African Journal of Microbiology Research Vol. 6(12), pp. 2869-2877, 30 March, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1170
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Cloning and characterization of the *pyrG* gene of *Pleurotus ostreatus* and *Pleurotus eryngii*

Yonggang Yin[1,2], Yu Liu[2], Shouxian Wang[2], Huamin Li[3], Liying Niu[2], Shuang Zhao[2], Xiaoli Geng[2], Ming Li[1]* and Feng Xu[2]**

[1]College of Horticulture, Agricultural University of Hebei, Baoding 071001, Hebei, China.
[2]Institute of Plant and Environmental Protection, Beijing Academy of Agriculture and Forestry Science, Beijing 100097, China.
[3]College of Biological Sciences, China Agricultural University, Beijing 100193, China.

Accepted 30 November, 2011

***Pleurotus* spp. are well known and important cultivated mushrooms. However, the *pyrG* gene in *Pleurotus* spp., which is used as a bio-safe selective marker in transformation systems, has not yet been characterized. In the present study, nested degenerated PCR was used to clone conserved fragments of the *pyrG* gene from *Pleurotus ostreatus* and *Pleurotus eryngii*. Chromosome walking technology was then used to obtain the full-length DNA sequence of *pyrG*. Finally, reverse transcriptase (RT) PCR was used to obtain the *pyrG* cDNA sequence. The cDNA sequence of *P. ostreatus* and *P. eryngii* were all 813 bp in length and encoded 246 amino acids. Comparison of the DNA sequences with cDNA sequences of both *P. ostreatus* and *P. eryngii* indicated that *pyrG* genes of these two strains consisted of two introns and their deduced amino acid sequence showed 93.09% similarity. Moreover, the 5'-flanking region of these two genes was analyzed.**

Key words: *Pleurotus ostreatus*, *Pleurotus eryngii*, *pyrG*, nested PCR, chromosome walking.

INTRODUCTION

Pleurotus ostreatus (Fr.) Kummer, the oyster mushroom, and *Pleurotus eryngii* are the most widely cultivated edible mushrooms. Many factors render *Pleurotus* spp. a good model for understanding biochemical and physiological processes. These include hydrophobins involved in hydrophobicity (Ma et al., 2008); medical effects (Gómez-Toribio et al., 2009); high lignincellulitic degrading activity (Rodríguez et al., 2008; Marques et al., 2010; Ruiz-Duenas et al., 2011; Lettera et al., 2011; Piscitelli et al., 2011); agro-industrial waste bioconversion (Songulashvili et al., 2006; Shabtay et al., 2009; Salvachúa et al., 2011); and toxic heavy metal biosorption activities (Pan et al., 2005) that have been applied to environmental protection. Hence, a homologous selective marker based efficient transformation system is demanded emergently for the functional analysis. The orotidine-5'-monophosphate (OMP) decarboxylase encoded by the *pyrG* gene, which is one of the best choice for the homologous selective marker, is the key enzyme in the uridine synthesis process. Kim et al. (1999) reported that the transformation of the *P. ostreatus pyrG* deficient strain by *ura3* from *Trichoderma reesei*. However, the transformation efficiency was too low. The homologous *pyrG* gene from *P. ostreatus* might enhance the transformation efficiency. Thus, it is imperative to isolate homologous *pyrG* from *Pleurotus* spp. in order to establish an efficient transformation system. To date, there has been, to the best of our knowledge, no report of cloning the *pyrG* gene from *Pleurotus* spp.

Nested PCR provide an efficient and high specific resolution for the cloning conserved genes using degenerate primers. The key feature of this method is to design two pairs (both inner and outer) of degenerate primers and the success rate will increase when the 1st PCR product obtained with the outer primer pair is adopted as the template in the 2nd PCR using the inner

*Corresponding author. E-mail: yyliming@hebau.edu.cn. Tel: +86 312 7528306. Fax: +86 312 7528306 or

**Co-corresponding author. E-mail: xxu1022@163.com. Tel: +86 10 51503432. Fax: +86 10 51503899

Table 1. Primers used in this study.

Primer name	Sequence (5'→3')	Reference
ITS1	TCCGTAGGTGAACCTGCGG	White et al., 1990.
ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990.
F1	ATHTTYGARGAYMGNAARTT	This study.
F2	GCNGAYATHGGNAAYACNGT	This study.
R1	TADATNCCNCKNCCNACDAT	This study.
R2	GGNGTNCKRTAYTGYTGNCC	This study.
RV-M	GAGCGGATAACAATTTCACACAGG	TaKaRa, Code No. D3880.
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	TaKaRa, Code No. D3887.
WF1	TGAACCCGATTACGAAGTCCCGGTGA	This study.
WF2	GGGGTAAGCCTACTGAAGAAA	This study.
WR1	ATCGTCGCAGGGCTTTCTTCAGTA	This study.
WR2	TCGTAATCGGGTTCATTGGG	This study.
C1	GTACATATTGTCGTTAGAACGCGTAATACGACTCA	TaKaRa, Code No. DRR015.
C2	CGTTAGAACGCGTAATACGACTCACTATAGGGAGA	TaKaRa, Code No. DRR015.
pyrG-F	ATGAGCTCAAAGGGAGTC	This study.
pyrG-R	TTACGATTTACACCTTTC	This study.

primer pair. Parvizi et al. (2005) reported the first detection of *Leishmania major* in peridomestic *Phlebotomus papatasi* using both nested PCR and semi-nested PCR of nuclear ITS ribosomal DNA from minicircle kinetoplast DNA. Aradaib and Majid (2006) established a simple and rapid method for detection of *Trypanosoma evansi* in the dromedary camel using a nested PCR. Botton et al. (2011) identified the *Pythium insidiosum* by nested PCR in cutaneous lesions of Brazilian horses and rabbits. These demonstrated the validity and efficiency of the cloning conserve fragment by using this strategy.

