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SHORT COMMUNICATION

Isolation and cloning of an aryl-aldehyde dehydrogenase gene from the white-rot fungus *Pycnoporus cinnabarinus* strain MUCL 39533

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ABSTRACT

Aims: The white rot fungus *Pycnoporus cinnabarinus* MUCL 39533 is able to reduce vanillic acid to vanillin. Reduction of vanillic acid to vanillin catalysed by the key enzyme aryl-aldehyde dehydrogenase has been reported. Here we report the isolation and cloning of aryl-aldehyde dehydrogenase from *P. cinnabarinus* strain MUCL 39533.
Methodology and results: An aryl-aldehyde dehydrogenase gene (*PcALDH*) was isolated from *P. cinnabarinus* by producing a partial cDNA sequence fragment of an aryl-aldehyde dehydrogenase gene through PCR. Degenerate PCR primers were designed based on codons corresponding to conserved amino acid regions of aryl-aldehyde dehydrogenases of several fungi and bacteria. The full-length *PcALDH* cDNA was obtained through Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) and Rapid Amplification cDNA Ends (RACE) PCR. *PcALDH* cDNA comprises an open reading frame of 1,506 bp that encodes a protein of 501 amino acids. The PcALDH predicted protein showed the highest amino acid sequence identity (84%) to ALDH from *Trametes versicolor*. *In silico* analysis of PcALDH indicated that it belongs to the ALDH super-family and Class 3 ALDH.
Conclusion, significance and impact study: *PcALDH* cDNA was successfully isolated and characterized. Important motifs identified from the highly conserved PcALDH protein indicated that it belongs to the aldehyde dehydrogenase super-family. The cDNA clone will be used in expression studies to confirm the catalytic function of the enzyme.

Keywords: Aldehyde dehydrogenase super-family, vanillin, vanillic acid

INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main component of vanilla, which is a widely used flavouring in the food industry (Priefert *et al*., 2001). Vanillin is produced from the beans of *Vanilla planifolia* Andrews, a member of the orchid family (Orchidaceae) (Berger, 2007). Vanillin from vanilla beans has a very tenacious, creamy, vanilla-like odour, is intensely sweet and forms white, needle-like crystals (Priefert *et al*., 2001). More than 12,000 tons of vanillin are produced per year, but natural vanillin makes up only 1% of the total (Walton *et al*., 2003).

Natural vanillin costs $ US 1,000 to 2,000 per kg to extract whereas chemically produced vanillin costs only $ US 800 per kg (Havkin-Frenkel and Belanger, 2008) but there is an increasing, consumer-led demand for natural vanillin (Priefert *et al*., 2001). This has lead to numerous studies on the synthesis of vanillin in microbes (Mitsui *et al*., 2010).

In filamentous fungi (*Aspergillus niger* and *Pycnoporus cinnabarinus*), vanillin is formed in a two-stage process in which ferulic acid is converted to vanillic acid then reduced to vanillin (Lesage-Meessen *et al*., 1996). According to Falconnier *et al*. (1994), the reduction of vanillic acid to vanillin can be catalysed by the extracellular aryl aldehyde dehydrogenase (ALDH) a result which has been confirmed by others (Frost, 2002). Similarly, the white rot fungus *P. cinnabarinus* is able to reduce vanillic acid to vanillin (Lesage-Meessen *et al*., 1996). Aryl aldehyde dehydrogenases are widespread amongst living organisms where they detoxify aromatic aldehydes to their corresponding carboxylic acids (Nakamura *et al*., 2010).

As far as we are aware, ALDH of *P. cinnabarinus* has yet to be elucidated or isolated/cloned. Our aim here is to

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isolate the gene and use it to produce bio-vanillin from vanillic acid.

MATERIALS AND METHODS

Strains

Pycnoporus cinnabarinus MUCL 39533 was obtained from the Mycotheque de l'Universite Catholique de Louvain, Louvain, Belgium. The strain was stored at –20 °C in 10% (v/v) glycerol. *Eschericia coli* JM109 was obtained from Promega (Fitchburg, WI, USA) and stored at −80 °C.

