Role of mating receptivity peptides in *Helicoverpa armigera*: Molecular and evolutionary perspectives

ABSTRACT

Previous studies have implicated the role of peptides/proteins in male accessory glands (MAGs), responsible for the induction of behavioral and reproductive changes in *Helicoverpa armigera* females. Our and severalother studieshave seen the MAG showed post-mating behaviors such as accelerated unreceptivity to re-mating and other post-mating traits. We have reported several peptides responsible for reduced mating receptivity, including a putative 4.9 kDa, a novel peptide with various functions in female post-copulatory reproductive behavior(PMR). In this study, we report a detailed analysis of the proteins from a molecular and evolutionary perspective. 5 Major classes of multigene family proteins were detected after database searches. All proteins were found to bind nucleic acids, possess signal peptides, and undergo posttranslational modification by seminal fluid proteins (SFPs).The Porphobilinogen synthetase protein (PBGS)was unique to the lepidopteran order. Our molecular studies support the observation that the molecular weight of MAG proteins varies significantly across species, suggesting the existence of different target sites or faster evolution of SFP. MAG proteins not only ensure male reproductive fitness by constantly evolving under postcopulatory sexual selection(PCSS),but also maintain species-specific barriers to fertilizationthus potentially contribute to the formation of new species and mediate reproductive conflicts between the sexes.

Keywords:*Helicoverpa armigera*, Male accessory glands (MAGs), Calmodulin, Gene families, Post-copulatory sexual selection (PCSS).

1. INTRODUCTION

Among insect pests, the order Lepidoptera represents a diverse and important group of agricultural pests. Lepidopteran, the world's major crop pests, is a Heliothine, which includes*Helicoverpa armigera* and is responsible for major agricultural losses in several parts of the world[1]. In the overall life cycle of insects, mating plays a crucial role, as it is essential for reproduction and evolution. Successful reproduction involves a range of behaviors, from finding an appropriate mate, courting,copulation, successful egg production (oviparousinsects)and fertilization following mating. Furthermore, factors such as copulation itself, the presence of eggs, sperm stored in spermathecae, or seminal factors usually peptides (SFPs) secreted by the male accessory glands (MAGs), may arrest re-mating in several insect species [2]. These

SFPS are synthesized, activated, or released at appropriate periods from the (MAGs), and elicit short- and long-term post-mating responses through proteins and neural networks in both sexes. The network spans from the female reproductive organs and abdominal ganglion to the brain and operates by activating receptors for SFPsand octopamine[3]. This activation leads to the modulation of various signals influencing arange of female's physiological behavior, organ structure, and hormone secretion such as enhanced nutrient intake,upregulation of reproduction-related genes, ovulation, and sperm storage[4,5].Furthermore, the actions of seminal proteins are prime targets for sexual conflict that impacts male and female fitness and have been proposed to evolve under postcopulatory sexual selection (PCSS). Profound remodeling of behavioral, physiological, and transcriptomic pathways in females of many insect taxa is induced by male SFPs[6].

These classes of proteins are found in seminal fluids across taxa ranging from arthropods to mammals, suggesting their conservation and importance in gametogenesis and reproduction. SFPs identified to date encompass a variety of protein categories, such as proteases/protease inhibitors, lectins, prohormone precursors, peptides, and protective proteins (e.g., antioxidants). The relative molecular weights of different SFPs also vary significantly, ranging from 36 amino acids in sex peptide (SP) to 200–400 amino acids in prohormone-like polypeptides and large glycoproteins [7].Several studies have shown thatMAG proteins inhibit mating receptivity in different female insects, viz., *Helicoverpa*[8],*Heliothis virescens*[9], *Lymantria dispar*[10], *Bombyx mori*[11] and several other species. More than one mechanism has been proposed to demonstrate the effects of male-derived substances on female mating receptivity. The physiologically active SP-like factor directly inhibits pheromone biosynthesis, which is released duringscotophase by pheromone biosynthesis activation neuropeptide (PBAN) [12] and subsequently, it also stimulates the production of juvenile hormones (JHs) by the corpora allata (CA)[13]. Since major differences in reproductive physiology exist between species of insects, seminal protein information from multiple insects could shed light on the evolutionary patterns of reproductive traits in insects.

Sexual reproduction requires coordinated contributions from both sexes to proceed efficiently. However, the reproductive strategies that the sexes adopt often have the potential to give rise to sexual conflict because they can result in divergent, sex-specific costs and benefits. SFPs mediate these conflictswhich occur at many levels, from molecular to behavioral[14]. Several lines of experimental evidence suggest that mating systems can impose selection that influences the evolution of non-sexual phenotypes, such as development, activity, metabolism, and nutrient homeostasis[15]. SFPare excellent examples to trace the operation of sexual conflict, since the proteins rapidly evolve under positive selection between species,further due to divergence, theyhave major rolesin contributing to reproductive isolation between populations.

The molecular characterization of peptides/proteins of MAGs that modulate female reproductive behavior has been reported for several insects [16,17], similar information on *H. armigera*, is emergingin the last decade using genomic and proteomic methods [18, 19]. Our previous study demonstrated various

physiological changes in female post-mating behaviour*H. armigera*,[20] In the present study, we mine data with respect to the biochemical, physiological, and molecular roles of MAG peptides which could shed light on the evolutionary mechanisms operational on these proteins in reproduction and related physiological changes in *H. armigera*insects.

2. MATERIALS AND METHODS

Insect rearing and MAG-duplex crude extract preparations and bioassays are described in Rama etal. (2024). Further, Proteomics analysis (Nano-LC–MS/MS analysis) is described in Rama etal. (2024) unpublished data under review.

2.1. PROTEIN BIOINFORMATICS ANALYSIS

Database searches

SWISS-PROTdatabase is the curated database of proteins (produced by the UniProt consortium) describing the functions, domain structure, subcellular location, post-translational modifications and functionally characterized variants. Whereas, TrEMBL consists of computer-annotated entries in SWISS-PROT-like format derived from the translation of all coding sequences (CDS) in the EMBL nucleotide sequence database, except for CDS already included in SWISS-PROT.

Proteome analysis of the MAG-duplex tissue from virgin male moths was carried out by Nano-LC–MS/MS to identify peptides/proteins involved in female behavior changes. Further,the database searches were performed using non-redundant(nr) databases from the SWISS-PROT and TrEMBL with the automated database-searching program using the MASCOT search engine version 2.4 (Matrix Science, Boston, MA, USA).

MAG protein phylogenetic tree construction and Multiple sequence alignment

Clustal programs are widely Bioinformatics methods for carrying out automatic multiple alignment of nucleotide or amino acid sequences that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences.The peptide sequences of MAG retrieved from the database were used to construct a phylogenetic tree to determine the possible evolutionary trajectory of these classes of proteins across the tree of life using the ClustalX program. The phylogenetic tree was constructed by the maximum likelihood (ML) method using the program Mega 5.05 (http://www.megasoftware.net/). Homologous relationships were determined using bootstrap analysis with 1000 replications.

Multiple protein sequences of CaM were aligned using the ClustalW method available through the server (http://www.clustal.org/).

2.2. PROTEIN INTERACTION NETWORK AND 3D STRUCTURE DEDUCTION

STRING is a database of known and predicted protein-protein interactions. The interactions include direct (physical) and indirect (functional) associations from knowledge of several organisms, and from interactions aggregated from other (primary) databases devired from computational predictions.

The protein-protein interactions of the PBGS protein were determined using STRING server (https://cn.string-db.org/, accessed on March 2024).

SWISS-MODE is a fully automated protein structure homology-modelling server. Possible three-dimensional models of unknown protein structure are built using a database of solved structures by protein threading scans the amino acid sequences. In each case, a scoring function is used to assess the compatibility of the sequence to the structure.AlphaFold2 predicts the 3D structure based on amino acid sequence of a novel protein and aligns it to the sequences of other similar proteins. by identifying sections of the sequence that tend to change together in evolutionary time, and thus are likely to be interacting and thus physically close.

