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Full Length Research Paper

Generation of a cDNA library reveals the presence of a *Ty-1*/copia transposon in *Phellinus rimosus* (Hymenochaetaceae, Basidiomycota)

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Lignocellulolytic fungi have significant potential as bio-convertors with a particular capacity to degrade recalcitrant natural composts, such as lignin. We generated a cDNA library of a lignocellulolytic fungus, the *Phellinus rimosus* (Hymenochaetaceae, Basidiomycota), and a typical species of polypore mushroom from the Brazilian semi-arid region. To the best of our knowledge, this is the first report ever to describe the existence of a *Ty1*-copia retrotransposon in the *P. rimosus* species. Given the taxonomic complexity in the Brazilian semi-arid region, our findings will be useful as they provide a molecular identity to this important parasite species of the semi-arid region of Brazil.

Key words: Lignocellulolytic fungi, biotechnology, *Phellinus rimosus, Ty-1 copia,* Brazilian semi-arid region, molecular marker.

INTRODUCTION

Most species of the order Hymenochaetales Oberwinkler (Basidiomycota) shows a pattern of tropical distribution and are able to degrade wood, causing white rot. To date, studies in Brazil involving such organisms have been limited to general taxonomic surveys, specific inventories or ecological studies (Drechsler-Santos et al., 2010; 2008; Góes-Neto and Groposo, 2005; Azevedo and Guerrero, 1993). There is a clear lack of studies aimed at functional genomics characterization of this species in Brazil, particularly in the semi-arid region.

The study of molecular diversity of native species of lignocellulolytic Hymenochaetales from the semi-arid region of Brazil, among them the *Phellinus rimousus*, can be carried out through the construction and analyses of cDNA libraries, which, in turn, will enable mapping of genes of biotechnological potential for the development of wood delignification processes, therefore, potentially posing as a strategy for pulp bleaching with a consequent reduction of damaging environmental impact (Martinez et al., 2004, Coehn et al., 2002, Mayer and Staples, 2002, Blanchette, 1991). Thus, the major aim of this study was to construct and analyze a cDNA library of the species *Phellinus rimosus*.

The cDNA library was generated and clones derived from the library were sequenced and analyzed *in silico* using bioinformatics tools. To the best of our knowledge, this is the first report ever to describe the existence of a *Ty1*-copia retrotransposon in the *P. rimosus* species.

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Given the taxonomic complexity in the Brazilian semi-arid region, our novel findings could be useful as they provide a molecular identity to this important parasite species of the semi-arid region of Brazil.

MATERIALS AND METHODS

Sample collection and processing

Phellinus rimosus basidiomata were removed from the trunk of live tree of *Cesalpinia pyramidalis* (Leguminosae) in the Brazilian semi-arid region (Ipirá, Bahia, Brazil, 12°10'49"S; 39°46'14"W) in an area of Caatinga vegetation. Basidiomata were then appropriately placed in a waxed paper bag and transported in a cooler to be immediately processed. A fresh portion of the tissue of the internal part of the pileus was used as source for extraction of total RNA. The remaining basidiomata were then dehydrated under a constant flow of warm air at 40°C in an electric dehumidifier and subsequently deposited in HUEFS (Herbarium of the *Universidade Estadual de Feira de Santana* - BA) under the number 118024 (http://herbario.uefs.br).

Taxonomic identification

The taxonomic identification of the host tree species was determined by comparison of identified vouchers in HUEFS with the aid of an expertise botanist in Leguminosae. In addition, it was based on the analysis of macro and micromorphological features as previously described by Ryvarden 1991, and host identity by Dreschler-Santos et al. Briefly, features included in our macro-analysis of dehydrated basidiomes comprised the type and longevity of basidiome colour, form, attachment, surface, and size of pileus (when present), as well as the colour and form of abhymenial margin (when present), and also the colour, fertility and size of hymenial margin, form and number of pores, form and thickness of dissepiments, the presence or the absence of black line, and stratification, colour, and size of tubes. Micro-analysis features included the type of the hyphal system, colour, wall, form, ramification, septation and diameter of context and tramal generative and skeletal hyphae; the presence or the absence of crystal hyphae, and extra-hymenial setae; the presence or the absence, colour, wall, and form of hymenial setae; presence or absence, colour, wall, and form of cystidioles; colour (in water and KOH), wall, form, size, and length/width ratio of spores. Macroscopic features were analyzed with the aid of a stereomicroscope and sections were made for the study of microscopic characteristics. Slide preparations were stained with 1% phloxine, 5% KOH, and Melzer's reagent was used to test the iodine reaction.

