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Motif and Evolutionary Analysis of Chitinase–An In Silico Approach

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A B S T R A C T

Chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin. Chitin is a component of cell walls of fungi and exoskeletal elements of some animals (including worms and arthopods). Chitinases are generally found in organisms that either need to reshape their own chitin or dissolve and digest the chitin of fungi or animals. Chitin is an abundant biopolymer that is relatively resistant to degradation and not digested by animals. Certain fishes are able to digest the chitin. Chitin digestion by animals requires bacterial symbionts and lengthy fermentation similar to cellulose digestion by ruminants. Chitinases are naturally occuring in many common foods. Cartilage chitinases are produced in the human body in response to allergies and asthma. Phylogenetic analysis is the study of evolutionary relationship among molecules, phenotypes and organisms. In this study, we use Clustal-W for Multiple sequence alignment, Motif finder and MEME for Motif analysis, and phylogenetic tree was drawn using MEGA software. The phylogenetic tree of chitinases (71 sequences) from prokaryotes, eukaryotes, vertebrates and invertebrates demonstrated divergence patterns. Motif analysis of chitinase showed Glyco-hydro-18 domain in eukaryotes, protist (except Amoeba), Glyco-hydro-19 and Chitin-bind-1 domain in plants and Putative and unfunctional motif in amoeba.

Introduction

Chitinases (EC 3.2.1.14) are enzymes that randomly hydrolyze the β-1,4 glycosidic bonds of chitin, a water-insoluble homopolymer compose of β-1,4-linked *N*-acetyl-d-glucosamine (GlcNAc**).** Chitinases are involved in many physiological and bioconversion processes (Gooday, 1999, Muzzarelia *et al*., 2012). It plays a major role in nutrition and parasitism in bacteria. In case of plants and vertebrates, it is involved in the defense mechanisms. Chitinases are involved in morphogenesis in

invertebrates, protozoa and fungi. Baculoviruses also produces chitinases for pathogenesis (Patil *et al*., 2000). Chitinases have been divided into 2 main groups. Endochitinases (E.C 3.2.1.14) and exo-chitinases. The endochitinases randomly split chitin at internal sites, thereby forming the dimer di-acetylchitobiose and soluble low molecular mass multimers of GlcNAc such as chitotriose, and chitotetraose. The exo-chitinases have been further divided into 2 subcategories: Chitobiosidases (E.C. 3.2.1.29), which are involved in catalyzing the progressive release of di-acetylchitobiose starting at the non-reducing end of the chitin micro fibril and 1-4-β-glucosaminidases (E.C. 3.2.1.30), cleaving the oligomeric products of endochitinases and chitobiosidases, thereby generating monomers of GlcNAc (Harman *et al.,* 1993; Sahai & Manocha, 1993). Chitin is a major constituent of the exoskeleton, or external skeleton, of many arthropods such as insects, spiders, and crustaceans (Dhaiya *et al*., 2006). Chitin is second most abundant polysaccharide in nature, and serves as an indispensable structural component for different organisms (Kittur & Tharanathan, 2003). N-acetyl chitooligosaccharides $(GlcNAc)_n$ and GlcNAc are hydrolysis products of chitin with a variety of physiological functions, such as immunostimulatory activity (Wang *et al*., 2011, Wang *et al* 2007), improvement of skin quality and alleviate osteoarthritis, respectively (Chen *et al.,* 2010, Matsumiya, 2004,). Chitinase are widely distributed in a variety of living organisms, such as bacteria, fungi, insects, plants, humans and animals and they have roles in various biological process (Patil *et al*., 2000).

Plant chitinases

Chitinases are constitutively present in plants, stems, seeds, flowers, and tubers. Plants are equipped with a variety of defense mechanisms to protect themselves against the attack of pathogens (Sharma *et al*., 2011). Plant chitinases provides the ability to self defend against phytopathogens. Plant chitinases are tissue specific and are synthesized when they are induced by infection of phytopathogens (Fukamizo, 2000). Chitinases have been implicated in plant resistance against fungal pathogens because their inducible nature and antifungal activities *in vitro* (Taira *et al*., 2002).

Taking into account the amino acid sequences, plant chitinases have been categorized into 5 or 6 classes. The key structure of the class I, II, and IV enzymes contains globular domains. While 8 α-helices and 8 β-strands form the class III and V plant chitinases. Plant chitinases are produced as pathogenesis-related proteins in plant self defense in response to the attack of phytopathogens, or by contact with elicitors such as chitooligosaccharides or growth regulators such as ethylene. There are some chitinases, which are expressed in response to environmental stresses (Fukamizo *et al.,* 2003).