3D protein structure ofPBGS protein was generated using https://swissmodel.ExPASy.org/servers.

3. RESULTS AND DISCUSSION

3.1. PROTEIN BIOINFORMATICS

Since *H. armigera* is not a model organism and there is no complete genome sequence available to date, proteomics is a novel and direct method for identifyingunannotated peptides/proteins [27].Ourproteomic study of male MAGs by the shotgun method validated the presence of 5 classes of peptides/proteins (Tables 1 and 2) when searched in the SWISS-PROT and TrEMBL databases.As depicted in the phylogenetic tree of the MAG proteinsin Figure 1, the proteins were grouped into clades a, b, c, and d respectively.The clade a-comprising the heat shock protein (HSP) cognate 3 of the Asian swallowtail, the speckled wood butterfly, the HSP of the cotton bollworm (a member of the Lepidoptera order), the monarch butterfly (Lepidoptera), the tsetse fly, and the oriental fruit fly (Diptera). clade b: HSC 70 cognate 3 of Indian jumping ants (Hymenoptera), small parasitoid wasps (Hymenoptera), uncharacterized proteins of honey bees (Hymenoptera), and mountain pine beetle (Diptera). clade-c comprising uncharacterized protein and uncharacterized protein fragment of the mountain pine beetle belonging to (Coleopteran) and finally the clade-d: uncharacterized protein of small parasitoid wasps, HSC 70cognate 3 of Indian jumping ant, uncharacterized proteins of honey bee belonging to (Hymenoptera).

A phylogenetic tree of the MAG proteins across different phyla grouped the calmodulin proteins into clades a, b, c, d, e, and f (Figure 2). Ascidians, or marine invertebrates, included sponges, sea urchins, sea peaches, sea squirts, and Amphioxus in their clades a, b, and c;clade-d of single-celled algae,

human/animal infectious protozoan producing African sleeping sickness and Chagas disease in humans, and clade-e of free-living nematodes and annelids (epi-endogenic earthworms);Potato late blight fungus, and two types of algae(giant kelp and brown algae), comprise clade-f and finally clade-g, h of insects-fruit fly, Carolina sphinx moth, owlet moths, and Fall armyworm.ClustalW alignment of calmodulin proteinin diverse organisms from corn to zebrafish demonstrated high conservation at various amino acid residues suggestive of its importance in Ca^{2+} mediated cellular processes(Figure 3).Functional annotation of the proteins revealed that the HSP and uncharacterized proteins were nucleic acid-bindingproteins;also,the proteins have a signal peptide and undergo posttranslational modification (UniProt). Finally, the proteins were present in the three orders of insects (Table 3). Several criteria are essential for classifying a protein as an SFP, and our preliminary analysis indicated that the peptides in our study satisfied a few of these criteria, necessitating future studies.

3.2 PROTEIN INTERACTION NETWORK AND 3D STRUCTURE DEDUCTION

Proteins are vital components of a cell's complex biological network of macromolecules, acting as molecular machines, sensors, transporters, and structural elements. Protein-protein interactions are dynamic, and adjust in response to different stimuli. Reproduction and related physiological mechanisms are subjected to several cues in insects hencea robust network provides considerable flexibility in functions. The protein interaction map of porphobilinogen synthase (PBGS) suggested a PPI enrichment p value of < 1.0e-16 (Figure 4). GO-annotation of the proteins suggested 11 proteins in the hub with 11 nodes converging on Heme biosynthesis. Further, to gain insight to the protein structure of the PBGS 3D model was generated using *Drosophila* PBGS X-ray structure.The 3D structure suggested a homology model 60.19% identity with 8 ligands (2 active sites).The alpha-fold model with high pLDDT-90, corroborated the accuracy of the predictions (Figure 5A & 5B).

Cumulatively, these research findings highlight the importance of these proteins in *H. armigera* reproduction and related physiological phenomena.

4. DISCUSSION

Mating in most species of insects leads to a transient or permanent loss of sexual receptivity in females. Our previous study supports the findings ofunreceptivity induced by MAG secretions in related *Helicoverpa zea* species [20]. These data implicatethat both sex-peptide (SP)-like and plasmatocyte-spreading peptide (PSP)-like factors may be present in the male accessory glands of *H. armigera*. The relative molecular weights of different SFPs varysignificantly, ranging from 36 amino acids in sex peptide (SP) to 200–400 amino acids in prohormone-like polypeptides and large glycoproteins [29]. This indicates two possible reasons: one is that their targets are different, and the other is the rapid evolution of proteins

involved in reproduction and related traits.The MAGs of many insect species secrete several reproductive proteins collectively named accessory proteins (Acps). These proteins, many of which are rapidly evolving, are essential for male fertility and represent formidable modulators of female post-mating behavior. The reproductive strategies that the sexes adopt often have the potential to give rise to reproductive sexual conflicts varying costsand benefits.

The MAG's *in silico* proteomics analysis identified diverse peptides and proteins, including: a. heat shock proteins, also known as chaperone proteins; b. delta-aminolevulinic acid dehydratase; c. proteins that bind calcium calmodulin; and d. unidentified proteins (Tables 1 & 2). Several of these proteins are key housekeeping proteins that are involved in various cellular pathways and have important functions in reproduction in both sexes. Multiple sequence alignment of insect MAGs using MUSCLE (3.8) is depicted in (Worddoc). The alignment highlights several amino acids were conserved in the C-terminal of the proteins suggesting their role in (PMR).Orthologous of HSP genes have been identified in male accessory glands of insects belonging to Lepidoptera, Diptera and Hymenoptera such as *H. armigera*[30], *Anopheles gambiae*[31], and *Apis mellifera*[32]. Chaperone proteins function as intercellular signaling molecules and facilitate sperm-oocyte interactions[33, 34]. HSC 70 proteins have sequence homology to hsp70 family proteins, which are expressed at relatively constant levels under normal conditions and can be induced, to some extent, by heat shock [35]. These proteins are highly expressed in the testes and ovaries of Drosophila, suggesting their roles in gametogenesis (Fly Base, http://flybase.org/). Porphobilinogen synthases (PBGSs) are evolutionarily conservedmetal-binding proteins (zinc and magnesium or potassium at the active site) that catalyze an early step in the biosynthesis of tetrapyrroles [37]. The current sequence database has a repository of 130 diverse organisms spanning Archaea, bacteria, eukaryotes, and metazoans. These enzymes form the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) enabling electron transfer from NADH to the respiratory chain. The proteins exhibit differences in kinetic, physiological, inhibitory and immunological activities. The PBGS of *Drosophila melanogaster* (DmPBGS) shows sequence homology to the two zinc-binding sites found in yeast and mammalian PBGS [38]. In *Pieris brassicae* this protein is reportedas a bilin-binding protein (BBP) in the hemolymph, fat body, epidermis and wings[39].Also, in animal tick insects with high quantities in fat body (16%) and ovaries (36%). Cumulatively,these studies implicate their role in reproduction-related physiological phenomena in insects.

As evident from the phylogenetic tree in Figure. 4, paralogs of the calmodulin protein were found in unicellular euglena, fungi, certain species of brown algae, *C.elegans*, in human parasites and insects. Calcium-dependentcalmodulinCaM)and protein phosphatase(CaMK) play essential role in the transduction of the intracellular calcium (Ca^{2+})-mediated signalingwhich regulates several cellular and physiological processes ranging from development to reproduction[40]. cAMP-dependent phosphorylation of the sperm axonemal component is the important intracellular trigger causing the initiation, activation, and hyperactivation of sperm motility facilitating fertilization in diverse organisms, such as tunicates, sea

urchins, mussels, salmonid fish and mammals [41, 42].*Euglena*reproduces through asexual reproduction method known as binary fission. In the *Euglena gracilis*euglenoid cells reproduce by mitosis and shows negative gravitactic behavior andCaM.2 is an essential element in the signal transduction cascades. Immunofluorescence of flagella,suggests that an actomyosin contractile system controlled by CaM similar to that in human sperm, enables various reproduction-related biological processes[43].Multifunctional CaM is widely distributed in fungi and has key roles in virulence, stress tolerance, growth, development, and fertility in a number of fungus, including *C. neoformans* [44], *S. pombe*[45], and *N. crassa*[46].Also,plant-pathogen interactions are mediated by these proteins [47, 48]. *Phytophthora infestans* an oomycete pathogen, including downy mildews are the causal agents of tomato and potato late blight disease. The fungi secrete hundreds of candidate effectors that contain the RXLR motif, which have been shown tosuppress plant immunity and effect $Ca^{2+/}$CaM signaling [49].

