The Journal of Microbiology, August 2009, p. 431-440 Vol. 47, No. 4
DOI 10.1007/s12275-008-0166-3

Identification and Characterization of a Class III Chitin Synthase Gene of *Moniliophthora perniciosa*, the Fungus That Causes Witches' Broom Disease of Cacao

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(Received July 2, 2008 / Accepted April 1, 2009)

Chitin synthase (CHS) is a glucosyltransferase that converts UDP-N-acetylglucosamine into chitin, one of the main components of fungal cell wall. Class III chitin synthases act directly in the formation of the cell wall. They catalyze the conversion of the immediate precursor of chitin and are responsible for the majority of chitin synthesis in fungi. As such, they are highly specific molecular targets for drugs that can inhibit the growth and development of fungal pathogens. In this work, we have identified and characterized a chitin synthase gene of *Moniliophthora perniciosa* (*Mopchs*) by primer walking. The complete gene sequence is 3,443 bp, interrupted by 13 small introns, and comprises a cDNA with an ORF with 2,739 bp, whose terminal region was experimentally determined, encoding a protein with 913 aa that harbors all the motifs and domains typically found in class III chitin synthases. This is the first report on the characterization of a chitin synthase gene, its mature transcription product, and its putative protein in basidioma and secondary mycelium stages of *M. perniciosa*, a basidiomycotan fungus that causes witches' broom disease of cacao.

***Keywords*:** cell wall, chitin, chitin synthase, witches' broom disease, cacao

The fungal cell wall is a dynamic structure that plays a fundamental role in fungal growth and development, as well as the interaction of fungi with their surroundings. It is directly involved in many important biological processes such as morphogenesis, antigenic expression, adhesion, and cell-cell interaction. The cell wall protects the cell from changes in osmotic pressure and other environmental stresses, allowing fungal hyphae to retain their shape and integrity (Bowman and Free, 2006).

Fungal cell walls are comprised of glycoproteins, with both N- and O-linked carbohydrates, and polysaccharides, mainly beta-1,3-glucan and chitin (Latgé, 2007). Chitin, the β-1,4-linked natural linear homopolymer of N-acetylglucosamine, is a structural endogenous carbohydrate, which constitutes, together with β-linked glucans, the highly crystalline, water insoluble components of the innermost region of hyphal cell wall of true fungi (Lewis, 1991). Chitin is crucial to the architecture and integrity of the fungal cell wall. When chitin synthesis is disrupted, the cell wall becomes malformed and osmotically unstable, which may result in cell death (Bago *et al*., 1996; Behr, 2003).

Chitin is produced by the integral membrane enzyme chitin synthase (CHS) (E.C. 2.1.4.16), a glucosyltransferase that catalyzes the irreversible transfer of N-acetylglucosamine residues from uridine diphosphate (UDP)-N-acetylglucosamine to a growing chitin chain (Durán *et al*., 1975). This enzyme was originally described by (Glaser and Brown, 1957), but the first gene encoding a fungal chitin synthase was isolated and characterized only about thirty years after the description of the enzyme's activity (Bulawa *et al*., 1986).

Multiple chitin synthase isoforms are produced by fungi, and at least six distinct classes of chitin synthases (class I-VI) are currently recognized based on their conserved primary, secondary, and tertiary structures (Choquer *et al*., 2004). Fungal chitin synthase isoenzymes may have different expre-

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ssion levels depending on the stage of the life cycle and cellular location. Class III chitin synthases are essential for fungal development since they are directly involved in cell wall biosynthesis and are responsible for the majority of chitin synthesis under normal growth conditions (Roncero, 2002; Ruiz-Herrera *et al*., 2002).

Chitin synthesis has been considered a promising target for anti-fungal agents, largely due to the structural integrity that chitin provides to the fungal cell (Behr, 2003). Disruption of some chitin synthase genes from *Saccharomyces cerevisae*, *Aspergillus fumigatus*, and *Neurospora crassa* significantly reduces total chitin synthesis and compromises cell wall integrity (Roncero, 2002).

Chitin synthases have been mainly studied in Ascomycota (Ruiz-Herrera *et al*., 2002; Choquer *et al*., 2004), and fully characterized basidiomycotan chitin synthase genes have been reported for only seven distinct species: one Ustilaginomycotina (*Ustilago maydis*) (Kamper *et al*., 2006; Weber *et al*., 2006); one Pucciniomycotina (*Puccinia graminis*) (Broeker *et al*., 2006); and five Agaricomycotina (*Agaricus bisporus*, *Coprinopsis cinerea*, *Filobasidiella neoformans* (Anamorph: *Cryptococcus neoformans*), and *Pleurotus ostreatus* (Sreenivasaprasad *et al*., 2000; Birren *et al*., 2003; Loftus *et al*., 2005; Nishihara *et al*., 2007).