RNA extraction

Total RNA was extracted using the RNeasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues, and Filamentous Fungi (Qiagen, Germantown, MD, USA). Next, we used the kit BD Creator Smart cDNA Library Construction (Clontech, Mountain View, CA, USA) to obtain the cDNA library. DNA fragments were fractioned using a HIS Trap column (FF crude, GE Healthcare, Waukesha, WI, USA). The specific profile of fractions was further verified by gel electrophoresis. Selected fragments were annealed to the vector pTZ57R/T (Figure 1) (Fermentas) following the manufacturer instructions. Transformation was performed using a BIO-RAD *Gene Pulser Cuvette* 0.2 cm electrod, employing electro-competent DH5ß cells (Invitrogen, Carlslab, CA, USA). Plating was carried out between the cells undergoing transformation and blank colonies were pre-selected. Twelve positive clones were used as template, for proper control of fragment sizes inserted in the transformation medium through a PCR reaction using the following primers (M13-F 5' GTAAAACGACGGCCAGT 3' and M13-R 5' CAGGAAACAGCTATGAC 3'). Vector extraction was accomplished through Mini-prep and sequencing reactions were processed by using *DYEnamic ™ ET* Dye Terminator Cycle Sequencing kit for *MegaBACE DNA* Analysis Systems (Amersham Biosciences, Sweden). Generated sequences were then converted to the FASTA file format and quality scores were simultaneously obtained with PHRED. Values equal or higher to 10 were discarded and final processing achieved with utilization of the CROSS-MATCH program. Sequences were then analyzed using Genbank/tBLASTx (NCBI, MD, USA) (Johnson et al., 2008).

Cloning procedures

First strand cDNA were synthesized using the Clontech BD SMART PCR cDNA Synthesis KIT (Mountain View, CA) as recommended by the manufacturer. 0.5 to 1 µg of total RNA was incubated at 72°C for 2 min with 1 µl 3' BD SMART CDS Primer II A (12 µM) and 1 µl BD SMART II A Oligonucleotide (12 µM) in a total volume of 5 µl. Then 2 µl 5X First-Strand Buffer, 1 µl DTT (20 mM); 1 µl dNTP Mix (10 mM of each dNTP), 1 µl BD PowerScript Reverse Transcriptase were added and the mixture was incubated at 42°C for 1 h in an air incubator. Next, 3 µl Biotin-dATP (Invitrogen), 3 µl Biotin-dCTP (Invitrogen), 1 µl 5'-NVVVVV-3' prime, 2 µl 5X First-Strand Buffer, 1 µl BD PowerScript Reverse Transcriptase were added, and the mixture was kept at 42°C for 30 min. For capture of the unfinished strand, the reaction was mixed with 600 µl of Streptavidine MagneSphere Paramagnetic Particles (Promega) and eluted as recommended by the manufacturer. For the cDNA amplification step, a 2 µl aliquot from the first strand synthesis was used in the LD PCR (Clontech). Each reaction was performed with 80 µl deionized water, 10 µl 10X BD Advantage 2 PCR Buffer, 2 µl 50X dNTP Mix (10 mM of each dNTP), 4 µl 5' PCR Primer II A (12 µM), 2 µl 50X BD Advantage 2 Polymerase Mix in a 98 µl total volume. The PCR reaction consisted of 18 to 25 PCR cycles at 95°C for 15 s, 65°C for 30 s, 68°C for 6 min, following with a final extension at 70°C for 10 min. After comparison of fragment sizes with those of model species, fragment sizes of some cDNA libraries were improved using cDNA size fractionation. These libraries were submitted to an "agarase step" after 18 cycles PCR. Double-stranded cDNA was separated on 1% low-melting agarose gel and the DNA ladder "lane" was stained and photographed. Two size fractions (< 1.2 kb and > 1.2 kb) were excised from the unstained cDNA "lane" based on the DNA ladder "lane". cDNAs were extracted from the gel slices with agarase (Fermentas). After a gelase digestion, the cDNA was precipitated with one volume of isopropanol. The pellets were dried and suspended in ribonuclease free water. Four to five additional PCR cycles were performed in order to improve the efficiency of ligation in pGEM®-T Easy Vector. One µl of the second strand product was cloned in pGEM®-T Easy Vector Systems (Promega) and transformed by electroporation in the DH10B T1 resistant strain of Escherichia coli (Invitrogen). Last, transformation products were platted on LB-ampicillin agar plates and incubated overnight at 37°C. White colonies were picked using a Qpix 2 XT biorobot (Genetix) and stored in 384 well plates at -80°C.