Brown algae in the eukaryotic lineage,is highly divergent from the animal, fungal, and green plant lineages with respect to photosynthesis and reproductive strategies. In the early stages of development, apolar fucoid zygotes possess no inherent cytoplasmic order and polar axis is generated during “axis formation”. This polarity may be facilitated by environmental stimuli [50]or by CaM[51]. CaM have beenimplicated in gametophyte and embryonic development, fertilization, zygotic photopolarization, and physiology in *S. japonica*.In*C. elegans.* Ca^{2+}signaling is coupled with sperm activation, although the underlying mechanism is inhibition rather than activation. Ca2+ orchestrates various events that prepare and facilitate gamete maturation, interaction, and ultimately, fertilization in *C. elegans* [52].The kcnl-2(tm1885) *C. elegans* mutant KCNL-2 null strain displayed analtered phenotype of egg-laying impairment. Six transmembrane domains, a potassium-selective pore, and a CaM-binding domain were identified by sequence analysis of the KCNL-2 protein, indicating a potential involvement for these proteins in reproduction [53].

CaM proteins have been reported in *Trypanosoma brucei*[54] and *Trypanosoma cruzi*[55]. They are associated with CaM-dependent protein kinase function in *Trypanosoma brucei*[56]. The paraflagellar rod (PFR) has an intricate extra-axonemal structure called the flagellum CaM. Axoneme and Ca^{2+}dependent signaling cascades are responsible for PFR activity [57] in these parasites. CaM is involved in the developmental phases of the *Trypanosoma brucei* life cycle.Manipulating intracellular Ca^{2+} homeostasis is proposed as a drug treatment strategy [58]. The CaM protein profiles of *T. cruzi* is complex and are differentially expressed at specific stages of development [59].Related to *Lumbricus terrestris, Lumbricus rubellus* is a species of lower invertebrate earthworm found in soils rich in organic matter (Annelida, Oligochaeta). Immunohistochemistry demonstrated the presence of CaM in L. terrestris' epidermis [60]. Moreover, data indicates that the worm consumes significant amount of the $Ca^{2+}$and then excreted from the body by the calciferous glands. Collectively, these findings imply that CaM is involved in the physiology of *Lumbricus* species.

In lower marine invertebrates, Ca^{2+} enables hyperpolarization of the plasma membrane through the efflux of K+ and subsequent cAMP synthesis, which contributes to the initiation of sperm motility and activation through the CaM/calmodulin kinase system(CaMK)[61]. In sea urchin eggs, calmodulin transiently stimulates the membrane guanylyl cyclase in a Ca2+-independent manner and activates a K (+)-selective channel that hyperpolarizes the sperm. Ca2+ regulates microtubule sliding in the flagella [62]. In *C. intestinalis*, a plasma membrane Ca^{2+}ATPase (PMCA), referred to as a sperm-activating and attracting factor (SAAF)-binding protein, functions as a CaM protein.SAAF activates the Ca^{2+}/CaM pathway and subsequently mediates the activation of sperm motility through an increase in cAMP [63]. Immunolocalization indicates the abundance of these proteins in the spinal cord and the gonads tissues.CaM functions as a high-fidelity signal transducer for more than 300 target proteins however,diversity among its four Ca^{2+}binding sites, called EF-hands(consists of four helix–loop–helix motifs arrayed in two lobes), contributes to the functional versatility of CaM. Each of the four EF-hand motifs have distinct biophysical and structural properties which is supported by their distribution spanning*Archea*to mammals.[64]. ClustalW analysis of the calmodulin protein across phyla revealed that 80% of the residues were conserved, highlighting its importance in Ca (2+)-mediated role is reproduction mechanisms.

HSPs, or endoplasmic reticulum chaperones, are essential for quality control and protein folding. The nucleotide-binding (NBD) and substrate-binding (SBD) domains' allosteric coupling, mediated by ATP, controls their chaperone activity (UniProt). HSP homologs were found in two lepidopteran species in the current investigation. In insects,Azuma [65], reported the protein is involved in the stimulation of different phases of the insulin signaling system, which is necessary for the proliferation of spermatocytes and higher quantities have been observed in the ovaries and testes. Rybczynski and Gilbert [66] report in *Manduca sexta*that neuropeptide prothoracicotropic hormone (PTTH) participates in the ecdysteroid hormone synthesis pathway by increasing synthesis of heat shock protein (HSP 70) cognate proteins.The eukaryotic elongation factor-1 (eEF1) complex plays central roles in peptide elongation during eukaryotic protein synthesis [67, 68]. The eEF1 complex consists of two functional parts, the eEF1A and eEF1B complexes. eEF1A, in its GTP-bound form, binds and delivers aminoacyl-tRNAs, whereas eEF1B ensures proper scaffolding of the eEF complex [68]. In the present study, homologs of eEF-1were identified in two species of noctuidaemoths. The elongation factor eEF1A is expressed in germ cells, indicating its role in gametogenesis [69].The protein is reported in bed bug *Cimex lectularius* [70]and in *Locusta migratoria*Zhou [71] report eEF-1 production increase during JH production, which enables additional protein synthesis a pre-requisite for egg formation.

Behaviors associated with reproduction are major contributors to the evolutionary success of organisms and are subject to many evolutionary forces, including natural and sexual selection and sexual conflict. As a consequence, behaviors and genes associated with reproduction are often under strong selection and evolve rapidly. Several hypotheses of adaptive evolution, such as sexual selection and sexual conflict,

have been proposed to explain this phenomenon. Sexual conflict is involved in many aspects of reproduction where evolutionary interests between males and females diverge [72]. A potential evolutionary outcome of sexual conflict is a continual co-evolutionary chase between the sexes where adaptations in one sex led to counter adaptations in the other sex [73, 74]. According to Arnqvist & Rowe [75]and Gavrilets [76], sexual conflict and sexually antagonistic co-evolution are potent and ubiquitous drivers of evolutionary change. SFPs have wide-ranging evolutionary implications onthe fitness of many insects. Sequence data from diverse taxonomic groups reveal that reproductive genes are rapidly evolving due to adaptive pressures [77]. In *Drosophila* comparisons of sequence data between speciesindicatefaster evolution of male reproductive genes thannon-reproductive genes [78, 79]. Data from diverse taxa indicate that SFP transferred at mating affect the reproductive successof both sexes and are also found to evolve rapidlyensuring species-specific barriers to fertilization, thus contribute to the formation of new species.

The fact that distinct molecules with comparable activities can occasionally be found in closely related insects suggests that these peptides have evolved rapidly in relation to insect reproduction. For example, Accessory gland-specific peptide 26Aa (Acp26Aa) [80] and Ovary-serine protease (OSP) [81] may act on ovaries, while SP is suggested to act directly on CA, hence, it becomes necessary to characterize the female behavior-modifying proteins/peptides of the individual insect species.Selection based on variations in pre, peri and post-mating behaviors can lead to reproductive isolation and incipient speciation. Studies focused on a single or few SFP genes have shown evidence of rapid adaptive evolution by positive selection [82,83]. Rowe et al. [84] report that 11 to 15% of all SFPs are under evolutionary constrain. This variation could be due to selective constraints acting on individual protein and its functions [85, 86]at the genetic level the response to selection could include the expansion of gene families.Since many SFPs are multigene families, gene duplication is proposed to play an important role during evolution [87]. Our MAG duplex data support this observation which revealed several peptides(list) belong to the multigene family of proteins. Hence, cumulative evidences from biochemical, physiological and molecular characteristics of MAG cocktailpeptides implicate their roles in reproduction and related physiological phenomena warranting future detailed analysis.

