permitting phylogenetic comparisons among distantly related species (Berbee & Taylor, 2001). The ITS1 and ITS2 regions, in contrast, can have more nucleotide variations since their transcripts are excised from the final rRNA fragments. Therefore, the ITS sequence including both ITS1 and ITS2, which are separated by the conserved short 5.8S rRNA, has been commonly used to infer phylogenetic relationships of closely related species as well as to assess the variability of a population, e.g. of geographically distant isolates (ecotypes).

The amplified and cloned partial rDNA 18S and ITS *Nf*b19 products (Table 1), yielding GenBank accessions EF491865 for the 18S and EF491864 for the ITS sequence, respectively, showed the highest level of similarity to corresponding sequences from *Phlebia* species according to FASTA search (<http://www.ebi.ac.uk/fastas33>). This notion was further confirmed by amplifying and cloning also the 18S and ITS regions of *Pr* 79: the resulting DNA fragments were of similar size to those obtained for *Nf*b19 (sequence accession EF491866 for *Pr* 79 18S, and EF491867 for *Pr* 79 ITS) (Table 1). While submitting the sequences, we noticed recent, unpublished sequence deposits of the same *Pr* 79

Fig. 4. Maximum-parsimony (MP) tree of 18S rRNA sequences from species of the fungal genera *Phlebia*, *Phlebiopsis*, *Phanerochaete*, *Nematoloma* and *Hypholoma*. The corresponding sequence of a heterobasidiomycete, *Cryptococcus* (*Filobasidiella*) *neoformans*, is displayed as outgroup (root). *Nf* b19 *Phlebia* sp. and *Phlebia radiata* 79, this study. The consensus tree of 27 taxa, inferred from 11 023 most parsimonious trees, is shown. Branches corresponding to partitions reproduced in less than 50 % of trees are collapsed. The consistency index was 0.834197 (0.683168), the retention index was 0.849765 (0.849765), and the composite index was 0.708872 (0.580533) for all sites and (in parentheses) parsimony-informative sites. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. The MP tree was obtained using the close-neighbour-interchange algorithm (Tamura *et al.*, 2007) with search level 4, in which the initial trees were obtained with the random addition of sequences (500 replicates). All alignment gaps were treated as missing data. There were a total of 1341 positions in the final dataset, out of which 62 were parsimony informative. Fungal species names are followed by sequence accessions (dbj, DDBJ; emb, EMBL; gb, GenBank).

(ATCC 64658) (accession AY946267 for 18S, and DQ056859 for ITS) that were 100 % and 99.9 % identical with our partial *Pr* 79 18S and ITS sequences, respectively.

The 18S and ITS sequences were further analysed by molecular evolutionary computing methods. Ribosomal SSU 18S partial (1324 nt) sequences of species belonging to the genera *Phlebia*, *Phlebiopsis* and *Phanerochaete* of the order Aphyllophorales, family Corticiaceae, and *Hypholoma* (*Nematoloma*) species of the order Agaricales, family Strophariaceae, were compared by applying both minimum-evolution and maximum-parsimony approaches (Fig. 4). The tree topologies were similar with both methods. The 18S sequence of the heterobasidiomycete yeast-like *Cryptococcus* (*Filobasidiella*) *neoformans* was used as an outgroup. The 18S tree reveals close positioning of *Nf* b19 to the *Phlebia radiata* clade branch, nearest to *Phlebia*

sp. strain DABAC9, which is not given any species-level identity. The latter fungus was recently isolated by plating aqueous extracts from a soil that was heavily contaminated with PAHs (D'Annibale *et al.*, 2006).

However, the more extensive ITS trees constructed with all the available ITS1-5.8S-ITS2 sequences of good quality and 520–560 nt in length from *Phlebia* spp. and members of closely related genera (Fig. 5) imply that *Nf* b19 may be either (i) another more diverged isolate belonging to the species *Phlebia acerina*, or (ii) a completely new *Phlebia* species positioned between the *P. acerina* and *P. radiata* clades. To finally clarify the taxonomic position of the *Nf* b19 isolate, it will be necessary to complement our molecular studies by classical fungal genetics, e.g. using mating experiments (Kausarud *et al.*, 2006).