The basidiomycete *Moniliophtora perniciosa* (Stahel) Aime and Phillips-Mora [previously known as *Crinipellis pernicosa* (Stahel) Singer)] is the causative agent of witches' broom disease of the cacao tree (*Theobroma cacao*), whose seeds are the source of chocolate (Aime and Phillips-Mora, 2005). It is the most important phytopathological problem of cacao-producing areas of the American continent, and has decimated the Brazilian cacao industry (Griffith *et al*., 2003). In the last few years, a number of studies have elucidated details of chitinase regulation (Lopes *et al*., 2008), chitin metabolic pathway (Pirovani *et al*., 2005), and the genetic variability of the fungus (Rincones *et al*., 2006). Additionally, the mitochondrial genome (Formighieri *et al*., 2008) and, more recently, the whole genome (Mondego *et al*., 2008) of the organism have been sequenced.

In this work we have identified and characterized for the first time, by primer walking in genomic DNA, a class III chitin synthase gene, the terminal region of its mature transcription product by cDNA analysis, and its putative protein product from the unusual pathogenic agaric *M. perniciosa* in basidioma and secondary mycelium stages.

Materials and Methods

Data mining and annotation of putative segments of chitin synthase gene in *M. perniciosa* Genome Project Database

Data mining and annotation of putative segments of chitin synthase gene in the *M. perniciosa* genome project database (http://www.lge.ibi.unicamp.br/vassoura) comprised five steps: (i) reads gathering, (ii) screening of collected reads, (iii) contigs assembly, (iv) screening of assembled contigs, (v) comparative sequence analysis of contigs and identification of putative homologs at both gene and protein levels (Fayyad, 1996).

The resulting reads of both text-based querying and sequence similarity searches were inspected. Those that may have doubtful or spurious information were eliminated and only high quality reads (*E*-value $\leq 1\times10e^{-5}$ and sequence with at least 250 contiguous bases with a Phred value ≥ 20) were considered significant and used for further analyses.

The assembly of the putative genes from segments derived from whole shotgun genomic sequencing was made using the program Phrap (Green, 2007). The formed contigs were then compared with complete sequences of genes and corresponding proteins of Basidiomycota stored in GenBank (NCBI) by similarity analysis using both BLAST version 2.2.16 (Altschul *et al*., 1997) and FastA version 3.46 (Pearson, 1990) to evaluate conserved and variable regions.

Primers design

After analysis of the putative segments of the chitin synthase gene in the *M. perniciosa* genome project database, several specific primers (sense and anti-sense) were designed and used to determine unknown regions of genomic DNA segments. Primers were manually designed and verified by comparative similarity analysis of Basidiomycota gene sequences using both BLAST version 2.2.16 and FastA version 3.46.

Fungal isolate and culture conditions

Isolate CCMB 000257 (Culture Collection of Microorganisms of Bahia-CCMB, Feira de Santana, BA, Brazil, http://www.uefs.br/ccmb), obtained from field-collected basidiomata in Experimental Station Sósthenes of Miranda (ESOMI-CEPLAC) (São Sebastião of the Passé, BA, Brazil, 12° 30' S, 38° 29' W), was used for the extraction of genomic DNA of *M. perniciosa*.

Genomic DNA and total RNA extractions

Genomic DNA was extracted largely according to Doyle and Doyle (1987), and total RNA was extracted using TRIZOL® following manufacturer instructions. Aliquots of DNA solutions were further deposited in the UEFS Microorganism DNA Bank (State University of Feira de Santana, Feira de Santana, Brazil).

PCR amplification of target segments

PCR reactions were performed to amplify cDNA segments from total RNA and unknown genomic DNA segments from known segments by primer walking (Parker *et al*., 1991) as well as using several combinations of primer pairs.

Cycle parameters were as follows: 1 initial denaturation cycle at 94°C for 3 min, followed by 35 denaturation cycles at 94°C for 45 sec and annealing at 61.6°C for 45 sec (Pchsic13.3F / Pchsic13.3R); 59.7°C for 45 sec (Pchsic34F / Pchsic34R), 65°C for 60 sec (all other primer pairs) and extension at 72°C for 45 sec, with a final extension cycle at 72°C for 10 min. Negative controls, containing all reagents except for *M. perniciosa* genomic DNA, were prepared in each of the three PCR replicates to exclude the possibility of contamination in the reagents.

Sequencing of PCR products

Purified PCR products of genomic and cDNA were directly sequenced with the same primers used in the PCR amplifi-

cation. PCR products were labeled with BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), and sequences were determined in an automatic sequencer (ABI Prism 3100, Applied Biosystems). At least three sequences for each direction (forward and reverse) were obtained from each segment to assure correct base calling in all positions.

Sequence analysis

Sequences derived from primer walking of genomic DNA and cDNA, and those previously analyzed from *M. perniciosa* genome database were assembled with SeqMan (Lasergene v. 7.0) (Burland, 2000). The entire edited consensus sequence was translated to all possible (six frames) protein sequences using EditSeq and submitted for comparative similarity analysis with complete protein and gene sequences of Basidiomycota deposited in NCBI/EMBL/DDDJ, using both BLAST (v. 2.2.16) and FastA (v. 3.46).