5.CONCLUSION

The female reproductive system either retains or metabolizes the target receptors of SFPs. The putative protein reported from our study is retained and modulated several physiological and biochemical traits of females. After extrapolating conclusions from the current study and several previous investigations, one may be tempted to propose that they have direct role in female unreceptivity. Delivering the putative peptide to a virgin female leads to layingof unfertilized eggs,however, mating is not a requirement.The extreme polyphagy of the two Heliothines*H. armigera* and *H. zea* is associated with extensive amplification and neofunctionalization of genes in response to host range, resistance, and adaptation coupled with versatile proteomic/transcriptional changes. Pearce et al. [88] suggested that the two

genomes remain very similar in terms of gene content and order; however, *H. armigera* is more polymorphic, and *H. zea* has lost several genes. Nonsynonymous/synonymous sites in the expanded gene families have rapidly diverged both between the two species and between paralogs of other insect orders.Phylogenetic analysis supports thisnotion, since the HSP protein and an uncharacterized protein were present in three orders of insects and calmodulin across six phyla in the tree of life.

PBGSsproteins haveimportant roles in basal physiologyand reproductive processes, which require aerobic respiration.This class of proteins is proposed to have species-specific sensitivity to certain active site-directed inhibitors. The protein enables enhanced antioxidant activity in mitochondria following oxidative stress during and after mating. Furthermore, fly models have demonstrated oxidative stress-mediated ovo-toxicity of VCH 4-vinylcyclohexene (VCH) [89]. Taken together, these data highlight the role of this protein in receptivity-related phenomena. The discovery of the PBGS protein exclusively to lepidopteran species in *H. armigera's* MAG, opens-up avenues for design of eco-friendly pest management strategies involving tetra-pyrrole biosynthesis inhibitors.

Remarkable biochemical, functional adaptability and conservation of CaM isreflected in the range of different types of selective constraints associated with the particular physiological function of the proteinfrom lower to higher eukaryotes which enables many intracellular Ca^{2+}dependent processes[90]. The findings from present study support the afore observationgiven the homologs are location several species. In contrast to higher plants, algae and early divergent land plants reproduce through flagellated cells. CaM participation during polar axis formation is demonstrated convincingly by the effects of artificially raising the cytoplasmic CaM concentration in developing *F.serratus* zygotes and by the suppression of photopolarization by CaMantagonists. It has been demonstrated that the CaMs and CMLs of brown algae have independently evolved from the well-studied green plant lineage, suggesting a distinct progenitor. It is well established that elevated intracellular Ca^{2+} plays roles in sperm motility, activation, and chemotaxis in both marine invertebrates and vertebrates. It's possible that the multigene family CaM in amphioxus developed separately from the multigene family in vertebrates. According to multiple researcher descriptions, shortened versions of CaM are almost exclusively found in green algae and parasitic protists like apicomplexans, kinetoplastides, or animal-pathogenic oomycetes. This suggests that the loss of CaM may be connected to specific adaptations in these species. Trypanosomatids have comparatively high calmodulin concentrations further, *T. cruzi*share 89% of amino acid sequence with vertebrate CaM, which could explain theirinfectious potential. Thus, the CaM protein has undergone various structural modifications, enabling distinct function/s in the respective phyla. It is hypothesized that during molecular evolution of CaM proteins (proteins with multiple interactions and roles)—interactions between gene duplication and natural selection events could have enabled such unique properties[91]. In the evolutionary tree of life, the gene has evolved more slowly in more recent species but quickly in ancestral species. While CaM has evolved slowly, fungi have evolved at faster rates.The plausible molecular events in the evolution of CaM are depicted in the Figure 6(A,B,C).This

supports the hypothesis of genes encoding reproduction-related proteins have a tendency to evolve rapidly. EF-1a nuclear genes have been characterized in diverseorganisms including shrimp [92], mice [93], humans [94], and honeybees [95], with 70% nucleotide and 80% protein conservation. EF-1alpha is essential for protein synthesis and apoptosis regulationfurther it interacts with actin and ubiquitin-dependent proteolysis pathway. While normally viewed as a single-copy gene in insects [96], the presence of two copies of EF-1a in bees raises the possibility that two copies are widespread, which could result from an ancient gene duplication event. Furthermore, the intron-less feature of the gene serves as a substrate for synonymous substitutions.

The tenets of molecular evolution research indicate that orthologousand paralogous nonsynonymous regions in the enlarged gene families are rapidly diverging. Our research result support this observationwhich found that a number of *H. armigera*-specific proteins were also found in other insect orders viz.,Difference in homologs of the HSP protein (protein1, 2) in Lepidopteran,*Drosophila* and tobacco hornworm, and the ascidian CaM homologs (1, 2, b fragments) amongst strains of amphioxus, sea peach, and sea urchin.A prominent focus of study on sexual conflict and sexually antagonistic co-evolution has been the *D. melanogaster* sex peptide (SP). It has developed for a single characteristic, linked to a single locus, to favor male reproductive success at the expense of female reproductive success. Our fertility and lifespan studies on mated and MAG-duplex extract-injected females [97, 20]corroborates the paradigm. Investigation showed twofold increase in fecundity of mated females and enhanced survival of virgin females. Fecundity and longevity are coupled and are negatively related decreasing the lifespan of virgin females because of diversion of resources for egg development and oviposition, leaving fewer resources for survival. Future research focused on finding homologous receptors/s, creating antagonists, and examining the evolutionary patterns of reproductive features in *H. armigera* will help establish structure-function relationship.

In summary,since major differences in reproductive physiology exist between species of insects, MAG-duplex peptidesknowledge could shed light on identifying the target sites of peptides in *H. armigera* female moth. Also, this could aid in the development of antagonists of target sites and eco-friendly pest control regimes directed towards sustainable agriculture.