Growth conditions

Pycnoporus cinnabarinus was grown on malt-extract agar (MEA) for 5 days after which the spores were transferred to and grown in basal medium containing 20 g/L maltose, 1.82 g/L di-ammonium tartarate, 0.5 g/L yeast extract, 0.2 g/L KH_2PO_4, 1.32 mg/L $CaCl_2 \cdot 2H_2O$, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 2.5 mg/L thiamine hydrochloride (Gross *et al*., 1990) for 10 days. Mycelia were collected, mixed and fragmented using a Waring™ blender. All steps were performed aseptically. Five millilitres of mycelial suspension was inoculated into 100 mL fresh basal medium in 250 mL Erlenmeyer flasks, incubated at 30 °C and shaken at 120 rpm. Three days later, 0.3 g/L vanillic acid was added and after 7 days of growth, the mycelia were harvested, frozen in liquid nitrogen and then kept at −80 °C (Falconnier *et al*., 1994; Ong *et al*., 2014).

Isolation and cloning of a partial aryl aldehyde dehydrogenase cDNA

Frozen mycelia were ground into powder and total ribonucleic acid (RNA) was isolated using TRIzol® reagent (Invitrogen/Life Technologies, Grand Island, NY 14072 USA). Reverse transcription (RT-PCR) was carried out using SuperScript™ III First-Strand Synthesis System (Invitrogen/Life Technologies, USA). The 20 µL reverse transcription mixture contained 1.5 µg of total RNA, 50 µM oligo$(dT)_{20}$, annealing buffer, 2× first-strand reaction mix and 200 U SuperScript™ III reverse transcriptase. Incubation was at 65 °C for 5 min, followed by 50 min at 50 °C for 50 min. Reactions were terminated by heating to 85 °C for 5 min. Partial *PcALDH* was amplified from cDNA fragments using Go Taq® Green Master Mix (Promega, USA). Twenty-five microlitres of the PCR reaction mixture contained 1× Go Taq® Green Master Mix, 1 µL of template cDNA and 100 µM each of pALDHF (5'-CCNTGGAAYTTYCCNYT-3') and pALDHR (5'-GGNCCRAADAGYTCYTC-3') degenerate primers. After an initial denaturation at 95 °C for 2 min, PCR conditions comprised 25 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 5 min. The partial *PcALDH* PCR products were cloned into a pGEM-T Easy vector (Promega, USA) and the ligation products transformed into competent cells of *Escherichia coli* JM109. Plasmids were sequenced using the universal primer M13.

Isolation and cloning of full length aryl-aldehyde dehydrogenase cDNA

Total RNA was isolated using TRIzol® reagent as described above, de-phosphorylated and de-capped. Ligation of RNA oligonucleotides (GeneRacer™ RNA oligo) to the de-capped mRNA then followed. Subsequently, reverse transcription was carried out using SuperScript™ III RT (Invitrogen, USA) to generate the GeneRacer™ cDNA. Amplification of 5' and 3' cDNA ends (RACE) was carried out using KOD Hot Start DNA polymerase (Merck KGaA, Darmstadt, Germany). Primers (10 µM) used for 5' RACE were GeneRacer™ 5' (Life Technologies, USA) (5'-CGACTGGAGCACGAGGACACT GA-3') and 10 µM RPRACE (5'-TGAGGTTGAAGGAGCC AGCAGGGAAT-3') primers whereas 3' RACE employed 10 µM GeneRacer™ 3' (5'-GCTGTCAACGATACGCTAC GTAACG-3') and 10 µM FPRACE (5'- GGTCCCCAGGTC TCGCAACAACAATACG-3') primers. In a 50 µL PCR reaction contained 1 U KOD Hot Start DNA polymerase (Merck KGaA, Germany), 10 µM of each primer, 10× KOD Hot Start DNA polymerase buffer, 25 mM $MgSO_4$, 2 mM dNTPs and 1 µL template cDNA. After initial denaturation at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 20 sec, annealing at 65 °C for 10 sec, extension at 70 °C for 20 sec with final extension at 70 °C for 5 min were performed. Finally, the full-length aryl-aldehyde dehydrogenase cDNA (*PcALDH*) fragment was amplified using 1 U KOD Hot Start DNA polymerase, 10 µM of F-ALDH (5'-AGCTGCCAGAAGGATCACC-3') and R-ALDH (5'-TGCTGTCAACGATACGCTAC-3') primers, 10× KOD Hot Start DNA polymerase buffer, 25 mM $MgSO_4$, 2 mM dNTPs and 1 µL template cDNA. The PCR conditions were as described above. The full-length *PcALDH* PCR products were cloned into pGEM-T Easy vector (Promega, USA). Subsequently, the ligation products were transformed into competent cells of *E. coli* JM109. Plasmids were sequenced using the universal primer M13.

