



Analysis of the ergosterol biosynthesis pathway cloning, molecular characterization and phylogeny of lanosterol 14 α -demethylase (*ERG11*) gene of *Moniliophthora perniciosa*

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Abstract

The phytopathogenic fungus *Moniliophthora perniciosa* (Stahel) Aime & Philips-Mora, causal agent of witches' broom disease of cocoa, causes countless damage to cocoa production in Brazil. Molecular studies have attempted to identify genes that play important roles in fungal survival and virulence. In this study, sequences deposited in the *M. perniciosa* Genome Sequencing Project database were analyzed to identify potential biological targets. For the first time, the ergosterol biosynthetic pathway in *M. perniciosa* was studied and the lanosterol 14 α -demethylase gene (*ERG11*) that encodes the main enzyme of this pathway and is a target for fungicides was cloned, characterized molecularly and its phylogeny analyzed. *ERG11* genomic DNA and cDNA were characterized and sequence analysis of the *ERG11* protein identified highly conserved domains typical of this enzyme, such as SRS1, SRS4, EXXR and the heme-binding region (HBR). Comparison of the protein sequences and phylogenetic analysis revealed that the *M. perniciosa* enzyme was most closely related to that of *Coprinopsis cinerea*.

Keywords: Basidiomycota, fungus, ergosterol, *Theobroma cacao*.

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Introduction

Cocoa (*Theobroma cacao* L.) cultivation has suffered significant production losses because of diseases that affect its crops (Pereira *et al.*, 1989; Purdy and Schmidt, 1996; Evans, 2007). Among the main pathogens that cause dis-

eases in cocoa, the basidiomycete *Moniliophthora perniciosa* (Stahel) Aime & Philips-Mora has received considerable attention because it is the causal agent of witches' broom disease (Griffith *et al.*, 1994, 2003; Aime and Phillips-Mora, 2005).

Moniliophthora perniciosa has biotrophic and saprophytic stages (Griffith *et al.*, 2003; Meinhardt *et al.*, 2006). The biotrophic stage is characterized by basidiospore infection in meristematic regions that leads to hypertrophy of infected tissues and the proliferation of abnormal axillary

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branches known as green brooms. In the saprophytic stage, basidiomata that sporulate on plant material are generated and dry brooms occur as a result of cell death in infected tissues (Ceita *et al.*, 2007), one of the characteristic symptoms of this disease. Oxalate production and calcium oxalate crystal accumulation also play a role in the pathogenesis of witches' broom disease (Rio *et al.*, 2008).

The action of azole-group antifungals has created new perspectives for controlling witches' broom in cocoa trees and has highlighted the importance of analyzing ergosterol biosynthesis in *M. pernicioso* in order to develop specific inhibitors of this pathway (McQuilken and Rudgard, 1988; Mota *et al.*, 2010). To accomplish this, it is essential to study the development of the disease from molecular and biochemical perspectives. In this sense, the sequencing of the *M. pernicioso* genome has led to the discovery of genes that are essential for metabolism and development in this species and has resulted in several studies that have focused on gene expression analysis (Formighieri *et al.*, 2008; Mondego *et al.*, 2008).

Analyses of the biotrophic and saprophytic stages of *M. pernicioso* revealed high gene expression levels of the cytochrome P450 superfamily, this altered expression included lanosterol 14 α -demethylase, a key enzyme in the ergosterol biosynthetic pathway in fungi that is a target for antifungals and is encoded by the *ERG11* gene (Rincones *et al.*, 2008; Pires *et al.*, 2009). Lanosterol 14 α -demethylase belongs to the CYP51 family of the cytochrome P450 superfamily, which is notable for being the only cytochrome P450 family that is present in all biological kingdoms (Waterman and Lepesheva, 2005; Lepesheva and Waterman, 2007).

Antifungals that affect the ergosterol biosynthetic pathway have been used for decades. Azole-group antifungals are potentially useful disease-modulating agents because of their specific binding to lanosterol 14 α -demethylase and their selective inhibition of the removal of the methyl group by this enzyme, which leads to the accumulation of unsaturated intermediates and to the depletion of ergosterol (Hof, 2001; Carrillo-Muñoz *et al.*, 2006; Sheng *et al.*, 2009). Ergosterol is an important component of the fungal cell membrane that regulates membrane fluidity and permeability (Barrett-Bee and Dixon, 1995; Lees *et al.*, 1995; Veen and Lang, 2005).

The *ERG11* gene sequences of a wide range of fungal species that are harmful to agricultural crops and human health have been determined and characterized. These species include *Antrodia cinnamomea* (Lee *et al.*, 2010), *Aspergillus fumigatus* (Mellado *et al.*, 2001; Warrilow *et al.*, 2010), *Botrytis cinerea* (Albertini *et al.*, 2002), *Candida albicans* (Lai and Kirsch, 1989; Park *et al.*, 2011), *Candida glabrata* (Kairuz *et al.*, 1994), *Cryptococcus neoformans* (Revankar *et al.*, 2004; Sheng *et al.*, 2009), *Penicillium digitatum* (Zhao *et al.*, 2007), *Malassezia globosa* (Kim *et al.*, 2011), *Monilia fructicola*, previ-

ously known as *Monilia fructicola* (Luo and Schnabel, 2008), *Pneumocystis carinii* (Morales *et al.*, 2003), *Saccharomyces cerevisiae* (Kalb *et al.*, 1987), *Oculimacula yallundae*, previously known as *Tapesia yallundae* (Wood *et al.*, 2001), *Uncinula necator* (Délye *et al.*, 1997) and *Ustilago maydis* (Lamb *et al.*, 1998). The *ERG11* gene also has an important role in the steroid biosynthetic pathways of bacteria, plants and mammals in which its gene product has the same metabolic role as its fungal counterpart (Rozman *et al.*, 1996; Bak *et al.*, 1997; Bellamine *et al.*, 1999; Jackson *et al.*, 2003; Pietila *et al.*, 2006).