Bacteria

Bacteria mainly produce chitinases in order to supply nitrogen and carbon as a source of nutrients or precursors and parasitism (Adrangi *et al.,* 2010, Dhaiya *et al*., 2006). They are used for degradation of chitin and its utilization as an energy source (Kupiec *et al.,* 1998). Chitinases play an important role in bacterial pathogenesis wherever host contains chitin (Busby *et al.,* 2012). Serratia marcescens, one of the best studied chitinolytic bacteria, has been reported producing mainly four types of chitinases ChiA, ChiB, ChiC, and CBP21 (chitin binding protein). All three chitinases belong to family 18 of glycosyl hydrolases with

(β/α) 8 TIM-barrel catalytic domain with approximately six sugar subsites (Aalten *et al*., 2000). ChiA and ChiB have multimodular organization, that is, have an N-terminal chitin binding module with a fibronectin like fold in ChiA or a C-terminal CBM5 module (Rathore *et al.,* 2015). The production of chitinases in bacteria is mainly for the degradation of chitin and its utilization as an energy source (Hamid *et al*., 2013).

Fungi

Chitinases have the ability of chitin digestion that constitutes a main compound of the cell wall in many of the phytopathogens such as fungi (Zarei *et al.*, 2011). Fungal chitinases play important role in nutrition, morphogenesis, and developmental process and are known to be produced at various stages during fungus growth. Fungal chitinases belong to GH18 family of glycoside hydrolases which showed little amino acid sequence similarity with class 3 plant chitinases. The fungal chitinases are divided into three groups, being chitinases A, B, and C on the basis of sequence and structural similarities. Fungal chitinase A is processive chitinase with a singular catalytic domain having deep substrate binding site and no CBMs. Type B is nonprocessive chitinases and they have a CBM or a serine/threonine rich domain on C-terminal of their catalytic domain. Type C fungal chitinases are also processive in nature due to their deep substrate binding site. They have a CBM on the N-terminal of catalytic domain. A special feature of fungal chitinase C is that it comprises several lysine motifs (LysM) also known as CBM 50. Fungal chitinase C is found to expressed in many mycoparasitic fungi. A study on mycoparasitic Trichoderma species showed that these enzymes are involved in degradation of both self and non-self-cell walls. Protection of self-cell wall in fungi is achieved by restricting the access to chitin by cell surface proteins (Rathore *et al*., 2015).

Mammalian chitinases

Mammals are not known to synthesize chitin or to metabolize it as a nutrient (Aronson & Funkhouser *et al.,* 2007). These chitinases belongs to the glycosyl hydrolases family 18. These enzymes are sub divided into chitinases like protein and true chitinases. True chitinases are involved in the chitin hydrolyzing activity but in case of chitinases like protein, they just involved only in binding of chitin. The latter do not have enzymatic activity. Chitotriosidase is a first human chitinase identified in gaucher patients. This enzyme is produced by macrophages and it has good antifungal properties. Chitinases were found even in human such as chitotriosidase and acidic mammalian chitinase. Humans express chitin degrading enzymes, known as chitinases but they don't biosynthesize. Two known human chitinases that have chitinolytic activity, acidic mammalian chitinase (AM Case) and chitotriosidase (CHIT-1), also many non- catalytically active chitinases called chi-lectins (Deeba *et al.*, 2016).

Insect chitinases

The enzyme production in insects is regulated by hormones during the transformation of larvae (Roopavathi *et al*., 2015). These enzymes act as degradative enzymes. Insect chitinase plays major role during this process (removal of external old skeleton). However, chitinase expression in insect is highly specific comes under hormonal control, avoiding its premature expression or over expression. In insect, ecdysis is a two step process, in which both

endo and exo-chitinases work together for transformation from larvae to the adult. Firstly, endochitinases breakdown cuticle into small subunits called chitooligosaccharides. These units of endochitinases, further hydrolyzed into N-acetylglucosamine (NAG) with the help of exo-chitinases (Merzendorfer, Zimoch *et al.*, 2003).

Materials and Methods

Database search

The chitinase sequence [accession no: 224983641] was retrieved from the NCBI database, and the similarities of the chitinase sequences are identified by performing BLASTp program.

Protein sequences alignment

Once the set of proteins sequences has been identified, sequences are typically aligned globally, that is, across their entire length, to construct a multiple sequence alignment (MSA). The Multi sequences alignment was carried out by Clustal-W.