Bioinformatics analyses

Comparative sequence analyses of PcALDH were performed using NCBI (National Center for Biotechnology Information) blastx (Basic Local Alignment Search Tool) programme (http://blast.ncbi.nlm.nih.gov/) (Altschul *et al*., 1997). The theoretical iso-electric point and mass of PcALDH were computed using Compute pI/Mw (http://web.expasy.org/compute_pi/) (Bjellqvist *et al*., 1993, 1994; Gasteiger *et al*., 2005). The signal peptide of PcALDH was predicted using Phobius (http://phobius.sbc.su.se/) (Kall *et al*., 2007). Disulphide bonds of PcALDH were predicted using DiANNA (http://clavius.bc.edu/~clotelab/DiANNA/) (Ferrè and Clote, 2005, 2006). Multiple sequence alignments of amino acid sequences of ALDHs were conducted using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

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(Larkin *et al*., 2007) and BoxShade server version 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

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1    ATGCCTGAGACCTACGTGCACAAGTTTGATACCCCCACCTTCAAGGGTGAGATTTCCGTG
1     M  P  E  T  Y  V  H  K  F  D  T  P  T  F  K  G  E  I  S  V
61   CCCCTCGGCCTCTACATCGACGGCAAGTGGGTCGACGGCAGCAGCGGCAACAAGATCGAT
21    P  L  G  L  Y  I  D  G  K  W  V  D  G  S  S  G  N  K  I  D
121  GTCATCAATCCCTCCAACGGAAAGCTCATCACGAAGATTACCGAGGGCTCGCCGAAGGAT
41    V  I  N  P  S  N  G  K  L  I  T  K  I  T  E  G  S  P  K  D
181  GTCGACCTCGCCGTCGCTGCTGCGCAGAAGGCGTTTGACACGACCTGGGGTCTGCACAAG
61    V  D  L  A  V  A  A  A  Q  K  A  F  D  T  T  W  G  L  H  K
241  AACGGTTTCGAGCGCGCGCGTCTCCTCCAGAAGCTCGGCGACCTGATGGAGCAGAACGCG
81    N  G  F  E  R  A  R  L  L  Q  K  L  G  D  L  M  E  Q  N  A
301  GACAAGCTCGCAGCACTCGAGGCGCTTGACAACGGCAAGACGTTCAACTGGGCAAGGACC
101   D  K  L  A  A  L  E  A  L  D  N  G  K  T  F  N  W  A  R  T
361  ACCGACATCGACCACTCCATCCGCTGCATCCAGTACTACGCTGGCTGGGCGGACAAAATC
121   T  D  I  D  H  S  I  R  C  I  Q  Y  Y  A  G  W  A  D  K  I