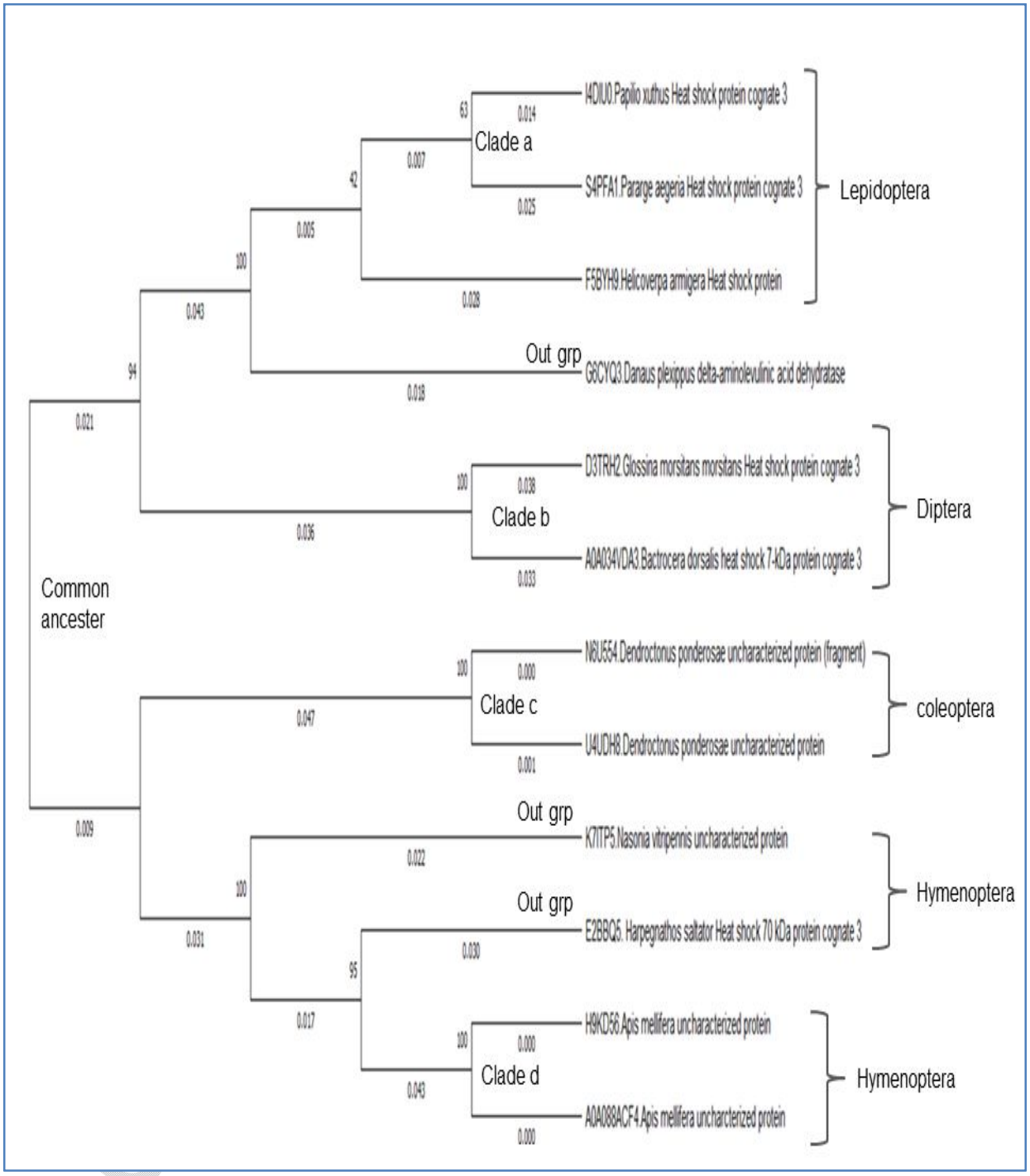


Fig. 1. Phylogenetic tree of MAG peptides across insect orders constructed using the ClustalX program.Constructed by the maximum likelihood (ML) method and homologous relationships were determined using bootstrap analysis with 1000 replications

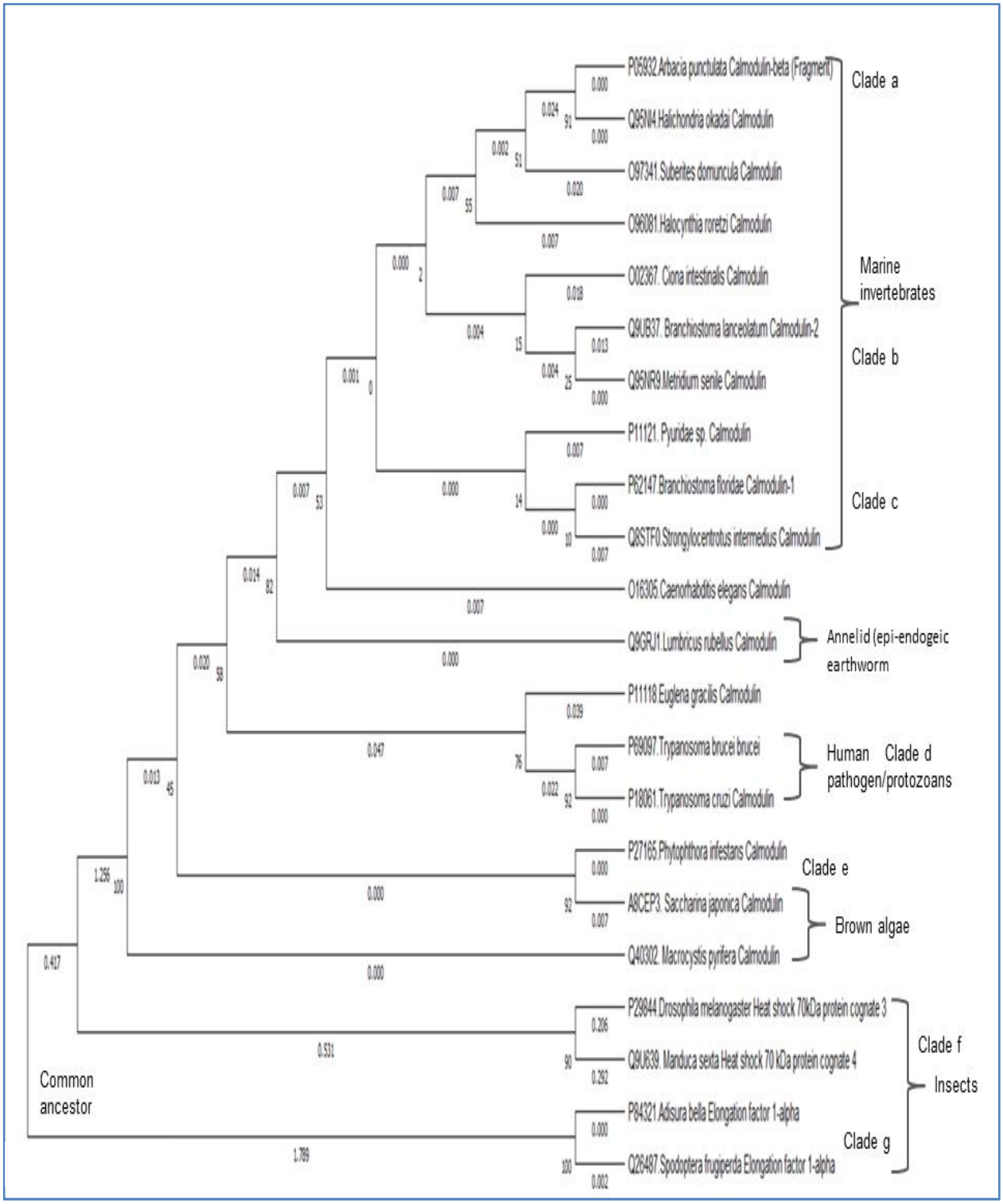


Fig. 2. Phylogenetic tree of the calmodulin protein across various organisms constructed using the ClustalX program. Constructed by the maximum likelihood (ML) method and homologous relationships were determined using bootstrap analysis with 1000 replications