Determination of exon-intron borders and the number, size, and sequence of introns present in the putative *M. perniciosa* chitin synthase gene were performed by intrinsic (*ab initio*) and extrinsic (comparative) methods. The intrinsic methods comprised an analysis performed on trained neural networks with exon-intron borders (Wang *et al*., 2008) and in AUGUSTUS (Stanke and Waack, 2003) with a previously built model for *M. perniciosa* to find gene models in the gDNA. The extrinsic methods encompassed a manual comparison to genomic DNA sequences of homologous genes of Basidiomycota with traditional sequence similarity tools and an automatic comparative method using GeneWise (Birney *et al*., 2004). A cDNA library of *M. perniciosa* was also analyzed to search for clones containing EST that could validate the gDNA data. Furthermore, we have experimentally determined the terminal region of the transcription product of the gene by cDNA analysis.

Analysis of conserved motifs and domains of the putative *M. perniciosa* chitin synthase protein was carried out using InterPro (Mulder and Apweiler, 2007).

Complete gDNA and amino acid sequences for *Moniliophthora perniciosa* class III chitin synthase, described in this paper, were assigned GenBank accession no. EU154354 and ABW09311, respectively. Partial cDNA derived from mRNA of basidioma and saprophytic mycelium stages were assigned GenBank accession no. FJ765527.

Table 1. *M. perniciosa* reads related to *Agaricus bisporus* CHS1 (emb|CAB96110.1), identified by BLASTx in the witches' broom genome project database (http://www.lge.ibi.unicamp.br/vassoura)

Read	Contig	*E*-Value
CP02-S3-000-118-A08-UC.G CP02-PF-096-002-C08-UC.F CP02-PF-096-002-C08-UC.G	3	$6e^{-37}$
CP02-S2-000-036-D04-EM.R CP02-S2-000-166-A03-CL.F CP02-S2-000-166-C10-UC.F	4	$1e^{-82}$
CP02-S1-000-007-A08-CL.F CP02-S2-038-242-G02-EM.F CP02-S2-000-180-A03-UC.F CP02-S2-000-138-A09-EM.R CP02-S1-000-015-C08-EM.F CP02-S2-000-032-G09-EM.R	5	$1e^{-88}$

Phylogenetic analyses

The data set consisted of the putative *M. perniciosa* chitin synthase (*Mop*CHS) and complete fungal protein sequences of chitin synthase with identity ≥50% to MopCHS in NCBI/EMBL/DDDJ, as determined by BLASTp 2.2.17 using the matrix BLOSSUM 80. Sequences were aligned using TCoffee (Notredame *et al*., 2000) and phylogenetic analyses were performed in PAUP 4.0b10 (Swofford, 2002), PHYLIP 3.6 (Felsenstein, 2005), and Mr. Bayes 3.1 (Ronquist and Huelsenbeck, 2003), using distance, maximum parsimony, maximum likelihood, and Bayesian methods. Trees were rooted in the branch whose terminal was *Tuber borchii* Kauffman (Ascomycota, Pezizomycetes) since Ascomycota is the sister group of Basidiomycota, and inside Ascomycota, since Pezizomycetes probably originated before corresponding groups of the other sampled species in the data set (James *et al*., 2006).

Mean distances and a neighbor-joining algorithm were used for distance analysis, unweighted parsimony for maximum parsimony analysis and JTT (Jones-Taylor-Thornton) +Γ rate model (alpha=0.5) were used for maximum likelihood and bayesian analyses. Three independent runs were conducted (each with four chains) for 1×10^6 generations, sampling every 100 generations for bayesian analysis. Clade robustness was assessed using bootstrap proportions (1,000 replicates) for distance, maximum parsimony, and likelihood

Table 2. Primers used for amplification of *M. perniciosa chs* gene. F, forward primer; R, reverse primer. All primers were specifically designed for this study

Primer	Sequence (5'-3')	Position in genomic DNA (first base)
Pchsic34-F	ggT gAA CgT gCA gCA TAC ACg	2,352
Pchsic34-R	gCT CCC ACT ggT CCT Tgg AAg	2,585
Pchsic13.3-F	CCA CCg TTg gTT gTT CAA CgC C	1,192
Pchsic13.3-R	gCC AAC gAA TgA TCC CCA Tgg	1,644
Pchs54-F	CTA CTA Tgg CgA ATC gCC Cg	1
Pchs54-R	CAT TTA gTT CCg TCT gCA ACA TCT g	3,422
ChsHindIII-F(13F)	Agg gcA ACT TCA TCA CCg AgT ATC C	475
ChsXholI-R (13-R)	CgT ggC TCg Agg gTgC ACC AgT Tg	1,097
ChsHindIII-F(10F)	ggg TAC gAA AgC TTg CgA TAA AgC C	2,936
ChsXholI-R (10-R)	gAA gTA CTC gAg ACA CTg TCA Tgg	3,381

analyses, and posterior probabilities proportions for Bayesian analysis. Trees were edited using TreeView 1.6.6 (Page, 1996).