421  TTGGGTCAGACGATCGAGACGTCCGAGGCCAAGCTCGTTTACGTCCGCCACGAACCCATC
141   L  G  Q  T  I  E  T  S  E  A  K  L  V  Y  V  R  H  E  P  I
481  GGAGTCGTGGCCCAGATCATCCCCTGGAACTTCCCCCTGCAAATGATGGCGTGGAAGATT
161   G  V  V  A  Q  I  I  P  W  N  F  P  L  Q  M  M  A  W  K  I
541  GGCCCCGCTCTCGCGACCGGCTGCTCGGTCATCCTAAAGCCTTCAGAATTCACTCCTCTC
181   G  P  A  L  A  T  G  C  S  V  I  L  K  P  S  E  F  T  P  L
601  ACCGCGCTCTTCGTGGCGCAGCTTATTCACGAGGCCGGATTCCCTGCTGGCTCCTTCAAC
201   T  A  L  F  V  A  Q  L  I  H  E  A  G  F  P  A  G  S  F  N
661  CTCATCAACGGCTACGGTCACGTCGTCGGCCAAGTACTTGCAGAGCACAATGGAATTGAG
221   L  I  N  G  Y  G  H  V  V  G  Q  V  L  A  E  H  N  G  I  E
721  AAGATTGCGTTCACCGGCAGCACGCTCGTCGGCCGCAAGATCATGGAGGCTTCCTCCAAA
241   K  I  A  F  T  G  S  T  L  V  G  R  K  I  M  E  A  S  S  K
781  TCCAACCTGAAGAAGGTCACGCTCGAGCTCGGCGGAAAGAGCCCAAGTATTGTCTTCGAC
261   S  N  L  K  K  V  T  L  E  L  G  G  K  S  P  S  I  V  F  D
841  GATGCGGACCTGGAGCAGGCGGTGAAGTGGGCGGCGTTCGGGATCTACTACAACCACGGG
281   D  A  D  L  E  Q  A  V  K  W  A  A  F  G  I  Y  Y  N  H  G
901  CAGTGCTGCTGTGCAGGCTCGCGTATCTTCGTGCATGAGAAAATTTACGACCAGTTCATG
301   Q  C  C  C  A  G  S  R  I  F  V  H  E  K  I  Y  D  Q  F  M
961  CAGCGCTTCACGGAGCACACCAAGTCGCTCAAGGTCGGCGACCCCTTCGACCCCGAGGTC
321   Q  R  F  T  E  H  T  K  S  L  K  V  G  D  P  F  D  P  E  V
1021 TACCAGGGTCCCCAGGTCTCGCAACAACAATACGACCGCATCCTGAGTTACATCAAGTCC
341   Y  Q  G  P  Q  V  S  Q  Q  Q  Y  D  R  I  L  S  Y  I  K  S
1081 GGAAAGGAGGAGGGTGCGACGATCGTCACCGGCGGCGAGCCGCTCGGCCAGGAGGGCTAC
361   G  K  E  E  G  A  T  I  V  T  G  G  E  P  L  G  Q  E  G  Y
1141 TACATCCAGCCCACGATCTTCACCGACGTGAAACCGAGCATGAAGATCGTGCGGGAGGAG
381   Y  I  Q  P  T  I  F  T  D  V  K  P  S  M  K  I  V  R  E  E
1201 ATTTTCGGGCCCGTCTGCGTCGTCATCAAGTTCAAGGACGACGACGACATCGTCGCGATG
401   I  F  G  P  V  C  V  V  I  K  F  K  D  D  D  D  I  V  A  M
1261 GCGAACGACACCATCTACGGCCTCGCGGCGTCAGTGTTTTCGCAGAACGTCACCCGCGCT
421   A  N  D  T  I  Y  G  L  A  A  S  V  F  S  Q  N  V  T  R  A
