Research Article

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

Geruza de Oliveira Ceita[1,4], Laurival Antônio Vilas-Boas[2], Marcelo Santos Castilho[3],
Marcelo Falsarella Carazzolle[5], Carlos Priminho Pirovani[6], Alessandra Selbach-Schnadelbach[4],
Karina Peres Gramacho[7], Pablo Ivan Pereira Ramos[4], Luciana Veiga Barbosa[4],
Gonçalo Amarante Guimarães Pereira[5] and Aristóteles Góes-Neto[1]

[1]*Laboratório de Pesquisa em Microbiologia, Departamento de Ciências Biológicas,*
*Universidade Estadual de Feira de Santana, Feira de Santana, BA, Brazil.*
[2]*Centro de Ciências Biológicas, Departamento de Biologia Geral, Universidade Estadual de Londrina,*
*Londrina, PR, Brazil.*
[3]*Laboratório de Bioinformática e Modelagem Molecular, Departamento do Medicamento,*
*Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, BA, Brazil.*
[4]*Laboratório de Biologia Molecular, Instituto de Biologia, Departamento de Biologia Geral,*
*Universidade Federal da Bahia, Salvador, BA, Brazil.*
[5]*Laboratório de Genômica e Proteômica, Departamento de Genética e Evolução,*
*Universidade Estadual de Campinas, Campinas, SP, Brazil.*
[6]*Centro de Biotecnologia e Genética, Departamento de Ciências Biológicas,*
*Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil.*
[7]*Laboratório de Fitopatologia Molecular, Centro de Pesquisas do Cacau, Ilhéus, BA, Brazil.*

## 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 14α-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*.

Received: April 11, 2014; Accepted: June 4, 2014.

## Introduction

Cocoa (*Theobroma cacao* L.) cultivation has suffered significant production losses because of diseases that affect its crops (Pereira *et al.*, 1989; Purdy and Schimidt, 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

Send correspondence to Aristóteles Góes-Neto. Laboratório de Pesquisa em Microbiologia, Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, Avenida Transnordestina, 44036-900 Feira de Santana, BA, Brazil. E-mail: arigoesneto@pq.cnpq.br.

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. perniciosa* 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. perniciosa* 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. perniciosa* revealed high gene expression levels of the cytochrome P450 superfamily, this altered expression included lanosterol 14α-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 14α-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 14α-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), *Monilinia 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 14α-demethylase, an enzyme that is essential for the survival and pathogenicity of *M. perniciosa*. 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 perniciosa* and sequence analyses

The sequences examined in this work were retrieved from the *Moniliophthora perniciosa* 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. perniciosa*. 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 14α-demethylase, and to compares 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 14α-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 µg 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 |
|---|---|---|---|
| *Aspergillus fumigatus* | ACF17705.1 | 515 | $5e^{-143}$ |
| *Candida albicans* | AAF00603.1 | 528 | $2e^{-135}$ |
| *C. dubliniensis* CD36 | CAX41448.1 | 528 | $5e^{-138}$ |
| *C. glabrata* | AAX39317.1 | 533 | $5e^{-139}$ |
| *C. tropicalis* | AAX39316.1 | 528 | $7e^{-135}$ |
| *Coprinopsis cinerea* | AAU01159.1 | 531 | 0 |
| *Cryptococcus neoformans* var. *neoformans* | AAF35366.1 | 550 | 0 |
| *C. gattii* | AEQ63274.1 | 550 | 0 |
| *Homo sapiens* | NP_000777.1 | 509 | $1e^{-196}$ |
| *Kluyveromyces lactis* | CAG99196.1 | 527 | $9e^{-137}$ |
| *Moniliophthora perniciosa* | JX915631 | 555 | —- |
| *Monilinia fructicola* | ACF06200.1 | 522 | $3e^{-130}$ |
| *Mus musculus* | NP_064394.2 | 503 | $5e^{-96}$ |
| *Mycobacterium tuberculosis* | CAB02394.1 | 451 | $8e^{-161}$ |
| *Pneumocystis carinii* | AAO38776.1 | 513 | $5e^{-148}$ |
| *Phanerochaete chrysosporium* | AAU01160.1 | 540 | 0 |
| *Pichia kudriavzevii* | ABY26735.1 | 528 | $9e^{-128}$ |
| *Saccharomyces cerevisiae* | AAB68433.1 | 530 | 7e-137 |
| *Trichoderma reesei* QM6a | EGR51226.1 | 526 | $6e^{-134}$ |
| *Ustilago maydis* 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 *M. perniciosa ERG11* cDNA (bp) |
|---|---|---|
| LanFNcoIfull (Forward) | ATGTCCCAtGgCGAAAACCTCAACGGTTC | 1 |
| LanFNheIfull (Forward) | CCTGTCATTGCtAgcGTACTCAATGTTCTCCG | 127 |
| LanFNheIdel1ahel (Forward) | GGCTCAGCTagcTCATACGGCAATGACCC | 223 |
| LanFNheIdel2ahel (Forward) | CTCTTTGcTaGcCGCGTCACTGTTGCC | 307 |
| LanRXhoI (Reverse) | GCGTCTCCTcgAGTGAATACGTCTAGGCGT | 1 591 |
| LanRNheI (Reverse) | GGCATCGTAATCATGcTAgcGTAGTTCTGAGC | 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.
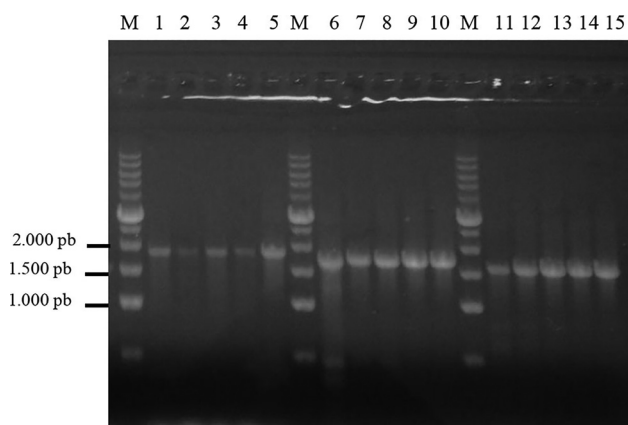
## Cloning of cDNA fragments of *ERG11*

