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Comparative genome wide analysis of the EF-hand containing calcium binding proteins in five edible mushrooms

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ABSTRACT

The current scenario required urgent attention for the nutritional security and environmental deterioration due to the agricultural wastes. Beneficial fungi are well known for the nutrient recycling, plant growth, soil health and degradation of the plant wastes. Notably, mushrooms, an edible fungus, are an excellent source of the proteins (essential amino acids); vitamins, medicinal properties and can be utilized as food and tonic. Though, several mushroom genomes sequenced in last decade, but the knowledge of the mushroom developmental biology is still meagre. Moreover, calcium is ubiquitous signaling agent in all the eukaryotes, which binds with different calcium binding proteins. The present study reports the genome wide comparative analysis of the EF-hand containing calcium binding proteins of five edible mushrooms viz. *Agaricus bisporous var. bisporous H97and Agaricus bisporous var. burnettii JB137-S8; Pleurotus ostreatus strain PC15*; *Lentinula edodes strain NBRC 111202*; *Schizophyllum commune strain H4-8; Coprinopsis cinerea strain okayama7#130.* The results demonstrated that EF-hand number ranges from two to five, while EF-hand containing proteins ranges from fourteen to seventeen in all the analysed mushroom genomes. Phylogenetic analysis showed that, EF-hand containing proteins fall into four groups. In general, Group I contain all the CaM and CMLs while Group II includes the CBL and NCS. Group III having all the PEF proteins and Group IV contains remaining proteins in which other functional domain was also present. The comparative information generated in this study could be helpful for functional characterization of the genes of five edible mushrooms and their relationship with important agronomic traits.

Keywords: Edible mushrooms, Calcium, EF-hand, Calcium binding proteins

Fungi are one of the key components of the ecosystem, which contribute to nutrient recycling, plant growth, soil health and degradation of plant waste (Morin *et al.*, 2012; Patyshakuliyeva *et al.*, 2015). Mushrooms belongs to the fungal kingdom. They are the rich source of the proteins, minerals and vitamins, and can be grown on agricultural wastes (Khader, 1999; Kumar *et al.*, 2013). Though, many species of mushrooms are edible but very few are being cultivated at commercial level (Kumar *et al.*, 2013). *Lentinula edodes* (shiitake mushroom) and *Pleurotus ostreatus* (Oyster) are the commonly cultivated mushrooms all over the world (Chang and Buswell, 1996; Szeto *et al.*, 2008; Qu *et al.*, 2016). Among edible mushrooms, *Agaricus bisporus*, commonly known as button mushroom, is the most common mushroom in India. *Schizophyllum commune* (wood-degrading fungus) and *Coprinopsis cinerea* (gray shag) are the excellent model to study the mushroom developmental biology (Ohm *et al.*, 2010; Stajich *et al.*, 2010).

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In biological system, calcium (Ca^{2+}) mediates an array of signalling events through Ca^{2+} binding proteins (Berridge, 1987; Gadd, 1994; Sanders *et al*., 2002; Brini *et al*., 2013). Calmodulin (CaM) is one of the key members of the intracellular Ca^{2+} binding protein, which interact with the other proteins and further regulate several signalling pathways and physiological processes (Hoeflich and Ikura, 2002). In fungal kingdom, CaM is well reported for its essentiality in hyphal growth and development as well as in germination and penetration in plant tissue (Ahn *et al*., 2003; Sato *et al*., 2004; Wang *et al*., 2006; Ahn and Suh, 2007). Notably, CaM was reported to modulate the expression of lignin-modifying enzyme in an edible mushroom *P. ostreatus* (Suetomi *et al*., 2014). Moreover, Penta EF-hand proteins were also reported in lower eukaryotes including fungi (Ohkouchi *et al.*, 2001). However, PEF can be categorized in Group I (ALG-2, peflin, and their plant, protists and fungal homologs), and Group II (sorcin, calpain and grancalcin subfamily) on the basis of the calcium binding loops of EF1 (Maki *et al*., 2002).

