

## POSITIONING OF INTRONS IN DIFFERENT LACCASE GENES, A RELEVANT TOOL FOR SOLVING PHYLOGENETIC POSITION AMBIGUITY OF *VOLVARIELLA VOLVACEA* LACCASE GENES

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### ABSTRACT

*Volvariella volvacea* (paddy straw mushroom) is a high temperature-loving mushroom with the shortest cropping cycle in the basidiomycete family Pluteaceae. This mushroom is cultivated extensively in many South-East Asian and African countries by employing both the outdoor and indoor cultivation techniques. The objective of this study is to analyze intron positions in different laccase genes of this mushroom. Primer pairs for laccase 1, 2, 3, 4, 5 and 6 were designed by using the mRNA sequences of these genes available in NCBI nucleotide database and the Primer 3 free software. These primers were used to amplify different laccase genes in one heterozygotic strain, Vv-01 and one putative homozygotic strain, BBSR-003 at an annealing temperature range of 53 to 58°C to obtain amplicons of requisite sizes. PCR products were then sequenced and annotated using the BioEdit and Artemis softwares. Out of 6 laccase genes, partial sequences of laccase 1, 2, 3 and 6 in the heterozygotic strain Vv-01 and partial sequence of laccase 3 in homozygotic strain BBSR-003 were obtained. The lcc 3 sequences of two strains, Vv-01 and BBSR-003 have been submitted to NCBI GenBank with accession numbers HQ687205 and JF313903, respectively and they showed differences at 10 different base positions. In lcc 3, there was an intron 8 found earlier in laccase genes from *Coprinopsis cinerea*, but same has not been spliced in published mRNA sequence of *V.volvacea*. This intron has to be spliced in strain Vv-01 to give a functional protein and by virtue of which, the phylogenetic position of lcc3 in laccase dendrograms is probably wrong. In lcc 1, 16 different introns have been recorded and the amino acid sequence of Vv-01 strain differs by one amino acid less than the published sequence of lcc1. No evidence of heterozygosity has been recorded in this sequence. The lcc 2 sequence also revealed 16 different introns. Although only a small portion of sequence could be validated but evidence for heterozygosity was found in strain Vv-01. The small sequence of lcc 2 in strain BBSR-003 also differed at some bases from strain Vv-01. At the protein sequence level, 26 amino acids of the published mRNA seemed to be derived from the wrong reading frame. This could be due to errors in sequencing, or that the strain used had a pseudo gene or a very original allele for lcc 2. Based on these observations, the position of laccase 2 in the dendrogram is likely to be changed. The sequence of lcc 6 helped in identifying 5 introns. The positions of introns in different laccase genes (lcc 1, lcc 2 and lcc 3) of *V. volvacea* grouped them to sub-family A.

**Keywords:** *Volvariella volvacea*; Phylogenetic; Laccase; Introns; Heterozygotic

## INTRODUCTION

*Volvariella volvacea* (Bull ex Fr.) Sing. belongs to the family Pluteaceae Kotl. & Pouz. of the Basidiomycetes [1]. It is an important edible mushroom of the tropics and subtropics. Literature available on this mushroom has emphasized more on its cultivation technology than its morphology [2], cytology [3], physiology [4, 5] and molecular biology [6]. This mushroom can produce an array of extracellular hydrolytic and oxidases enzymes - e.g. endo-1,4- $\beta$ -gucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91),  $\beta$ -glucosidase (EC 3.2.1.21) and laccase (EC 1.10.3.2) [6-11]. The copper binding regions and the N-terminal amino acid sequences have also been used to generate complete sequence of six laccases from this mushroom [12, 13] and out of these, lac1 and lac4 have been suggested to play roles in morphogenesis in this mushroom [12, 13]. The complete mRNA sequences of six laccases in this mushroom have also been obtained in the past, which are also available in NCBI database.

The genus *Volvariella* comprises about 50 species worldwide [14], including the cultivated *V. volvacea* (paddy straw mushroom). Using the 'strips' recognized by Singer [15], several morphological/ecological groups have been differentiated within this genus. Specifically, the *V. volvacea*-group has been identified with pileus >50 mm in diameter, with darkly colored and usually grey-brown basidiospores. Based upon the nLSU data, Moncalvo *et al.* [16] placed *Volvariella* in a very distinct position to *Pluteus*, clustering with *Fistulina* Bull. and *Schizophyllum* Fr. Similarly in a later study [17] using six genes, *Volvariella* (*V. gloiocephala*) was placed as the sister group of *Pluteus* along with *Melanoleuca*, traditionally classified in the *Tricholomataceae* R. Heim ex Pouzar [15], and were together placed with members of *Amanitaceae*, *Pleurotaceae* Kuhner, the aquatic basidiomycete *Limnoperdon* G.A. Escobar and some other 'orphan' agaric genera (*Tricholomopsis* Singer, *Cantharocybe* H.E. Bigelow & A.H. Sm., *Macrocystidia* Joss) in one major group named the 'Pluteoid' clade. Likewise Binder *et al.*, [18] got a similar topology for *Pluteus*, *V. gloiocephala* and *Melanoleuca* with *Amanita* Pers. as members of the core Pluteoid clade with *Tricholomopsis* and *Cantharocybe* outside of the clade. In a recent study based upon molecular data from nuclear ribosomal genes (nSSU, ITS, nLSU), *Volvariella* has been described as polyphyletic, and *Volvariella sensu stricto* has been placed with some hygrophoroid genera (*Camarophyllum*, *Cantharocybe*), representing as a sister group of the Pluteoid clade and a new generic name, *Volvopluteus* has been proposed with species as *earlei* and *gloiocephalus*, keeping these last ones in Pluteoid clade [19]. Compared to the Agaricoid clade, the respective positions of *Pleurotus ostreatus* and *V. volvacea* are still unclear.