1321 TTGTCGACCGCGCATAAGATCCGCGCCGGCACCGTCTGGGTCAACACCGCGAACATGCTC
441   L  S  T  A  H  K  I  R  A  G  T  V  W  V  N  T  A  N  M  L
1381 TACCCGCAGGTGCCGTTCGGCGGCTACAAGCAGTCCGGCATCGGTCGCGAGCTCGGCGAG
461   Y  P  Q  V  P  F  G  G  Y  K  Q  S  G  I  G  R  E  L  G  E
1441 TACGCCCTCGCAAACTACACCGCCGTCAAGGCCGTCCAGGTCAACTTGCACCTCAACCTG
481   Y  A  L  A  N  Y  T  A  V  K  A  V  Q  V  N  L  H  L  N  L
1501 TCATGA
501   S  -
```

Figure 1: The full-length 1,506 bp sequence of the cDNA of aryl-aldehyde dehydrogenase of *Pycnoporus cinnabarinus* MUCL 39533 (GenBank Accession KP342121). Capital letters represent nucleotides while capital letters marked in bold show the translated amino acid sequence. Boxed 'ATG' and 'TGA' represent translational start and stop codons respectively.

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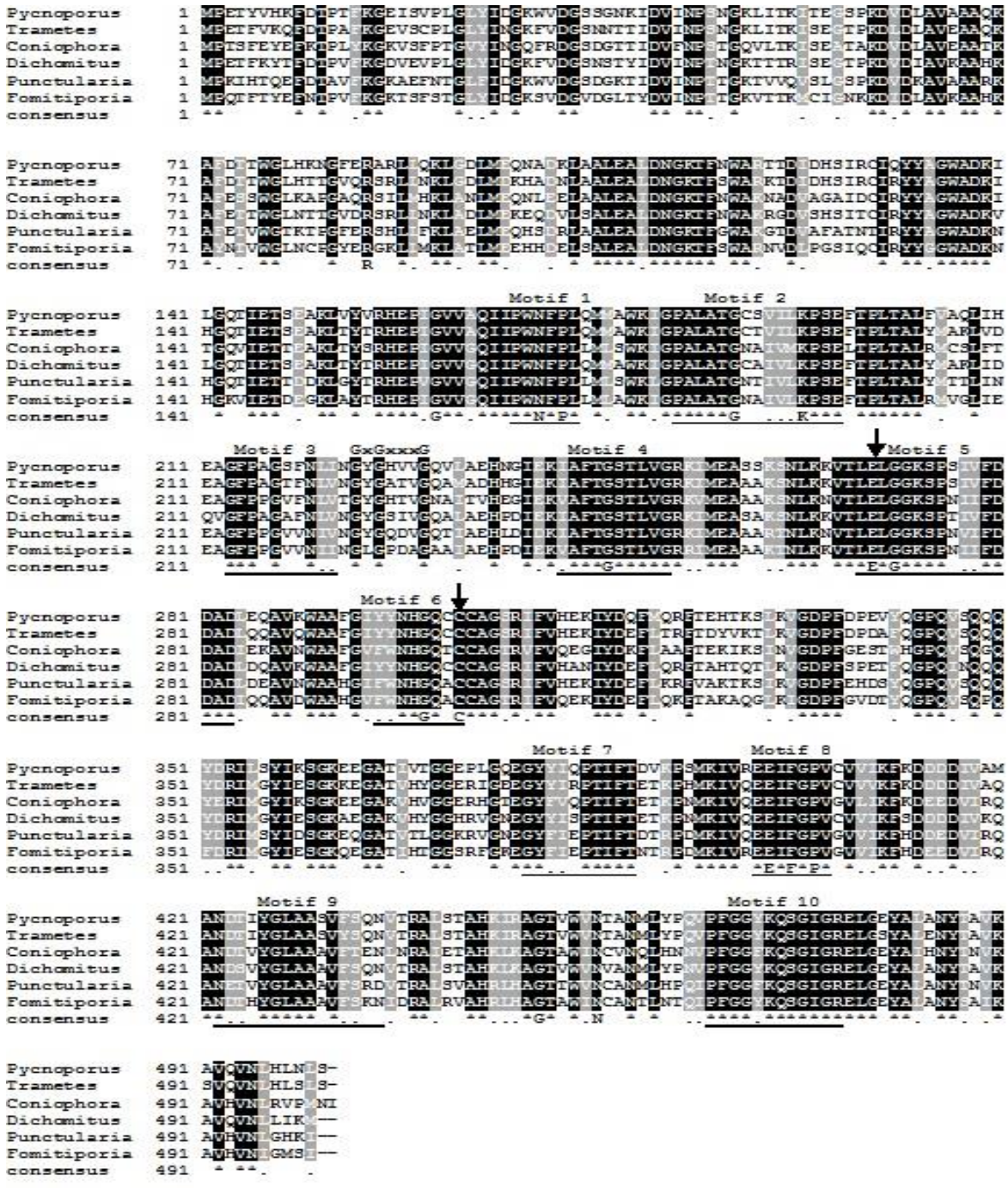

Figure 2: Amino acid sequence alignments ALDH of: *Pycnoporus cinnabarinus* (this study, GenBank Accession KP342121), *Trametes versicolor* (GenBank accession no. EIW65328.1), *Coniophora puteana* (GenBank accession no. EIW75974.1), *Dichomitus squalens* (GenBank accession no. EJF56132.1), *Punctularia strigosozonata* (GenBank accession no. EIN03470.1) and *Fomitiporia mediterranea* NAD-dependent ALDH (GenBank accession no. EJD04639.1). Asterisks and full stops indicate residues with identity and weak similarity, respectively. Invariant residues are putative active sites common to the ALDH super family and are indicated by arrows. The 16 residues conserved in the ALDH super family are indicated by capital letters. The positions of the ten motifs in the ALDH super family are shown at the top. The glycine motif involved in NAD(P)$^+$ binding, G-x-G-x-x-G, is also shown (x positions without a clear residue consensus).

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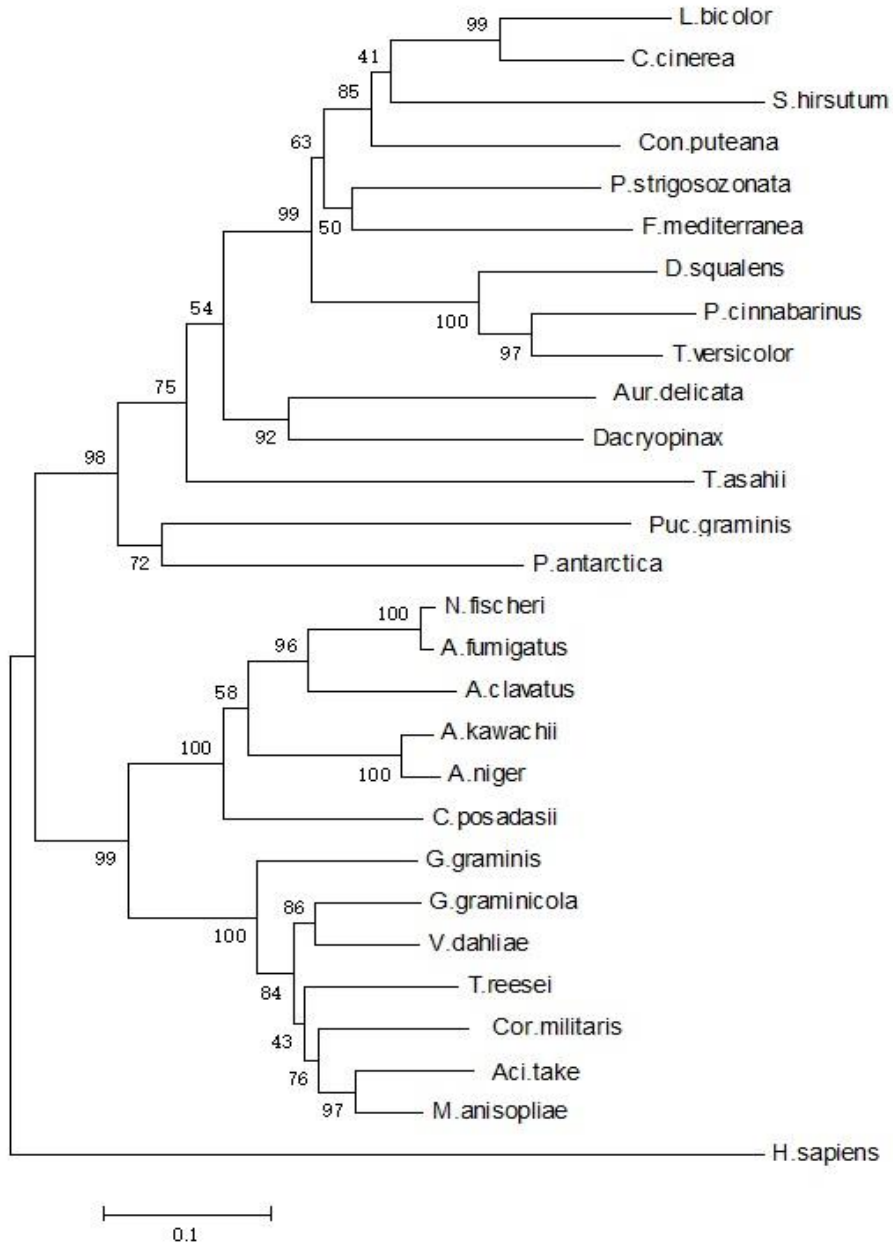