Availability of the sequenced genome is a key resource for genetic/molecular diversity analysis as well as to dissect the agronomically important genes (Li *et al*., 2014). In last era, several mushroom genome were sequenced, however, *in silico* analysis of different genes were reported in different mushrooms such as heme peroxidases (Ruiz-Dueñas *et al*., 2011) and MYB transcription factor in *P. ostreatus* (Wang *et al*., 2018); lanostanoids in *Ganoderma* (Suarez-Medellin *et al*., 2016). Recently, Zhou *et al*. (2024) analysed bZIP genes and explored its role in heat stress and development of *P. ostreatus.* But so far, no reports are available regarding the comparative analysis of the EF-hand containing proteins in different edible mushrooms. The present study reports the genome wide comparative analysis of the EF-hand containing proteins in five edible mushrooms viz. *A. bisporus var. bisporus* H97, *A. bisporus var. burnettii* JB137-S8*, P. ostreatus* strain PC15*, L. edodes* strain NBRC 111202*, S. commune* strain H4-8 and *C. cinerea* strain okayama7#130.

MATERIAL AND METHODS

Identification of EF-hand containing proteins in five edible mushrooms

In order to identify the CaM and other EF-hand containing proteins, we have downloaded the amino acid sequence of *Neurospora crassa* (OR74A), different EF-hand containing proteins *i.e,* Calmodulin (CaM-APB97098.1), Neuronal calcium sensor 1 (NCS1-XP_957456.1), calcium/calmodulin-dependent protein kinase (CDPK1-XP_962989.3), myosin regulatory light chain (cdc4-XP_962359.3) and Penta EF hand protein (PEF-XP_011393017.1) genes from the NCBI database (https://www.ncbi.nlm.nih.gov/). Further these sequences were used as queries to perform the BLASTP and tBLASTn search against the five edible mushrooms *i.e. A. bisporus var. bisporus* H97, *A. bisporus var. burnettii* JB137-S8, *P. ostreatus* strain PC15, *L. edodes* strain NBRC 111202, *S. commune* strain H4-8 and *C. cinerea* strain okayama7#130*,* genome databases. Moreover, the gene prediction was performed using the HMM-based gene-finder tool i.e Fgenesh (http://www.softberry.com/) using the different selected organism database as a specific gene-finding parameter. Further the genes were confirmed for the presence of the EF-hand (Ca^{2+} binding domain) (SM00054 and PF00036, PF13499) and other domain using the SMART (http://smart.embl-heidelberg.de/) and Pfam tool (https://pfam.xfam.org/search/sequence). The sequences, which were not having the EF hand domain(s) were removed and not included for further analysis.

Identification of conserved Domain and Motif Analysis

In order to functionally characterise the identified protein, conserved domains in the EF hand containing proteins were predicted and analysed using the Interpro database (https://www.ebi.ac.uk/interpro/search/sequence-search). In addition, MEME suit tool (http://meme-suite.org/) was used to find out the novel conserved motifs in the Ca^{2+} binding protein sequences. Protparam tool (https://web.expasy.org/protparam/) was used to determine the amino acid length, molecular weight (MW) and isolelectric point (pI).

Multiple Sequence Alignments and Phylogenetic tree construction

All the selected mushroom's EF hand domain containing protein sequences and related fungus protein sequences of (*Neurospora crassa* OR74A and *Dictyostelium discoideum* AX4) were aligned using the ClustalW programme implemented in the MEGA 6.06 software with default parameter (Tamura *et al.*, 2013). The identified CaM protein sequences and other fungal CaM proteins were aligned in a separate file. Further, the multiple aligned separate files were subjected to phylogenetic tree reconstruction using Neighbor-joining (NJ) method with 1000 bootstrap replicates, poissons distribution and pairwise deletion and other parameter to understand the evolutionary relationships among the different Ca^{2+} binding proteins in the five edible mushrooms.