Results and Discussion

Data mining in *M. perniciosa* Genome Project Database

Data mining in the *M. perniciosa* Genome Project Database resulted in 72 reads with the formation of 19 contigs. None of these contigs harbored the entire putative gene encoding a chitin synthase, but some had *E*-values ($E \leq 10e^{-10}$) that were statistically significant. Three contigs (iContig3, iContig4, and iContig5) presented highly significant identity ($E \leq 10e^{-37}$) to *A. bisporus* (emb|CAB96110.1) chitin synthase protein (Table 1), and were successfully mapped on it, resulting in a 76% discontinuous coverage with two gaps in the median area of the putative *Mopchs* gene.

The putative translation initiation codon of *M. perniciosa* chitin synthase was determined by similarity, comparing the initial portion of iContig5 with the initial portion of the homologous genes of *A. bisporus* (emb|CAB96110), *P. graminis* (gb|ABB70409), and *P. ostreatus* (dbj|BAF37219). Although we could not confidently identify a motif corresponding to a TATA box either in our sequence or in other sequenced basidiomycotan chitin synthases (Sreenivasaprasad *et al*., 2000; Birren *et al*., 2003; Loftus *et al*., 2005; Broeker *et al*., 2006; Kamper *et al*., 2006; Weber *et al*., 2006; Nishihara *et al*., 2007), we noticed an AT-rich region of six nucleotides (TTAATT) located 39 bp upstream of the putative translation initiation codon. This region may be the promoter region of the gene since eukaryotic genes present transcription initiation signals (an AT-rich 6-7 nucleotide region) 20 to 40 bases upstream of the translation initiation codon (Smale and Kadonaga, 2003).

The translation of iContig3 (frame +2), revealed a QRRRW motif, considered as the signature of fungal chitin synthases