In this report, we describe the first identification, molecular characterization, cloning and phylogenetic analysis of the *ERG11* gene that encodes lanosterol 14 α -demethylase, an enzyme that is essential for the survival and pathogenicity of *M. pernicioso*. The results presented here should be useful in identifying antifungal drugs active against this enzyme and, consequently, in controlling witches' broom disease in cocoa trees.

Materials and Methods

Ergosterol pathway in *Moniliophthora pernicioso* and sequence analyses

The sequences examined in this work were retrieved from the *Moniliophthora pernicioso* Genome Sequencing Project database (www.lge.ibi.unicamp.br/vassoura/) and were used as the primary source for identifying genes of the ergosterol biosynthetic pathway in *M. pernicioso*. With this database, it was possible to obtain the genomic consensus sequences that were predicted to encode the main enzymes of this pathway, such as lanosterol 14 α -demethylase, and to compare these sequences with those from other organisms.

The genes were accurately analyzed using *ab initio* programs for gene prediction, such as AUGUSTUS (Stanke *et al.*, 2006) and GeneMark (Ter-Hovhannisyan *et al.*, 2008). These analyses were then combined with the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990) to align the sequences with those of phylogenetically related species. This approach allowed the identification of expressed regions, the presence and number of introns, and of regions bordering the *ERG11* gene. BioEdit software (v. 7.1.3) (Hall, 1999) was used to align the predicted genomic DNA and cDNA sequences with sequences determined by sequencing.

Signal peptide prediction was done using SignalIP 4.0 software (Petersen *et al.*, 2011), and probable transmembrane domains were determined using the Phobius program (Kall *et al.*, 2007). The ProtoParam tool was used to analyze the physical and chemical parameters of the proteins, such as the theoretical isoelectric points (pIs) and molecular masses (Gasteiger *et al.*, 2005). Additionally, the conserved domains, functional sites and protein family

were determined using PROSITE software (Sigrist *et al.*, 2009).

The lanosterol 14 α -demethylase sequences of other organisms were identified in GenBank and analyzed using the Protein BLAST (Blastp) tool. The *M. perniciosa* sequence was used as a reference (Table 1).

M. perniciosa strain and culture conditions

The 948F strain of *M. perniciosa* (Ilhéus, Bahia, Brazil), which was kindly provided by the Phytopathology Laboratory at the Cocoa Research Center (Centro de Pesquisas do Cacau da Comissão Executiva do Plano da Lavoura Cacaueira - CEPEC/CEPLAC, Ilhéus, Bahia, Brazil), was used in the experimental procedures. The strain was inoculated in Petri dishes containing Sabouraud agar solid medium (Sigma-Aldrich) to maintain mycelial discs and in YPD liquid medium (1% yeast extract, 1% peptone and 2% dextrose) (Sigma-Aldrich) and incubated statically at 27 °C for 15 days to obtain an adequate mycelial mass for genomic DNA and total RNA extraction.

Genomic DNA extraction

Total DNA was extracted from *M. perniciosa* mycelia according to the protocol described by Raeder and Broda (1985). The DNA concentration was determined spectrophotometrically (Novaspec II, Pharmacia Biotech) and the quality and quantity of the extracted DNA were analyzed in 1% agarose gels.

RNA isolation and reverse transcription

Total mycelial RNA was obtained using a RiboPure kit (Ambion) according to the manufacturer's recommendations. The first cDNA strand was produced from 1-3 μ g of total RNA using the RevertAid H Minus M-MuLV Reverse Transcriptase kit (Fermentas Life Sciences) according to the manufacturer's recommendations.

Primer design and amplification conditions

Specific primers for PCR and RT-PCR were designed based on the predicted *ERG11* sequence in *M. perniciosa* (Figure S1). We aimed to obtain complete fragments of DNA and cDNA (1,668 bp) using the primers LanFNcoIfull and LanRXhoI. A fragment (1,496 bp) that lacked the signal peptide was obtained using the primers LanFNheIfull and LanRNheI. A fragment (1,400 bp) that lacked the first transmembrane helix was obtained with the primers LanFNheIdel1ahel and LanRNheI. Lastly, a fragment (1,316 bp) that lacked the regions corresponding to the first and second transmembrane helices was constructed using the primers LanFNheIdel2ahel and LanRNheI (Table 2).

Amplifications were done using a Mastercycler Gradient thermocycler (Eppendorf) with variable annealing temperatures. The following program was used: 3 min at 94 °C, 35 cycles that included 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C and a final extension of 5 min at 72 °C.