Several methods have been described for the isolation of unknown regions on either side of the conserved fragment; they are collectively known as genome walking or chromosome walking techniques. The merits of this technique based on the PCR are fast and less labour intensive. It includes a digestion step, end elongation and two final PCR, one for linear amplification and a second for exponential amplification (Acevedo et al., 2008). Chromosome walking method requires either a specific cassette (LeCleir et al., 2004), a specific modification of a universal cassette (Nthangeni et al., 2005), or a special adaptor (Siebert et al., 1995) to make different restriction enzyme libraries. Oubrahim et al. (2005) reported the isolation and sequencing of the 5'-flanking region of mouse caspase-12 gene by chromosome-walking technique to investigate how caspase-12 was transcriptionally and translationally regulated. Acevedo et al. (2008) established an improved genome walking method to the clone of complete genes for novel hydrolytic enzymes from Antarctic sea water bacteria. These inspired us to clone the full length fragment of *pyrG* gene including open reading frame (ORF) and promoter, terminator regions by using combination of the nested PCR and chromosome walking.

This present study was initialed to clone and characterize the *pyrG* gene of *P. ostreatus* and *P. eryngii*, which encodes orotidine-5'-monophosphate decarboxylase (OMPDC). The *pyrG* genes were cloned from monokaryotic *P. ostreatus* and *P. eryngii* strains by a combination of nested PCR and chromosome walking and were then characterized. To our knowledge, this is the first report describing the *pyrG* genes of *P. ostreatus* and *P. eryngii*.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

P. ostreatus No. 2106 dikaryotes strain and *P. eryngii* No. 7 dikaryotes strain were obtained from the Institute of Plant and Environmental Protection, Beijing Academy of Agriculture and Forestry Science, Beijing, China. Basidiospores from the fruiting body were distributed on the potato dextrose agar (PDA) plates. Then the plates were incubated at 25°C till small colony appeared. The colonies were transferred to PDA slants and then validated by the clamp connections by visualizing under a microscope (BX51; Olympus, Tokyo, Japan). The monokaryotic strains were maintained on PDA slants at 25°C and then were used for isolation of genomic DNA. *Escherichia coli* DH10B cells and pGEM-T (Promega, USA) were used as the host cell type and cloning vector, respectively. *E. coli* DH10B harboring the recombined vectors were cultured at 37°C on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 μg/ml; Sigma-Aldrich, St. Louis, MO, USA).

Extraction of genomic DNA

Fungal genomic DNA was extracted following the methods described by Doyle and Doyle (1987), with some modifications. DNA purity was estimated using $A_{260}/A_{280}/A_{230}$ extinction ratios and an ultraviolet (UV) spectrophotometer (LabTech UV-1000; LabTech, Columbia, MO, USA).

Table 2. Gene accession number in this study.

Name of DNA sequences	GenBank accession number
ITS sequence of *Pleurotus ostreatus* strain 2106	HQ286595
ITS sequence of of *Pleurotus eryngii* strain no. 7	HQ286596
pyrG gene DNA sequence of *Pleurotus ostreatus* strain 2106	JF795324
pyrG gene DNA sequence of *Pleurotus eryngii* strain no. 7	JF795325
pyrG gene cDNA sequence of *Pleurotus ostreatus* strain 2106	HQ267384
pyrG gene cDNA sequence of *Pleurotus eryngii* strain no. 7	HQ267383
upstream region of *pyrG* gene of *Pleurotus ostreatus* strain 2106	JN977548
upstream region of *pyrG* gene of *Pleurotus ostreatus* strain 2106	JN604981
downstream region of *pyrG* gene of *Pleurotus eryngii* strain no. 7	JN604982
downstream region of *pyrG* gene of *Pleurotus eryngii* strain no. 7	JN604983

ITS gene amplification, cloning and sequencing

The entire stretch of ITS1-5.8S-ITS2 was amplified using the universal primers ITS1 (forward) and ITS4 (reverse) (Innis et al., 1990) (Table 1). PCR was performed in a final volume of 50 µl containing 50 ng genomic DNA, 0.2 mmol/l dNTPs (Takara), 2.5 µl 10× PCR buffer (Takara), 80 pmol of each primer, and 1 U *Taq* DNA polymerase. The reaction conditions included an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 56°C, and 30 sec at 72°C, followed by a single extension step of 7 min at 72°C. All analyses were performed in a Mastercycler® gradient (Eppendorf, Hamburg, Germany). In the present study, high-fidelity Ex *Taq* polymerase (Takara) was used. The ITS gene PCR products were purified following the Nucleic Acid Recycling and Purification Kit protocol (Tiangen Biotech, Beijing, China). The purified PCR products and pGEM-T plasmid vectors (Promega, USA) were ligated overnight at 4°C. Recombined plasmids were then transformed into *E. coli* DH10B cells by electroporation (BTX ECM 830; Holliston, MA, USA) at 500 V for 17 ms. Positive clones were detected by the appearance of white colonies. Recombinant plasmids were isolated from overnight cultures by alkaline lysis according to the method described by Sambrook and Maniatis (1989). Three positive clones were selected for sequencing using the primer set RV-M/M13-47 (Table 1) in both directions with an ABI377 automatic sequencer (Applied Biosystems, Foster City, CA, USA). GenBank accession numbers of the ITS sequences were shown in Table 2.

Cloning of conserve region of *pyrG* gene using nested PCR

Two pairs of degenerate primers (F1/R1 and F2/R2, shown in Table 1) were designed on the basis of the conserved sequences of eukaryotic *pyrG* genes among *Coprinus cinereus*, *Schizophyllum commne* and *Lentinula edodes,* and were used for nested PCR amplification. Using the primers above, nested PCR was performed under the following conditions: the 1st PCR reaction mixture (50 µl) contained 50 ng genomic DNA, 1×PCR buffer (Mg^{2+} plused, Takara, Japan), 0.2 mmol/l dNTP, 80 pmol of primer F1, 80 pmol of primer R1 and 1.5 U Ex *Taq* polymerase (Takara, Japan). The amplification program was as follows: 1 cycle of 94°C for 2 min, 40 cycles of 94°C for 30 sec, 45°C for 60 sec and 72°C for 40 sec, then a final extension at 72°C for 5 min. 1 µl of the 1st PCR product was then used as the template in a 2nd PCR, using the same reaction mix and amplification conditions except that primers F1 and R1 were replaced with primers F2 and R2. The purification and sequencing procedures were the same as those described for the ITS fragment.