RESULTS

Identification of the EF hand containing proteins in five edible mushrooms

The number of EF-hand ranged from two to five in the EF-hand containing proteins of the analyzed genome of five edible muhsrooms. The genome wide comprehensive analysis identified seventeen EF-hand containing proteins in *A. bisporus var. bisporus* H97, *P. ostreatus* and *A. bisporous var. burnettii* JB137-S8, while sixteen in *L. edode* and *S. commune*. In *C. cinerea* contain only fourteen EF-hand containing proteins. Notably, six genes of 4EF-hand containing proteins were found in all the mushroom except *P. ostreatus* (5 genes) and *C. cinerea* (4 genes). Moreover, the number of 3-EF hand containing proteins in *S. commune*; *C. cinerea* and *L. edodes* was three and one, respectively, while, *A. bisporous* and *P. ostreatus* having two genes. Five genes in *S. commune* and *C. cinerea* and 6 genes in *A. bisporous var. burnettii JB137-S8;* and *L. edodes* contain the 2EF-hand, while *A. bisporous var. bisporous H97 and P. ostreatus* contain 7 and 8 genes, respectively. Interestingly, *L. edodes* contain three genes of Penta-EF hand proteins while other mushroom contains two genes (Table 1).

P. ostreatus and *C. cinerea* contain two CaM genes while others having only one CaM. Moreover, six CMLs were observed in all the mushrooms except *C. cinerea* which contain four CMLs. Notably, singular CBL gene was found in all the mushrooms. However, all the mushrooms contain one NCS gene except *A. bisporus* and *S. commune* which have two NCS genes (Table 1).

Multiple sequence alignement and phylogenetic analysis of EF-hand-containing proteins

All the identified EF-hand containing proteins were aligned by Clustal W, followed by phylogenetic tree construction using neighbour-joining method. The Group I of the tree contained all the CaM and CMLs of the five edible mushrooms. However, Group I also included few other domain containing proteins i.e. Sc-OD5 (WD40), Cc-OD3 (Transmembrane domain), Ab-H-OD5 (ORC5 domain), Po-OD5 (Polyketide synthase), Po-OD6 (ATPsyth). Moreover, all the NCS and CBL genes of the five edible mushrooms constituted the Group II. Interestingly, the PEF proteins clustered in Group III and remaining EF-hand and other domain containing proteins constitutes the Group IV (Fig.1).

Table 1. The characteristics of EF-hand containing proteins in five edible mushrooms

Accession/ NCBI ID	Coding	EF-hand No.	Amino acid	pI
***Agaricus bisporus* var. bisporous H97**				
XP_006456046.1	Ab-H-CaM	4	149	4.17
AEOK01000196.1	Ab-H-CML1	4	178	4.44
AEOK01000002.1	Ab-H-CML2	4	167	4.72
XP_006456047.1	Ab-H-CML3	4	246	4.44
XP_006453904.1	Ab-H-CML4	4	167	4.72
XP_006454041.1	Ab-H-CML5	2	208	4.78
AEOK01000005.1_1	Ab-H-CML6	2	208	4.78
XP_006460004.1	Ab-H-NCS1	3	190	4.74
AEOK01000066.1	Ab-H-NCS2	3	203	4.85
XP_006459824.1	Ab-H-CBL	4	175	4.46
XP_006458254.1	Ab-H-PEF1	4	218	6.22
XP_006460581.1	Ab-H-PEF2	4	249	5.12
AEOK01000005.1_2	Ab-H-OD1	2	641	9.24
XP_006454131.1	Ab-H-OD2	2	692	9.22
XP_006462756.1	Ab-H-OD3	2	645	5.5
XP_006457912.1	Ab-H-OD4	2	627	8.31
AEOK01000202.1	Ab-H-OD5	2	667	6.59
XP_007331882.1	Ab-J-CaM	4	149	4.17
XP_007331737.1	Ab-J-CML1	4	173	4.54
XP_007325923.1	Ab-J-CML2	4	167	4.72
AEOL01000219.1	Ab-J-CML3	4	178	4.44
AEOL01000014.1	Ab-J-CML4	2	208	4.78
XP_007325211.1	Ab-J-CML5	2	208	4.78
AEOL01000018.1	Ab-J-CML6	4	167	4.72
XP_007329544.1	Ab-J-NCS	3	190	4.74
AEOL01000120.1	Ab-J-NCS2	3	204	4.92
XP_007326525.1	Ab-J-PEF1	4	218	6.22
XP_007333378.1	Ab-J-PEF2	4	249	5.12
XP_007333662.1	Ab-J-CBL	4	175	4.46
AEOL01000009.1	Ab-J-OD1	2	671	9.17
XP_007325783.1	Ab-J-OD2	2	694	9.18
XP_007329726.1	Ab-J-OD3	2	645	5.56
XP_007326087.1	Ab-J-OD4	2	627	8.31
Pleurotus ostreatus				
KDQ23290.1	Po-CaM	4	149	4.15
KDQ26002.1	Po-CML1	4	146	4.35
KDQ31500.1	Po-CML2	4	165	4.68
KDQ31029.1	Po-CML3	2	205	4.78
KDQ23366.1	Po-CML4	2	141	4.52
AYUK01000010.1_2	Po-CML5	2	179	4.54
AYUK01000010.1_1	Po-CML6	4	674	5.15
KDQ29224.1	Po-NCS	3	190	4.83
KDQ28789.1	Po-CBL	4	175	4.57
KDQ33816.1	Po-PEF1	3	257	6.54
KDQ28389.1	Po-PEF2	3	227	5.32
KDQ31163.1	Po-OD1	2	704	9.12
KDQ25557.1	Po_OD2	2	645	6
KDQ33378.1	Po-OD3	2	663	7.69
AYUK01000001.1	Po-OD4	2	1288	8.82
AYUK01000003.1	Po-OD5	2	484	5.61
AYUK01000007.1	Po-OD6	3	382	4.9