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 14α-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 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).



**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 ≥ 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 14α-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 14α-demethylase

Determination of the sequence of the *ERG11* transcript allowed prediction of the primary structure of lanosterol 14α-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. perniciosa* have been identified by genomic data mining. Our results indicate that the *M. perniciosa* 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 *Kluveromyces 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-
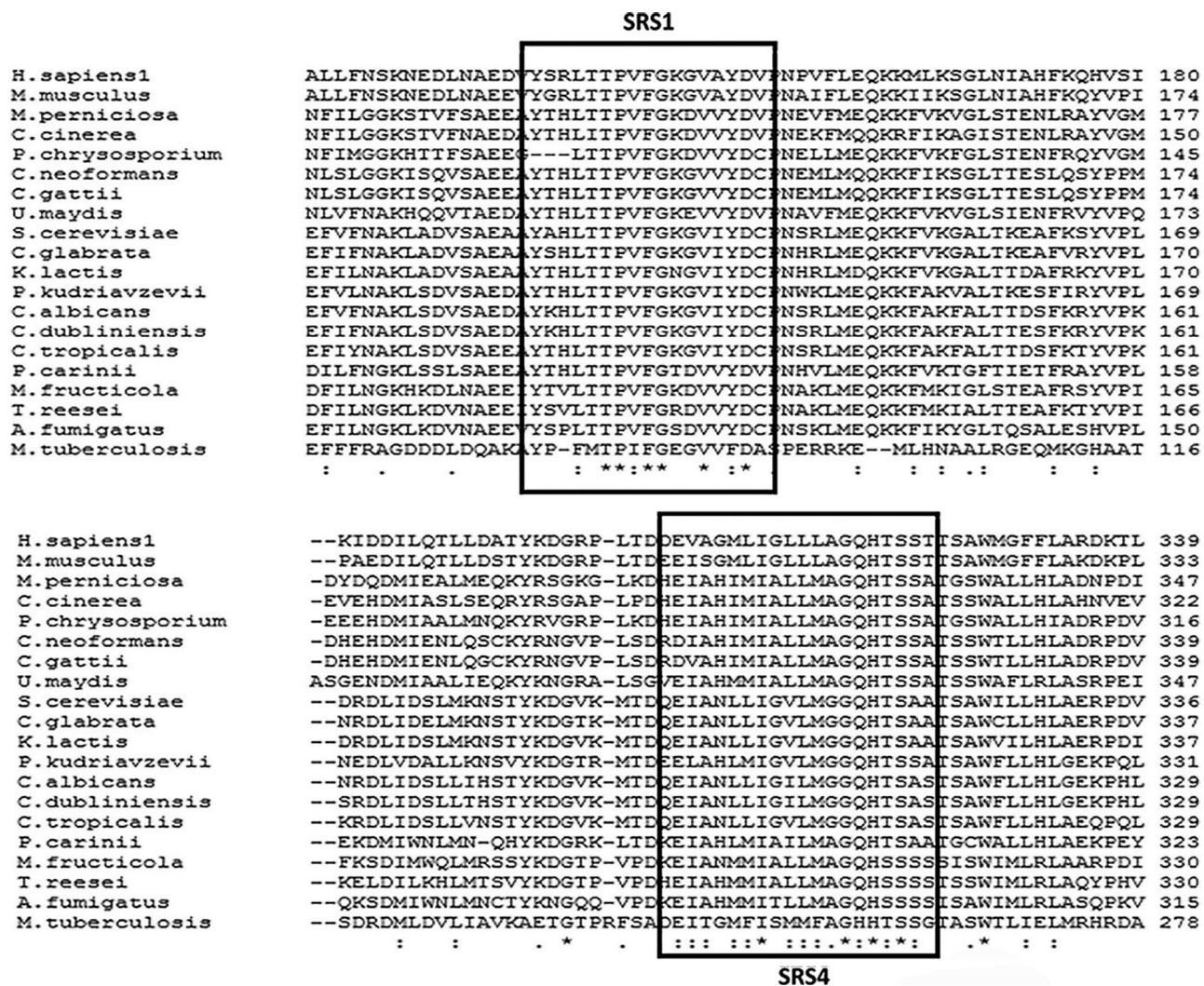