Cloning of the flanking region of *pyrG* gene using chromosome walking

To obtain upstream and downstream flanking regions of the conserved fragment, LA-PCR *in vitro* Cloning Kit (DRR015, Takara, Japan) was used according to the protocol. Four walking primers (WF1 and WF2 for amplification of upstream from conserved fragment, WR1 and WR2 for amplification of the downstream from conserved fragment, shown in Table 1) were designed on the basis of the conserved sequences obtained above. Corresponding site of the walking primers are shown in Figure 1. The genomic DNAs of monokaryotic *P. ostreatus* and *P. eryngii* strains were digested separately with six restriction enzymes (*Hin*d III, *Sal* I, *Bam*H I, *Eco*R V, *Pst* I and *Eco*R I, Takara, Japan) and ligated to each corresponding linker (provided by the kit) as shown in Figure 1. 1st PCR was performed by using the walking primers, the cassette primers (provided with the kit) and the cassette-ligated genomic DNA fragments as the templates (Figure 1). The 1st PCR reaction mixture (50 µl) contained: 50 ng template DNA, 1 × LA PCR buffer (Mg^{2+} plused, Takara, Japan), 0.2 mmol/l dNTP, 80 pmol of cassette primer C1, 80 pmol of walking primers WF1 or WR1 and 1.5 U Takara LA *Taq* (Takara, Japan). Amplification conditions were as follows: 1 cycle of 94°C for 60 s, 32 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 80 s and then a final extension at 72°C for 5 min. 1 µl of the 1st PCR product was used as the template in a 2nd PCR using the same reaction mix and amplification conditions, except that cassette primer C2 replaced cassette primer C1 and specific primers WF2 or WR2 replaced WF1 or WR1, respectively (Figure 1). The purification and sequencing procedures were the same as those described for the ITS fragment.

Amplification of *pyrG* gene DNA sequences

The full-length *pyrG* gene including upstream region, conserved fragment and downstream region were analyzed by DNAMAN Version 5.2.9 software (Lynnon BioSoft, Canada). The *pyrG* gene DNA sequence was obtained according to the deduced open reading frame (ORF) based on the analysis. Two specific PCR primers (*pyrG*-F/*pyrG*-R, shown in Table 1) were designed for the amplification of *pyrG* gene DNA fragment. The PCR amplification was performed using the reaction mixture as described in 1st PCR mixture for conserved fragment, except that the specific primers (*pyrG*-F/*pyrG*-R) replaced F1/R1. The PCR parameters were as follows: preheating at 95°C for 5 min; then 30 cycles of 95°C for 30 s, 56°C for 60 s and 72°C for 60 s. The purification and sequencing procedures were the same as those described for the c ITS fragment. GenBank accession numbers of the *pyrG* gene DNA