Accession/ NCBI ID	Coding	EF-hand No.	Amino acid	pI
Lentinula edodes				
GAW06928.1	Le-CaM1	4	151	4.11
GAW06925.1	Le-CaM2	4	145	4.09
GAW04059.1	Le-CML1	4	205	5.33
BDGU01000175.1	Le-CML2	4	148	5
BDGU01000366.1	Le-CML3	4	167	4.38
GAW08047.1	Le-CML4	2	228	5
BDGU01000552.1	Le-CML5	2	172	4.83
GAW07420.1	Le-CML6	2	141	4.52
GAW07682.1	Le-NCS	3	201	4.91
GAV99290.1	Le-PEF1	4	241	4.75
GAV99289.1	Le-PEF2	4	244	4.77
GAW06283.1	Le-PEF3	4	219	5.76
GAW02187.1	Le-CBL	4	175	4.65
GAW05963.1	Le-OD1	2	1170	6.15
GAW06793.1	Le-OD2	2	1024	5.44
GAW08851.1	Le-OD3	2	669	7.73
Schizophyllum commune				
XP_003025993.1	Sc-CaM	4	149	4.17
ADMJ01000106.1	Sc-CML1	3	162	4.2
XP_003026110.1	Sc-CML2	2	141	4.6
XP_003037483.1	Sc-CML3	2	198	4.8
XP_003031735.1	Sc-CML4	4	210	4.9
XP_003026327.1	Sc-CML5	4	165	4.68
ADMJ01000301.1	Sc-CML6	4	211	5.17
ADMJ01000008.1	Sc-NCS1	3	190	4.91
XP_003038032.1	Sc-NCS2	3	190	4.91
XP_003036958.1	Sc-CBL	4	175	4.67
XP_003030756.1	Sc-PEF1	4	176	5.3
XP_003030758.1	Sc-PEF2	3	169	5.25
ADMJ01000024.1	Sc-OD1	2	705	8.46
XP_003037306.1	Sc-OD2	2	670	9
XP_003034526.1	Sc-OD3	2	652	5.66
XP_003038248.1	Sc-OD4	2	696	8.57
ADMJ01000317.1	Sc-OD5	4	848	6.66
Coprinopsis cinerea				
XP_001834963.2	Cc-CaM1	4	149	4.15
XP_001835120.2	Cc-CaM2	3	115	4.19
AACS02000001.1	Cc-CML1	3	235	8.47
XP_001829473.1	Cc-CML2	4	164	4.62
XP_001835011.2	Cc-CML3	2	141	4.52
XP_001829115.1	Cc-CML4	2	202	4.71
XP_001837073.2	Cc-NCS	3	178	4.97
XP_001839327.2	Cc-CBL	4	175	4.46
XP_001829781.1	Cc-PEF1	3	235	5.46
XP_001833409.1	Cc-PEF2	4	216	5.49
XP_001829210.2	Cc-OD1	2	705	8.85
XP_001833080.1	Cc-OD2	2	658	8.44
AACS02000012.1	Cc-OD3	4	623	5.17
XP_001835979.2	Cc-OD4	2	647	5.73