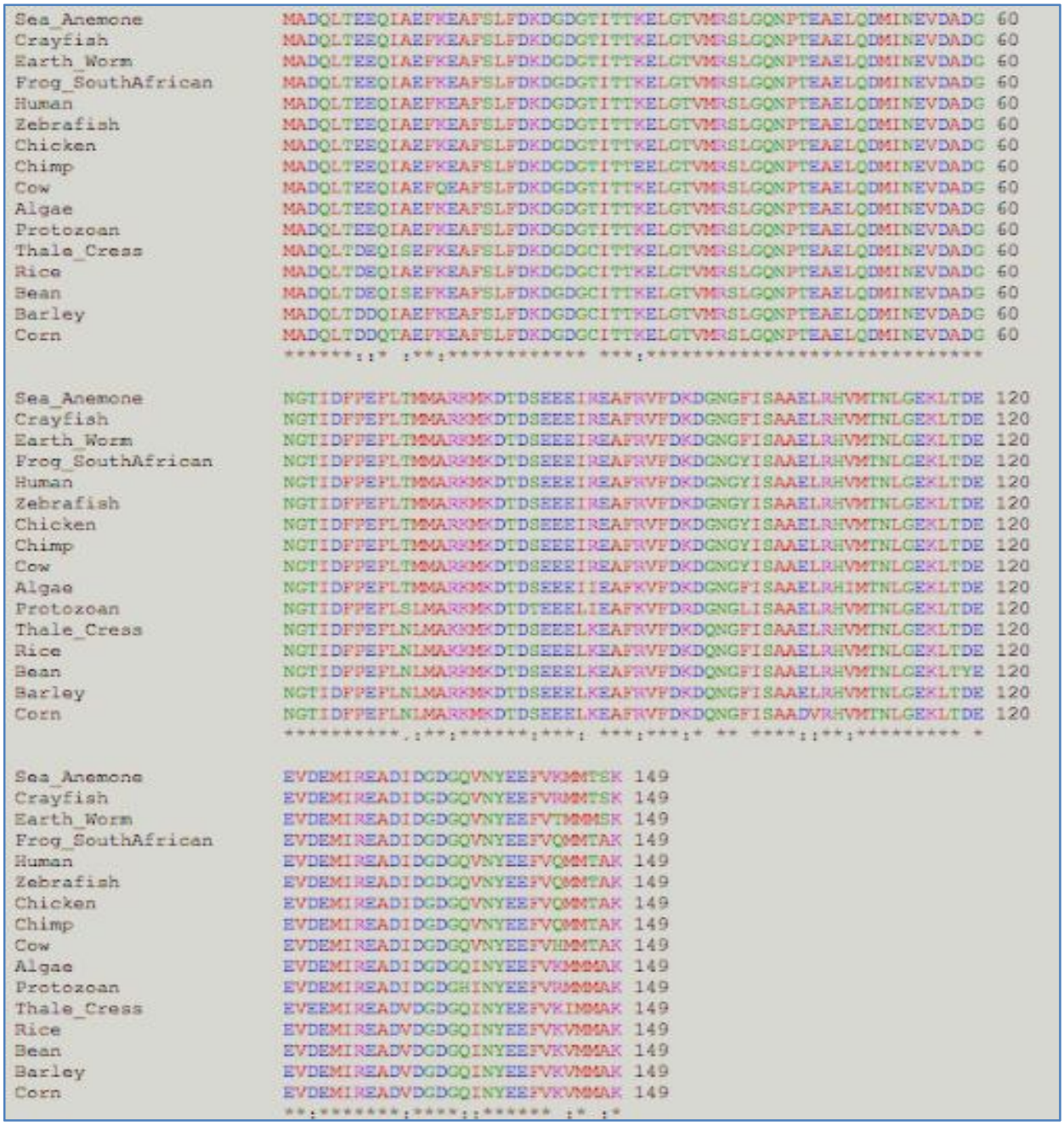


Fig. 3. ClustalW alignment of calmodulin protein sequences from different eukaryotic species from corn to zebrafish to protozoans highlighting the conservation between extremely diverse species

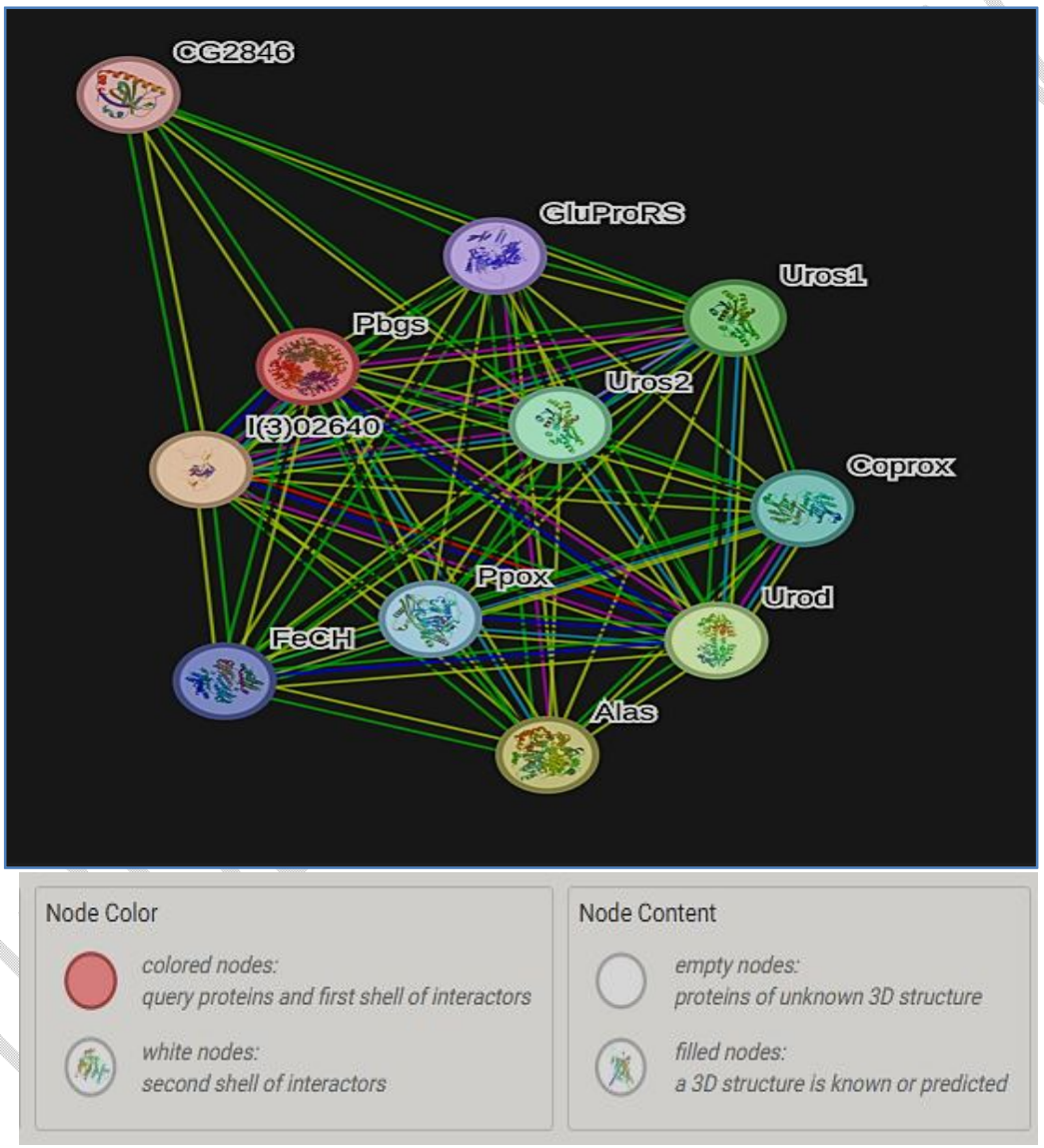
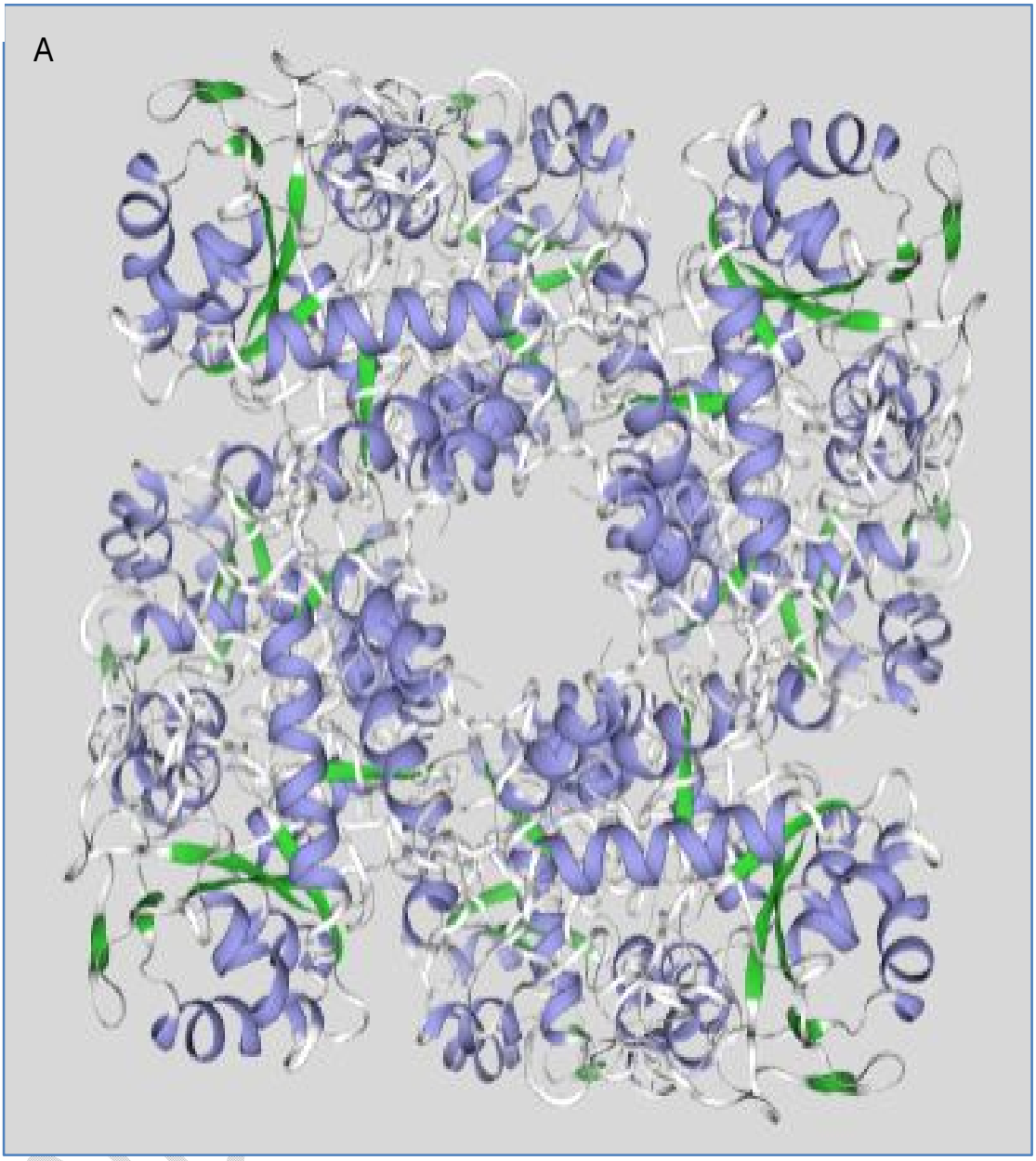