RESULTS AND DISCUSSION

Our first step was to clone specific inserts in to the

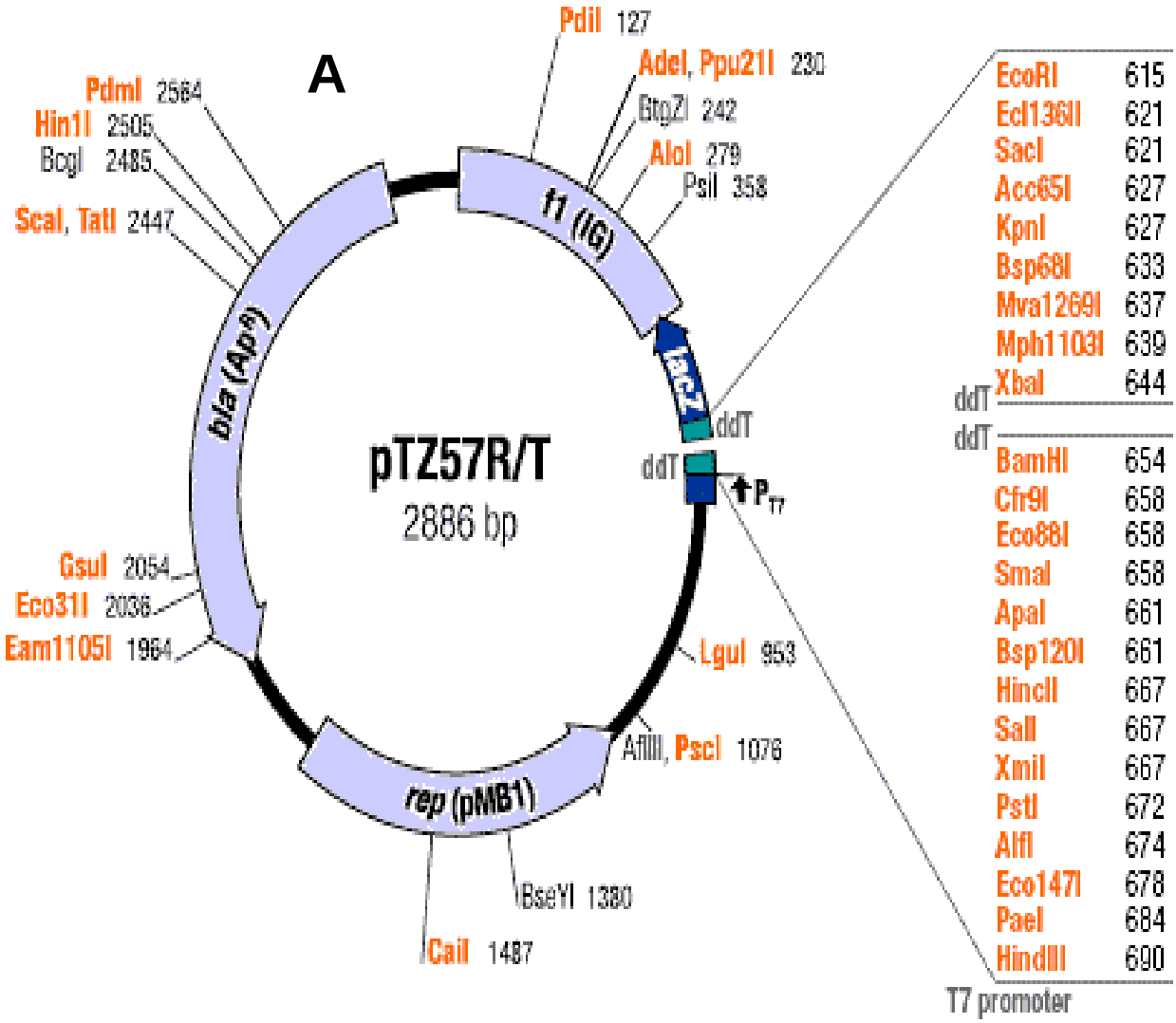

Figure 1A. Schematic diagram of the vector pTZ57R/T. This vector was used in the cloning of fragments amplified by RT-PCR.

pTZ5R/T vector. To this end, a total of 86 inserts were cloned into the pTZ57R/T vector shown in Figure 1A. They had an average size of 665.8 bp (116-1782) and a PHRED valued of ≥ 10 (Table 1). Among the analyzed sequences, most of them demonstrated significant similarity with rRNA sequences of species belonging to the genus *Phellinus* (Table 1), in agreement with other studies (Grant et al., 2006, Martinez et al., 2006). Next, as shown in Figure 1B, total RNA samples of the species *P. rimosus* were efficiently extracted**.** PCR of the colonies revealed that the majority of cloned fragments had a size superior to 500 bp as demonstrated in Figure 2. Importantly, these results support the robustness of methods employed in this study and also demonstrate the efficiency of our bacterial transformation.

We also found that most of the sequences had an E value near zero, indicating a high degree of similarity between the compared sequences. It also demonstrates that alignment did not occur merely by chance. Furthermore, results shown in Table 1 demonstrate that clone F06 shares a high degree of homology (75%) with the coding gene of the small rRNA subunit of *Phellinus kawakamii,* a species that is phylogenetically related to the *Phellinus rimosus* (Larsson et al., 2006). The samples (field-collected basidiomata) were undoubtedly identified as *Phellinus rimosus*, based on morphology and comparison with well-identified vouchers in HUEFS from the same region (additional details are in materials and methods section). Furthermore, *Phellinus rimosus* exhibits host recurrence (Cesalpinia spp.) in the Caatinga vegetation of Brazilian Semi-arid region (Drescheler-Santos, 2010) and our samples were collected in this host (more specifically, Cesalpinia pyramidalis). In addition, the morphological identification of this voucher specimen was also reviewed (Drechsler-Santos et al., 2009). Surprisingly, the D05 clone showed significant

Table 1. cDNA library clones of the *Phellinus rimosus* present significant homology to sequences deposited in *GenBank*. Surprisingly, clone D05 corresponds to a RT coding gene segment of a retrotransposon providing a novel way of molecular identity for this species.