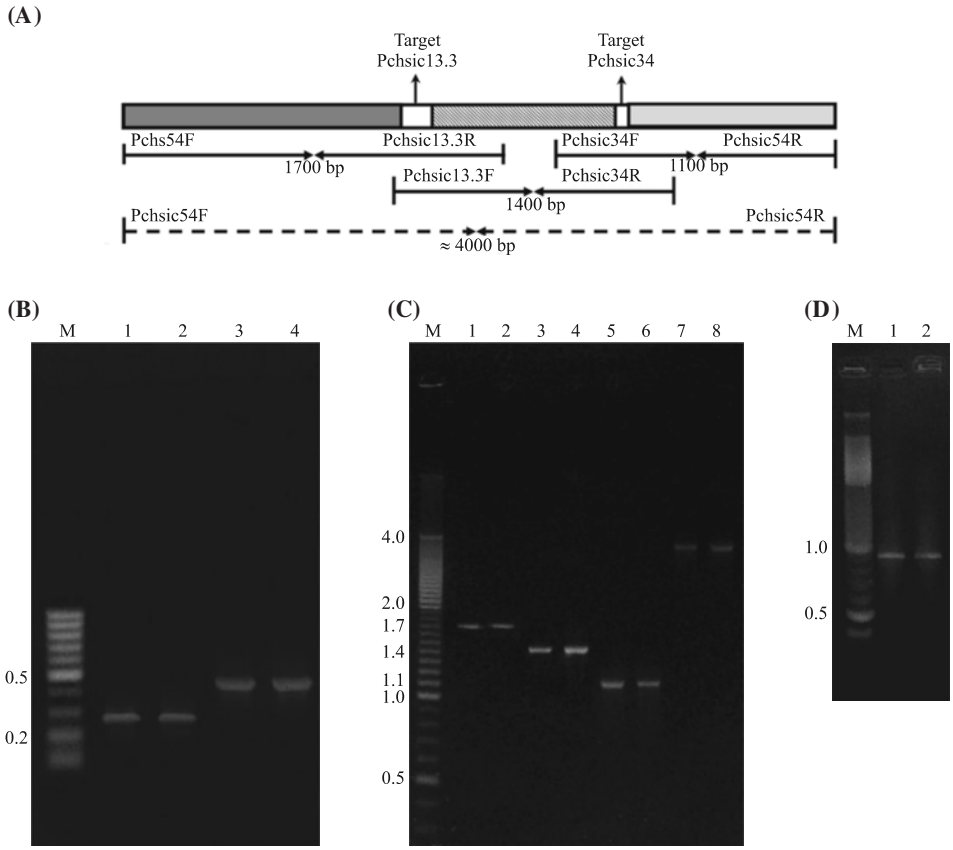

Fig. 1. The *M. perniciosa* chitin synthase gene (*Mopchs*). (A) Outline of the *Mopchs*. Boxes represent icontig5 (dark grey), icontig3 (hatched), and contig4 (light grey) as well as initially unknown regions (white), which were subsequently determined by primer walking. Pairs of arrows correspond to the segments that were amplified and sequenced in this work. Forward/reverse primers and the size of the expected segment are indicated above and below each pair of arrows, respectively. (B) Electrophoretic analysis of PCR products of initially unknown regions shown in 1A of the *Mopchs.* A total of 3 μl of each PCR product were applied in wells of a 1.0% agarose gel. 1 and 2, Segments of about 290 bp obtained with primer pair Pchsic34F and Pchsic34R. 3 and 4, 480 bp PCR product obtained with primer pair CHSiC13.3F and CHSiC13.3R. (C) Eletrophoretic analysis of PCR products of upstream, medial, and terminal regions of the *Mopchs*. 1 and 2, Pchs54-F/Pchsic13.3-R amplified a segment of approximately 1,700 bp. 3 and 4, Pchsic13.3F/PchsiC34R amplified a segment of approximately 1,400 bp. 5 and 6, PchsiC34F/Pchs54F amplified a segment of approximately 1,100 bp. 7 and 8, Pchs54F/Pchs54R amplified a segment of approximately 3,500 bp. (D) Electrophoretic analysis of PCR products of terminal region of *Mopchs* cDNA derived from basidioma and saprophytic mycelium RNA. 1, Badidioma; 2, Saprophytic mycelium.

Table 3. Features of exons and introns of the *M. perniciosa* chitin synthase gene

Exon			Intron			
No.	Size (bp)	Position	No.	Size (bp)	Position	Splice site (5'-3')
1	275	1-275	1	53	276-328	***GTGAGC...CCTCAG***
2	237	329-565	2	55	566-620	***GTAAGT...GGCCAG***
3	391	621-1011	3	52	1012-1063	***GTTTGT...CTATAG***
4	168	1064-1231	4	55	1232-1286	***GTAAGC...CATCAG***
5	199	1287-1485	5	55	1486-1540	***GTAAGT...CCACAG***
6	350	1541-1890	6	56	1891-1946	***GTAAGT...TTCTAG***
7	81	1947-2027	7	53	2028-2080	***GTAAGT...CTCCAG***
8	132	2081-2212	8	52	2213-2264	***GTAAGT...TTCTAG***
9	119	2265-2383	9	51	2384-2434	***GTGCGA...GAATAG***
10	200	2435-2634	10	60	2635-2694	***GTGAGC...ACTTAG***
11	29	2695-2723	11	51	2724-2774	***GTACGT...CTCTAG***
12	102	2775-2876	12	51	2877-2927	***GTACGT...TCCTAG***
13	405	2928-3332	13	57	3333-3389	***GTACGT...TGACAG***
14	51	3390-3440	-	-	-	-

(Ruiz-Herrera *et al*., 2002), and is absolutely conserved among translated sequences of basidiomycotan chitin synthase genes. Previous reports have suggested that the QRRRW motif comprises the catalytic domain of the enzyme, as mutations that disrupt this region result in loss of chitin synthase activity (Nagahashi *et al*., 1995).

Determination of the coding sequence of a chitin synthase gene in *M. perniciosa*

After sequence annotation in the *M. perniciosa* Genome Project Database database, primer pairs (Table 2) were designed to amplify the unknown fragments (Fig. 1A). As expected, the smallest fragment, about 290 bp, was generated with the primer pair Pchsic34F and Pchsic34R, whereas the primer pair Pchsic13.3F and Pchsic13.3R produced a larger fragment of approximately 480 bp (Fig. 1B).

The amplification of the whole gene was carried out with different combinations of primer pairs, resulting in contiguous segments of distinct sizes (Fig. 1C). These amplicons, of approximately 1.7, 1.4, and 1.1 kb, comprised the upstream, medial, and terminal regions of the *M. perniciosa* chitin synthase gene, respectively, and were obtained with primer pairs Pchs54F/Pchsic13.3R, Pchsic13.3F/Pchsic34R, and Pchsic34F/Pchs54R. The entire gene of approximately 3.5 kb was also obtained with a single amplification, using the primer pair Pchs54F/Pchs54R. The amplified segments were sequenced three times in both directions (forward and reverse), and corroborated the results of the previous experiments as well as those from data mining of the *M. perniciosa* project database. Contig assembly of sequenced segments suggested the presence of at least one chitin synthase gene in *M. perniciosa*.