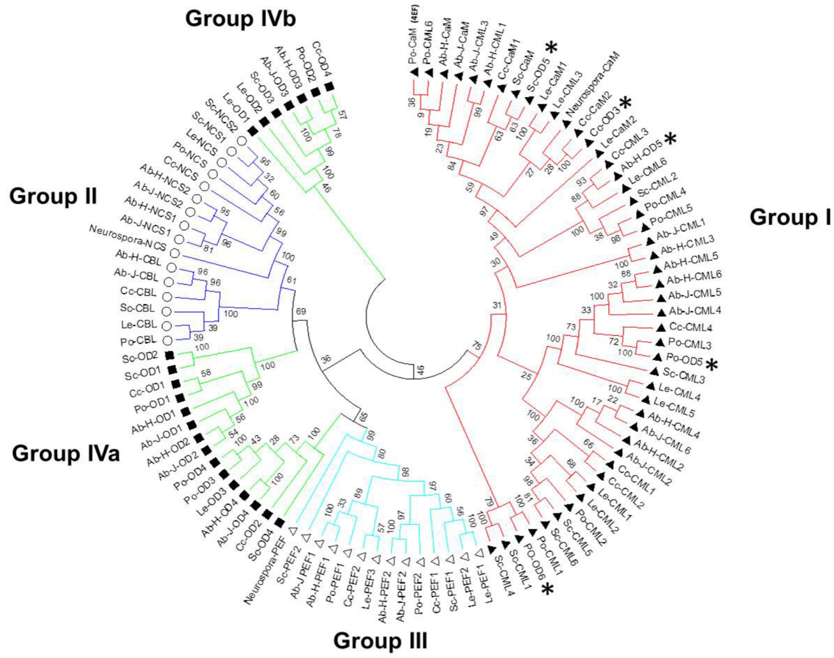


Fig. 1. Unrooted phylogenetic tree of the EF-hand containing proteins of the five edible mushrooms. All the identified EF-hand containing proteins were aligned using the ClustalW programme and subjected to phylogenetic tree reconstruction using Neighbor-joining (NJ) method with 1000 bootstrap replicates using MEGA 6.06 software. CaM: Calmodulin; CBL- Calcineurin B like protein, NCS: Neuronal Ca2+ sensor (NCS) protein; PEF: Penta-EF-hand (PEF) proteins; OD: EF-hand proteins which contain other functional domain. *Asterisk symbol indicates the presence of OD proteins in Group I. Ab-H: *Agaricus bisporus var. bisporus H97;* Ab-J: *A. bisporus var. burnettii JB137-S8*; Po: *Pleurotus ostreatus strain PC15*; Le: *Lentinula edodes strain NBRC 111202*; Sc: *Schizophyllum commune strain H4-8;*Cc: *Coprinopsis cinerea strain okayama7#130;* NC: *Neurospora crassa;* Dd:*Dictyostelium discoideum*