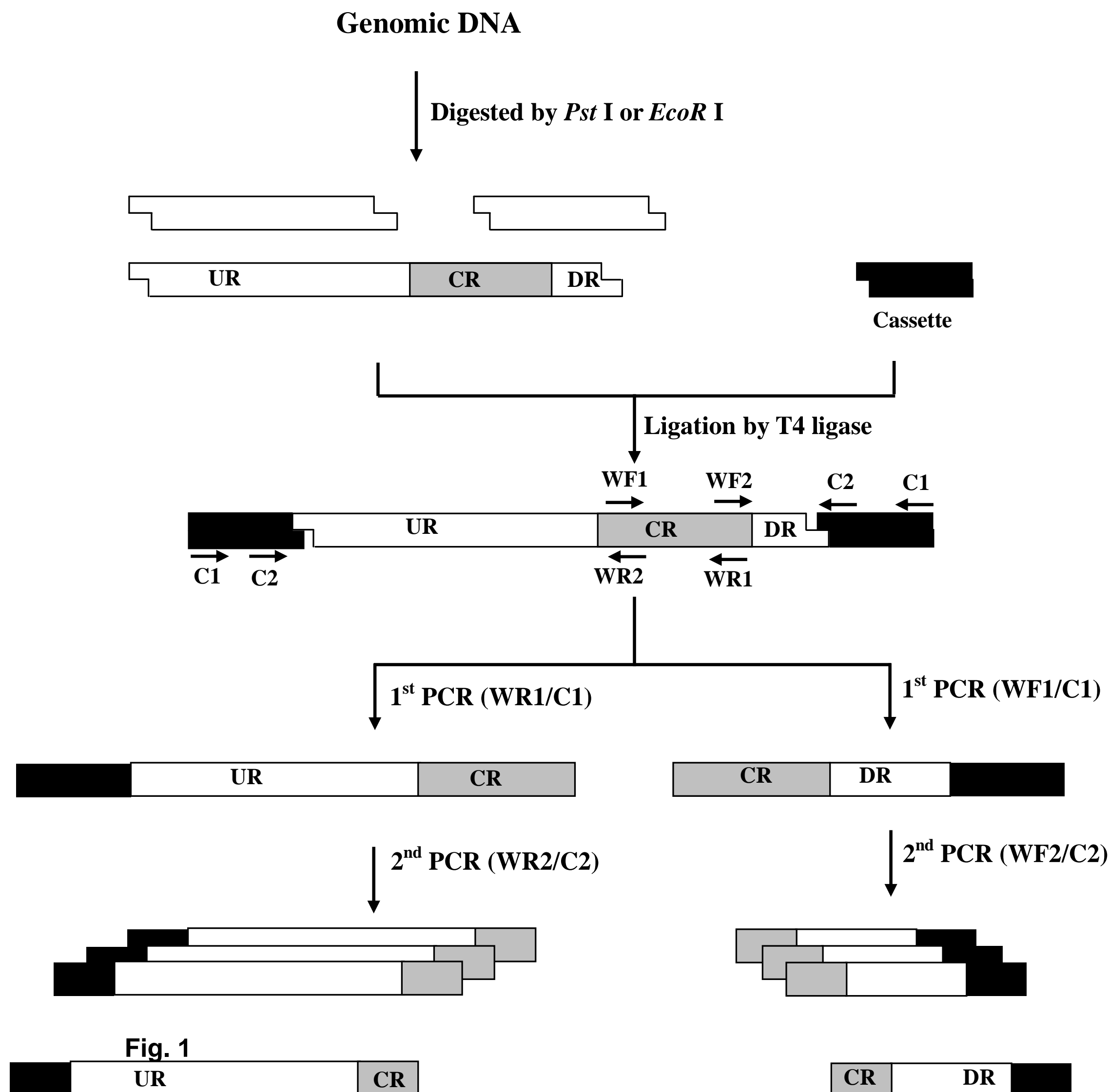


Figure 1. Sketch of chromosome walking used in the present study. UR, upstream region of the conserved partial *pyrG* fragment; CR, conserver region of *pyrG* gene; DR, downstream region of the conserved partial *pyrG* fragment.

sequences, upstream and downstream DNA sequences are shown in Table 2.

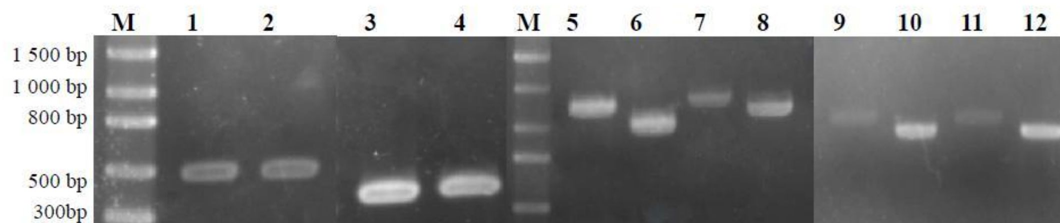
Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from the powdered mycelia of *P. ostreatus* and *P. eryngii* using the QIAGEN RNA/DNA Mini Kit (QIAGEN, Germany). The total RNA was reverse-transcribed with an oligo (dT) primer and moloney murine leukemia virus reverse transcriptase (Ferments, UK). The synthesized cDNA was subjected to PCR amplification with the *pyrG*-F and *pyrG*-R primers (Table 1). The PCR mixture and parameters were as described with *pyrG* gene DNA sequences. The purification and sequencing procedures were the same as those described for the ITS fragment. GenBank accession numbers of the *pyrG* gene cDNA sequences are shown in Table 2.

Characterization and phylogenetic analysis of *pyrG*

The obtained *pyrG* gene sequences were compared with those in DDBJ/EMBL/GenBank using the basic local alignment search tool (BLAST) (Altschul et al., 1990). Additionally, sequences were aligned using Clustal_X (Thompson et al., 1997). The distances were calculated according to the two-parameter method (Kimura, 1980). The phylogenetic tree was inferred using the neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis was based on 1,000 re-samplings; MEGA4.0 (Tamura et al., 2007) was used for all analyses. The *pyrG* gene sequences of the fungi type strains were obtained from DDBJ/EMBL/GenBank.

a



b

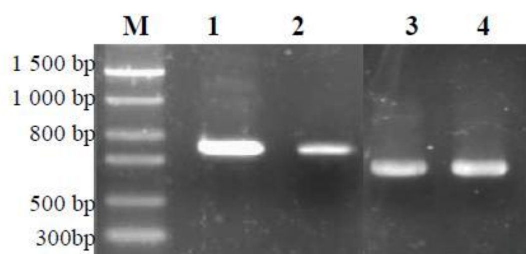


Figure 2 (a): PCR amplification of multiple steps used for nested PCR and chromosome walking. M, 1 kp DNA plus ladder; Lane 1, 1st PCR amplification of conserved fragment of *pyrG* gene amplified from *P. ostreatus* using F1/R1 as primers; Lane 2, 1st PCR amplification of conserved fragment of *pyrG* gene amplified from *P. eryngii* using F1/R1 as primers; Lane 3, 2nd PCR amplification of conserved fragment of *pyrG* gene amplified from *P. ostreatus* using F2/R2 as primers; Lane 4, 2nd PCR amplification of conserved fragment of *pyrG* gene amplified from *P. eryngii* using F2/R2 as primers. Lane 5, 1st PCR amplification of region upstream from the conserved fragment of *P. ostreatus* using C1/WR1 as primers; Lane 6, 2nd PCR amplification of region upstream from the conserved fragment of *P. ostreatus* using C2/WR2 as primers; Lane 7, 1st PCR amplification of region upstream from the conserved fragment of *P. eryngii* using C1/WR1 as primers; Lane 8, 2nd PCR amplification of region upstream from the conserved fragment of *P. eryngii* using C2/WR2 as primers; Lane 9, 1st PCR amplification of region downstream from the conserved fragment of *P. ostreatus* using C1/WF1 as primers; Lane 10, 2nd PCR amplification of region downstream from the conserved fragment of *P. ostreatus* using C2/WF2 as primers; Lane 11, 1st PCR amplification of region downstream from the conserved fragment of *P. eryngii* using C1/WF1 as primers; Lane 12, 2nd PCR amplification of region downstream from the conserved fragment of *P. eryngii* using C2/WF2 as primers. (b): M, 1 kp DNA plus ladder; Lane 1, *pyrG* gene DNA sequences from *P. ostreatus*; Lane 2, *pyrG* gene DNA sequences from *P. eryngii*; Lane 3, *pyrG* gene cDNA sequence from *P. ostreatus*; Lane 4, *pyrG* gene cDNA sequence from *P. eryngii*.

RESULTS

Sequence analysis of the ITS gene

Because ITS sequences can accumulate mutations at a faster rate than the 5.8, 18 or 28S rRNA genes, the ITS region is typically the most useful for molecular systematics at the species or within species levels (Wang et al., 2011). In the present study, the ITS1-5.8S-ITS2 gene sequences of *P. ostreatus* and *P. eryngii* (about 680 bp in length) were amplified from the genomic DNA extracted from mycelia of these strains. These two ITS gene sequences were then compared with those in DDBJ/EMBL/GenBank, which generated significant hits with *P. ostreatus* and *P. eryngii*, respectively. Thus, these two strains were used as material for the following studies.