Recently, the laccase genes have been used for species identification and establishment of evolutionary relationships, where other molecular markers have failed [20]. This group of enzymes (benzenediol: oxygen oxidoreductase, EC 1.10.3.2.) play important role in catalyzing the single electron transfer from various organic compounds and concomitant four-electron reduction of oxygen to water, and they are found widely spread in plants, bacteria, insects and fungi. Laccase gene has also been used for studying presence of basidiomycetes in a forest soil and saprotrophic fungi have been reported to be less spread through the soil horizons than the mycorrhizal ones. [21]. The diversity of the laccase genes have again been used in phylogenetic perspectives of *Botryosphaeria rhodina* (Ascomycota: fungi) and some related taxa [22], and likewise the multi-gene family of *Coprinopsis cinerea* has been studied, which has been divided into two sub-families A and B [23]. In the laccase phylogeny, three principal clades, comprised

of sub-family A and B from *C. cinerea*, and sub-family C from *Pleurotus ostreatus* PoxA3 and *A. bisporus* lcc1 and lcc2 laccases have been proposed [24-26]. In clade A, the position of *V. volvacea* is very peculiar, which suggests that these laccases can belong to a new clade, distinct from clade A and supports the idea of an early divergence of these genes from the other laccase genes of clade A. The present study was conducted to determine the variations in the positions of introns in laccase genes of *V. volvacea*, and identify their relationship with the positions of introns in laccase genes of other related species, and to draw the evolutionary relationships between the laccase genes of related species.

## MATERIALS AND METHODS

***V. volvacea* strains and their DNA extraction.** The strains Vv-01 and BBSR-003, originally collected from Coimbatore (Tamil Nadu) and Bhubaneswar (Orissa), the Southern coastal region of India, were used in the study. The strains were grown separately at  $32 \pm 2^\circ\text{C}$  for 5 days on Malt Extract Agar medium petridishes and the well grown mycelia was scaped and used for genomic DNA extraction. DNA was extracted by using Illustra DNA extraction Kit from GE Healthcare, UK by following the protocol provided by the manufacturer.

**Primers for laccase gene amplification:** The primer pairs for six laccase genes (lcc1, lcc2, lcc3, lcc4, lcc5 and lcc6) of *V. volvacea* were designed based on the mRNA sequences of these genes (Accession Nos. lac1-AY249052.1, lac2-AY338483.1, lac3-AY338484.1, lac4-AY338486.1, lac5-AY338485.1 and lac6-AY338487.1) available in NCBI nucleotide database using Primer 3.0 (version 0.4.0) free software available on internet. These primers were synthesized from Sigma Life Science, France (Table 1).

**PCR amplification of laccase genes:** PCR amplification of different genes was performed in reaction mixture of 25  $\mu\text{l}$ ; comprising 5X PCR buffer 5  $\mu\text{l}$  (Promega, Medison, WI), dNTP 3.5  $\mu\text{l}$  (1.2 mM each), bovine serum albumin 0.5  $\mu\text{l}$  (10 mg/ml), Taq DNA polymerase 0.2  $\mu\text{l}$  (5 U/ $\mu\text{l}$ , Promega M830B), primer pairs @ 2  $\mu\text{l}$  each (10  $\mu\text{M}$ ) and DNase/RNase- free water 10.8  $\mu\text{l}$  and template DNA 1  $\mu\text{l}$  (50 ng/ $\mu\text{l}$ ). The PCR reaction was performed in Master Cycler Gradient (Eppendorf) with initial denaturation at  $95^\circ\text{C}$  for 5 min, followed by 35 cycles of  $95^\circ\text{C}$  for 1 min,  $55^\circ\text{C}$  for 5 min and  $72^\circ\text{C}$  for 2 min 30 sec, and by final extension at  $72^\circ\text{C}$  for 4 min, with a lid heating option at  $110^\circ\text{C}$ . The PCR amplified products (amplicons) were run on 1.2% agarose gel prepared in 1.0% TBE buffer and the gel was run at 70 V for 1 hour 30 min in 0.5% TBE buffer. The gel was visualized under gel doc system from BioRad.

PCR amplification of different genes was optimized by using the annealing temperature gradients of 2 to  $6^\circ\text{C}$  depending on the performance of the initial PCR amplification of the different laccase genes. The annealing temperature giving the highest product intensity at appropriate position (product size) under UV light was selected as the annealing temperature for further PCR reactions.

**Sequencing of PCR amplicons:** The amplicons along with the corresponding primer pairs (forward and reverse) were sent to BECKMAN COULTER GENOMICS, UK for sequencing and the received sequences were further processed.

**Table 1:** Source and sequences of primers designed for different laccase genes

Laccase gene	mRNA Accession No. in NCBI GenBank	mRNA GI No. in NCBI GenBank	Primers	Sequence (5' to 3')
Laccase 1	AY249052.1	37732219	Vv1FL1 Vv4RL1	CCGATGAAGTTGGGACATTC TCGCAATCACAATCACAGTTC
Laccase 2	AY338483.1	37791150	Vv1FL2 Vv4RL2 Vv12FL2 Vv42RL2	CCTTGCTCAACACCCTTACC GCCAGAGACTGGGTAGACG CTGCTGCACAACAGAGCTTC AATACCAGAGGACGCGAGAC
Laccase 3	AY338484.1	37791152	Vv1FL3 Vv4RL3	TGCGAGCGTAACTCAATGTC GCAAAGCTCATCCCAAGAAG
Laccase 4	AY338486.1	37791156	Vv1FL4 Vv4RL4 Vv15FL4 Vv45RL4 Vv05FL4	ATATCTGCTCGGCCATCTTG GACAGAGCTGCTTCCATTCC TCATTCTGGGGTTATCCTG TAGAAGCGGGTTTCTCCTTG CTCGTCGCGGGTAAATATC
Laccase 5	AY338485.1	37791154	Vv1FL5 Vv4RL5	CAGTGCAATTTTGGTCAACG AAGTGTCCGATCCACTGTCC
Laccase 6	AY338487.1	37791158	Vv1FL6 Vv4RL6 Vv01FL6 Vv35RL6	TGGAACAGCCTCTCACACAC ATTGACACCAGGTCTGAGG CAACACCCTCACTGTCTGTT CACAAAGGTCATCCCACTCC
Laccase 3 (for remaining portion)	Received genome sequence of lcc3	NA	Vv2FL3 Vv3RL3 Vv2RL3 Vv3FL3	AGCCCGTTGACAACTACTGG GGGACGGCATTCTTCTATGAG AGTTGTCAACGGGCTGATTG TTGATTGGGATATGCTGCAC
Laccase 1 (for remaining portion)	Received genome sequence of lcc1	NA	Vv18FL1 Vv2FL1 Vv18RL1 Vv2RL1 Vv3RL1 Vv08FL1 Vv45RL1	GGCTGATTTTCGATCTCTTGC CCGTTATCAATAGGCCAACG AATCAGCCTCATGCGATACC TTGATAACGGAATGCGTCAG CCTAGGGTCTTGCTCGAGTTC TTGAGCTCGCATACGTTGAC TCTGAAGCGGGGATAGAAAA