Fig. 4. Protein interaction map of the PBGS protein using STRING, highlighting the network of proteins involved in Heme biosynthesis pathway

Fig. 5A.



Homology model of PBGS protein(Ribbon structure)with 8 ligands(2 active sites)with 60.19% identity to Drosophila PBGS

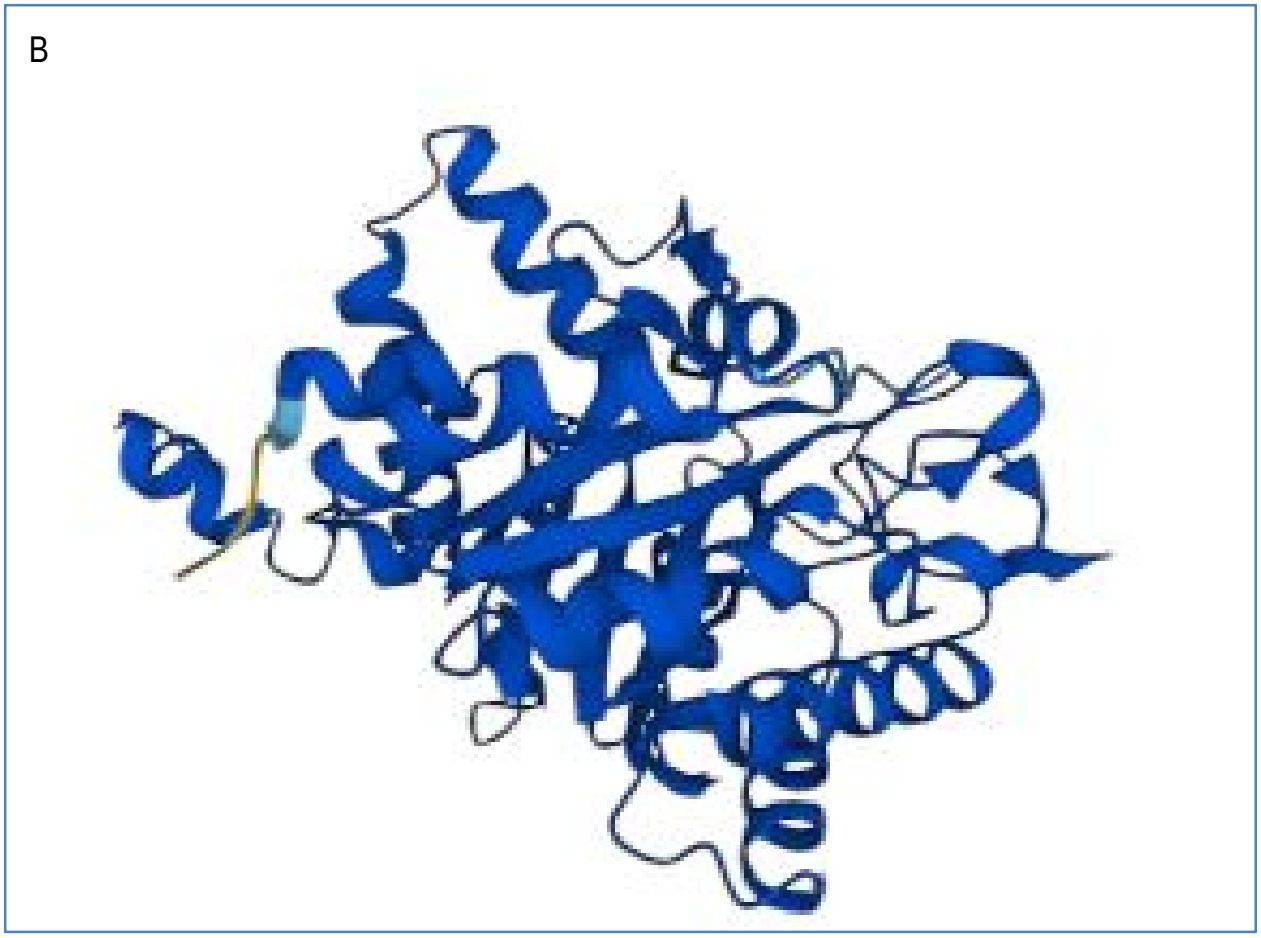


Fig. 5B.Alpha fold model(High pLDDT-90)

Fig. 5A &B.3D protein model of PBGSprotein (Ribbon structure) using the SWISS-MODEL homology-modeling pipeline