Cloning and sequencing analysis of the *pyrG* gene

A fragment approximately 320 bp in size was amplified by nested PCR using degenerate primers and genomic DNA from monokaryotic *P. ostreatus* and *P. eryngii* strains as templates (Figure 2a). The sequences of the conserved fragment were compared with those in DDBJ/EMBL/GenBank, which generated significant hits with partial OMPDC sequences from *C. cinereus* and *S. commune*.

The unknown sequences upstream and downstream of the conserved region of *pyrG* were obtained by chromosome walking. Two fragments approximately 1,000 and 1,200 bp in size were amplified from upstream the conserved regions of *P. ostreatus* and *P. eryngii*, respectively, when *Pst* I was used to digest the genomic DNA (Figure 2a). Two fragments approximately 1,100 and

a

```
-592          CCATATATCCTCAACGCCCTCCCTCGCACGTGAATATCGACACCCTCGT
-543  CGCACAGGTAAATCAGACCACTCATGTCCAGCGCCGAGCGTTGATTCTCCGCGA
-489  GTCCGGCATCGTGGTTCCCCGCAATGAAGACTTTATGCTGGTGTGGGAGTGACC
-435  GCAGCCAATCTAGCGTCTTCTGCAACTCCTCTGCGGAGCCAGAATGAGTCAAGT
-381  CTCCTGCGTGCACAAAGACATCGCCTTGTGGAATGTCAGAGAGCGGCAGTATGT
-327  GGTTATGGGTGTCTGAAACACAGACGAGGCGTATCTTCGTATGGTCAGTTAGGG
-273  GCAGTACTGTGGGGGGTAGTGGAGCGTGTCGCAGTAGATGATAGATTGTCGAGA
-211  GTACGAAGGTTGTAGATGAATCCTCTAATCTAGCACGTTTTGAGGCGCGTTCAAT
-164  ACTCTCCGCCTCGGTTTCGTGTTTGAGAGACATGATCGAAGTTATTTTGCGAGGT
-109  GTAAGATCACGCGCTTAGATGATAGATTTCGCAGCCCCTGTCTTTCACTAAATCC
 -54  CCAGAGCCATGTCGACGGTTCATCCACTTCCCGAAGGCCCCTACTCTCTACCATC
   1  ATGAGCTCAAAGGGAGTCCAAGCATTGCCATATGCCCAAAGGGCTGACAATTAC
   1   M  S  S  K  G  V  Q  A  L  P  Y  A  Q  R  A  D  N  Y
```

b

```
-671                                              ACTTTGAGAGACATG
-656  ATCAAAGTTATTTTGCGAGATGGCAAGATCATGCGCCAGACTGCTCTTGGGTTCG
-601  AAGCATCACCATCTCGAGGTTTCCCACGGTTGACTCCCAGTTCTCGAGGCTCATC
-546  GTCGGCCACGCACAGGCTTCCAATACCTCTCTTGCACTACGACAAGGGAAGGCA
-492  GCACGGTCTCCTACCAGGGGGTGCTCCTTCTTTAAGGAGCTTACATGAAGTTCAC
-437  GCCCTCTGAGTCCTGGAAGGACTATAGACTTCCCGGTGTCGGTGCTCCCTTGGTA
-382  AAGGAGCTTAAAGTGGATGGTGTATCCTGCTTGAAACTTCCTTCGCTATACCTCT
-327  ATGTCCAACACTACCCGATCAACCATTATCATACCCACCGTCCTTGCCCGCCTCG
-272  GGAACTGCTCCAAACACGGCCCAACCCCAACCTGCCCAACCCAACCCATATCAA
-218  CTGGGTGCAACGTTAACTCGTTGTACTCAACCCACCGCGTCTTAGTAGCTTTAGC
-163  GTACTTGTTGTGGTATATAAATGCAGACATTAATAAAAATAAAAATAAAAGGCA
-109  AGATCACGTGCTTGGTAGATGTTAGATTTCACAGCCCCTGTCTTTCACTAAAACC
 -54  CCGGAGCCATGTCGAAGGTTCATCCACTTCCCGAGGGCACCTACTTTCAACCATC
   1  ATGAGCTCAAAGGGAGTCCAGGCATTGTCATATGTCCAAAGGGCTGACAACTAC
   1   M  S  S  K  G  V  Q  A  L  S  Y  V  Q  R  A  D  N  Y
```

Figure 3. a: Nucleotide sequence and deduced amino acid sequence of *P. ostreatus pyrG* gene. The deduced amino acid sequence is indicated below the respective codon. Intron sequences are in lower-case letters; all the other sequences are in upper-case letters. The putative TATA box and CAAT box in the promoter region are single-underlined and the transcription initiation sites are double-underlined. b: Nucleotide sequence and deduced amino acid sequence of *P. eryngii pyrG* gene. The deduced amino acid sequence is indicated below the respective codon. Intron sequences are in lower-case letters; all the other sequences are in upper-case letters. The putative TATA box and CAAT box in the promoter region are single-underlined and the transcription initiation sites are double-underlined.

1,100 bp in size was amplified from downstream of the conserved regions of *P. ostreatus* and *P. eryngii*, respectively, when *Eco*R I was used (Figure 2a). The full-length fragments including the conserved sequences and flanking regions were analyzed by DNAMAN Version 5.2.9 software (Lynnon BioSoft, Canada). Based on the analysis, approximately 920 bp of the *pyrG* DNA fragment was amplified by using *pyrG*-specific primers (*pyrG*-F/*pyrG*-R, shown in Table 1) designed according to the ORF of *pyrG* gene (Figure 2b). When RT-PCR technique was used, approximately 810 bp of the *pyrG* cDNA fragment was amplified (Figure 2b). Comparison of the full-length *pyrG* DNA sequences with cDNA sequences from *P. ostreatus* and *P. eryngii* revealed that the *pyrG* gene contained two introns. The cDNA sequence of *pyrG* from *P. ostreatus* show 95.20% similarity with that of *pyrG* from *P. eryngii*.