Fig. 5. Evolutionary relationships of *Phlebia* spp. (47 taxa) according to their ribosomal ITS sequences (ITS1-5.8S-ITS2). Species from the closely related fungal genera *Phanerochaete* and *Phlebiopsis*, and four representatives from the agaric genus *Hypholoma* (*Nematoloma*) were included for taxonomic comparison. The MP tree was rooted with the corresponding ITS sequence from the heterobasidiomycete *Cryptococcus* (*Filobasidiella*) *neoformans*. The consensus tree inferred from 270 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index is 0.537627 (0.496850), the retention index is 0.774045 (0.774045), and the composite index is 0.416147 (0.384585) for all sites and (in parentheses) parsimony-informative sites. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. The MP tree was obtained using the close-neighbour-interchange algorithm (Tamura *et al.*, 2007) with search level 4, in which the initial trees were obtained with the random addition of sequences (500 replicates). All alignment gaps were treated as missing data. There were a total of 683 positions in the final dataset, out of which 326 were parsimony informative. Fungal species names are followed by sequence accessions (dbj, DDBJ; emb, EMBL; gb, GenBank).

Our ITS trees indicated furthermore that *Phlebia* sp. DABAC9 (D'Annibale *et al.*, 2006) is another isolate of the species *P. acerina* (Fig. 5). Five of the six ITS sequences retrieved for *P. radiata* collapsed within one branch including our *Pr* 79 ITS, implying that also the *P. radiata* CBS, AFTOL and Japanese isolates are of the same origin as our *P. radiata* 79 (ATCC 64658).

It has recently been demonstrated that strains of *Phlebia acerina* together with *Phlebia lindtneri* and several *Phlebia brevispora* isolates are capable of degrading chlorinated dibenzo-*p*-dioxins (Kamei *et al.*, 2005; Kamei & Kondo, 2005). Moreover, the same authors have reported the ability of the *P. lindtneri* isolate USDA GB1027 to biotransform chloronaphthalenes and other PAHs (Mori *et al.*, 2003). Interestingly in this respect, the basidiomycete *Nf* b19, which was found and isolated in Bariloche (Argentina), the *Phlebia* sp. DABAC from Italy, and six strains of *P. acerina* from Japan, which all possess promising degradative capabilities concerning the removal of hazardous organopollutants, are most closely related to the *P. radiata* clade (Fig. 5). Accordingly, the recently isolated saline-tolerant fungus *Phlebia* sp. MG-60 (Kamei *et al.*, 2007) and *P. lindtneri* USDA GB1027, both Japanese isolates, branch next to the *P. radiata* and *P. acerina* clades. As noted before in an ITS-phylogenetic study on *Phanerochaete* spp. (De Koker *et al.*, 2003), the genus *Phlebia* is noticeably polyphyletic, which is also evident from our ITS and 18S trees outside the *P. acerina*, *P. radiata* and *P. brevispora* clades (Figs 4 and 5).

According to the 18S and ITS sequence phylogeny, the only other representative of the genus *Nematoloma*, *N. longisporum* isolate AFTOL 1893, clusters with strains of the genus *Hypholoma* (Figs 4 and 5). In the class Homobasidiomycotina, the genus *Hypholoma* (*Nematoloma*) belongs to the order Agaricales, family Strophariaceae, whereas the genus *Phlebia* is classified to the order Aphyllophorales, family Corticiaceae (Hibbett & Thorn, 2001).

Previous reports on the efficiency of *Pr* 79 and *Nf* b19 in decomposition and mineralization of synthetic lignin (DHP) (Lundell *et al.*, 1990; Moilanen *et al.*, 1996; Hofrichter *et al.*, 1999) and similarities between the catalytic properties of MNP2 of *Nf* b19 (Hofrichter *et al.*, 1998), and MNP2 and MNP3 of *Pr* 79 (Karhunen *et al.*, 1990; Hofrichter *et al.*, 2001; T. K. Lundell and others, unpublished results) can now be explained by the evident taxonomic proximity of the fungal isolates, implying favourable features for environmental biotechnology. These enzymic data are furthermore supported by very recent cloning of two additional long MNP-encoding genes from another *Phlebia* isolate, *Phlebia* sp. MG-60 (Kamei *et al.*, 2007). The latter MNP2 and MNP3 show high sequence identity and phylogenetic branching next to the *Nf* b19 MNP2 and *P. radiata* MNP2 (Fig. 2).

Since the basidiomycete *Nf* b19 was originally isolated from a typical agaric fruiting body growing on wood (Hofrichter

& Fritsche, 1997), it can be assumed that both *N. frowardii* and the b19 *Phlebia* sp. strain were present in the decaying log, but only the latter organism survived during the cultivation and preservation procedures. According to the molecular identification on protein-encoding genes (*mnp*, *lip*, *lac*, *gapdh*), and ribosomal 18S rRNA and ITS sequences, we recommend designating the basidiomycete isolate as *Phlebia* sp. *Nf* b19 until a systematic reidentification of the fungus has been accomplished.

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