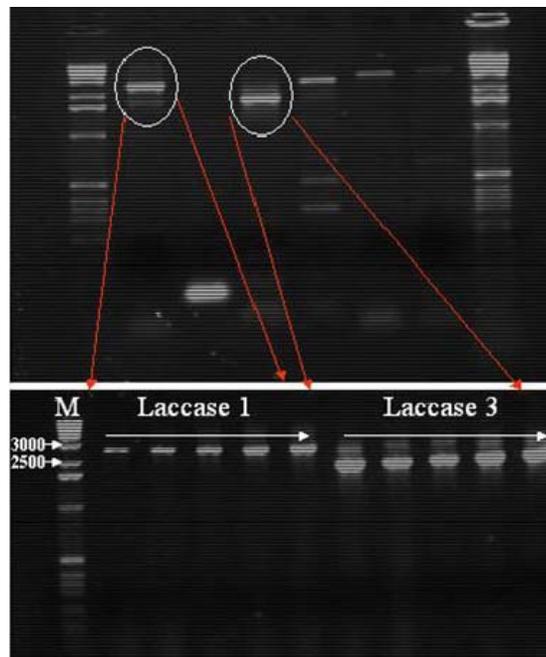
**Analysis of sequences and annotation for intron positioning:** The received sequences were edited using the BioEdit free software. This software facilitates visualization of the nucleotide sequences along with their peaks on chromatogram. The poor quality sequences were first deleted, followed by blast searches to verify the genes. The confirmed cleaned sequences were saved and compared with the available mRNA sequence using CAP contig. The comparison involved opening of the cleaned sequences from both forward and reverse primers of respective laccase genes along with mRNA sequence together inside the same window. The sequences obtained from both forward and reverse primer were matched with the sequence of mRNA to identify the location of different introns in the laccase genome. Further annotations for finding amino acid sequences and the intron positions at the amino acid level were performed using the Artemis free Software. The annotated nucleotide and amino acid sequence obtained from different laccase genes were prepared in Sequin for their submission to NCBI data base as the new sequences.

**Statistical Analysis:** The intron profile of each laccase gene was scored as '1' for presence and '0' for absence of intron. A putative ancestor laccase gene with no intron was added. A

combined binary data matrix for all the laccase genes was constructed. The binary data matrix was entered in the Paup package version 4.0 beta [27] and data was analysed using DOLLO parsimony [28]. Bootstrapping was carried out with 1000 replications.

## RESULTS AND DISCUSSION

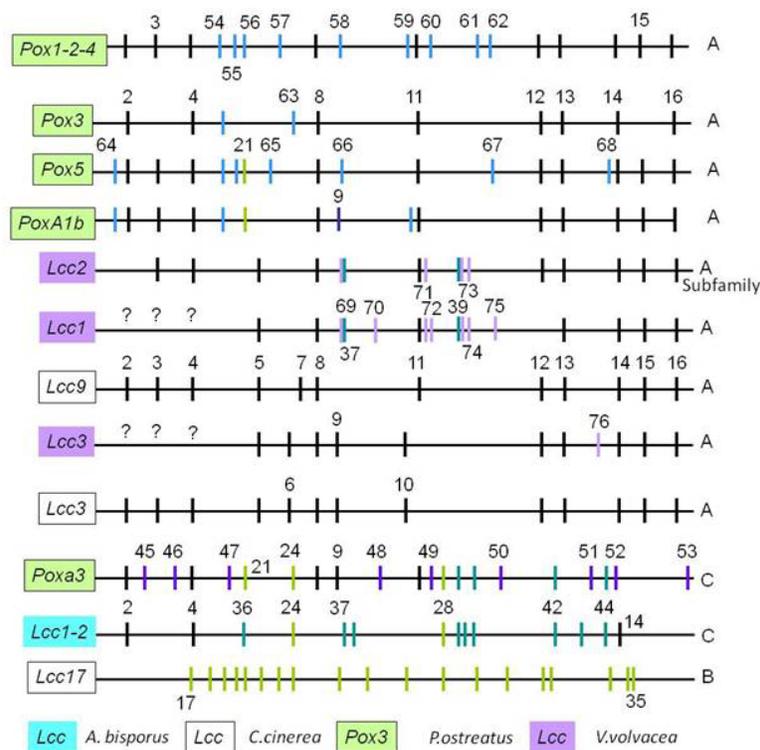
**Amplification of laccase genes:** Out of the six pairs of primers corresponding to the six different laccase genes of strain Vv-1, unique single bands of desired sizes were obtained only for laccase genes 1 and 3. For laccase 2, only low molecular weight fragments (probably primers dimers) were obtained, while in the case of laccase 4, multiple amplicons were obtained, which indicates non-specificity of the primers. For laccase 5 and 6 genes, the amplicons of desirable size were obtained but were in very low concentration. In order to improve the amplification efficiency, the PCR was performed at an annealing temperature of  $57 \pm 2^\circ\text{C}$  and amplicon's band intensities increased with the increase in the annealing temperature (Fig 1). Similarly, attempts were made for laccase 4 and 5 genes at annealing temperatures of  $57 \pm 2^\circ\text{C}$  and  $54 \pm 1^\circ\text{C}$ , respectively but no further improvement in amplicon quality (intensity /purity) was recorded, hence the primer pairs were rejected due to low specificity. The redesigned primers of laccase 2 and 6 genes were tried at annealing temperatures of  $58 \pm 2^\circ\text{C}$  and laccase 2 exhibited fairly good amplification at almost all temperatures ( $56\text{-}60^\circ\text{C}$ ), while laccase 6 gene exhibited amplification at only  $59$  and  $60^\circ\text{C}$  annealing temperatures. In case of homozygotic strain BBSR-003, out of 4 pair of primers (laccase1, 2, 3 & 6) attempted at  $57 \pm 1^\circ\text{C}$  annealing temperatures, appropriate amplification were obtained only for laccase 3 gene.