Table 1 - CYP51 proteins deposited in GenBank used for sequence and phylogenetic analyses.

| Organism | Accession number | Length (aa) | E-value |
|---|------------------|-------------|--------------------|
| <i>Aspergillus fumigatus</i> | ACF17705.1 | 515 | 5e ⁻¹⁴³ |
| <i>Candida albicans</i> | AAF00603.1 | 528 | 2e ⁻¹³⁵ |
| <i>C. dubliniensis</i> CD36 | CAX41448.1 | 528 | 5e ⁻¹³⁸ |
| <i>C. glabrata</i> | AAX39317.1 | 533 | 5e ⁻¹³⁹ |
| <i>C. tropicalis</i> | AAX39316.1 | 528 | 7e ⁻¹³⁵ |
| <i>Coprinopsis cinerea</i> | AAU01159.1 | 531 | 0 |
| <i>Cryptococcus neoformans</i> var. <i>neoformans</i> | AAF35366.1 | 550 | 0 |
| <i>C. gattii</i> | AEQ63274.1 | 550 | 0 |
| <i>Homo sapiens</i> | NP_000777.1 | 509 | 1e ⁻¹⁹⁶ |
| <i>Kluyveromyces lactis</i> | CAG99196.1 | 527 | 9e ⁻¹³⁷ |
| <i>Moniliophthora perniciosa</i> | JX915631 | 555 | — |
| <i>Monilinia fruticola</i> | ACF06200.1 | 522 | 3e ⁻¹³⁰ |
| <i>Mus musculus</i> | NP_064394.2 | 503 | 5e ⁻⁹⁶ |
| <i>Mycobacterium tuberculosis</i> | CAB02394.1 | 451 | 8e ⁻¹⁶¹ |
| <i>Pneumocystis carinii</i> | AAO38776.1 | 513 | 5e ⁻¹⁴⁸ |
| <i>Phanerochaete chrysosporium</i> | AAU01160.1 | 540 | 0 |
| <i>Pichia kudriavzevii</i> | ABY26735.1 | 528 | 9e ⁻¹²⁸ |
| <i>Saccharomyces cerevisiae</i> | AAB68433.1 | 530 | 7e ⁻¹³⁷ |
| <i>Trichoderma reesei</i> QM6a | EGR51226.1 | 526 | 6e ⁻¹³⁴ |
| <i>Ustilago maydis</i> 521 | EAK84840.1 | 561 | 1e ⁻¹⁶⁹ |

Table 2 - Primers used to amplify the *Moniliophthora perniciosa* *ERG11* gene.

| Primer | Sequence (5' - 3') | First nucleotide position in <i>M. perniciosa</i> <i>ERG11</i> cDNA (bp) |
|----------------------------|---|--|
| LanFNcoIfull (Forward) | ATGTCCCA tGg CGAAAACTCAACGGTTC | 1 |
| LanFNheIfull (Forward) | CCTGTCATTG CtAgc GTACTCAATGTTCTCCG | 127 |
| LanFNheIdel1ahel (Forward) | GGCTCAG CTagc TCATACGGCAATGACCC | 223 |
| LanFNheIdel2ahel (Forward) | CTCTTT GcTaGc CGCGTCACTGTTGCC | 307 |
| LanRXhoI (Reverse) | GCGTCTC CtAg TGAATACGTCTAGGCGT | 1 591 |
| LanRNheI (Reverse) | GGCATCGTAATCAT GcTAgc GTAGTTCTGAGC | 1 627 |

The variable temperatures used to amplify the genomic DNA were: 56 °C, (positions 1, 6 and 11), 57.9 °C (positions 2, 7 and 12), 60 °C (positions 3, 8 and 13), 62.7 °C (positions 4, 9 and 14) and 64 °C (positions 5, 10 and 15) (Figure 1). The temperatures used to amplify the cDNA were: 57.9 °C (positions 1, 5 and 9), 60 °C (positions 2, 6 and 10), 62.7 °C (positions 3, 7 and 11) and 64 °C (positions 4, 8 and 12) (Figure 2).

The PCR products were visualized on 1% agarose gels containing ethidium bromide (0.5 µg/mL) and the gel was photographed using an EDAS 290 photodocumentation system (Kodak).

Sequencing of PCR products

The DNA and cDNA sequences that corresponded to *ERG11* were confirmed by sequencing the purified PCR products on a MegaBACE 1000 capillary sequencer (Amersham Biosciences - GE Healthcare) using a DYEnamic ET dye terminator kit (GE Healthcare) according to the manufacturer's instructions.