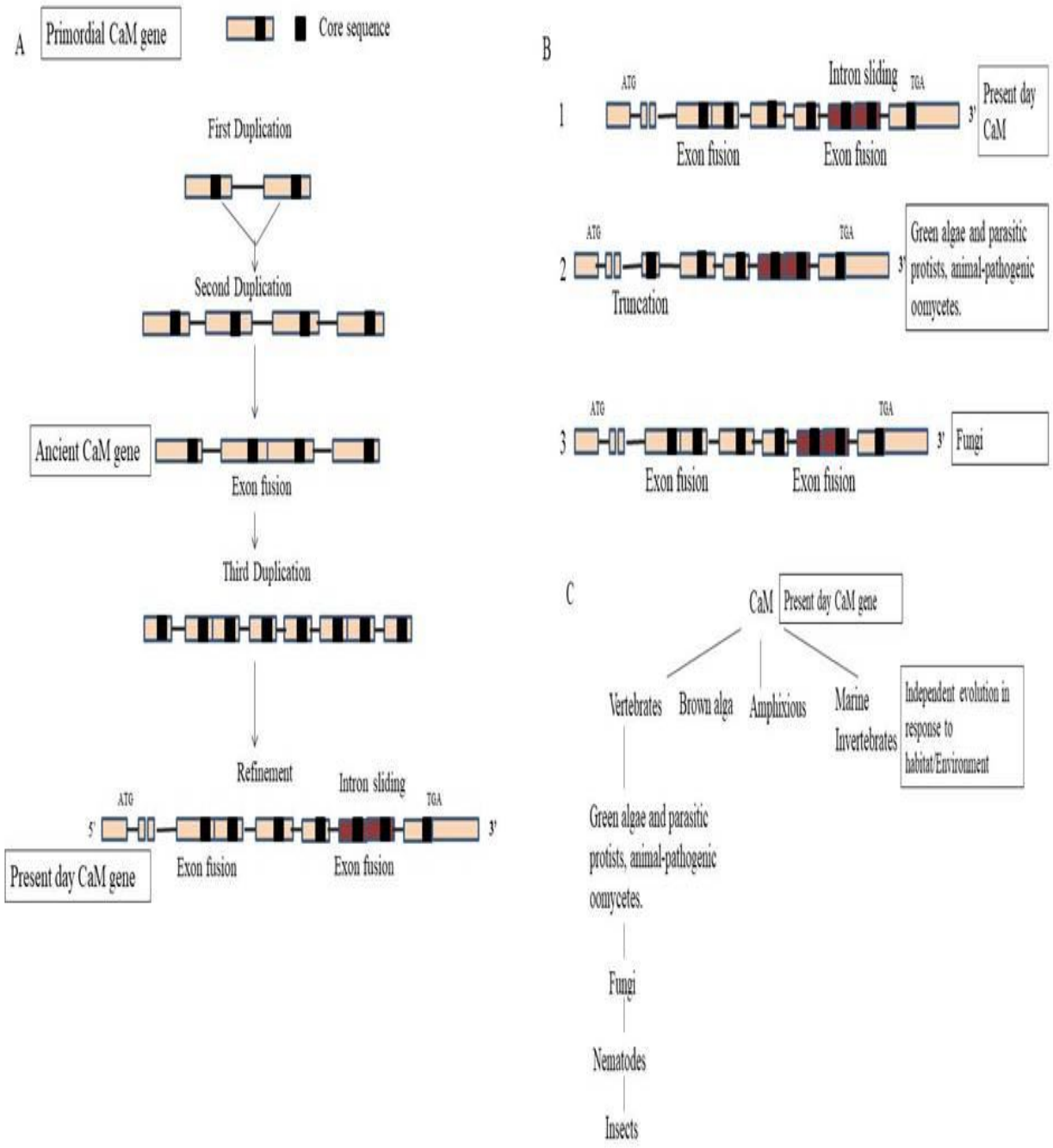


Fig. 6A. Plausible mode of genesis of CaM gene, B. Molecular adaptation CaM gene, C.Molecular evolution enabling adaptation.

Table 1.*In silico*proteome analysis of MAG duplexes of *H. armigera* virgin males using the SWISS-PROT database across phyla.

Sl.no	Accession	Description	Proteins	Unique Peptides
1	P29844	*Drosophila melanogaster* Heat shock 70 kDa protein cognate 3	1	3
2	Q9U639	*Manduca sexta* Heat shock 70 kDa protein cognate 4	1	3
3	P62147	*Branchiostoma floridae* Calmodulin-1	1	0
4	Q9UB37	*Branchiostoma lanceolatum* Calmodulin-1	1	0
5	O96081	*Halocynthia roretzi* Calmodulin	1	0
6	O16305	*Caenorhabditis elegans* Calmodulin	1	0
7	O02367	*Ciona intestinalis* Calmodulin	1	0
8	Q9GRJ1	*Lumbricus rubellus* Calmodulin	1	0
9	Q40302	*Macrocystis pyrifera* Calmodulin	1	0
10	Q95NR9	*Metridium senile* Calmodulin	1	0
11	P27165	*Phytophthora infestans* Calmodulin	1	0
12	P11121	*Pyuridae sp*. Calmodulin	1	0
13	A8CEP3	*Saccharina japonica* Calmodulin	1	0
14	O97341	*Suberites domuncula* Calmodulin	1	0
15	P05932	*Arbacia punctulata* Calmodulin-beta (Fragment)	1	0
16	P11118	*Euglena gracilis* Calmodulin	1	0
17	Q95NI4	*Halichondria okadai* Calmodulin	1	0
18	Q8STF0	*Strongylocentrotus intermedius* Calmodulin	1	0
19	P69097	*Trypanosoma brucei brucei*	1	0
20	P18061	*Trypanosoma cruzi* Calmodulin	1	0
21	P84321	*Adisura bella* Elongation factor 1-alpha	1	0
22	Q26487	*Spodoptera frugiperda* Elongation factor 1-alpha	1	0

Table 2.*In silico* proteome analysis of MAG duplexes of *H. armigera* virgin males using the TrEMBLE database in insects

Sl. No	Accession	Description	Proteins	Unique Peptides
1	D3TRH2	*Glossina morsitans morsitans* Heat shock protein cognate 3	1	0
2	E2BBQ5	*Harpegnathos saltator* Heat shock 70 kDa protein cognate 3	1	0
3	F5BYH9	*Helicoverpa armigera* Heat shock protein	1	0
4	G6CYQ3	*Danaus plexippus* Delta-aminolevulinic acid dehydratase	1	0
5	I4DIU0	*Papilio xuthus* Heat shock protein cognate 3	1	0
6	K7ITP5	*Nasonia vitripennis* Uncharacterized protein	1	0
7	N6U554	*Dendroctonus ponderosae* Uncharacterized protein (Fragment)	1	0

8	H9KD56	*Apis mellifera* Uncharacterized protein	1	0
9	S4PFA1	*Pararge aegeria* Heat shock protein cognate 3	1	0
10	U4UDH8	*Dendroctonus ponderosae* Uncharacterized protein	1	0
11	A0A088ACF4	*Apis mellifera* Uncharacterized protein	1	0
12	A0A034VDA3	*Bactrocera dorsalis* Heat shock 70 kDa protein cognate 3	1	0

Table 3. Summary of functional properties of MAG peptides identified in the study based on GO ontology

Sl. no	Peptide/protein	Category of protein based on GO ontology	Present in Lepidoptera (a), Diptera (b) Hymenoptera (c)	Post-transcriptional modification (Yes/no)	Signal peptide (Yes/no)
1	*Glossina morsitans* morsitans Heat shock protein cognate 3	Nucleotide binding	a,b,c	Yes	Yes
2	*Harpegnathos saltator* Heat shock 70 kDa protein cognate 3	Nucleotide binding	a,b,c	Yes	Yes
3	*Helicoverpa armigera* Heat shock protein	Nucleotide binding	a	Yes	Yes
4	*Danaus plexippus* Delta-aminolevulinic acid dehydratase	Nucleotide binding	a,b,c	Yes	Yes
5	*Papilio xuthus* Heat shock protein cognate 3	Nucleotide binding	a,b,c	Yes	Yes
6	*Nasonia vitripennis* Uncharacterized protein	Nucleotide binding	c	Yes	Yes
7	*Dendroctonus ponderosae* Uncharacterized protein (Fragment)	Nucleotide binding	Data not available	Yes	Yes
8	*Apis mellifera* Uncharacterized protein	Nucleotide binding	Data not available	Yes	Yes
9	*Pararge aegeria* Heat shock protein cognate 3	Nucleotide binding	a,b,c	Yes	Yes
10	*Dendroctonus ponderosae* Uncharacterized protein	Nucleotide binding	b	Yes	Yes
11	*Apis mellifera* Uncharacterized protein	Nucleotide binding	c	Yes	Yes

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DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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