Motif Analysis

For the aligned protein sequences, motif were identified by incorporating the sequences in motif search [http://www.genome.jp/tools/motif/] tool and MEME version (4.11.2) [http://meme-suite.org/tools/meme] tool. Protein domain prediction including determination of glyco-hydro-18 (GH-18), glyco-hydro-19 (GH-19), chitin-bind-1 were identified by this tool.

Phylogenetic analysis

The phylogenetic tree was constructed based on the Maximum likelihood method by using MEGA software version (7.0.18) with the Poisson-correction for multiple amino acid substitutions. From the motif results, the tree was constructed based on the domain glyco-hydro-18 and glyco-hydro-19. For multiple sequence alignments, the Clustal-W was used to aligned the protein sequences. The domains were obtained from the multiple sequence alignment. Values used for pairwise alignments were gap opening penalty 8 and gap extension penalty 0.1. Values for multiple alignment were gap opening penalty 8, gap extension penalty 0.2.

Results and Discussion

Identification of domain from various organism of chitinase protein

Literature search from NCBI and BLAST (blastp) searches led to the identification of chitinase protein sequences from 50 organisms. It provides an overview of all sequences retrieved, that includes species specification, common protein aliases, and NCBI accession numbers. Our analysis rendered new insights into the evolutionary relationships of the chitinase protein from various organisms. Fifty sequences (50) of chitinase proteins from various organisms were used in this phylogenetic study. Figure (1) demonstrates multiple sequence alignment that was determined by the clustal-W tool. The tree were generated based on maximum likelihood method by MEGA software.

The maximum likelihood tree was yielded in the phylogenetic analysis with generally >50% bootstrap support. The tree included chitinase from variety of archea, bacteria, eukaryotes and viruses. In all cases, the three methods produced very similar topologies. This is an unrooted tree. The tree was constructed from an alignment of

complete chitinase sequences. Based on their primary structures, endochitinases have been categorized into 2 families, 18 and 19 glycosyl hydrolases. In this analysis family 18 chitinases are present in bacteria, fungi, mammals, and viruses whereas the glyco-hydro-19 (GH-19) and chitin-bind-1 are widely present in plants .They were identified by the performing motif search and MEME tool. Therefore, they are likely to have evolved from different ancestors. Figure (4) represents the motif location for the chitinase sequences on MEME tool. Figure (5) demonstrates the maximum likelihood phylogenetic tree of chitinase sequences. The bootstrap values are shown at each node. The phylogenetic tree was separated into two cladograms. Among the different species, 30 motif regions and domains were identified is shown in figure (2) and (3).

Sequence alignment

Clustal-W is a multiple sequence alignment tool for the alignment of protein sequences. Clustal-W calculates the best match for the input sequences based on the parameters entered and generates an easy to interpret report. This sequence alignment report displays the optimal alignment score, the alignment between sequences in a form such that the identities, similarities and differences can be clearly seen and a guide tree of the evolutionary relationships of aligned sequences (Figure 1)**.**

Motif search

The domain regions present in given chitinase sequences were identified using motif search tool (Figure 2).

Motif Identification

This motif location were identified from the conserved region among the chitinase sequences.The coloured portion represents the motif region. The height of the letter indicates its relative frequency at the given position in the motif (Figure 3).

Fig.1 Represents the multiple sequence alignment by the Clustal-W.