Sequence of the 5'-flanking region

For the 5'-flanking region of *P. ostreauts*, there was one typical TATA box (TAAAT) and one CAAT boxes (CAAT) located in the 5'- flanking region (Figure 3a). The TATA

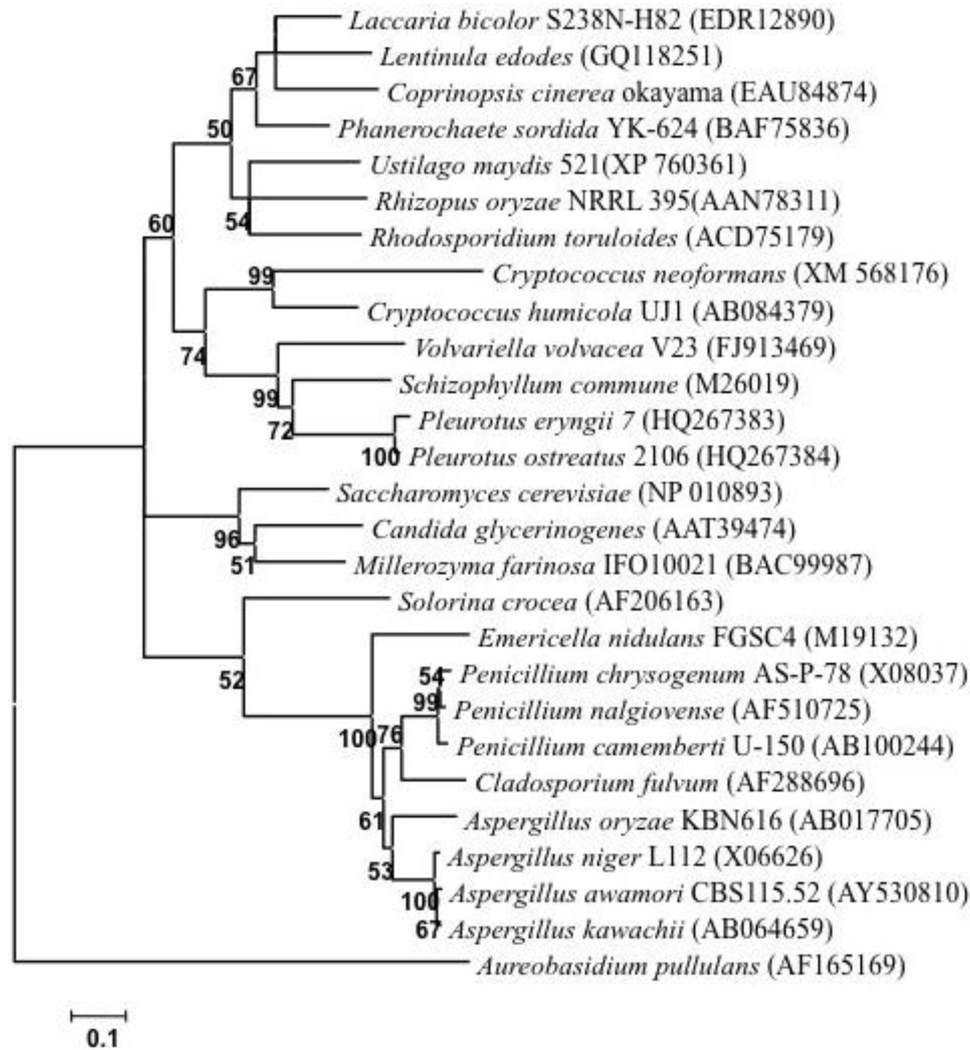


Figure 4. Phylogenetic tree based on deduced amino acid of *pyrG* gene sequences comparison obtained using the neighbour-joining method, showing the phylogenetic position of *P. ostreatus* and *P. eryngii*. Numbers at branching points represent bootstrap values from 1000 replicates. Only values greater than 50% are shown. Bar, 0.1 substitutions per nucleotide position.

box was observed 57 bp upstream from the start codon, while the CAAT boxes were located 164 bp upstream of the initiating ATG codon. For the 5'-flanking region of *P. eryngii*, there were two typical TATA box (TATATAAA, 143 bp upstream from the start codon; TAAAA, 57 bp upstream from the start codon) and one CAAT boxes (CCAAT, 523 bp upstream from the start codon) located in the 5'- flanking region (Figure 3b). Using the promoter prediction software (http://www.fruitfly.org/seq_tools/promoter.html), three possible core promoter regions were found at the 5'-flanking region of *P. eryngii* which were located -169~ -120 bp, -144~ -95 bp and -84~ -35 bp upstream from the start codon (Figure 3b). Comparing the upstream region of *P. ostreatus* with that of *P. eryngii*, one possible core promoter region was found at the 5'- flanking region of *P. ostreatus* which was located -84~ -35 bp upstream from the start codon (Figure 3a).

Characterization and phylogenetic analysis of the deduced PyrG amino acid sequence

The predicted amino acid sequence consisted of 246 amino acids and showed a high similarity to known fungal *pyrG* sequences. The deduced amino acid sequence of *pyrG* from *P. ostreatus* showed a 93.09% similarity to that of *pyrG* from *P. eryngii* with 16 amino acid differences.

A phylogenetic tree using the deduced amino acid sequences, which had high similarities to the published OMPDC, was constructed based on genetic distance (Figure 4). The deduced sequence of *pyrG* from *P. ostreatus*, together with that from *P. eryngii*, showed the highest similarities to that of *S. commne* (61.4 and 59.1%

respectively) and *Volvariella volvacea* (60.3 and 57.8% respectively). In contrast, they showed a lower similarity to that of *C. cinerea*.