**Figure 3** - *Moniliophthora perniciosa* CYP51 alignment with other homologous CYP51 sequences. Conserved amino acid residues that correspond to the heme-binding site (HBR), substrate recognition sites (SRS1 and SRS4) and EXXR motifs are shown in boxes. The arrow indicates the conserved cysteine residue. *Homo sapiens 1 - H. sapiens* isoform 1 (NP_0000777.1), *Mus musculus - Mus musculus* (NP_064394.2), *Moniliophthora perniciosa - Moniliophthora perniciosa* (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), *Kluveromyces lactis, Kluveromyces 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).

```
H.sapiens1      Q---KKCYLEQKTVCG-----ENLPPLTYDQLK-DLNLLDRCIKETLRIRPPIMIMMRMA 390
M.musculus      Q---EKCYLEQKAVCG-----EDLPPLTYDQLK-DLNLLDRCIKETLRIRPPIMTMMRMA 384
M.perniciosa    A---EALYQEQVKHFSNP--DGSFRSMSYEELR-ELPVLDSVIRETLRVHPPIHSIMRYV 401
C.cinerea       AYVSEALYNEQVEHFSNP--DGSFRPLTYDELR-ALPILDSVIRETLRMHPPIHSIMRYV 379
P.chrysosporium A----EALYEEQVKHFRQS--DGSWRTPEYEELK-ELPVLDSVIRETLRIHPPIHSIMRAV 370
C.neoformans    V---EALYQEQKQKLGNP--DGTFRDYRYEDLK-ELPIMDSIIRETLRMHAPIHSIYRKV 393
C.gattii        V---EALYQEQKEKLGNP--DGTFRDYKYEDLK-ELPIMDSIIRETLRMHAPIHSIYRKV 393
U.maydis        I---EELYEEQLNVYSDG--HGGLRELDYETQKTSVPLLDAVVKETLRIHPPLHSIMRYV 402
S.cerevisiae    Q---QELYEEQMRVL-----DGGKKELTYDLLQ-EMPLLNQTIKETLRMHHPLHSLFRKV 387
C.glabrata      Q---EELYQEQMRVL-----NNDTKELTYDDLQ-NMPLLNQMIKETLRIHHPLHSLFRKV 388
K.lactis        Q---EKLYEEQMKVL-----DNGTKELTFDLLQ-EMPLLNQTIKETLRIHHPLHSLFRKV 388
P.kudriavzevii  Q---EEIYREIQSVLG----ENFERELTYDDLQ-KLDLVNATIKETLRIHMPLHSIFRKV 383
C.albicans      Q---DVIYQEVVELLKEK--GGDLNDLTYEDLQ-KLPSVNNTIKETLRMHMPLHSIFRKV 383
C.dubliniensis  Q---DAIYQEVVELLKEK--GGDLNDLTYEDLQ-KLPSVTNTIKETLRMHMPLHSIFRKV 383
C.tropicalis    Q---DDLYEELTNLLKEK--GGDLNDLTYEDLQ-KLPLVNNTIKETLRMHMPLHSIFRKV 383
P.carinii       I---KLLLEEQKRVFG-----DNLDDLTYDNLK-DMELLSYVIKETLRIHPPLHSIIRKV 374
M.fructicola    M---EELYQEQIEVLG-----ADLPDLKYEDLS-KLTLHQNILKETLRMHHPIHSIMRKV 381
T.reesei        V---EDLYQEQIRVLG-----ADLPPLQYEHLA-KLPLCQAIVKETLRINAPIHSIMRKV 381