**Figure 1:** PCR amplification of different laccase genes of *V.volvacea*.

a) From left to right, surrounded by 1 Kb ladder (Life Technologies), Laccase 1 to 6. b) DNA ladder, Laccase 1 annealing temperature from  $55$  to  $59^\circ\text{C}$ , Laccase 3 annealing temperature from  $55$  to  $59^\circ\text{C}$

**Intron positions in different laccase genes:** The CAP contig of laccase 1 gene revealed a total of sixteen introns in addition to the possibility of additional introns at the beginning of the gene. The number of introns was also sixteen in laccase 2 gene. However, in same stretch of gene length, it had intron 12, which was absent in lcc 1. The three introns (70, 72 and 75), which existed in lcc1 were missing in this gene. Laccase 2 exhibited two introns (3 and 4) in the genome region, where sequence is missing in lcc1 and lcc3 (Fig. 2). A total of eleven introns could be documented in the same gene length of laccase 3 from strain Vv-1, out of which only six matched with the positions of introns in lcc1 and seven in lcc2 (Fig. 3). The small partial sequence of lcc6 we obtained exhibited only five introns (position 72, 39, 73, 74 and 77, a new position between 62 and 75) and their location was not considered in laccase gene classification nor in the phylogenetic analyses between laccases of different mushroom species. In a similar study carried out on *Coprinopsis cinerea*, the number of introns in different laccase genes varied from what we have recorded in the present study [23]. It was 7 in lcc1, while 19 in lcc17 [23, 29].



**Figure 2.** Relative positions of introns in different laccase genes of different sub-families. Nomenclature of introns 2 to 35 [23], 36 to 44 (*A. bisporus* lcc1 and lcc2), 45 to 68 (*P. ostreatus* Poxa3, Pox1-2-4, Pox3, Pox5), 69 to 76 (*V. voluacea* lcc1 and lcc3). *P. ostreatus* gene nomenclature corresponds to: Pox1(LACC9), Pox2 (LACC10), Pox3 (LACC4), Pox4 (LACC1), Pox5 (LACC11), PoxA1b (LACC6) and Poxa3 (LACC2)

The position of introns in different laccase genes of different mushrooms was compared and the laccases belonging to sub-family A (Lcc3 and Lcc9) from *Coprinopsis cinerea* exhibited a closer relationship with respect to positions of introns in their genes than laccase genes belonging to sub-family B or C (Fig. 2). Fig. 3 presents the comparative sequences of laccase 2 gene of strain Vv-01 of *V. voluacea* with positions of 16 introns to the published mRNA

sequence from some other strain. In this figure the gray boxes indicate bases that still need to be confirmed. Some of the ambiguous results were due to the heterozygosity of this gene (two sequences of gene) in the heterokaryotic strain Vv-01.

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Vv1_1cc2 ~~~~~
AY338483 TGGTTTGGGA CTCTCAAGCA TCTCACAGCA GTGAAAAGCA ATGGCCTTCT CGTCGTCGCG AATGCAATCC

Vv1_1cc2 ~~~~~-CTGST GCACAACAGA
AY338483 TTGCTCAACA CCCTTACCCT CATGATAACC TTGATGGGTT TGGGTGTTAG CTATGCTGCT GCACAACAGA

Vv1_1cc2 GCTTCCACAC CCTGGAGTTG ACTAATGATG AGGTTAACCC TGATGGTTTC CAGCGCGATG CGGTCCTCGT
AY338483 GCTTCCACAC CCTGGAGTTG ACTAATGATG AGGTTAACCC TGATGGTTTC CAGCGCGATG CGGTCCTCGT

Vv1_1cc2 CAATGGCGGC TTATTTGGAG CGTCAATCAC AGGCCAAAAG GTCTGTATTA TAGTGTAGCA CTCGACAATG
AY338483 CAATGGCGGC TTATTTGGAG CGTCAATCAC AGGCCAAAAG GTCTGTATTA TAGTGTAGCA CTCGACAATG

Vv1_1cc2 CCCTGATCTT TCATCATTAG GGAGATGAGT TCGTCATTGA AGTTGACAAT CAACTCACTG ACTCCCTCCT
AY338483 CCCTGATCTT TCATCATTAG GGAGATGGGT TCGTCATTGA AGTTGACAAT CAACTCACTG ACTCCCTCCT

Vv1_1cc2 CCGCAAGAGT ACATCCATCG TAAGTACAAA GTCATGCACC ATTTTTCGGG CTTGATGGAG GCTCACAAT
AY338483 CCGCAAGAGT ACATCCATC- ~~~~~

Vv1_1cc2 TATTACAAT AGCATTGGCA CGGGTTGTTT CAACGTGACA GCGCATGGGC TGATGGTCCC GCATTGCTCA
AY338483 ~~~~~-CATTGGCA CGGGTTGTTT CAACGTGACA GCGCATGGGC TGATGGTCCC GCATTGCTCA

Vv1_1cc2 CCCAATGCCC GATCGCTCCT GGCATACCTT TCACTTATCG GTTCACTGCT ACTGAGGAAG CTGGAACATT
AY338483 CCCAATGCCC GATCGCTCCT GGCATACCTT TCACTTATCG GTTCACTGCT ACTGAGGAAG CTGGAACATT

Vv1_1cc2 CTGGTACCAC TCGCACTTGG GTACGCCTGC GTTCACAGCG ATGGCGTCTA GCATTGTGTA ATAATTTTTG
AY338483 CTGGTACCAC TCGCACTTGG ~~~~~

Vv1_1cc2 TAGATGCTCA ATATTGCGAT GGACTTCGAG GCCCCTTCAT CATTATGAT CCAAACGACC CTCACCTTGG
AY338483 ~~-ATGCTCA ATATTGCGAT GGACTTCGAG GCCCCTTCAT CATTATGGT CCAAACGACC CTCACCTTGG

Vv1_1cc2 TCTCTACGAC GTTGATAATG GTAGGTCCAA GTTTACTGAT ACGAGTCTCT CGACTTGCTC ATACTCTTAT
AY338483 TCTCTACGAC GTTGATAATG ~~~~~

Vv1_1cc2 GCCAACAGAG GACACGATCA TCACCTGGC CGACTGGTAT CGTAAGTGAC CCACAACCTT ACCTCCACAA
AY338483 ~~~~~AG GACACGATCA TCACCTGGC CGACTGGTAT C~~~~~