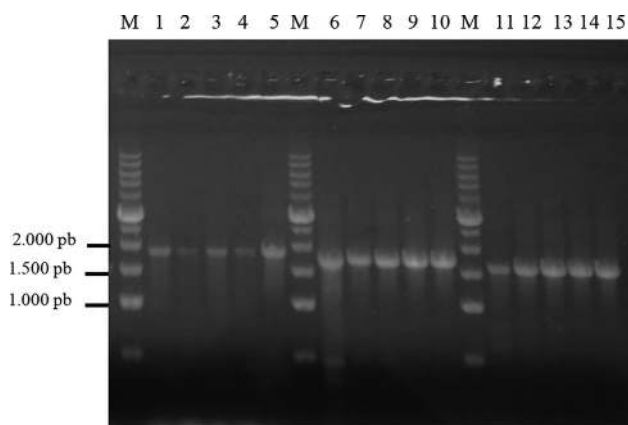


Figure 1 - Agarose gel electrophoresis of PCR products of *Mper ERG11* amplified from genomic DNA. The reactions were done with an annealing temperature gradient. M - molecular weight marker (1 kb; Amresco). 1-5 - genomic PCR products with no region corresponding to the signal peptide (1,858 bp), 6-10 - genomic PCR products without the first transmembrane insertion (1,712 bp), 11-15 - genomic PCR products without the first and second transmembrane insertions (1,575 bp).

Cloning of cDNA fragments of *ERG11*

cDNA fragments of *M. perniciosa ERG11* were cloned into the pET-28a vector (Novagen) and recombinant plasmids that contained the *ERG11* gene were transformed in *E. coli* Rosetta (DE3) that were grown in LB medium (1% Tryptone, 1% NaCl and 0.5% yeast extract) that contained appropriate antibiotics for selection. The identities of positive clones were confirmed by PCR and sequencing and were subsequently used for expression studies and the purification of lanosterol 14 α -demethylase.

Sequence alignment and phylogenetic analysis

Sequences obtained from the NCBI database (Table 1) and the predicted sequence from *M. perniciosa* were used to perform alignments with BioEdit (Hall, 1999) and ClustalW2 (Thompson *et al.*, 1994) and a data matrix was used for the phylogenetic analyses. Twenty sequences were analyzed and *Mycobacterium tuberculosis* was used as an outgroup.

Maximum parsimony analysis (MP) was done with PAUP 4.0b10 software (Swofford, 2002) using Fitch parsimony (unordered characters and equal weighting) and *branch-swapping* heuristic search algorithms. The search

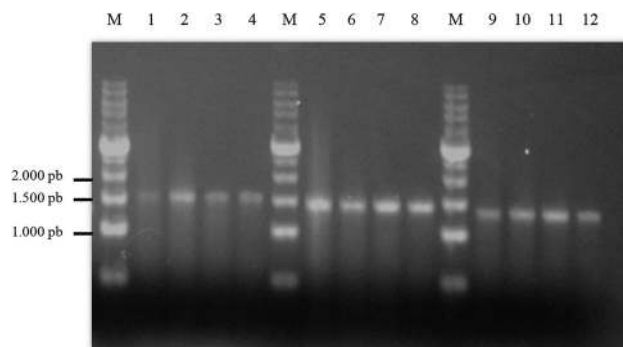


Figure 2 - Agarose gel electrophoresis of PCR products of *ERG11* amplified from cDNA. The reactions were done with an annealing temperature gradient. M - molecular weight marker (1 kb; Amresco). 1 - cDNA products (1,496 bp) with no region corresponding to the signal peptide, 5-8 - cDNA products (1,400 bp) without the first transmembrane insertion and 9-12 - cDNA products (1,316 bp) without the first and second transmembrane insertions.

consisted of 1,000 replicates in which data were randomly added using the tree bisection and reconnection (TBR) algorithm; 15 trees per replicate were saved and all *gaps* were treated as missing data. A strict consensus tree was then obtained and the relative clade support was determined using the bootstrap - BS test (Felsenstein, 1985). Only values that were $\geq 75\%$ are shown and the retrieved trees were edited using TreeView v.1.6 software (Page, 1996).

Results

Isolation and characterization of the *ERG11* gene in *M. perniciosa*

The complete *ERG11* gene that was amplified from genomic DNA contained 2,030 base pairs (bp) while the gene that was amplified from cDNA was 1,668 bp long. The gene product with no region corresponding to the signal peptide contained 1,858 bp (Figure 1) while the product obtained by RT-PCR contained 1,496 bp (Figures 2 and S2).

Removal of the regions that corresponded to transmembrane insertions 1 and 2 generated genomic PCR products of 1,712 bp and 1,575 bp, respectively (Figure 1) and cDNA products of 1,400 and 1,316 bp, respectively (Figure 2), as previously predicted by our bioinformatic analysis. The reliability of the DNA and cDNA base composition of *ERG11* was confirmed by sequencing and demonstrated the accuracy of the gene prediction models that we used. Sequence analysis showed that *M. perniciosa ERG11* has eight exons and seven introns (positions 166-215, 333-385, 509-561, 1027-1076, 1250-1301, 1771-1821 and 1915-1967).

Comparison of the lanosterol 14 α -demethylase amino acid sequence of *M. perniciosa* with those of other organisms indicated that it shared the greatest identity with the sequences of *Coprinopsis cinerea* (accession no. AAU01159.1) and *Phanerochaete chrysosporium* (AAU01160.1) (71%), *C. neoformans* (AAF35366.1) and *Cryptococcus gatti* (AEQ63274.1) (57%) and *U. maydis* (EAK84840.1) (55%). Comparative analysis of the sequences yielded an E-value of 0.0 and the identity values exceeded the existing average of the amino acid sequences of CYP51 of lower eukaryotes (41%) (Lepesheva and Waterman, 2007).