A.fumigatus     L---EELYQEQLANLGPAGPDGSLPPLQYKDLD-KLPFHQHVIRETLRIHSSIHSIMRKV 371
M.tuberculosis  Y---AAVIDELDELYGDG------RSVSFHALR-QIPQLENVLKETLRIHPPLIILMRVA 328
                   *                  :.:      :      :****:. .:  : *  .
                                                         EXXR

H.sapiens1      ------------------------DNPASGEKFAYVPFGAGRHRCIGENFAYVQIKTIWS 470
M.musculus      ------------------------DNPASGEKFAYVPFGAGRHRCVGENFAYVQIKTIWS 464
M.perniciosa    EGIAAQALKTYVDE-NGEKIDYGFGAVSKGTESPYQPFGAGKHRCIGEQFAYLQLGTLIA 515
C.cinerea       NGVAAQAYSTYVDG-NGEKIDYGFGAVSKGTESPYQPFGAGKHRCIGEQFAYLQLGTLIA 493
P.chrysosporium EGVAAQAYKQYDDA-EGAKVDFGFGLVSKGTDSPYQPFGAGRHRCIGEQFAYLQLGTIIS 484
C.neoformans    KGFAAAAMVQYT---KAEQVDYGFGSVSKGTESPYQPFGAGRHRCVGEQFAYTQLSTIFT 505
C.gattii        KGFAAAAMAQYT---KAEQVDYGFGSVSKGTESPYQPFGAGRHRCVGEQFAYTQLSTIFT 505
U.maydis        T--TAAAMQDSG----EDKQDFGFGMISTGANSPYLPFGAGRHRCIGEQFAYLQIGVILA 516
S.cerevisiae    ---DSASSYSVG-----EEVDYGFGAISKGVSSPYLPFGGGRHRCIGEHFAYCQLGVLMS 485
C.glabrata      ---DAASSSAAGG----DEVDYGFGAISKGVSSPYLPFGGGRHRCIGELFAYCQLGVLMS 487
K.lactis        ---DAASSYASN-----EKVDYGFGSISKGVSSPYLPFGGGRHRCIGEHFAYMQLGTILS 486
P.kudriavzevii  KSIDGGISLPAEGENAKETVDYGFGKISKGVASPYLPFGGGRHRCIGEPFAYTQLGTLLV 490
C.albicans      AAKANSVSFNSS-----DEVDYGFGKVSKGVSSPYLPFGGGRHRCIGEQFAYVQLGTILT 485
C.dubliniensis  AAKANSVSFNSS-----DEVDYGFGKISKGVSSPYLPFGGGRHRCIGEQFAYVQLGTILT 485
C.tropicalis    DTKASAVSFNSE-----DTVDYGFGKISKGVSSPYLPFGGGRHRCIGEQFAYVQLGTILT 485
P.carinii       -----EKNTEDS-----DKIDYGYGLVTKGAFSPYLPFGAGRHRCIGEQFAYMQLGTIIT 470
M.fructicola    SG---RVIGNDQDE---EFQDYGYGMISKGASSPYLPFGAGRHRCIGEQFANVQLITIMA 482
T.reesei        SPNAPLMVRNDEEE---EKVDYGYGIVSKGAASPYLPFGAGRHRCIGEHFANLQLQTIVC 485
A.fumigatus     AT------KEQEND---EVVDYGYGAVSKGTSSPYLPFGAGRHRCVGAAFAIMQIKAIFS 469
M.tuberculosis  ------------------------RQEDLLNRWTWIPFGAGRHRCVGAAFAIMQIKAIFS 409
                 .:  ***.*:***:*    **   ::  .:
                                         HBR  ↑
```
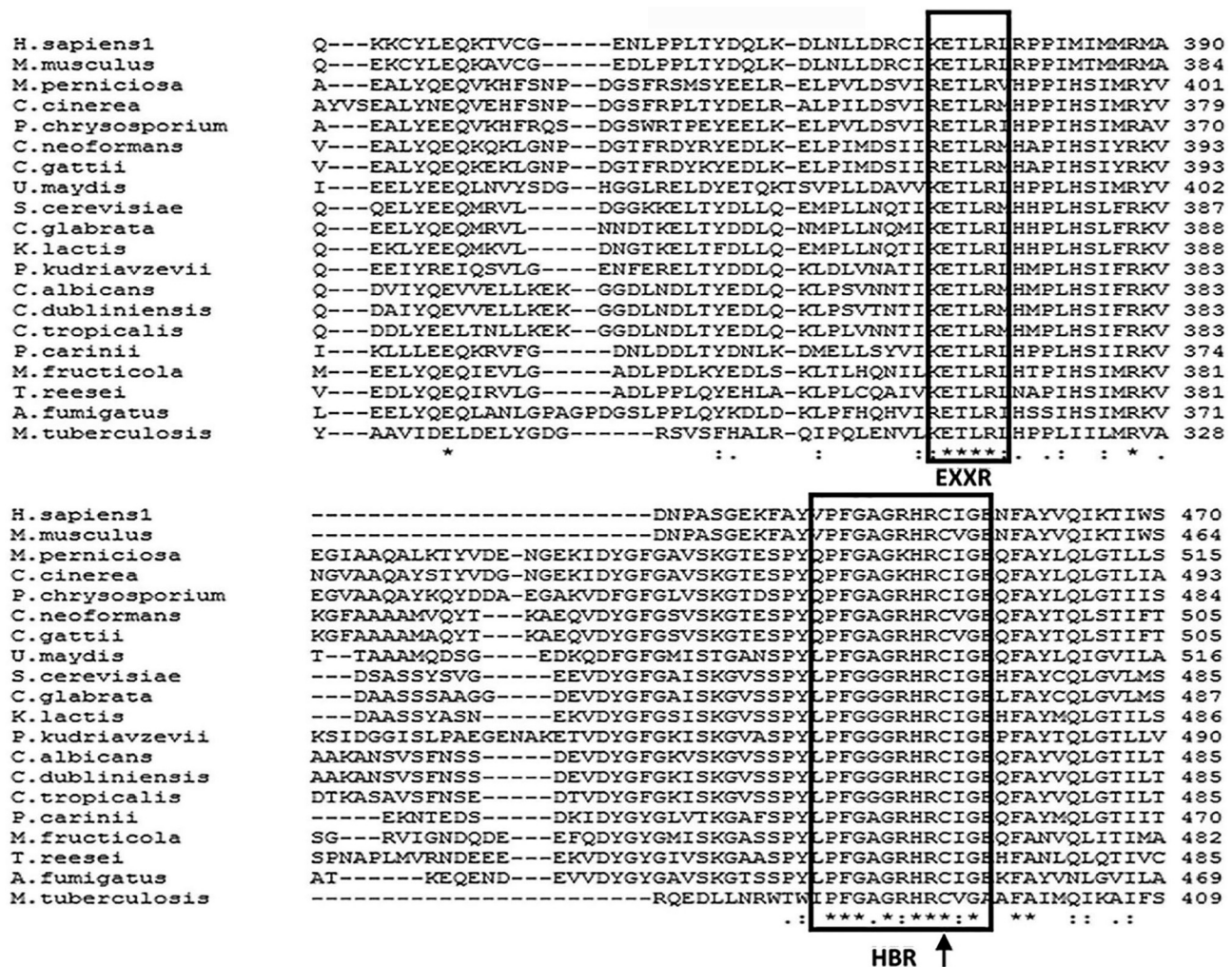
**Figure 3 cont.** -