Vv1_1cc2 CACAATAACT AACGCATACT CACAGACACT CCTGCTGAAC TGCTCAGCGG TGAATGTTC GTCTCCTTCT
AY338483 ~~~~~-ACACT CCTGCTGAAC TGCTCAGCGG TGAAT~~~~

Vv1_1cc2 TCCTGCAGGC TTTCACTAT TTACCCCTCA TTTTATAGCT CCCCTCAATC CACGTTGATT AACGGTTTGG
AY338483 ~~~~~-CT CCCCTCAATC CACGTTGATT AACGGTTTGG

Vv1_1cc2 GCCGCTCATC GAGCGACTTC ACTGCTCCTC TTGC~~~~~ CAAGGCCTCA AGTACCGCAT
AY338483 GCCGCTCATC GAGCGACTTC ACTGCTCCTC TTGCAGTCGT CAATGTTGAG CAAGGCCTCA AGTACCGCAT

Vv1_1cc2 GAGGTTGATC TCAATCTCCT GCGATCCAAA CTGGATCTTC TCCATCGAGG GACACGAGTT GACTGTCAAT
AY338483 GAGGTTGATC TCAATCTCCT GCGATCCAAA CTGGATCTTC TCCATCGAGG GACACGAGTT GACTGTCAAT

Vv1_1cc2 GAGGCTGATG GTATCAGCGT TCAAACCTGTT ACTGTCACCT CGTTGCAAAAT CTTTGTGGT GGGTAACCTT
AY338483 GAGGCTGATG GTATCAGCGT TCAAACCTGTT ACTGTCACCT CGTTGCAAAAT CTTTGTGGT ~~~~~

Vv1_1cc2 CTACCAATTC TATATATCCT AGCTGAGTTA TAAAACCCGW TTATTAGGTC AAAGATACTC GTTTGTGGTA
AY338483 ~~~~~-GTC AAAGATACTC GTTTGTGGT~~~~

Vv1_1cc2 CGCTCAAATC TATCGCAGCA TACTTGGAAA CATCCCTAAT ATCTGCCTAA TARTCCACG CTAACCAACC
AY338483 ~~~~~-CTCCACG CCAACCAACC

Vv1_1cc2 AGTCGGCAAC TACTGGATCC GTGCCAACCC GAACAAGGGA CCGACTGGGT TCGGCAACAA CATCAACAGC
AY338483 AGTCGGCAAC TACTGGATCC GTGCCAACCC GAACAAGGGA CCGACTGGGT TCGGCAACAA CATCAACAGC

Vv1_1cc2 GCCATCTTGA GGTACCAAGG AGCACCTATT GCAGACCCAA CTGGAGCAGG ACTTCAAGAT GCTCTCAACC
AY338483 GCCATCTTGA GGTACCAAGG AGCACCTATT GCAGACCCAA CTGGAGCAGG ACTTCAAGAT GCTCTCAACC

Vv1_1cc2 GCTTGGCCGA GCCCAATCTG CACGTAAGTC CCGTAACATT TATAGTGAA CTTTGTGCT GATGTTGCAT
AY338483 GCTTGGCCGA GCCCAATCTG CAC~~~~~

Vv1_1cc2 TAGCCCTCG TCAATCCCG AGTGTAAGG GCTCACTCCA ACTTTCGGAT TGGTCTTGGT CGTTAACGCC
AY338483 ~~-CCCTCG TCAATCCCG AGT~~~~~

Vv1_1cc2 CTCTAATTTA ATTTATAGCC TGGTCTCGCC CAGATCAATG GTGCTGATAT CGACATAGTG ATCAACATTG
AY338483 ~~~~~-CC TGGTCTCGCC CAGATCAATG GTGCTGATAT CGACATAGTG ATCAACATTG

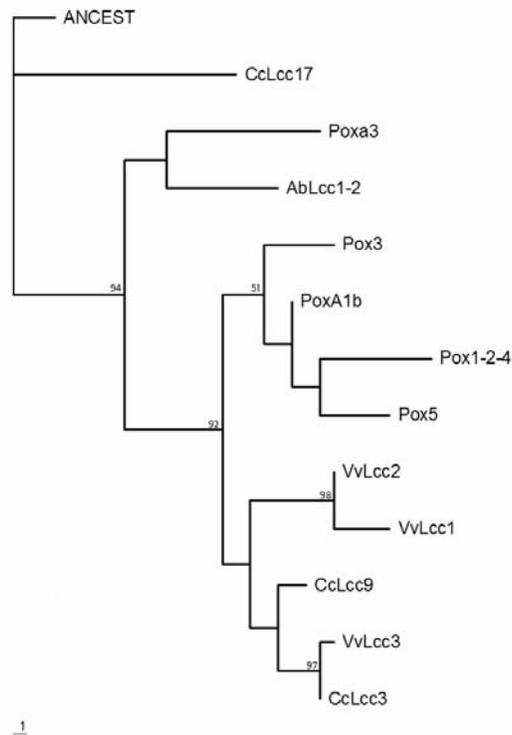
Vv1_1cc2 GCTTCGTAAG TCCCTCATTC CCCTCTCCCT CACCCCACTC TGGTTGACTC TCTCTGGCCA CTGCACACAC
AY338483 GCTTC~~~~~

Vv1_1cc2 TAGTCCGGAG GACTGTTCAA CATCGCCGGC ACATCKTACA CATCCCCGA CGTTCGGTCC TTGCTTCAAA
AY338483 ~~-TCCGGAG GACTGTTCAA CATCGCCGGC ACATCTTACA CATCCCCGA CGTTCGGTCC TTGCTTCAAA
    
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**Figure 3:** Sequence of laccase 2 gene of strain Vv-01 of *V. voluacea* with positions of 16 introns, compared to published mRNA sequence.

a) Letters in bold indicate the position of forward and reverse primers (Vv1FL2 and Vv42RL2). b) Grey boxes indicate bases that must be confirmed

A gap underlined corresponds to a base pair defect in the published mRNA sequence, which might have arisen in consequence of frame shift resulting from a first gap in the sequence (probably localised 40 to 80 bp before in the sequence missing in this work). The amino acid translation of the sequence before the identified gap should be LGSLMPSGSYIEL (which is very similar to laccase 1 homologous sequence: VSSLLPSGSYIEL) instead of IRLAHAQRILYRV.