Figure 3: Phylogenetic tree of aryl-aldehyde dehydrogenases (ALDHs). The tree was constructed by neighbour-joining. The scale indicates an evolutionary distance of 0.1%. Bootstrap values (%) are indicated at the nodes. The GenBank accession numbers of the ALDH sequences are as followed: *Pycnoporus cinnabarinus* (this study, KP342121), *Trametes versicolor* (EIW65328.1), *Dichomitus squalens* (EJF56132.1), *Punctularia strigosozonata* (EIN03470.1), *Coniophora puteana* (EIW75974.1), *Fomitiporia mediterranea* (EJD04639.1), *Laccaria bicolor* (XP_001883222.1), *Coprinopsis cinerea* (XP_001834689.1), *Auricularia delicata* (EJD50206.1), *Dacryopinax* sp. (EJT99149.1), *Stereum hirsutum* (EIM91726.1), *Puccinia graminis* (XP_003333586.1), *Trichosporona sahii* (EJT46540.1), *Glomerella graminicola* (EFQ35179.1*), Pseudozyma antarctica* (GAC71960.1), *Neosartorya fischeri* (XP_001258260.1), *Aspergillus fumigatus* (XP_751026.1), *Aciculosporium take* (BAF57023.1), *Trichoderma reesei* (EGR48076.1), *Verticillium dahlia* (EGY19997.1), *Aspergillus clavatus* (XP_001268240.1), *Metarhizium anisopliae* (EFZ02176.1), *Coccidioides posadasii* (EFW13700.1), *Aspergillus kawachii* (GAA87440.1), *Cordyceps militaris* (EGX90970.1), *Aspergillus niger* (XP_001392844.1), *Gaeumannomyces graminis* (EJT79328.1) and *Homo sapiens* (NP_000680.2).

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RESULTS AND DISCUSSION

Cloning and sequence analysis

RNA was extracted from cultures 4 d after induction with vanillic acid. The first cDNA strand was produced from RNA via reverse transcription. A partial *PcALDH* fragment (710 bp) was amplified from the cDNA strands using the degenerate primers pALDHF and pALDHR. The resulting cDNA fragment was then translated and compared with protein databases of NCBI using blastx. The fragment had 64% identity to *Aspergillus fumigatus* Af293 (GenBank Accession XP_751026.1) ALDH mRNA at the amino acid level suggesting that the sequence encoded part of the ALDH gene of *P. cinnabarinus*.