Codon usage in the *pyrG* gene of *P. ostreatus* and *P. eryngii*

Varying patterns of codon usage have been observed in different organisms and preference is thought be connected to the abundance of relevant tRNA molecules (Fei et al., 2006). However, no report has yet been published on the codon usage of *Pleurotus* spp. Results showed that there was no particular preference for pyrimidine (Py) and purine (Pu) in both *P. ostreatus* and *P. eryngii*. Within the codons for Arg and Pro, thymine (T) and glycine (G) were not as frequently used as the third base in both *P. ostreatus* and *P. eryngii*. The TAA codon as terminator is most frequently observed in filamentous fungi and was also found in the *pyrG* gene.

DISCUSSION

The use of antibiotic in filamentous fungi has been universal and several antibiotics, such as *hph*, G418 and benomyle, have been used as selective marker, which might arouse an allergic reaction in the organism. Recently, bio-safe selective markers have been developed to avoid the side effects related to antibiotic use. *pyrG* gene is the kind of marker which has been widely used in the transformation of the filamentous fungi. Thus, in the present study, the full-length DNA sequences of *pyrG* from *P. ostreatus* and *P. eryngii* were isolated for the possible use in the safe and efficient transformation of *Pleurotus* spp..

With respect to the eukaryote, CAAT-box and TATA-box are important for the initiation of gene transcription. Some genes may contain TATA-like sequences; others only have AT-rich sequences. Likewise, CAAT-sequences are described for some fungal genes, but are often absent (Ballance, 1986). *pyrG* genes obtained in the present study all contained CAAT-box and TATA-like sequences as expected. It should be noted that the -169~ -120 bp and -144~ -95 bp of predicted possible core promoter region were overlapped. This region contained two consecutive TATA-like box (TATA and TAAAT) and an AT-rich sequence (-135~ -114 bp). This information might indicate that this region was the true functional core promoter.

The *pyrG* gene coding for the enzyme orotidine-5'-phosphate decarboxylase (OMPdecase), which converts orotidine-5'-monophosphate (OMP) to uridine-5'-monophosphate (UMP) in the last enzymatic step of the pyrimidine biosynthetic pathway leading to UMP synthesis, has been cloned and sequenced in several fungi. Phylogenetic analysis revealed a high degree of evolutionary conservation. Comparison of the deduced protein sequences of *P. ostreatus* and *P. eryngii* with other OMPD protein indicated a higher similarity with protein sequences of other fungi including some fruiting body-forming fungi, as expected. The first intron of *P. ostreatus* and *P. eryngii* located between 63 and 64 aa and the second located between the second and third nucleotides of the condon for amino acid 100. While, in *pyrG* of *S. commune*, which showed highest similarities with that of *P. ostreatus* and *P. eryngii*, had two introns, the first intron located between amino acids 62 and 63, and the second intron located between the first and second nucleotides of the codon for amino acid 100 (Froeliger et al., 1989). *pyrG* gene from *P. ostreatus* and *P. eryngii* showed lower similarities with *L. edodes* reported by Bao et al. (2009) and *V. volvacea* reported by Niu et al. (2010). Moreover, in the filamentous fungi *Aspergillus nidulans* (Oakley et al., 1987), *A. niger* (Wilson et al., 1988) and *Penicillium chrysogenum* (Cantoral et al., 1988), the most remarkable feature of this gene was the presence of an intron between the first and second nucleotides of the codon for amino acid 53. The equivalent gene from *Neurospora crassa* had no introns but contained an insertion of 103 amino acids, between 135 and 238, that was unique to this fungus (Glazebrook et al., 1987).

In summary, *pyrG* may be used as both a positive and negative selection gene in transformation of *Pleurotus* spp.. In the present study, the full-length encoding sequences of *pyrG* from *P. ostreatus* and *P. eryngii* were cloned, and then were characterized. This information will serve to create a pyrimidine auxotrophic mutant of *Pleurotus* spp. by either deletion or disruption of *pyrG* for the establishment of a safe and effective transformation system.

ACKNOWLEDGEMENT

We thank the reviewers for the helpful comments and improvement of the text. This work was supported by the grant of Institute of Plant and Environmental Protection, Beijing academy of Agriculture and Forestry Science (Grant No. CXJJ2010A01) and the Beijing Municipal Committee of CPC Organization Department (Grant No. 2011A002020000005).

REFERENCES

Acevedo JP, Reyes F, Parra LP, Salazar O, Andrews BA, Asenjo JA (2008). Cloning of complete genes for novel hydrolytic enzymes from Antarctic sea water bacteria by use of an improved genome walking technique. J. Biotechnol., 133: 277-286.

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

Aradaib IE, Majid AA (2006). A simple and rapid method for detection of *Trypanosoma evansi* in the dromedary camel using a nested polymerase chain reaction. Kinetoplastid Biol. Dis., 5:2.

Ballance DJ (1986). Sequences important for gene expression in filamentous fungi. Yeast, 2: 229–236.

Bao DP, Feng AP, Zhang MY, Tan Q, Chen MJ, Niu LY, Pan YJ (2009). Cloning of *le-pyrG*, an orotidine-5'-monophosphate decarboxylase encoding gene from *Lentinula edodes*. Acta Edulis Fungi 16: 17-20.

Botton SA, Pereira DI, Costa MM, Azevedo MI, Argenta JS, Jesus FP, Alves SH, Santurio JM (2011). Identification of *Pythium insidiosum* by nested PCR in cutaneous lesions of Brazilian horses and rabbits. Curr. Microbiol., 62: 1225-1229.

Cantoral JM, Barredo JL, Alvarez E, Diez B, Martin JF (1988). Nucleotide sequence of the *Penicillium chrysogenum pyrG* (orotidine-5'-phosphate decarboxylase) gene. Nucleic. Acids Res., 16: 8177.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19: 11-15.

Fei X, Zhao MW, Li YX (2006). Cloning and sequence analysis of a glyceraldehyde-3-phosphate dehydrogenase gene from *Ganoderma lucidum*. J. Microbiol., 44: 515-522.