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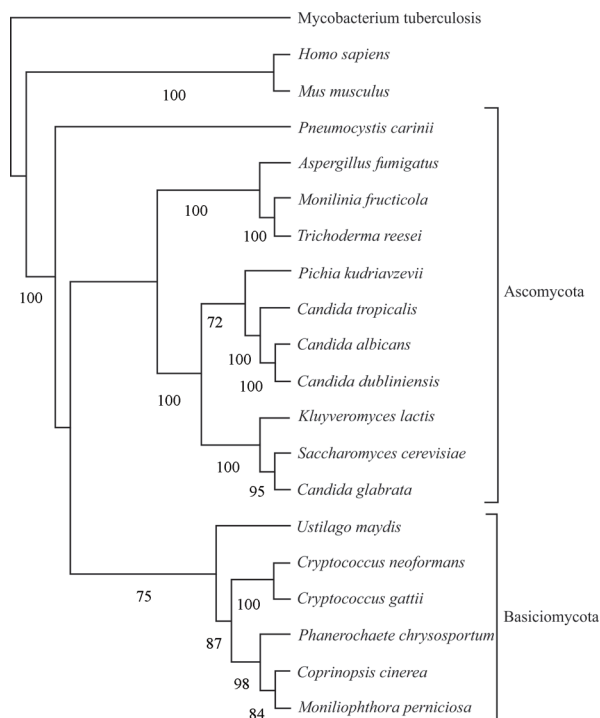
**Figure 4** - The most parsimonious tree obtained from the Maximum Parsimony analysis based on the amino acid sequence of lanosterol 14 α-demethylase. The numbers below the branches indicate bootstrap support (in %). Length: 2,700 steps.