Clone	Homology	Size (Bp)	Access (Hits)	E-value
A08	*Phellinus laevigatus* small subunit ribosomal RNA gene	812	AF230363 AF465617 L47584	3e-22 9e-04 0.003
A12	*Laccaria bicolour* S238N-H82 hypothetical protein	485	XM_001880189 XM_001830397 XM_002475341	4e-14 1e-13 5e-12
B03	Uncultured soil basidiomycete ITS1, 5.8S rRNA gene and ITS2	328	FM866368 DQ873641 EF011124	0.010 0.014 0.014
C02	*Clavibacter michiganensis* subsp. Michiganensis	881	AM711867 AM849034	0.021 0.069
C07	*Aspergillus oryzae* RIB40 DNA, SC026	629	AP007159	0.68
C10	*Coprinopsis cinerea* okayama7#130 hypothetical protein	929	XM_001829195 XM_002395956 XM_770463	1e-07 1e-06 1e-04
D05	*Zingiber officinale* retrotransposon putative reverse transcriptase (RT) gene	585	Q983234	9e-33
D10	*Phellinus laevigatus* small subunit ribosomal RNA gene	962	AF230363	0.51
E11	*Phellinus laevigatus* small subunit ribosomal RNA gene	562	AF230363 EF204913 FJ591062	9e-11 6e-09 5e-07
F02	*Coprinopsis cinerea* okayama7#130 hypothetical protein	558	XM_001834637 XM_001883187	2e-17 1e-15
F06	*Phellinus kawakamii* 25S large subunit ribosomal RNA gene	536	AY059028 AM269847 AM269856	3e-25 4e-25 4e-25

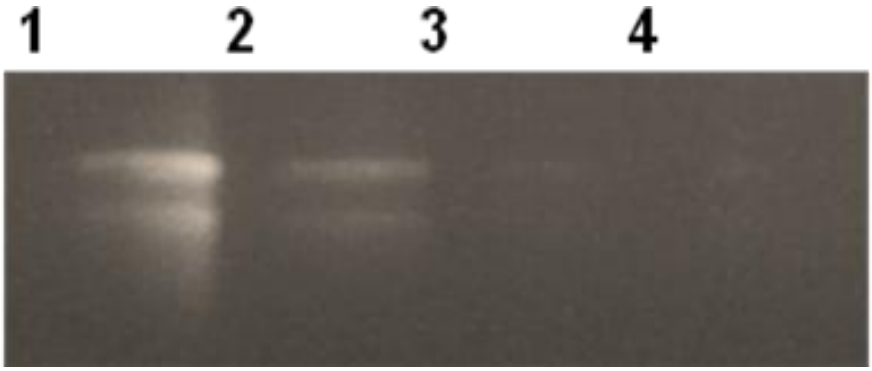

Figure 1B. Effective purification of RNA samples after treatment with DNase I. The first 2 lanes (Lanes 1 and 2) correspond to the first elution of the column, whiles Lanes 3 and 4 to the second elution column.

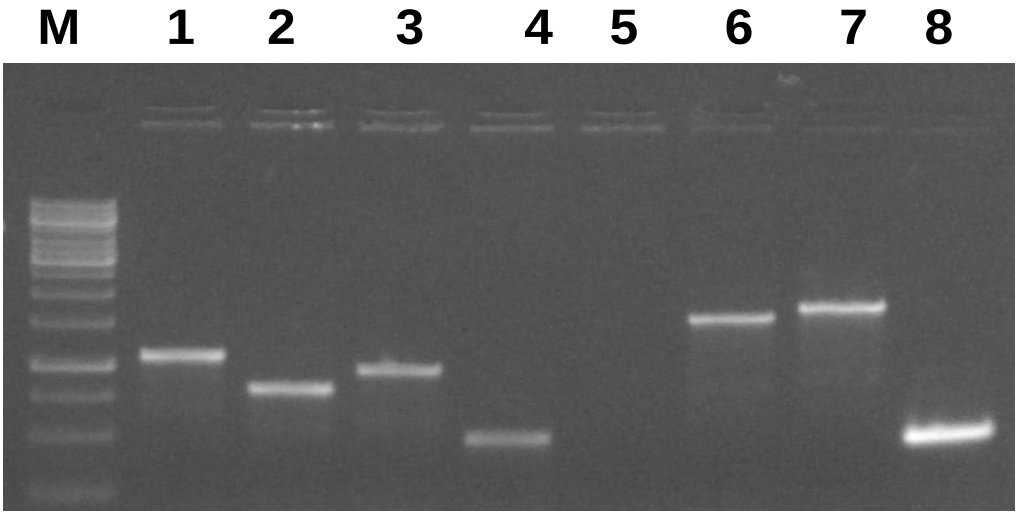

Figure 2. Robust amplification of cDNA molecules. Molecules obtained from colonies of the *P. rimosus* library were of different molecular weights, with most fragments having molecular weights equal or superior to 500 bp (Lanes 1-8= colonies; M = molecular weight marker 1 Kb).