**Figure 4:** One of the eight most parsimonious cladograms obtained by Dollo parsimony deduced out of 75 introns positions in laccases genes of three sub-families of four basidiomycetes. Thirty intron positions were parsimony-informative characters. Bootstrap values are from 50 % majority-rule consensus tree. Sub-family B - CcLcc17, sub-family C- Poxa3 and AbLcc1-2, sub-family A – all other genes, ANCEST – hypothetical ancestor gene with no intron.

Eight most parsimonious cladograms deduced from the positions of introns in twelve laccase genes belonging to three sub-families from four different basidiomycetes, revealed formation of eleven different clades (Fig. 4). In this dendrogram, Lcc3 from *V. volvacea* occupied the same clade as Lcc3 from *C. cinerea* in 97% of the bootstraps. Lcc1 and Lcc2 from *V. volvacea* also formed one clade with more than 98% of the bootstraps. Lcc9 from *C. cinerea* belonging to sub-family A formed a separate clade from Lcc3 from same mushroom [23]. The four laccases (Pox1-2-4, Pox5, PoxA1b and Pox3) of *P. ostreatus* belonging to sub-family A formed a clade in 97% of the bootstraps. All sub-family A laccase genes formed a clade supported by 92 bootstraps and sub-family A and C laccases formed clade supported by 94 bootstrap value. The only laccase (Lcc17) from *C. cinerea* belonging to sub-family B shared only 3 intron positions with genes of sub-family A and C (Fig. 2). The results indicate high

similarities between laccases of one sub-family belonging to a particular species and their distinctness from laccases of other sub-families either from the same or other related species. Laccases from *V. volvacea* share into two groups as already shown with protein sequences. In neighbour joining tree of the deduced amino acid sequences of *C. cinerea*, lcc2, lcc3, lcc12, lcc13 and lcc14 were placed in one clade [23]. In Hoegger's study [24], the neighbour joining tree of basidiomycete laccases based on realigned sequences has also clustered Vvo lac3 with Cci Lac3, as in present study. In Fig. 4, VvLcc2 and VvLcc1 clustered outside of the clade along with CcLcc9, VvLcc3 and CeLcc3, but another of the eight most parsimonious cladograms obtained, have placed these two genes as the sister group of the remainder of the sub-family A laccases.

**Sequence variations in lcc3 of two strains:** The nucleotide sequence obtained for laccase 3 genes of two strains (Vv-01 and BBSR-003) varied at ten different nucleotide positions. Strain Vv-01 exhibited heterozygosity at 10 different places, which are presented as R, Y, R, Y, R, Y, R, Y, R and Y, which mean A or G for R and C or T for Y. However, at these locations, the nucleotides in strain BBSR-003 were A, T, G, T, G, C, A, C, G and C respectively. This proves heterozygosity of this mushroom at the nucleotide level of this laccase gene (Fig 5). However, at the amino acid sequence level, there was only one difference: strain Vv-01 had D or N (i.e. Aspartic acid or Asparagine) noted X (i.e. any amino acid) in Fig. 6, while strain BBSR-003 had N means Asparagine.

The present study has mainly analyzed the variations in number and position of introns in different laccases of *V. volvacea* and compared that variation to other known laccases of *A. bisporus*, *P. ostreatus* and *C. cinerea*. The study has generated some valuable information like the grouping of *V. volvacea* laccases (lcc1, lcc2 and lcc3) in sub-family A and confirmation of clustering VvLcc3 with CcLcc3. In the present study, there was an intron 8, found earlier in lcc3 from *C. cinerea* but has not been spliced in the published mRNA sequence. This intron has to be spliced in strain Vv-01 to give a functional protein and by virtue of which, the published phylogenetic position of lcc3 in the laccase dendrograms is probably wrong. In lcc1, 16 different introns have been recorded and the sequence of Vv-01 strain differs by one amino acid from the published sequence of lcc1. No evidence of heterozygosity has been recorded in this sequence. The lcc2 gene also revealed 16 different introns. Although only a small portion of sequence could be validated but that also proved some heterozygosity in strain Vv-01. The small sequence of lcc 2 in strain BBSR-003 also revealed differences at some bases from strain Vv-01. At the protein sequence level, there were clearly 26 amino acids of the published mRNA, which were on the wrong reading frame. Maybe it is due to errors in sequencing, or the strain used had a pseudo gene or a very original allele for lcc 2. Due to these factors, the position of laccase 2 in the dendrogram will be changed. The sequence of lcc6 has helped in identification of 5 introns. The positions of introns in different laccase genes (lcc1, lcc2 and lcc3) of *V. volvacea* showed that they belong to sub-family A. Although not the main objective, the present study has proved heterozygosity in strain Vv-01 of *V. volvacea*, which has not been shown in earlier studies carried out on this species.