Structural prediction and physicochemical parameters of lanosterol 14 α -demethylase

Determination of the sequence of the *ERG11* transcript allowed prediction of the primary structure of lanosterol 14 α -demethylase, which consisted of 555 amino acids; the predicted molecular mass was 62 kDa and the theoretical pI was 6.34. The *ERG11* ORF in *M. perniciosa* exhibited two putative, highly hydrophobic transmembrane domains located near the N-terminal region of the protein at amino acid positions 33-55 and 62-84, which suggested a

probable insertion in the endoplasmic reticulum membrane (Lepesheva and Waterman, 2007). In addition, signal peptide prediction identified a cleavage site between amino acid positions 46 and 47 and suggested that this protein was directed to the endoplasmic reticulum through the secretory pathway.

Analysis of CYP51 in *M. perniciosa* identified an iron-binding domain at amino acid positions 493-502 that is characteristic of proteins belonging to the cytochrome P450 superfamily.

Sequence alignment and detection of conserved domains

The alignment of *ERG11* sequences from different organisms allowed the identification of several conserved domains that are characteristic of P450 proteins and, more specifically, of CYP51 proteins (EC 1.14.13.70). The C-terminal region of these proteins contains a binding domain that is common to the heme group (FxxGxxxCxG). This domain was identified in *M. perniciosa* CYP51 and contained a conserved cysteine residue at amino acid position 500 that is responsible for the binding of iron to this protein (Figure 3). The SRS1 and SRS4 domains (substrate recognition regions) are relatively well conserved in CYP51.

The SRS1 (YxxF/L(I)xxPxFGxxVxF/YD/a) and SRS4 regions (GQ/hHT/sS) of *M. perniciosa* CYP51 were very similar to those of other organisms (Figure 3). The SRS1 region (B' helix/B' loopC) forms the upper surface of the substrate binding cavity of P450 and the replacement of some conserved residues in this region can lead to partial or complete loss of CYP51 activity in *Homo sapiens* and *Mycobacterium tuberculosis* (Zhao *et al.*, 2007). The SRS4 domain is located in the C-terminal region of the P450 I-helix, which forms the right wall of the distal surface of the substrate binding cavity (Lepesheva and Waterman, 2007). The EXXR motif, which is characteristic of the K-helix of CYP51 (Kim *et al.*, 2011), is also highly conserved (Figure 3).

Phylogenetic analysis

Phylogenetic analysis based on protein sequence data allowed assessment of the evolutionary relationships among CYP51 proteins of 20 organisms (Figure 4). Maximum parsimony analysis yielded three equally parsimonious trees, each with 2,700 steps (TreeBase access TB2:S14729). The maximum parsimony result was very similar to the maximum likelihood analysis (not shown), with the branches that clustered bacteria, fungi and animals being clearly distinct. The formation of these clusters agreed with the accepted phylogeny for the major kingdoms of organisms.

Species of the phylum Ascomycota were grouped in one clade and those belonging to the Basidiomycota were grouped in another (Figure 4). This sister group relation-

ship for CYP51 phylogeny agreed with the general taxonomy of fungal species (James et al., 2006).

Discussion

Many of the genes that comprise the ergosterol biosynthetic pathway in *M. pernicioso* have been identified by genomic data mining. Our results indicate that the *M. pernicioso* genes that are part of this pathway show great similarity with those of the Basidiomycota, such as *Coprinopsis cinerea* (*ERG1*, *ERG2*, *ERG9*, *ERG11*, *ERG24*, *ERG25* and *ERG26*), *Laccaria bicolor* (*ERG5*, *ERG6* and *ERG7*) and *Cryptococcus gatti* (*ERG4*) (Martin

et al., 2008; Stajich et al., 2010; D'Souza et al., 2011). These data demonstrate the conservation of genes and biosynthetic pathways that are essential for the survival of related organisms.

The biosynthetic pathways for ergosterol and steroids have been studied mostly in model organisms such as *Kluyveromyces lactis* and *Saccharomyces cerevisiae*, and in those that cause public health problems, such as *Aspergillus fumigatus*, *Candida albicans*, *C. dubliniensis*, *C. glabrata* and *C. tropicalis* (Goffeau et al., 1996; Dujon et al., 2004; Jones et al., 2004; Nierman et al., 2005; Butler et al., 2009). However, there is little information on the steroid bio-