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 be 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. carinni* 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. neorformans* 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 14α-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.

## Acknowledgments

## References

Aime MC and Phillips-Mora W (2005) The causal agents of witches' broom and frost pod rot of cacao (chocolate, *Theobroma cacao*) from a new lineage of Marasmiaceae. Mycologia 97:1012-1022.

Albertini C, Thebaud G, Fournier E and Leroux P (2002) Eburicol 14α-demethylase gene (CYP51) polymorphism and speciation in *Botrytis cinerea*. Mycol Res 106:1171-1178.

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403-410.

Bak S, Kahn RA, Oisen CE and Halkier BA (1997) Cloning and expression in *Escherichia coli* of the obtusifoliol 14α-demethylase of *Sorghum bicolor* (L.) Moench, a cytochrome P450 orthologous to the sterol 14α demethylases (CYP51) from fungi and mammals. Plant J 11:191-201.

Barrett-Bee K and Dixon G (1995) Ergosterol biosynthesis inhibition: A target for antifungal agents. Acta Biochim Pol 42:465-480.

Bellamine A, Mangla AT, Nes WD and Waterman MR (1999) Characterization and catalytic properties of the sterol 14α-demethylase from *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 96:8937-8942.

Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, *et al.* (2009) Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459:657-662.

Carrillo-Muñoz AJ, Giusiano G, Ezkurra PA and Quindós G (2006) Antifungal agents: Mode of action in yeast cells. Rev Esp Quim 19:130-139.

Ceita GO, Macedo JN, Santos TB, Alemanno L, Gesteira AS, Micheli F, Mariano AC, Gramacho KP, Silva DC, Meinhardt LW, *et al.* (2007) Involvement of calcium oxalate degradation during programmed cell death in *Theobroma cacao* tissues triggered by the hemibiotrophic fungus *Moniliophthora perniciosa*. Plant Sci 173:106-117.

D'Souza CA, Kronstad JW, Taylor G, Warren R, Yuen M, Hu G, Jung WH, Sham A, Kidd SE, Tangen K, *et al.* (2011) Genome variation in *Cryptococcus gattii*, an emerging pathogen of immunocompetent hosts. MBio 2:e00342-10.

Délye C, Laigret F and Corio-Costet MF (1997) Cloning and sequence analysis of the eburicol 14α-demethylase gene of the obligate biotrophic grape powdery mildew fungus. Gene 195:29-33.

Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, De Montigny J, Marck C, Neuvéglise C, Talla E *et al.* (2004) Genome evolution in yeasts. Nature 430:35-44.

Evans HC (2007) Cacao diseases - The trilogy revisited. Phytopathology 97:1640-1643.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Formighieri EF, Tiburcio RA, Armas ED, Medrano FJ, Shimo H, Carels N, Góes Neto A, Cotomacci C, Carazzolle MF, Sardinha-Pinto N, *et al.* (2008) The mitochondrial genome of the phytopathogenic basidiomycete *Moniliophthora perniciosa* is 109 kb in size and contains a stable integrated plasmid. Mycol Res 112:1136-1152.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD and Bairoch A (2005) Protein identification and analysis tools on the ExPASy Server. In: Walker JM (ed) The Proteomics and Protocols Handbook. Humana Press, Totowa, pp 571-607.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, *et al.* (1996) Life with 6000 genes. Science 265:2077-2082.

Griffith GW, Bravo-Velasquez E, Wilson FJ, Lewis DM and Hedger JN (1994) Autecology and evolution of the witches' broom pathogen (*Crinipellis perniciosa*) of cocoa. In: Blakeman JP and Williamson B (eds) The Ecology of Plant Pathogens. CAB International, Wallingford, pp 245-265.

Griffith GW, Nicholson J, Neininger A and Birch R (2003) Witches' brooms and frosty pods: Two major pathogens of cacao. New Zeal J Bot 41:423-435.

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Res 41:95-98.

Hof H (2001) Critical annotations to the use of azole antifungals for plant protection. Antimicrob Agents Chemother 45:2987-2990.

Jackson CJ, Lamb DC, Marczylo TH, Parker JE, Manning NL, Kelly DE and Kelly SL (2003) Conservation and cloning of CYP51: A sterol 14α-demethylase from *Mycobacterium smegmatis*. Biochem Biophys Res Commun 301:558-563.

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J, *et al.* (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature 19:818-822.

Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, *et al.* (2004) The diploid genome sequence of *Candida albicans*. Proc Natl Acad Sci USA 101:7329-7334.