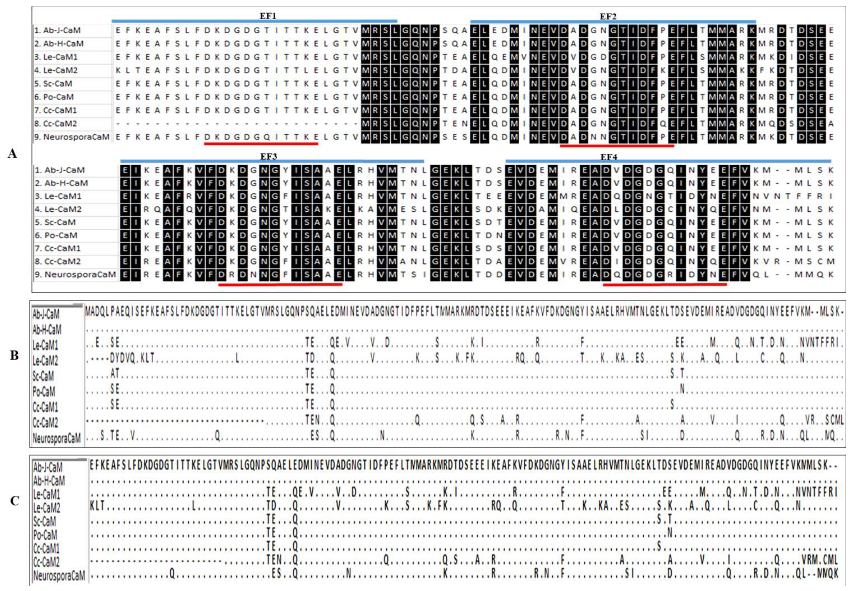


Fig. 2. Multiple Sequence alignment of the Calmodulin (CaM) proteins of edible mushrooms. A. Sequences were aligned by Clustal W program. Four EF-hands were depicted by blue line while the red line indicates Calcium binding loop. Black boxes depict the highly conserved residues i.e 100%. Variations among the amino acid residue of the (B) CaM genes and (C) EF-hand calcium binding loop region. Ab-H: *Agaricus bisporus var. bisporus H97;* Ab-J: *A. bisporus var. burnettii JB137-S8*; Po: *Pleurotus ostreatus strain PC15*; Le: *Lentinula edodes strain NBRC 111202*; Sc: *Schizophyllum commune strain H4-8;*Cc: *Coprinopsis cinerea strain okayama7#130*

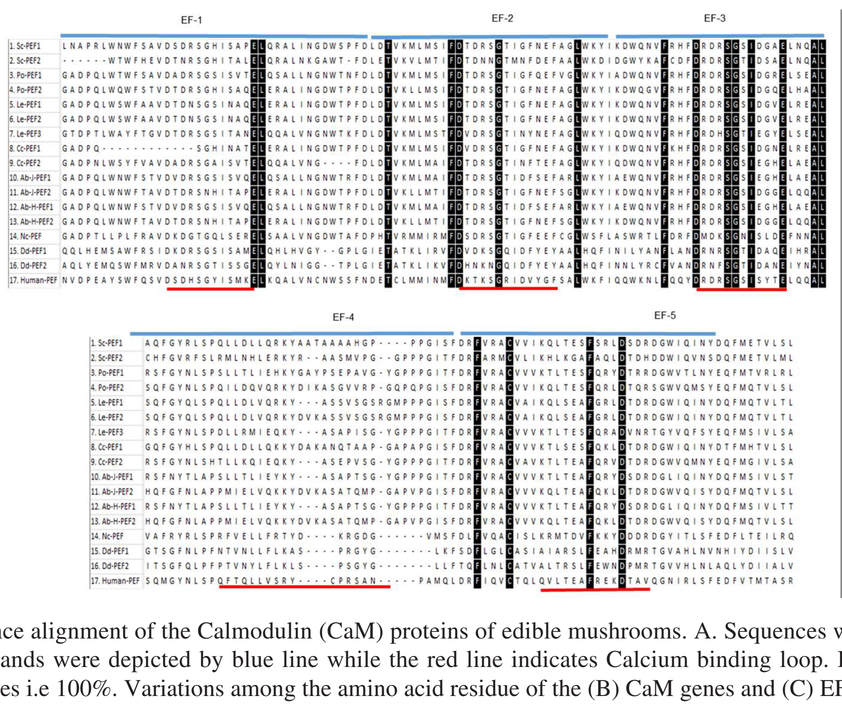
Moreover, CaM genes of the all mushrooms aligned using Clustal W method to analyse the similarity and difference among the edible mushrooms along with CaM of *N. crassa*. The results demonstrated that sixty-eight amino acid residues were highly conserved (100%) in all the members (Fig.2 A). Though, the variations in amino acid residues were also observed in EF-hand motifs of the CaM. The variations in CaM of the mushrooms ranges from six to thirty-one as compare with CaM of *A. bisporus* (Fig. 2B). While the variations ranged from four to thirty in EF-hand of the CaM of the other mushrooms when compared with *A. bisporous*. The highest variations were observed for the CaM1 (28) and CaM2 (30) of *L. edodes.* While, lowest for the CaM of *P. ostreatus* (4) and *S. commune* (5). The *C. cinerea* CaM1 and CaM 2 shows variation at four and twenty-one positions, respectively. The *A. bisporus* CaM differ from the *N. crassa* at twenty-three positions (Fig. 2C).