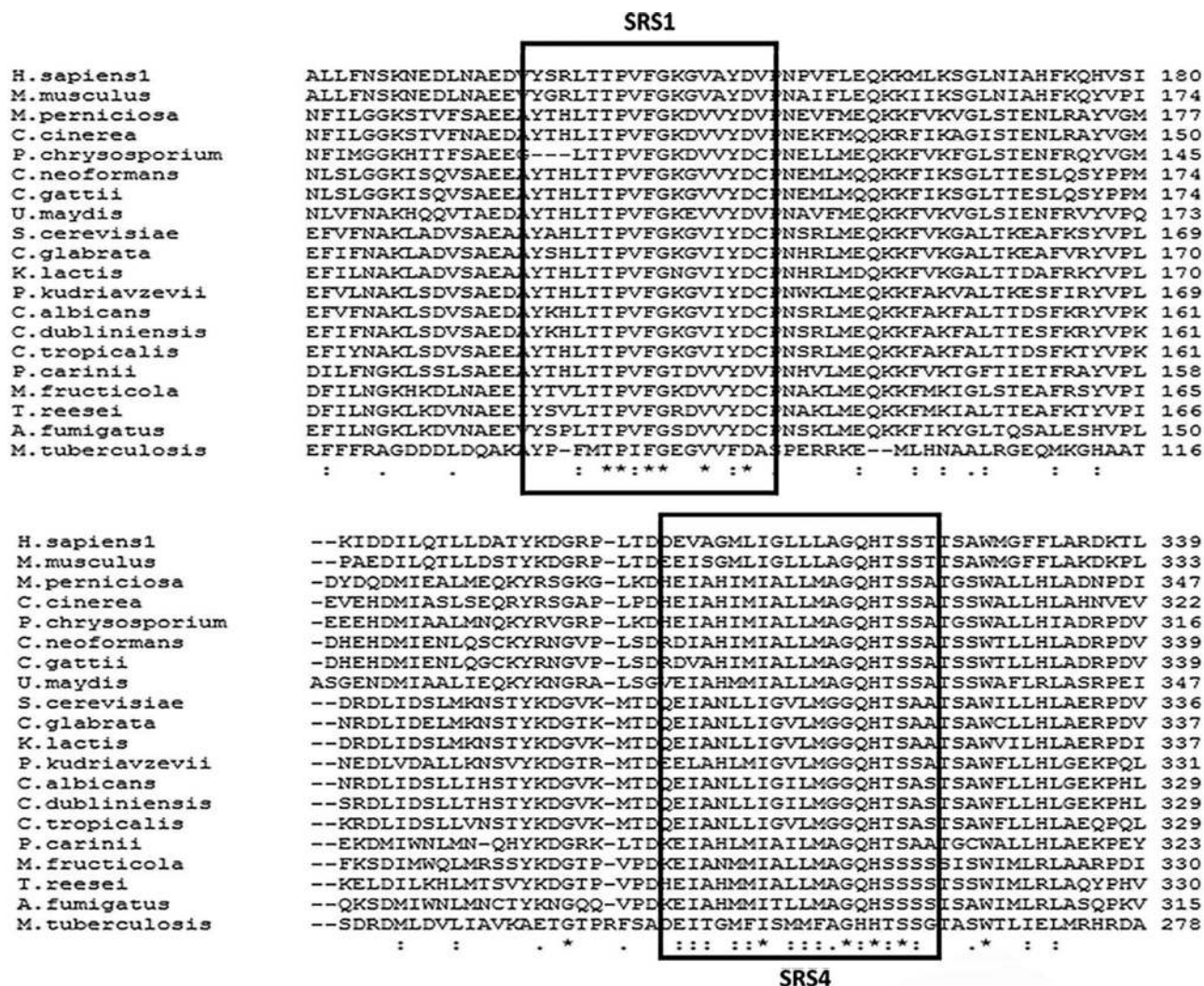


Figure 3 - *Moniliophthora pernicioso* CYP51 alignment with other homologous CYP51 sequences. Conserved amino acid residues that correspond to the heme-binding site (HBR), substrate recognition sites (SRS1 and SRS4) and EXXR motifs are shown in boxes. The arrow indicates the conserved cysteine residue. *Homo sapiens 1* - *H. sapiens* isoform 1 (NP_0000777.1), *Mus musculus* - *Mus musculus* (NP_064394.2), *Moniliophthora pernicioso* - *Moniliophthora pernicioso* (JX915631), *Coprinopsis cinerea* - *Coprinopsis cinerea* (AAU01159.1), *Phanerochaete chrysosporium* - *Phanerochaete chrysosporium* (AAU01160.1), *Cryptococcus neoformans* - *Cryptococcus neoformans* var. *neoformans* (AAF35366.1), *C. gattii* - *Cryptococcus gattii* (AEQ63274.1), *Ustilago maydis* - *Ustilago maydis* 521 (EAK84840.1), *Saccharomyces cerevisiae* - *Saccharomyces cerevisiae* (AAB68433.1), *Candida glabrata*, *Candida glabrata* (AAX39317.1), *Kluyveromyces lactis*, *Kluyveromyces lactis* (CAG99196.1), *Pichia kudriavzevii* - *Pichia kudriavzevii* (ABY26735.1), *Candida albicans* - *Candida albicans* (AAF00603.1), *Candida dubliniensis* - *Candida dubliniensis* CD36 (CAX41448.1), *Candida tropicalis* - *Candida tropicalis* (AAX39316.1), *Pneumocystis carinii* - *Pneumocystis carinii* (AAO38776.1), *Monilinia fructicola* - *Monilinia fructicola* (ACF06200.1), *Trichoderma reesei* - *Trichoderma reesei* QM6a (EGR51226.1), *Aspergillus fumigatus* - *Aspergillus fumigatus* (ACF17705.1), *Mycobacterium tuberculosis* - *Mycobacterium tuberculosis* (CAB02394.1).