Froeliger EH, Ullrich RC, Novotny ChP (1989). Sequence analysis of the *URAI* gene encoding orotidine-5'-monophosphate decarboxylase of *Schizophyllum commune*. Gene, 83: 387 393

Glazebrook JA, Mitchell K, Radford A (1987). Molecular genetic analysis of the *pyr*-4 gene of *Neurospora crassa*. Mol. Gen. Genet., 209: 399-402.

Gómez-Toribio V, García-Martín AB, Martínez MJ, Martínez AT, Guillén F (2009). Induction of extracellular hydroxyl radical production by white-rot fungi through quinone redox cycling. Appl. Environ. Microbiol., 75: 3944-3953.

Innis MA, Gelfand DH, Sninsky JJ, White TJ (1990). PCR Protocols: A Guide to Methods and Applications, eds. Academic Press, Inc., New York.

Kim BG, Magae Y, Yoo YB, Kwon ST (1999). Isolation and transformation of uracil auxotrophs of the edible basidiomycete *Pleurotus ostreatus*. FEMS Microbiol. Lett., 181: 225-228.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol., 16: 111-120.

LeCleir GR, Buchan A, Hollibaugh JT (2004). Chitinase gene sequences retrieved from diverse aquatic habitats reveal environment-specific distributions. Appl. Environ. Microbiol., 70: 6977–6983.

Lettera V, Del Vecchio C, Piscitelli A, Sannia G (2011). Low impact strategies to improve ligninolytic enzyme production in filamentous fungi: The case of laccase in *Pleurotus ostreatus*. C. R. Biol., 334: 781-788.

Ma AM, Shan LJ, Wang HJ, Du ZP, Xie BJ (2008). Partial characterization of a hydrophobin protein Po.HYD1 purified from the oyster mushroom *Pleurotus ostreatus*. World J. Microbiol. Biotechnol., 24: 501-507.

Marques G, Gamelas JA, Ruiz-Dueñas FJ, del Rio JC, Evtuguin DV, Martínez AT, Gutiérrez A (2010). Delignification of eucalypt kraft pulp with manganese-substituted polyoxometalate assisted by fungal versatile peroxidase. Bioresour. Technol., 101: 5935-5940.

Niu LY, Bao DP, Chen MJ, Tan Q (2010). Cloning and sequence analysis of the *vv-pyrG* gene encoding orotidine-5'-momophosphate decarboxylase in *Volvariella volvacea*. Acta. Agric. Shanghai., 26: 33-37.

Nthangeni MB, Ramagoma F, Tlou MG, Litthauer D (2005). Development of a versatile cassette for directional genome walking using cassette ligation mediated PCR and its application in the cloning of complete lipolytic genes from *Bacillus* species. J. Microbiol. Methods, 61: 225–234.

Oakley CE, Weil CF, Kretz PL, Oakley BR (1987). Cloning, mapping and molecular analysis of the *pyrG* (orotidine-5'-phosphate decarboxylase) gene of *Aspergillus nidulans*. Gene, 53: 293-298.

Oubrahim H, Wang J, Stadtman ER, Chock PB (2005). Molecular cloning and characterization of murine caspase-12 gene promoter. PNAS., 102: 2323-2327.

Pan XL, Wang JL, Zhang DY (2005). Biosorption of Pb (II) by *Pleurotus ostreatus* immobilized in calcium alginate gel. Process Biochem., 40: 2799-2803.

Parvizi P, Mauricio I, Aransay AM, Miles MA, Ready PD (2005). First detection of *Leishmania major* in peridomestic *Phlebotomus papatasi* from Isfahan province, Iran: comparison of nested PCR of nuclear ITS ribosomal DNA and semi-nested PCR of minicircle kinetoplast DNA. Acta. Trop., 93: 75–83.

Piscitelli A, Del Vecchio C, Faraco V, Giardina P, Macellaro G, Miele A, Pezzella C, Sannia G (2011). Fungal laccases: Versatile tools for lignocellulose transformation. C. R. Biol., 334: 789-794.

Rodríguez E, Ruiz-Dueňas FJ, Kooistra R, Ram A, Martínez ĂT, Martínez MJ (2008). Isolation of two laccase genes from the white-rot fungus *Pleurotus eryngii* and heterologous expression of the *pel*3 encoded protein. J. Biotechnol., 134: 9–19

Ruiz-Dueñas FJ, Fernández E, Martínez MJ, Martínez AT (2011). *Pleurotus ostreatus* heme peroxidases: An in silico analysis from the genome sequence to the enzyme molecular structure. C. R. Biol., 334: 795-805.

Sambrook J Maniatis T (2001). Molecular cloning: A laboratory manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Salvachúa D, Prieto A, López-Abelairas M, Lu-Chau T, Martínez AT, Martínez MJ (2011). Fungal pretreatment: An alternative in second-generation ethanol from wheat straw. Bioresour. Technol., 102: 7500-7506.

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995). An improved PCR method for walking in uncloned genomic DNA. Nucleic. Acids. Res., 23: 1087–1088.

Shabtay A, Hadar Y, Eitam H, Brosh A, Orlov A, Tadmor Y, Izhaki I, Kerem Z (2009). The potential of *Pleurotus*-treated olive mill solid waste as cattle feed. Bioresour. Technol., 100: 6457-6464.

Songulashvili G, Elisashvili V, Wasser S, Nevo E, Hadar Y (2006). Laccase and manganese peroxidase activities of *Phellinus robustus* and *Ganoderma adspersum* grown on food industry wastes in submerged fermentation. Biotechnol. Lett., 28: 1425-1429.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The clustal_X windows interface: flexible strategies for multiple sequence alignement aided by quality analysis tools. Nucleic Acid Res., 25: 4876-4882.

Wang P, Liu Y, Yin YG, Jin HJ, Wang SX, Xu F, Zhao S, Geng XL (2011). Diversity of microorganisms isolated from the soil sample surround *Chroogomphus rutilus* in the Beijing region. Int. J. Biol. Sci., 7: 209-220.

Wilson LJ, Carmona CL, Ward M (1988). Sequence of the *Aspergillus niger pyrG* gene. Nucleic. Acids Res., 16: 2339.