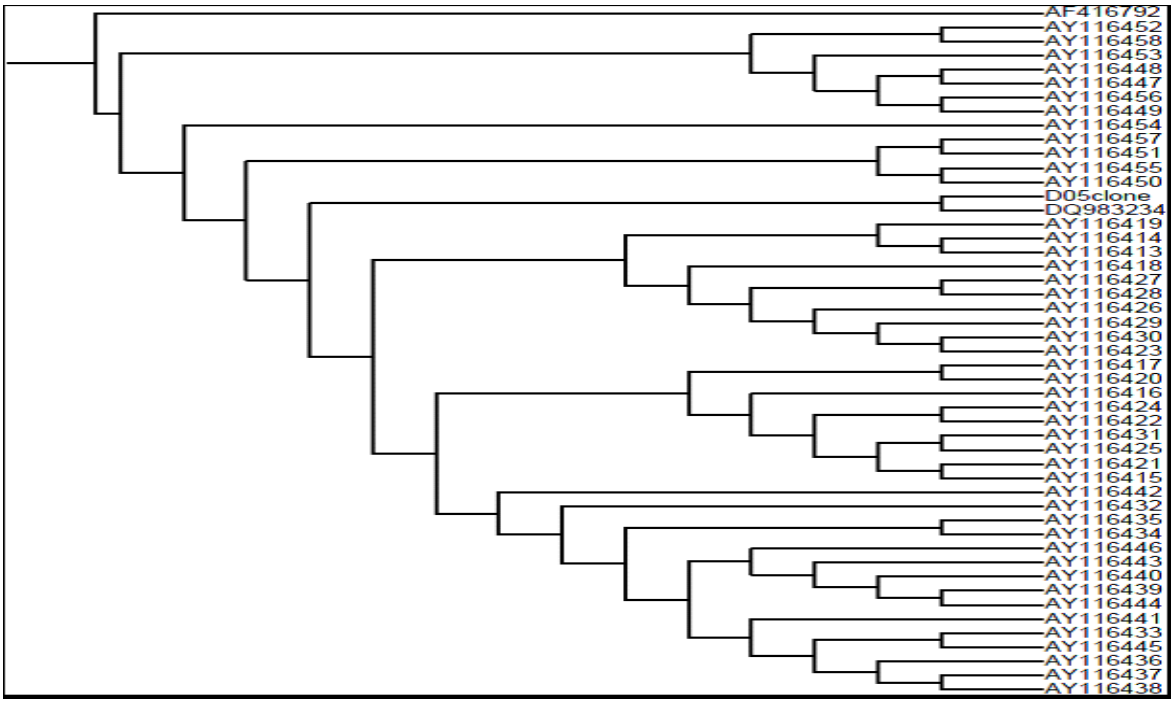

Figure 3. Phylogenetic tree based on sequences of Diez et al. 2003, D05 clone and *Zingiber officinale* DQ983234 (least squares distance matrix method, outgroup with Ty1copia RT retrotransposon of *Musa* sp. AAB Group clone 3B, AF416792).

homology with sequences of Reverse Transcriptase (RT) of plants (*Zingiber officinale* DQ983234, *Cicer arietinum* AJ535749 and *Beta nana* AJ489197). This clone corresponds to a RT coding gene segment of a retrotransposon, previously described by Díez et al. (2003), which is a new RT class (that is, *Ty1*/*copia)*, characteristic of the Basidiomycota, and related to the RT type *Ty1*/*copia* of plants and not of fungi. To further confirm the identification of this clone we conducted a phylogenetic analysis (least squares distance matrix method, outgroup with Ty1copia RT retrotransposon of *Musa* sp. AAB Group clone 3B AF416792) using the sequences in Díez et al. (2003) that led to the same information from our molecular biology studies (Figure 3 and Table 2). To the best of our knowledge, this work is the first report to demonstrate the existence of a type

Table 2. Correspondence between codes in phylogenetic tree (Figure 3) and sampled taxa.