Two gene-specific primers based on the partial cDNA sequence were designed (FPRACE and RPRACE) and used in RLM-RACE (RNA ligase-mediated rapid amplification of 5'- and 3'- cDNA ends) to clone the full length cDNA of *PcALDH*. RACE-PCR amplification yielded 5' and 3' flanking regions of ~720 bp and ~630 bp respectively. Sequencing the entire construct showed a full length *PcALDH* cDNA of 1.7 kbp consisting of a 5' un-translated region, a 3' poly (A) tail and an open reading frame of 1,506 bp encoding ALDH (GenBank Accession KP342121) (Figure 1). The deduced amino acid sequence contained 501 residues with a calculated molecular mass of 55.04 kDa and a p*I* of 6.20. PcALDH contained three disulphide bonds and the absence of a signal peptide indicated that it was an intracellular protein.

The deduced amino acid sequence was used to perform BLAST searches which clearly showed that it belonged to the ALDH super family. The best matches were to: (a) hypothetical protein BN946_scf184803.g29 of *Trametes cinnabarina* (GenBank Accession CDO69831.1) (98% identity); (b) ALDH of *Trametes versicolor* (GenBank Accession EIW65328.1) (84% identity) and (c) ALDH of *Dichomitus squalens* (GenBank Accession EJF56132.1) (78% identity). Other matches of more than 65% identity were to ALDH of *Punctularia strigosozonata* (GenBank Accession EIN03470.1), NAD-dependent ALDH of *Fomitiporia mediterranea* (GenBank Accession EJD04639.1) and ALDH of *Coniophora puteana* (GeneBank Accession EIW75974.1). Sixteen amino acid residues were fully conserved in more than 95% of the sequences (Perozich *et al.*, 1999; Lo and Chen, 2010; Yao *et al.*, 2012) (Figure 2). PcALDH has two active-site residues Glu268 and Cys296 that are responsible for catalytic activity (Lo and Chen, 2010). A glycine motif, presumably involved in $NAD(P)^+$ binding, GXGXXXG (GYGHVVG) was found between motifs 3 and 4. A phylogenetic tree based on 1,000 replicates (Figure 