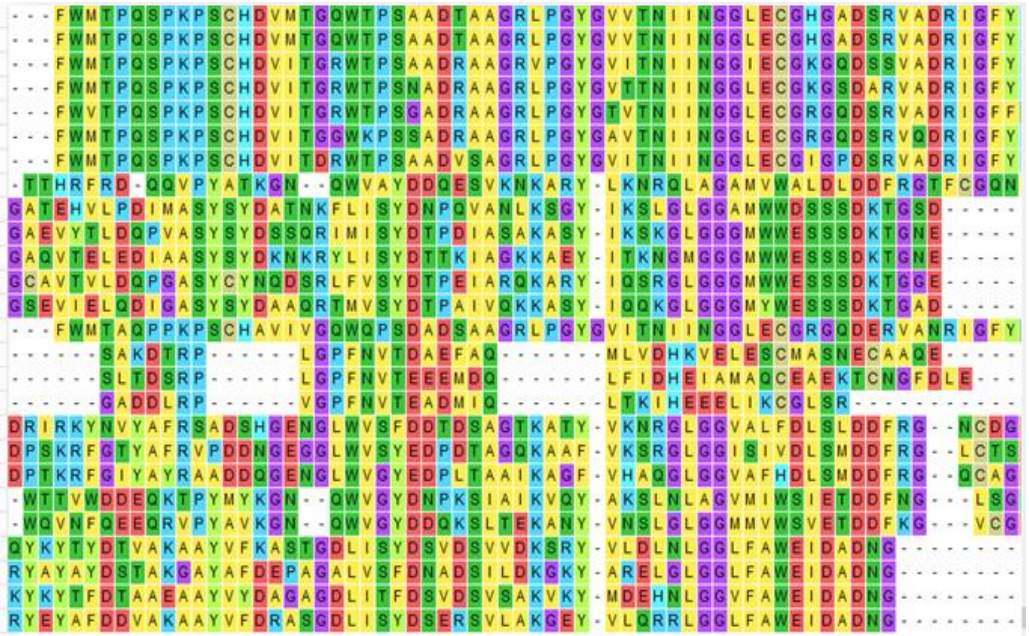


Fig.2 Represents the number of domain regions present in given chitinase sequences from the motif search tool. Among 50 species, 30 motif regions are identified in the given chitinase sequences.