According to all of the experimental (primer walking) and extrinsic and intrinsic theoretical computational procedures, the *M. perniciosa* chitin synthase gene (*Mopchs*) is 3,443 bp long, organized in 14 exons and 13 introns (Table 3) that produce a cDNA with an ORF of 2,739 bp and a 913 amino acid-long predicted protein. All of the introns resembled those of other basidiomycotan chitin synthase genes [*Coprinopsis cinerea* (Birren *et al*., 2003), *Cryptococcus neoformans* (Fung *et al*., 2004), *Pleurotus ostreatus* (Nishihara *et al*., 2007), and *Puccinia graminis* (Broeker *et al*., 2006), as they were bordered by GT/AG and were between 50 to 60 bp in length. Moreover, an EST from one clone (CP02-EC-001-013-A09-UE.F) of the *M. perniciosa* saprotrophic mycelium cDNA library corresponded exactly to the exons 11 and 12 of the gDNA sequence (in the positions 2694-2875).

We have also experimentally characterized the terminal region of the transcription product of this gene by determining the sequence of cDNA from RNA isolated from both basidiomata and secondary mycelium. The characterized cDNA segment, whose translation corresponded to the C-terminal region of the protein, comprised the exons 10∼14, including the exact translation stop site. Moreover, the comparison of this cDNA segment with previously determined genomic DNA confirmed the exact position of exon-intron boundaries and, therefore, the existence of introns 10∼13.

As well as for *A. bisporus* (Sreenivasaprasad *et al*., 2000) and *P. ostreatus* (Nishihara *et al*., 2007) class III chitin synthase genes, the homologous gene in *M. perniciosa* is also expressed in both basidioma and secondary mycelium stages (Fig. 1D). Although we did not perform a quantitative analysis of the transcriptional expression pattern of the *Mopchs* gene, all the four phylogenetic analyses (Supplementary Fig. 1A) showed a very close relationship between *M. perniciosa* class III CHS and both *A. bisporus* and *P. ostreatus* class III CHS, suggesting that *Mopchs* gene is also probably more actively expressed in basidioma stage.

Prediction of topological organization of *M. perniciosa* chitin synthase

The predicted *M. perniciosa* protein has a mass of approximately 102 kDa with a *pI*=8.43 in pH 7.0, similar to most other known basidiomycotan chitin synthases that are also basic (7.1∼8.9) and have molecular weights of 100±10 kDa (Table 4).

Topology prediction of *Mop*CHS using TMHMM (v2.0) (Krogh *et al*., 2001) suggested the presence of eight trans-

Table 4. Features of the basidiomycotan chitin synthase sequences

Species	Molecular mass (kDa)	p*I*	Charge in pH 7.0	Access no. and reference
M. perniciosa	102,76	8.511	11.777	ABW09311 This study
A. bisporus	102,26	8.230	8.591	CAB96110.1 Sreenivasaprasad *et al.* (2000)
C. cinerea	98,22	7.834	5.824	EAU87177.1 Birren *et al.* (2003)
C. cinerea2	93,13	8.972	18.101	EAU84753.1 Birren *et al.* (2003)
C. neoformans	101,27	8.419	10.912	EAL20616.1 Fung *et al.* (2004)
C. neoformans2	85,38	8.526	11.578	XP_570882.1 Loftus *et al.* (2005)
C. neoformans3	104,44	8.175	8.117	AAW43575.2 Loftus *et al.* (2005)
P. ostreatus	105,20	8.412	10.265	BAF37219.1 Nishihara *et al.* (2007)
P. graminis	96,63	8.667	15.031	ABB70408.1 Broeker *et al.* (2006)
P. graminis2	109,35	7.176	1.809	ABB70409.1 Broeker *et al.* (2006)
U. maydis	106,00	11.009	40.527	P30598 Kamper *et al.* (2006), Weber *et al.* (2006)

membrane helices, six of them with a posterior probability of approximately 1.0, and two with a probability of about 0.6. Topological predictions for the other known basidiomycotan chitin synthases revealed similar results: seven C-terminal transmembrane helices with five to six of them with probability close to 1.0 (Table 5). Interestingly, previous reports have noted that chitin synthases of euascomycetes also display a concentration of transmembrane helices at the C-terminus of the protein (Choquer *et al*., 2004).

Identification of class III conserved peptide motifs

Three highly conserved motifs, widely considered to be chitin synthase signatures, are present in *Mop*CHS: EDRXL, QRRRW, and QXXEY. The QXFEY motif, common in fungi

Table 5. Transmembrane helix prediction for chitin synthases of Basidiomycota

Species	No. of putative transmembrane regions (Probability ≈ 0.6)	No. of putative transmembrane regions (Probability ≈ 1.0)	Inicial position of putative transmembrane regions in protein sequence (bp)	Access No.
M. perniciosa	8	6	559	ABW09311 This study
A. bisporus	7	6	526	CAB96110 Sreenivasaprasad *et al.* (2000)
C. cinerea	7	5	487	EAU87177 Birren *et al.* (2003)
C. cinerea2	7	6	454	EAU84753 Birren *et al.* (2003)
C. neoformans	7	6	535	EAL20616 Fung *et al.* (2004)
C. neoformans2	7	6	389	XP_570882 Loftus *et al.* (2005)
C. neoformans3	7	6	570	AAW43575 Loftus *et al.* (2005)
P. ostreatus	7	6	558	BAF37219 Nishihara *et al.* (2007)
P. graminis	7	6	541	ABB70408.1 Broeker *et al.* (2006)
P. graminis2	7	7	593	ABB70409 Broeker *et al.* (2006)
U. maydis	7	6	574	P30598 Kamper *et al.* (2006), Weber *et al.* (2006)