Further, multiple sequence alignment analysis of the PEF proteins of the five edible mushrooms aligned with the PEF of *Neurospora crassa*, *Dictyostelium discoideum* and human to check the identity among them. The PEF proteins shows three to four EF-hand motifs in their sequences using Interproscan or SMART tool but alignment with the other PEF proteins demonstrated five EF-hand motifs. The results indicated that nineteen amino acids were conserved in the five EF-hand motifs among all the members. The conserved residue was also observed in the calcium binding loop of the different EF-hand motifs viz. Glutamate (E) in EF1; Aspartate and Glycine (D,G) in EF2; Aspartate, Serine, Glycine, Isoleucine, Glutamate (D, S, G, I, E) in EF3 and Aspartate (D) in EF5 motif (Fig. 3).

DISCUSSION

CaM, CMLs, CBL and NCS in the five edible mushrooms

CaM is a distinctive multifunctional Ca^{2+} sensor, which binds and alters the activities of the target



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proteins in response to increased Ca^{2+} concentration. In *Arabidopsis*, the difference at amino acid level was one to five amino acid residues of the four CAM isoforms (McCormack and Braam, 2003; McCormack *et al*., 2005) and further this minute variation can alter the effect on target protein binding (Yamniuk and Vogel, 2005). CaM is one of the essential factors for the fungal germination, development, hyphal growth and metabolism (Juvvadi and Subramanyam, 2004; Sato *et al*., 2004; Wang *et al*., 2006; Ahn and Suh, 2007). Mutational study of the calmodulin (cmd) gene in *Neurospora crassa*, revealed its essentiality for normal vegetative growth, sexual developlement and ultraviolet survial (Laxmi and Tamuli, 2016). Disruption of Yeast CaM gene results into the recessive lethal mutation, which further indicates its essentiality in fungal growth (Davis *et al*., 1986). In *P. ostreatus*, CaM overexpression and RNAi analysis indicates its effect on expression of lignin modifying enzymes (Suetomi *et al.*, 2014). In this study, one CaM gene was identified in all the mushrooms except *P. ostreatus* and *C. cinereal*, which contained two CaM genes.

In general, CMLs share low level of similarity i.e. less than 50 per cent with the CaM (McCormack and Braam, 2003; Boonburapong and Buaboocha, 2007). For example, CMLs share at least 16% sequence similarity with CaM in *Arabidopsis* (McCormack and Braam, 2003). In addition, CMLs number is also varied in the different plants viz. in *Arabidopsis* (50) and rice (32) (McCormack and Braam, 2003, Boonburapong and Buaboocha, 2007). In woodland strawberry genome (*Fragaria vesca*), 4 CaM and 36 CMLs were identified through genome wide analysis (Zhang et al., 2016). In the CMLs, EF-hand ranges from one to six in plants (McCormack and Braam, 2003; Boonburapong and Buaboocha, 2007). Here, in all five edible mushrooms EF hand ranges from two to four in the CML genes. Moreover, CBLs are closely related with NCS proteins and in most of the non-plant species singular CBL gene is present (Beckmann *et al*., 2016). Similarly, here, we have observed single gene of CBL in five edible mushrooms, which was closely related with the respective NCS gene and come together in one group (Group II) in the phylogenetic tree.

Penta-EF-hand (PEF) proteins contain five EF-hand motifs and belongs to the class of Ca^{2+}-binding proteins (Maki *et al*., 2002). However, PEF proteins were also reported in lower eukaryotes including *Dictyostelium discoideum* (cellular slime mold) (Ohkouchi *et al*., 2001) and *Saccharomyces cerevisiae* (Yeast) (Maki *et al*., 1997; Vernarecci *et al*., 2007). PEF proteins involved in regulation of the polarized growth as well as in stress response in *S. cerevisiae* (Vernarecci *et al*., 2007). Here, two PEF proteins were observed in all the mushrooms except *L. edodes*, which contained three genes. In conclusion, the findings of the present study will be helpful to explore the role of CaM and calcium binding protein genes in different edible mushrooms.

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