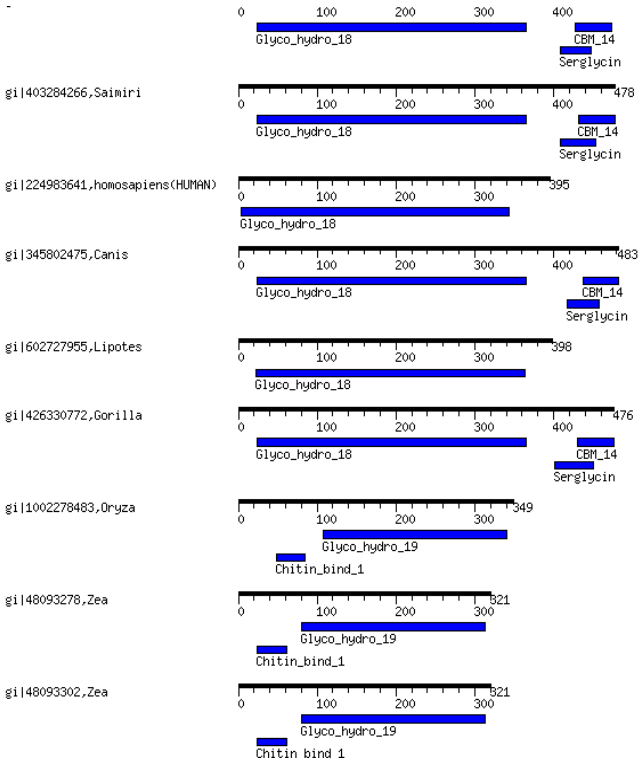


Fig.3 Demonstrates the conserved region of given sequence

Fig.4 Represents the motif location for the various chitinase sequences

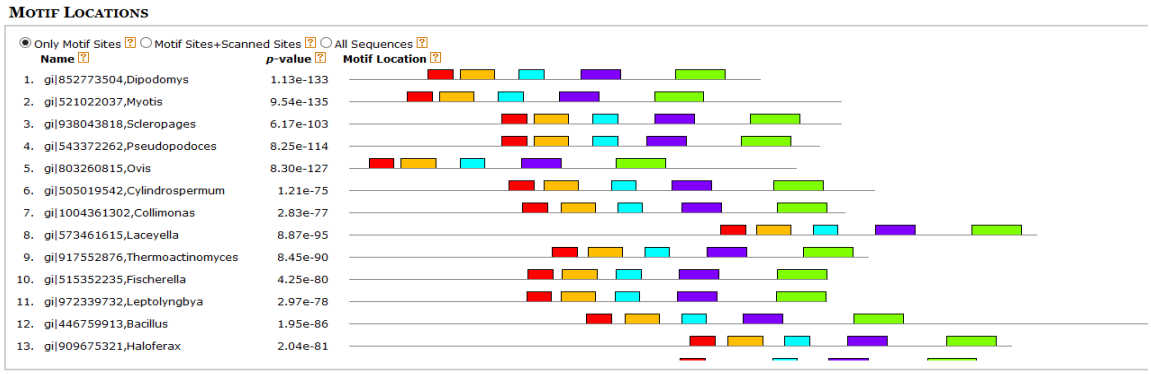
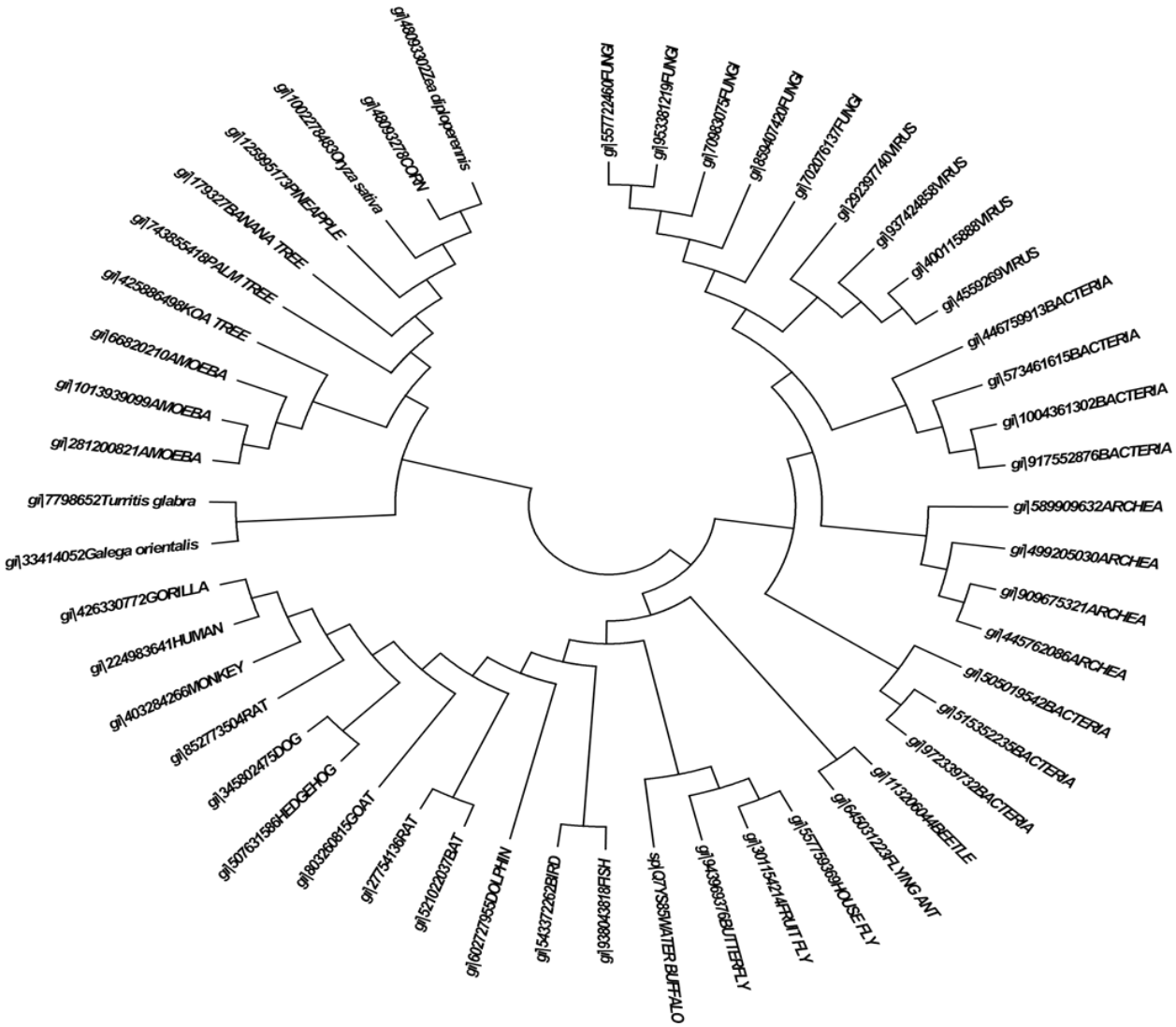


Fig.5 Molecular Phylogenetic analysis by Maximum Likelihood method



Identification of special motif from the conserved region

A total of 30 conserved motifs were queried within chitinase protein sequences using MEME tool. The motifs have been represented in different colors. The chitinase proteins are arranged according to their position in the phylogenic tree (Figure 4).

Phylogenetic tree of chitinase from various organism

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-6374.7221) is shown. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 50 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 99 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Figure 5).

Conclusion

The phylogenetic analysis is used to identify the evolutionary relationship among various organisms. This study is based on the overall *in silico* evaluation of chitinase protein. We conclude that the Glycosyl hydrolases-18 (GH-18) and CBM which is a domain obtained from the motif results are widely present in eukaryotes whereas, on the other hand Glycosyl hydrolases-19 (GH-19) and chitin-bind-1 were present in prokaryotes. In contrast to the above, protist like amoeba have the presence of putative, unfunctional domains (Table 1).

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