Code	Taxon
D05 clone	*Phellinus rimosus*
AY116413	*Pisolithus microcarpus*
AY116414	*Pisolithus microcarpus*
AY116429	*Laccaria bicolour*
AY116430	*Laccaria bicolour*
AY116428	*Laccaria bicolour*
AY116423	*Pisolithus microcarpus*
AY116426	*Laccaria bicolour*
AY116427	*Laccaria bicolour*
AY116419	*Pisolithus microcarpus*
AY116418	*Pisolithus microcarpus*
AY116415	*Pisolithus microcarpus*
AY116421	*Pisolithus microcarpus*
AY116422	*Pisolithus microcarpus*
AY116424	*Pisolithus microcarpus*
AY116420	*Pisolithus microcarpus*
AY116445	*Pisolithus* sp
AY116433	*Pisolithus* sp
AY116434	*Pisolithus* sp
AY116436	Pisolithus sp
AY116438	*Pisolithus* sp
AY116437	*Pisolithus* sp
AY116416	*Pisolithus microcarpus*
AY116425	*Pisolithus microcarpus*
AY116431	*Laccaria bicolour*
AY116417	*Pisolithus microcarpus*
AY116442	*Pisolithus* sp
AY116441	*Pisolithus* sp
AY116443	*Pisolithus* sp
AY116440	*Pisolithus* sp
AY116435	*Pisolithus* sp
AY116432	*Pisolithus* sp
AY116444	*Pisolithus* sp
AY116446	*Pisolithus* sp
AY116439	*Pisolithus* sp
AY116447	*Eucalyptus globulus*
AY116449	*Eucalyptus globulus*
AY116456	*Eucalyptus globulus*
AY116448	*Eucalyptus globulus*
AY116453	*Eucalyptus globulus*
AY116458	*Eucalyptus globulus*
AY116452	*Eucalyptus globulus*
AY116450	*Eucalyptus globulus*
AY116455	*Eucalyptus globulus*
AY116451	*Eucalyptus globulus*
AY116454	*Eucalyptus globulus*
AY116457	*Eucalyptus globulus*
AF416792	*Musa* sp. AAB Group clone 3B (OUTGROUP)

Ty1-copia retrotransposon, identified through its RT characteristic in the *P. rimosus* species of the order Hymenochaetales of Basidiomycota. This is very important since the Ty1-copia is normally found in yeasts and plants and not in fungi. Thus, our novel finding allows for the application of such knowledge as a molecular marker (Díez et al., 2003) for the genetic studies of populations of this species, an important species that is a parasite of typical trees of the Brazilian semi-arid region and belonging to a taxonomic complex of very difficult delimitation and interpretation (Drechsler-Santos et al., 2010). Transposons are DNA segments characterized by their ability to move inside the genome. They can cause significant impact in the chromossomal organization, gene expression patterns, as well as induce somaclonal variations in fungi and plants (Daboussi 1997; Hirochika et al., 1996). Retrotransposons move through an intermediary RNA by utilizing RT for reverse transcription and insertion of cDNA into the genome (Daboussi, 1997). LTR retrotransposons are further characterized by two repeated terminals and two ORFs that are similar to the retroviruses genes *gag* and *pol*, which are subdivided into two main categories: *Ty1-copia e Ty3-gypsy* (Daboussi, 1997). *Ty1-copia* elements occur mainly in yeasts and mycelial Ascomycota, but apparently, are very rare in Basidiomycota, having only been described in very small number of species (Díez et al., 2003).

Therefore, our exciting findings provide a way of establishing a molecular identity for the *Phellinus rimosus*, a tree parasite species of high significance for the Brazilian semi-arid region, particularly the Northeast Caatinga. The presence of RT that is characteristic of plants, and the presence of a *Ty-1 copia* transposon in the *Phellinus rimosus* might further suggest that this species could have potential biotechnology applications as a bioconversor in the area of wood delignification. Additional studies will now be required to fully test the utilization of *Phellinus rimosus* as a tool in wood delignification for reduction of the damaging effects to the environment of such process.