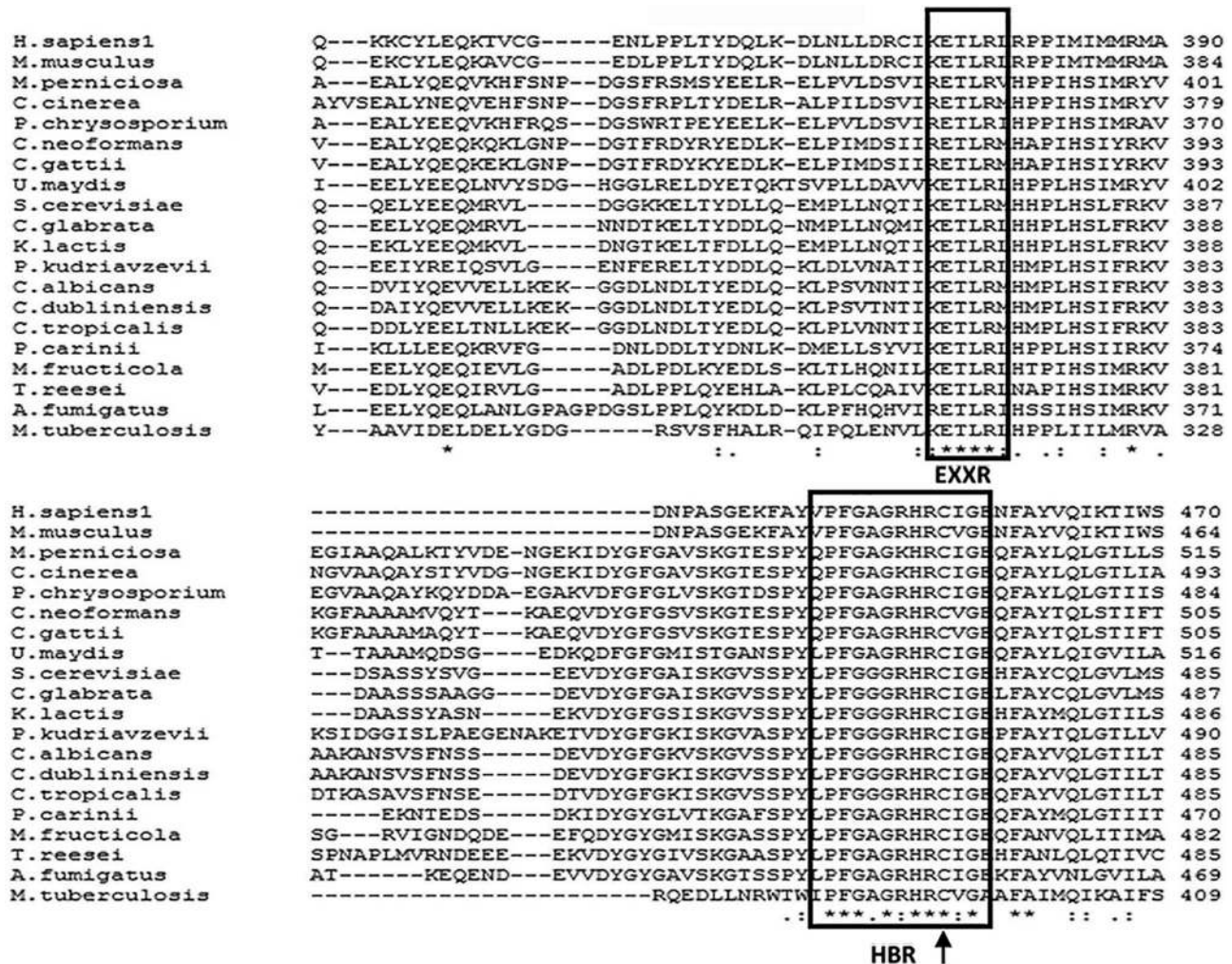


Figure 3 cont. -

synthetic pathways of phytopathogenic filamentous fungi (except for *A. fumigatus*). Gaps in our knowledge concerning the genes of this pathway in some organisms reflect a lack of genomic data or the need for further studies related to sequences that have already been deposited in genomic databases and EST libraries. The results of the present study fill a gap in our knowledge of this metabolic pathway. This pathway is particularly important for the development of *M. perniciosa* and may be useful in designing new antifungal drugs.

The detection of *ERG11* gene expression during the mycelial stage of *M. perniciosa*, as shown here, suggests that mycelial development requires intense steroidal activity. *ERG11* is highly expressed since ergosterol is an essential sterol. Ergosterol is the major component of the fungal cell membrane and is essential as a bioregulator of membrane fluidity, asymmetry and integrity (Barrett-Bee and Dixon, 1995; Carrilo-Muñoz *et al.*, 2006). The membranes of eukaryotic cells act as barriers between the inside of the cell or the lumen of organelles and the corresponding exter-

nal environment. These membranes also contain proteins that selectively transport molecules or act as enzymes in different metabolic activities (Veen and Lang, 2005).

Macroarray analysis of the differential expression of *ERG11* during *M. perniciosa* development, which was part of effort to construct a cDNA library for *M. perniciosa*, indicated that this gene was hyperexpressed during the white mycelial stage and had a lower level of expression during the primordial stage, prior to the formation of basidiomata (Pires *et al.*, 2009). This finding indicated that expression levels of this gene vary during fungal development.