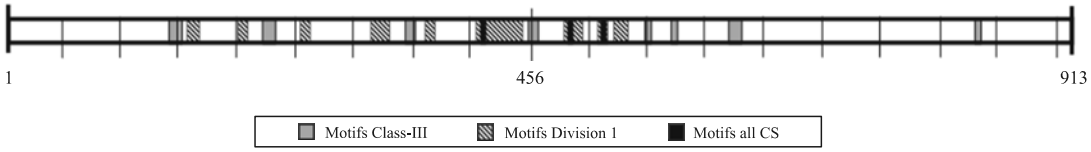

Fig. 2. Consensus motifs for all class III and Division 1 chitin synthases in *M. perniciosa* CHS.

and nematodes, has also been reported as being highly conserved in insects (Merzendorfer and Zimoch, 2003). The predicted *Mop*CHS have all the class III motifs described previously (Ruiz-Herrera *et al*., 2002): (D/E)YPVP(T/S)(A/P)I(Q/L)SA, RTLHGVM(Q/L)N(V/I)RDI, LNPE(I/V)C, PLEQYFHG, RMFF, WF(A/S)LA, LQF(I/V)LALGNRPK, FRT

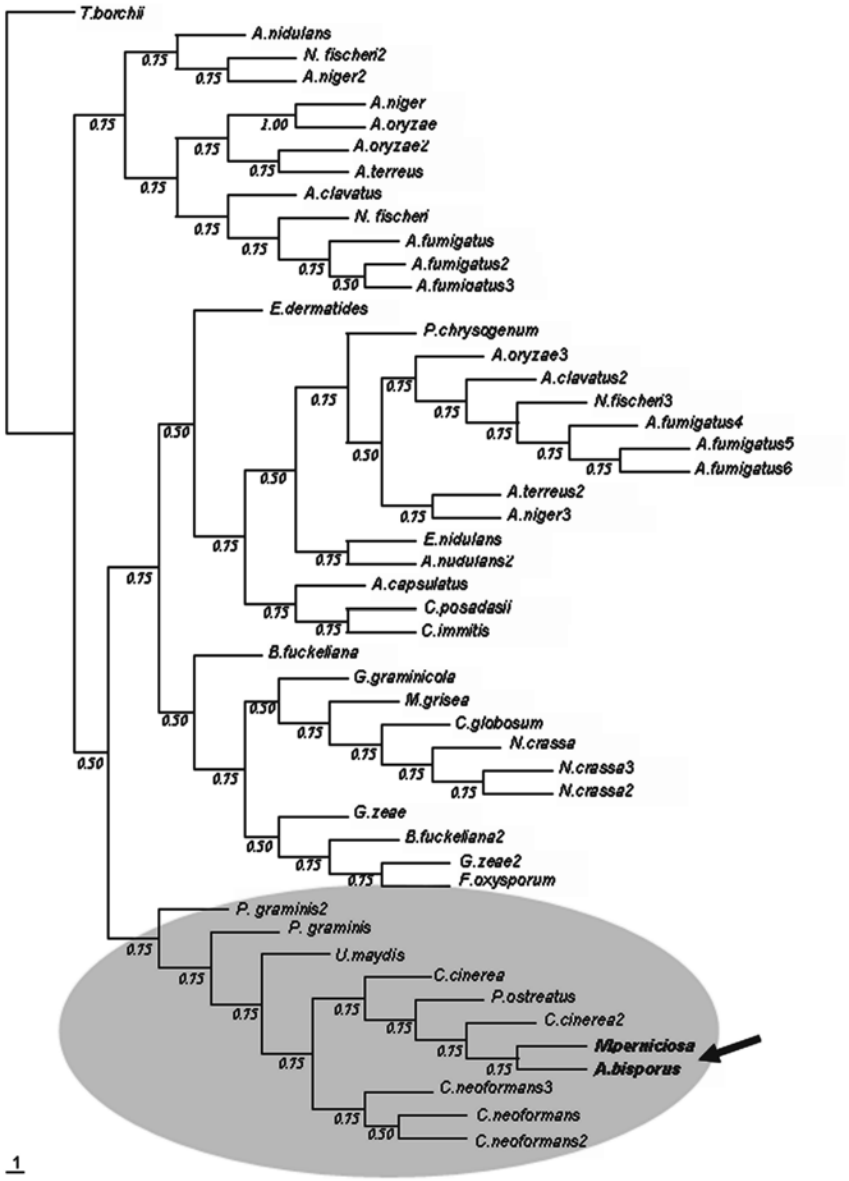

Fig. 3. Consensus tree of the four distinct available phylogenetic methods (Bayesian, Distance, Maximum Likelihood, and Maximum Parsimony) based on chitin synthase amino acid sequences of Dikarya (Basidiomycota+Ascomycota). Basidiomycota species are shown inside grey ellipse. Arrow indicates (*M. perniciosa*, *A. bisporus*) group.