Kairuz PB, Zuber JP, Jaunin P, Buchman TG, Bille J and Rossier M (1994) Rapid detection and identification of *Candida albicans* and *Torulopsis* (*Candida*) *glabrata* in clinical specimens by species-specific nested PCR amplification of a cytochrome P-450 lanosterol-α-demethylase (L1A1) gene fragment. J Clin Microbiol 32:1902-1907.

Kalb VF, Woods CW, Turi TG, Dey CR, Sutter TR and Loper JC (1987) Primary structure of the P450 lanosterol demethylase gene from *Saccharomyces cerevisiae*. DNA 6:529-537.

Kall L, Krogh A and Sonnhammer EL (2007) Advantages of combined transmembrane topology and signal peptide prediction- the Phobius web server. Nucleic Acids Res 35:429-432.

Kim D, Lim YR, Ohk SO, Kim BJ and Chun YJ (2011) Functional expression and characterization of CYP51 from dandruff-causing *Malassezia globosa*. FEMS Yeast Res 11:80-87.

Lai MH and Kirsch DR (1989) Nucleotide sequence of cytochrome P450 L1A1 (lanosterol 14α-demethylase) from *Candida albicans*. Nucleic Acids Res 17:804.

Lamb DC, Kelly DE, Manning NM, Hollomon DW and Kelly SL (1998) Expression, purification, reconstitution and inhibition of *Ustilago maydis* sterol 14α-demethylase (CYP 51; P450). FEMS Microbiol Lett 169:369-373.

Lee CH, Hsu KH, Wang SY, Chang TT, Chu FH and Shaw JF (2010) Cloning and characterization of the lanosterol 14α-demethylase gene from *Antrodia cinnamomea*. J Agr Food Chem 58:4800-4807.

Lees ND, Skaggs B, Kirsch DR and Bird M (1995) Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae* - A review. Lipids 30:221-226.

Lepesheva GI and Waterman MR (2007) Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim Biophys Acta 3:467-477.

Luo CX and Schnabel G (2008) The cytochrome P450 lanosterol 14α-demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilia fructicola* field isolates from Georgia. Appl Environ Microb 74:359-366.

Martin F, Aerts A, Ahrén D, Brun A, Danchin EG, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, *et al.* (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature 452:88-92.

McQuilken MP and Rudgard SA (1988) Sensitivity of *Crinipellis periciosa* to two triazole fungicides in vitro and their effect on development of the fungus in cocoa. Plant Pathol 37:499-506.

Meinhardt LW, Bellato CM, Rincones J, Azevedo RA, Cascardo JCM and Pereira GAG (2006) *In vitro* production of biotrophic-like cultures of *Crinipellis perniciosa*, the causal agent of Witches' broom disease of *Theobroma cacao*. Curr Microbiol 52:191-196.

Mellado E, Guerra TMD, Estrela MC and Tudela JLR (2001) Identification of two different 14α-sterol demethylase related genes (cyp51A and cyp51B) in *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol 39:2431-2438.

Mondego JMC, Carazzolle MF, Costa GGL, Formighieri EF, Parizzi LP, Rincones J, Cotomacci C, Carraro DM, Cunha AF, Carrer H, *et al.* (2008) A genome survey of *Moniliophthora perniciosa* gives new insights into Witches' broom disease of cacao. BMC Genomics 9:1-25.

Morales IJ, Vohra PK, Puri V, Kottom TJ, Limper AH and Thomas CF (2003) Characterization of a lanosterol 14α demethylase from *Pneumocystis carinii*. Am J Resp Cell Mol 29:232-238.

Mota SGR, Barros TF and Castilho MS (2010) *In vitro* screening and chemometrics analysis on a series of azole derivatives

with fungicide activity against *Moniliophthora perniciosa*. J Braz Chem Soc 21:510-519.

Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, *et al.* (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438:1151-1156.

Page RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357-358.

Park HG, Lee IS, Chun YJ, Yun CH, Johnston JB, Montellano PRO and Kim D (2011) Heterologous expression and characterization of the sterol 14α-demethylase CYP51F from *Candida albicans*. Arch Biochem Biophys 509:9-15.

Pereira JL, Ram A, Figueiredo JM and Almeida LCC (1989) Primeira ocorrência de vassoura-de-bruxa na principal região produtora de cacau do Brasil. Agrotrópica 1:79-81.

Petersen TN, Brunak S, Heijne G and Nielsen H (2011) SignalIP 4.0: Discriminating signal peptides from transmembrane regions. Nat Methods 10:785-786.

Pietila MP, Vohra PK, Sanyat B, Wengenack NL, Raghavakaimal S and Thomas CF (2006) Cloning and characterization of CYP51 from *Mycobacterium avium*. Am J Resp Cell Mol 35:236-240.