3) was generated using the neighbor-joining method and ALDH of *Homo sapiens* as the outlier. High bootstrap values supported the classification of each monophyletic group. As expected, Ascomycota and Basidiomycota clustered together into a group separate from other fungal ALDHs. Also, the *PcALDH* of *P. cinnabarinus* (Polyporaceae, Basidiomycota) was placed exactly where the taxonomic data suggested it should be.

ALDH catalysis involves acylation and deacylation. During acylation, a cysteine nucleophile interacts with the carbonyl carbon of aldehyde forming a thiohemiacetal intermediate, followed by hydride transfer from a tetrahedral thiohemiacetal intermediate to the pyridine ring of $NAD(P)^+$. Then, deacylation occurs involving hydrolysis of the resulting thioester intermediate (Marchal *et al*., 2001; Lo and Chen, 2010; Munoz-Clares *et al*., 2011; Tsybovsky *et al*., 2013). Glu residues play important roles in activation of water molecules (Marchal *et al*., 2001). Activation of the catalytic Cys upon binding of the cofactor to the enzyme is via a local conformational rearrangement involving the Glu and cofactor (Wang and Weiner, 1995; Marchal *et al*., 2001). Glu268 and Cys296 of PcALDH are potential active site residues since they correspond to Glu268 and Cys302 of human liver ALDH and are conserved in the aligned enzymes (Figure 2) (Abriola *et al*., 1990; Wang and Weiner, 1995). Based on this evidence, we conclude that PcALDH is a typical member of the ALDH superfamily enzymes and that PcALDH may be a key enzyme in the conversion of vanillic acid to vanillin in *P. cinnabarinus*.

CONCLUSION

PcALDH cDNA was isolated and characterised. Motifs identified from the highly conserved PcALDH protein indicated that it belongs to the aldehyde dehydrogenase super-family. The cDNA clone will be used in expression studies to confirm the catalytic function of the enzyme.

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