(S/R)LV. It also harbors nine motifs that are specific for Division 1: EF(T/SAK)X(L/M)(T/R)YXA(A/VC)T(C/V/S), LAEDRILC(F/W/Y)(E/D)(L/V)(V/A), T(M/Y/S)YNE(D/E/N), WXK(I/V)XVXX(I/V)XDG, (L/I)(L/I/V)(D/E)(A/V/C)GT, F(C/V)(L/M)K(E/Q/A)XNXKK(L/I)NSH)R/L)W, TDVP, PLV(A/Y)XQNFEYK(MI/L)SNILDK(P/T)(L/TV)ESX(F/M)G(Y/F/H)(I/V)(S/T)VLP(G/A)A(F/L)(S/C)AYR, E(F/L)(I/V)-XQRRRW(L/I)N(G/Q)X(FL/M)(F/A)A (Fig. 2).

The (S/T)WGTKG motif, which occurs in *Mop*CHS, likely plays an important role in the catalytic activity of the enzyme since substitution of tryptophan (W) and the second threonine (T) diminishes enzymatic activity (Merzendorfer, 2006) and is probably related to the processivity of glycosyltransferases (Choquer *et al*., 2004). Moreover, all conserved regions that are typical of chitin synthases (Pfam 01644, Pfam 03142, Pfam 08407, COG 1215) (Latgé, 2007) also occur in the putative *Mop*CHS.

Sequence similarity comparison with other completely sequenced basidiomycotan and ascomycotan chitin synthases in protein databases permitted the construction of a well-aligned matrix of 50 distinct sequences with pairwise *E*-values=0.0, as well as subsequent phylogenetic analyses with high confidence.

Besides the good alignment, coverage values of the aligned region between *Mop*CHS and known sequences are strongly significant, varying from 81% to 100%. Against the *A. bisporus* CHS (emb|CAB96110.1) the identity value was 79%, and 67% against *P. ostreatus* CHS (dbj|BAF37219.1) with coverages of 98% and 100%, respectively. Analysis of conserved regions in the Pfam database (Pfam v21.0/HMMs - http://pfam.janelia.org) revealed three domains of highly significant statistical values. *A. bisporus* CHS (Q9P4U1) was one of the 41 components of Pfam 08407, corroborating the identity between that experimentally determined protein and *Mop*CHS.

Phylogenetic relationships of *M. perniciosa* chitin synthase with other Dikaryan chitin synthases

Basidiomycotan chitin synthases formed a distinct, well-resolved, and strongly supported monophyletic clade with maximum values of both bootstrap and Bayesian posterior probabilities in all the phylogenetic analyses performed, regardless of the available distinct methods used (distance, maximum parsimony, maximum likelihood, and Bayesian inference (Supplementary Fig. 1), as depicted in the consensus tree of all the four methods (Fig. 3).

All representatives of the Agaricales Underw. (*A. bisporus*, *C. cinerea*, *P. ostreatus*, *M. perniciosa*) grouped together, forming a less inclusive and highly supported monophyletic clade in all performed analyses as well as in the comprehensive study of James *et al.* (2006), which used a multi-locus dataset of six nuclear rRNA and protein-coding genes sampled in 199 fungal species. Moreover, *M. perniciosa* chitin synthase (*Mop*CHS) is more closely related to that of *A. bisporus* (emb|CAB96110) than to any other completely sequenced basidiomycotan chitin synthases, consistent with the analysis of conserved motifs and domains in protein sequences.

In summary, sequence analyses of the *M. perniciosa* genome project database, followed by PCR amplification of target segments by primer walking in genomic DNA, subsequent complete sequencing of the gDNA, and the terminal region of cDNA indicated that a chitin synthase gene was identified and characterized in this important phytopathogenic fungus. The gene is 3,443 bp long with 14 exons and 13 introns, resulting in a cDNA with an ORF of 2,739 bp that encodes a 913 amino acid-long protein. The gene product has extensive conserved areas and is highly similar to the completely sequenced class III chitin synthases of other basidomycotan fungi, especially *Agaricus bisporus*. This is the first reported characterization of a chitin synthase gene, its partial mature transcription product, and its putative protein in *M. perniciosa*. We are currently predicting, by homology modeling, the three-dimensional structure of that enzyme.

Acknowledgements

We would like to thank all the people that contributed directly or indirectly to this work and, especially the Graduate Program in Biotechnology (PPGBiotec UEFS, http://www2.uefs.br/ppgbiotec), Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB, http://www.fapesb.ba.gov), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, http://www.capes.gov.br), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, http://www.cnpq.br) for financial support.

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