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REFERENCES

Azevedo CPL, Guerrero RT (1993). Estudo biossistemático de espécies do gênero Hymenochaete (Basidiomycetes) no Rio Grande do Sul. Insula, 22: 143-176.

Blanchette RA (1991). Delignification by wood-decay fungi. Annu. Rev. Phytopathol, 29: 381-403.

Coehn R, Persky L, Hadar Y (2002). Biotechnological application and potential of wood-degrading mushrooms of the genus Pleurotus. Appl. Microbiol. Biotechnol., 58: 582-594.

Daboussi MJ (1997). Fungal transposable elements and genome evolution. Genetica, 100: 253–260.

Díez J, Beguiristain T, Le Tacon F, Casacuberta JM, Tagu D (2003). Identification of Ty1-copia retrotransposons in three ectomycorrhizal basidiomycetes: evolutionary relationships and use as molecular markers. Curr. Genet., 43: 34–44.

Drechsler-Santos ER, Gibertoni TB, Góes-Neto, A, Cavalcanti MAQ (2009). A re-evaluation of the lignocellulolytic Agaricomycetes from the Brazilian semi-arid region. Mycotaxon, 108: 241-244.

Drechsler-Santos ER, Groposo C, Loguercio-Leite C (2008). New records of lignocellulytic fungi (Basyidiomycetes) from the Atlantic Rain Forest in State of Santa Catarina, Brazil. Hoehnea, 35: 57-61.

Drechsler-Santos ER, Santos PJP, Gibertoni TB, Cavalcanti MAQ (2010). Ecological aspects of Hymenochaetaceae in an area of Caatinga (semi-arid) in Northeast Brazil. Fungal Diversity, 42: 71-78.

Drechsler-Santos ER, Santos PJP, Gibertoni TB, Cavalcanti MAQ (2010). Ecological aspects of Hymenochaetaceae in an area of Caatinga (semi-arid) in Northeast Brazil. Fungal Diversity, 42: 71-78

Góes-Neto A, Groposo C. 2005. Hymenochaetales. Hymenochaetoid clade. Hymenochaetaceae. Version 10 June 2005. http://tolweb.org/Hymenochaetoid_clade/20547/2005.06.10 in The Tree of Life Web Project, http://tolweb.org/ (Visited on August 26, 2010).

Grant S, Grant WD**,** Cowan DA, Jones BE**,** Ma Y, Ventosa A, Heaphy S (2006). Identification of eukaryotic open reading frames in metagenomic cDNA libraries made from environmental samples. Appl. Environ. Microbiol., 72: 135-143.

Hirochika H, Sugimoto K, Otsuki Y, Tsugawa H, Kauda M (1996). Retrotransposons of rice involved in mutations induced by tissue culture. Proc Natl Acad. Sci. USA, 93: 7783–7788.

Johnson M, Zaretskaya, I, Raytselis Y, Merezhuk Y, McGinnis S, Madden, TL (2008). "NCBI BLAST: a better web interface" Nucleic Acids Res., 36: W5-W9.

Larsson KH, Parmasto E, Fischer M, Langer E, Nakasone KK, Redhead AS (2006). Hymenochaetales: a molecular phylogeny for the hymenochaetoid clade. Mycologia, 98: 926-936.

Martinez D, Larrondo LF, Putnam N (2004). Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnol., 22: 695-700.

Martinez RJ, Mills HJ, Story S, Sobercky PA (2006). Prokaryotic diversity and metabolically active microbial population in sediments from an active mud volcano in the Gulf of Mexico. Environ. Microbiol., 8: 1783-1796.

Mayer AM, Staples RC (2002). Laccase: new functions for an old enzyme. Phytochemistry, 60: 551-565.

Ryvarden L (1991). Genera of Polypores-Nomenclature and Taxonomy. Synopsis Fungorum 5, Fungiflora, Oslo.