Pires ABL, Gramacho KP, Silva DC, Góes-Neto A, Silva MM, Muniz-Sobrinho JS, Porto RF, Villela-Dias C, Brendel M, Cascardo JCM, *et al.* (2009) Early development of *Moniliophthora perniciosa* basidiomata and developmentally regulated genes. BMC Microbiol 9:e158.

Purdy LH and Schimidt RA (1996) Status of cacao witches' broom: Biology, epidemiology, and management. Annu Rev Phytopathol 34:573-594.

Raeder U and Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17-20.

Revankar SG, Fu J, Rinaldi MG, Kelly SL, Kelly DE, Lamb DC, Keller SM and Wickes BL (2004) Cloning and characterization of the lanosterol 14α-demethylase (*ERG11*) gene in *Cryptococcus neoformans.* Biochem Biophys Res Commun 324:719-728.

Rincones J, Scarpari LM, Carazzolle MF, Mondego JMC, Formighieri, EF, Barau JG, Costa GGL, Carraro DM, Brentani HP, Vilas-Boas LA, *et al.* (2008) Differential gene expression between the biotrophic-like and saprotrophic mycelia of the witches' broom pathogen *Moniliophthora perniciosa*. Mol Plant Microbe Int 21:891-908.

Rio MCS, Oliveira BV, Tomazella DPT, Silva JAF and Pereira GAG (2008) Production of calcium oxalate crystals by the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom disease of cacao. Curr Microbiol 56:363-370.

Rozman D, Stromstedt M, Tsui LC, Scherer SW and Waterman MR (1996) Structure and mapping of the human lanosterol 14α-demethylase gene (CYP51) encoding the cytochrome P450 involved in cholesterol biosynthesis: Comparison of exon/intron organization with other mammalian and fungal CYP genes. Genomics 38:371-381.

Sheng C, Miao Z, Ji H, Yao J, Wang W, Che X, Dong G, Lu J, Guo W and Zhang W (2009) Three-dimensional model of lanosterol 14α-demethylase from *Cryptococcus neoformans*: Active-site characterization and insights into azole binding. Antimicrob Agents Chemother 53:3487-3495.

Sigrist CJA, Cerutti L, Castro E, Langendijk-Genevaux PS, Bulliard V, Bairoch A and Hulo N (2009) PROSITE, a protein domain database for functional characterization and annotation. Nucleic Acids Res 38:161-166.

Stajich JE, Wilke SK, Ahrén D, Au CH, Birren BW, Borodovsky M, Burns C, Canback B, Casselton LA, Cheng CK, *et al.* (2010) Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). Proc Natl Acad Sci USA 107:11889-11894.

Stanke M, Keller O, Gunduz I, Hayes A, Waack S and Morgenstern B (2006) AUGUSTUS: Ab initio prediction of alternative transcripts. Nucleic Acids Res 34:435-439.

Swofford DL (2002) PAUP - Phylogenetic Analysis Using Parsimony (and other methods). Version 4.0b10. Sinauer Associates, Sunderland, MA.

Ter-Hovhannisyan V, Lomsadze L, Chernoff YO and Borodovsky M (2008) Gene prediction in novel fungal genomes using an ab initio algorithm with unsupervised training. Genome Res 18:1979-1990.

Thompson JD, Higgins DG and Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weigh matrix choice. Nucleic Acids Res 22:4673-4680.

Veen M and Lang C (2005) Interactions of the ergosterol biosynthetic pathway with other lipid pathways. Biochem Soc Trans 33:1178-1181.

Warrilow AGS, Melo N, Martel CM, Parker JE, Nes WD, Kelly SL and Kelly DE (2010) Expression, purification and characterization of *Aspergillus fumigatus* sterol 14α demethylase (CYP51) isoenzymes A and B. Antimicrob Agents Chemother 54:4225-4234.

Waterman MR and Lepesheva GI (2005) Sterol 14 α-demethylase, an abundant and essential mixed-function oxidase. Biochem Biophys Res Commun 338:418-422.

Wood HM, Dickinson MJ, Lucas JA and Dyer PS (2001) Cloning of the CYP51 gene from the eyespot pathogen *Tapesia yallundae* indicates that resistance to the DMI fungicide prochloraz is not related to sequence changes in the gene encoding the target site enzyme. FEMS Microbiol Lett 196:183-187.

Zhao L, Liu D, Zhang Q, Zhang S, Wan J and Xiao W (2007) Expression and homology modeling of sterol 14α-demethylase from *Penicillium digitatium*. FEMS Microbiol Lett 277:37-43.

## 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.