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lcc3_Vv1      TTGCGAGCGTAACTCAATGTCCTATTGTCCCATCAGAGTCTTTCCTCTATRAATTCACCG
lcc3_BBSR003  -----TCAATGTCCTATTGTCCCATCAGAGTCTTTCCTCTATAATTCACCG
                *****
lcc3_Vv1      TTCCAGACCAGGCTGGGACTTCTGGTATCATTCTCATYTGTTAGTGGGRCCCGCCTA
lcc3_BBSR003  TTCCAGACCAGGCTGGGACTTCTGGTATCATTCTCATYTGTTAGTGGGGCCCGCCTA
                *****
lcc3_Vv1      GTACCCTGCATTCCGATCGGTTTATTGACGTCGACCCTRACAGCTACACAGTATTGCGAT
lcc3_BBSR003  GTACCCTGCATTCCGATCGGTTTATTGACGTCGACCCTGACAGCTACACAGTATTGCGAT
                *****
lcc3_Vv1      GGTCTCAGGGGCCCGCTYGTCTTTATGATCCTGATGACCCTCATCGACACCTGTACGCC
lcc3_BBSR003  GGTCTCAGGGGCCCGCTCGTCGTTTATGATCCTGATGACCCTCATCGACACCTGTACGCC
                *****
lcc3_Vv1      GAGTTAGTTCATTTATTGCAAGGCRGACTCTAACCATATAATAGATATGATATAGACGAY
lcc3_BBSR003  GAGTTAGTTCATTTATTGCAAGGCRGACTCTAACCATATAATAGATATGATATAGACGAC
                *****
lcc3_Vv1      GGTGCGCATCATGATGTGGCTGAATCAATGCCACCCCGATTCTTACGTTGCCCATAGAAT
lcc3_BBSR003  GGTGCGCATCATGATGTGGCTGAATCAATGCCACCCCGATTCTTACGTTGCCCATAGAAT
                *****
lcc3_Vv1      CCACAATCATAACTCTTTCGGACTGGTATGAGTCCACTGGACTAATCCCGCTCATAGAC
lcc3_BBSR003  CCACAATCATAACTCTTTCGGACTGGTATGAGTCCACTGGACTAATCCCGCTCATAGAC
                *****
lcc3_Vv1      TGAGCTTCTGCACAAGGTACCATGTAAAGGCACCGGTTGCAGGACTTCARCCCTACACCTA
lcc3_BBSR003  TGAGCTTCTGCACAAGGTACCATGTAAAGGCACCGGTTGCAGGACTTCACCTACACCTA
                *****
lcc3_Vv1      TGTCCACGCTTATAAACGGAAGGGGAGATATGCCGGAGGCCCTAACGTGCCTCTTGAGG
lcc3_BBSR003  TGTCCACGCTTATAAACGGAAGGGGAGATATGCCGGAGGCCCTAACGTGCCTCTTGAGG
                *****
lcc3_Vv1      TGATTAACGTAGTCCCTGGAACAAGATATCGCTTCCGCCTAGTATCAATGGTTTGCGATC
lcc3_BBSR003  TGATTAACGTAGTCCCTGGAACAAGATATCGCTTCCGCCTAGTATCAATGGTTTGCGATC
                *****
lcc3_Vv1      CYAACTACATCTTTTCCATAGACGGACACGAACCTGGTATGCTATGCTCTTGCATAAAAGG
lcc3_BBSR003  CCAACTACATCTTTTCCATAGACGGACACGAACCTGGTATGCTATGCTCTTGCATAAAAGG
                *****

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**Figure 5:** Variations in partial nucleotide sequences of laccase 3 from strains Vv-01 and BBSR-003 of *V. volvacea*