The strong conservation of regions SRS1, SRS4 and the EXXR motif of lanosterol 14 α -demethylases among organisms belonging to different groups indicated their importance in the recognition of basal substrates. Despite the variability among CYP51 sequences, the basic structure of this protein needs to be maintained in order to retain its catalytic activity, and this structure is determined by the HBR and the more conserved substrate recognition sites (SRS1 and SRS4) (Lepesheva and Waterman, 2007; Zhao *et al.*,

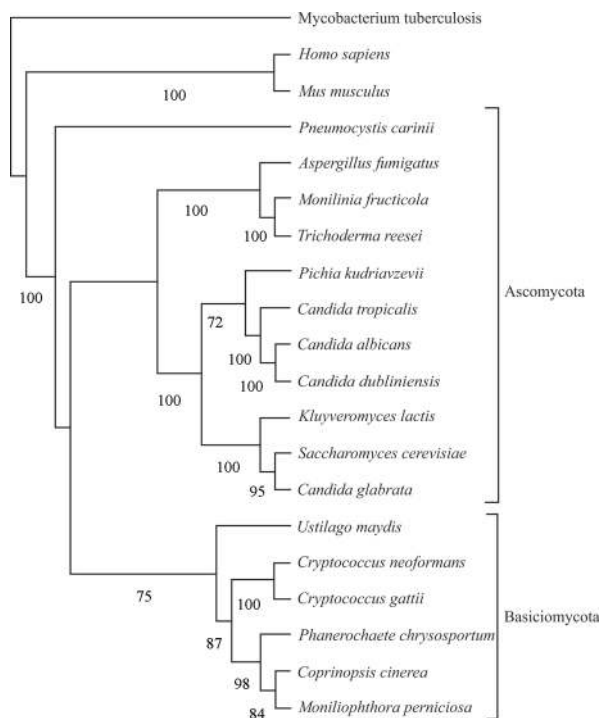


Figure 4 - The most parsimonious tree obtained from the Maximum Parsimony analysis based on the amino acid sequence of lanosterol 14 α -demethylase. The numbers below the branches indicate bootstrap support (in %). Length: 2,700 steps.

2007). These CYP51 regions are conserved in fungi from different phyla (Figure 3). The identity between the CYP51 sequences and the low E-values (Table 1) were indicators of the high similarity between the CYP51 orthologs of these phylogenetically related organisms.

As with *M. perniciosa*, introns have also been observed in the homologous sequences of other basidiomycetes such as *C. cinerea*, *C. neoformans* and *P. chrysosporium*, that contain six, eight and six introns, respectively (Mellado *et al.*, 2001; Revankar *et al.*, 2004; Warrilow *et al.*, 2010). *Ustilago maydis* is the only Basidiomycete that lacks introns in the *ERG11* gene (Revankar *et al.*, 2004). Furthermore, introns are usually not found in Ascomycota such as *C. albicans*, *P. carinii* and *S. cerevisiae*. The average size of the *ERG11* introns in *M. perniciosa* was 52 bp, as estimated for other genes analyzed by genome sequencing (Mondego *et al.*, 2008). In contrast to the positional variation of *ERG11* introns among organisms that may reflect evolutionary events in the P450 superfamily, the HBR, which is highly conserved in this family of proteins, has not suffered recombination events or exon shuffling (Rozman *et al.*, 1996).

In phylogenetic analysis, the formation of well-defined clusters confirmed the accepted phylogeny for the major kingdoms of organisms. Fungi formed two distinct clades. The organisms that showed higher identity values with the CYP51 sequences of *M. perniciosa* (*C. cinerea*, *P. chrysosporium*, *C. gattii*, *C. neoformans* and *U. maydis*)

formed a distinct clade that included *M. perniciosa* and had a significant bootstrap value (Figure 4); this finding indicates that *Mper ERG11* is phylogenetically closer to *C. cinerea ERG11* than to all homologs of the other Basidiomycota with well-characterized sequences.

The cloning of *M. perniciosa* CYP51 in expression vectors will enable the recombinant production of lanosterol 14 α -demethylase and should provide sufficient protein for elucidation of the structure of this enzyme. A similar approach applied to other enzymes in this pathway should contribute to the design and optimization of more effective drugs against this phytopathogenic fungus.

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Internet Resources

- AUGUSTUS software, <http://augustus.gobics.de> (11 Jan 2012).
- GeneMark, <http://exon.biology.gatech.edu/> (11 Jan 2012).
- SignalIP 4.0 software, <http://www.cbs.dtu.dk/services/SignalP/> (11 Jan 2012).
- Phobius program, <http://www.ebi.ac.uk/Tools/pfa/phobius/> (11 Jan 2012).
- ProtoParam tool, <http://web.expasy.org/protparam/> (11 Jan 2012).
- PROSITE software, <http://prosite.expasy.org/> (11 Jan 2012).

Supplementary Material

The following online material is available for this article:

Figure S1 - Positions of the primers used to amplify the DNA sequences.

Figure S2 - Nucleotide and amino acid sequence of the ERG11 gene of M.

The material is available as part of the online article from <http://www.scielo.br/gmb>.

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