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lcc3_BBSR003  ---QCPIVPSSEFLYNFTVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPPDPHRHLYDI
lcc3_Vv01     ASVTQCPIVPSSEFLYXFTVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPPDPHRHLYDI
                *****
lcc3_BBSR003  DDESTIITLSDWYHVKAPVAGLQPTPMSTLINGRGRYAGGPNVPLEVINVPGTRYRFRLL
lcc3_Vv01     DDESTIITLSDWYHVKAPVAGLQPTPMSTLINGRGRYAGGPNVPLEVINVPGTRYRFRLL
                *****
lcc3_BBSR003  VSMVCDPNYIFSIDGHELTIIIEADGVNTKPLTVDSIQIFAGQRYSFILNANQPVDNYWIR
lcc3_Vv01     VSMVCDPNYIFSIDGHELTIIIEADGVNTKPLTVDSIQIFAGQRYSFILNANQPVDNYWIR
                *****
lcc3_BBSR003  ANPNLGTGTGFTNGINSAILRYEGAPLQDPTTPLVPSVNPLVESNLRNLIENAVPGQPFPG
lcc3_Vv01     ANPNLGTGTGFTNGINSAILRYEGAPLQDPTTPLVPSVNPLVESNLRNLIENAVPGQPFPG
                *****
lcc3_BBSR003  GADININLFTDFDWDMLHFTVNGSPYIPPNAPVLLQILSGTYNAQDLMPAGSIIELPSNK
lcc3_Vv01     GADININLFTDFDWDMLHFTVNGSPYIPPNAPVLLQILSGTYNAQDLMPAGSIIELPSNK
                *****
lcc3_BBSR003  VIEISMPGGSPGSPHPFHLHGHTFDVIRSTDTQEYNFVDPVRRDVVNTGFETDNVTIRFT
lcc3_Vv01     VIEISMPGGSPGSPHPFHLHGHTFDVIRSTDTQEYNFVDPVRRDVVNTGFETDNVTIRFT
                *****
lcc3_BBSR003  TDNPGPWILHCHIDWHLEVGLAVVFAEDIPRISTPAPPPSW----
lcc3_Vv01     TDNPGPWILHCHIDWHLEVGLAVVFAEDIPRISTPAPPPSWDEL
                *****

```

**Figure 6:** Variations in amino acid sequences of laccase 3 from strains Vv-01 and BBSR-003 of *V. volvacea*

We compared intron positions present in sub-family A laccase genes in *A. bisporus* and *C. cinerea* from Agaricoid clade (VI) and *P. ostreatus* and *V. volvacea* from Pluteoid clade (III) [17]. From this comparison, it is clear that only intron position 2 was shared exclusively by *P. ostreatus* and the Agaricoid clade laccases. However, data in the present study is insufficient to indicate whether this intron position is present in *V. volvacea*. In contrast, intron positions 5, 6 and 10 are shared exclusively by *V. volvacea* and Agaricoid clade laccases. VvLcc3 clustered with Agaricoid clade VI sub-family A genes while *P. ostreatus* laccase genes formed a sister clade. This could indicate that ancestor genes from sub-family A laccases from *V. volvacea* and Agaricoid species could have diverged more recently than from ancestor genes of *P. ostreatus* sub-family A laccases. However VvLcc1 and VvLcc2 share intron position 37 and 39, which are present in laccase sub-family C and in Polyporus sub-family A laccases (Billette C. unpublished result). Then they could belong to the clade containing Polyporus laccase in sub-family A. Moreover, position of these two genes vary in the different cladograms we have obtained. Present finding is not sufficient to elucidate the respective phylogenetic positions of *V. volvacea*, *P. ostreatus* and Agaricoid clade species. To build further species phylogenetic trees, it is important to use sequences from *V. volvacea* and from *Volvopluteus gloiocephalus*, since they don't cluster together as demonstrated by Justo *et al.* [19].

The present findings have implications on other issues such as the role of intron positions in phylogenetic studies and the use of nucleotides sequence variability in exons flanking the specific introns for species identification, as it was done in species discrimination in Sclerotiniaceae [20]. The sequence of conserved copper binding amino acids of novel laccase genes also has the potential to help in resolving speciation conflict in several closely related species, where other molecular techniques have yielded confusing inferences, like in the case of Sclerotiniaceae [20]. The concept suggests redefining different genera by identifying these genes and concomitantly supports reclassification of misleading fungal species [30]. The study carried out by Castilho *et al.* [22] has reinforces the laccase diversity and interspecific variation in this genetic pool. As one of the most important criteria in phylogenetic systematics is the issue of homology [31], and out of different types of homologies, laccases fall in the category of paralogy, which means that they originate from gene duplication events. For example, the laccase amino acid sequences have been used for phylogenetic study and the molecular evolution studies can still further help in finding the laccases of industrial importance even in still unknown species.

## REFERENCES

- [1] Singer R. (1975). The Agaricales in modern taxonomy, 3<sup>rd</sup> ed., Verlag J. Cramer, Vaduz, Pp. 912.
- [2] Chang S.T., Yau C.K. (1971). *Volvariella volvacea* and its life history. *Amer. J. Bot.* 586: 552-567.
- [3] Li S.F., Chang S.T. (1979). Variation in the homothallic basidiomycetes *Volvariella volvacea*. *Mush. Sci.* 10: 171-184.
- [4] Philippoussis A. *et al.* (2001). Bioconversion of agricultural lignocellulosic wastes through the cultivation of edible mushroom *Agrocybe aegerita*, *Volvariella volvacea* and *Pleurotus* spp., *World J. Microbiol. Biotechnol.* 17:191-200.
- [5] Akinyele B.J., Adetuyi F.C. (2005). Effect of agro wastes, pH and temperature variation on the growth of *Volvariella volvacea*. *African J. Biotechnol.* 4: 1390-1395.

- [6] Buswell J.A., Chen M. (2005). Cultivation, biochemical, molecular biological and medicinal aspects of the culinary-medicinal straw mushroom *Volvariella volvacea* (Bull.:Fr.) Singer (Agaricomycetidae). *Int. J. Medicinal Mushroom*. 7:157-166.
- [7] Cai Y.J. *et al.* (1994). Cellulases and hemicellulases of *Volvariella volvacea* and the effect of Tween 80 on enzyme production. *Mycol. Res.* 98:1019-1024.
- [8] Cai Y.J. *et al.* (1998).  $\beta$ -Glucosidase components of the cellulolytic system of the edible straw mushroom, *Volvariella volvacea*. *Enzyme Microbiol. Technol.* 22: 122-129.
- [9] Cai Y.J. *et al.* (1999). Production and distribution of endoglucanase, cellobiohydrolase, and  $\beta$ -glucosidase components of the cellulolytic system of *Volvariella volvacea*, the edible straw mushroom. *Appl. Environ. Microbiol.* 65: 553-559.
- [10] Thurston C.F. (1994). The structure and function of fungal laccases. *Microbiol.* 140: 19-26.
- [11] Ahlawat O.P. *et al.* (2008). Profile of the extra cellular lignocellulolytic enzymes activities as a tool to select the promising strains of *Volvariella volvacea* (Bull. ex Fr.) Sing. *Indian J Microbiol.* 48: 389-396.
- [12] Chen S.C. *et al.* (2004a). Biochemical and molecular characterization of laccase from edible straw mushroom, *Volvariella volvacea*. *Eur. J. Biochem.* 271: 318-328.
- [13] Chen S.C. *et al.* (2004b). Molecular cloning of a new laccase from the edible straw mushroom *Volvariella volvacea*: Possible involvement in fruit body development. *FEMS Microbiol. Lett.* 230: 171-176.
- [14] Kirk P.M. *et al.* (2008). Anisworth & Bisby's dictionary of fungi, 10<sup>th</sup> edn., Pp. 340, CAB International, Wallingford, U.K.
- [15] Singer R. (1986). The Agaricales in modern taxonomy, 4<sup>th</sup> edn., Pp. 981. Koeltz Scientific Books, Koenigstein, Germany.
- [16] Moncalvo J.M. *et al.* (2002). One hundred and seventeen clades of euagarics. *Mol. Phylogenet. Evol.* 23: 357-400.
- [17] Matheny P.B. *et al.* (2006). Major clades of Agaricales: a multigenetic overview. *Mycologia.* 98: 982-995.
- [18] Binder M. (2010). Amylocorticiales ord. Nov. and Jaapiales or. Nov.: early-diverging clades of Agaricomycetidae were dominated by corticioid forms. *Mycologia* 102: 865-880.
- [19] Justo A. *et al.* (2011). Phylogeny of the Pluteaceae (Agaricales, Basidiomycota): taxonomy and character evolution. *Fung. Biol.* 115: 1-20.
- [20] Hirschhauser S., Frohlich J. (2007). Multiplex PCR for species discrimination of Sclerotiniaceae by novel laccase introns. *Intl. J. Food Microbiol.* 118: 151-157.
- [21] Luis P. *et al.* (2004). Diversity of laccase genes from basidiomycetes in a forest soil. *Soil Biol. Biochem.* 36: 1025-1036.
- [22] Castilho J.D. *et al.* (2009). On the diversity of laccase gene: A phylogenetic perspective from *Botryosphaeria rhodina* (Ascomycota: Fungi) and other related taxa. *Biochem Genet.* 47: 80-91.
- [23] Kilaru S. *et al.* (2006). The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide in to two distinct sub-families. *Curr. Genet.* 50: 45-60.
- [24] Hoegger P.J. *et al.* (2006). Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS J.* 273: 2308-2326.
- [25] Courty P.E. *et al.* (2009). Phylogenetic analysis, genomic organization, and expression analysis of multicopper oxidases in the ectomycorrhizal basidiomycete *Laccaria bicolor*. *New Phytol.* 182: 736-750.

- [26] Lettera V. *et al.* (2010). Identification of a new member of *Pleurotus ostreatus* laccase family from mature fruiting body. *Fungal Biol.* 114: 724-730.
- [27] Swofford, D. L. (1998). PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.
- [28] Young, D. A. (1981). "Are the Angiosperms Primitively Vesselless." *Systematic Botany* 6(4): 313-330.
- [29] Hoegger P.J. *et al.* (2004). The laccase gene family in *Coprinopsis cinerea* (*Coprinus cinereus*). *Curr. Genet.* 45: 9-18.
- [30] Hawksworth D.L. (2004). Fungal diversity and its implications for genetic resource collections. *Studies in Mycology* 50: 9-18.
- [31] Henning W *et al.* (1999). Phylogenetic systematic. University of Illinois Press, USA 253.