Identification and Expression Analysis of Diapause Hormone and Pheromone Biosynthesis Activating Neuropeptide (DH-PBAN) in the Legume Pod Borer, *Maruca vitrata* Fabricius

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Abstract

Neuropeptides play essential roles in a variety of physiological responses that contribute to the development and reproduction of insects. Both the diapause hormone (DH) and pheromone biosynthesis activating neuropeptide (PBAN) belong to the PBAN/pyrokinin neuropeptide family, which has a conserved pentapeptide motif FXPRL at the C-terminus. We identified the full-length cDNA encoding DH-PBAN in *Maruca vitrata*, a major lepidopteran pest of leguminous crops. The open reading frame of Marvi-DH-PBAN is 591 bp in length, encoding 197 amino acids, from which five putative neuropeptides (DH, PBAN, α-subesophageal ganglion neuropeptide (SGNP), β-SGNP and γ-SGNP) are derived. Marvi-DH-PBAN was highly similar (83%) to DH-PBAN of *Omphisa fuscidentalis* (Lepidoptera: Crambidae), but possesses a unique C-terminal FNPRL motif, where asparagine has replaced a serine residue present in other lepidopteran PBAN peptides. The genomic DNA sequence of Marvi-DH-PBAN is 6,231 bp in size and is composed of six exons. Phylogenetic analysis has revealed that the Marvi-DH-PBAN protein sequence is closest to its homolog in Crambidae, but distant from Diptera, Coleoptera and Hymenoptera DH-PBAN, which agrees with the current taxonomy. DH-PBAN transcripts were present in the head and thoracic complex, but absent in the abdomen of *M. vitrata*. Real-time quantitative PCR assays have demonstrated a relatively higher expression of Marvi-DH-PBAN mRNA in the latter half of the pupal stages and in adults. These findings represent a significant step forward in our understanding of the DH-PBAN gene architecture and phylogeny, and raise the possibility of using Marvi-DH-PBAN to manage *M. vitrata* populations through molecular techniques.

Introduction

The life cycle of insects consists of different developmental stages as well as various physiological processes, such as metamorphosis, diapause, eclosion, mating and reproduction. Neuropeptides play vital regulatory roles in a wide range of these biological activities in insects [1]. Diapause hormone (DH) and pheromone biosynthesis activating neuropeptide (PBAN) are two critical neuropeptides encoded by a single precursor and belong to the PBAN/pyrokinin family, or the FXPRL peptide family [2], due to their conserved last five C-terminal sequence that is the minimum requirement for physiological activity [3–5]. In moths, the DH neuropeptide has been reported to trigger embryonic diapause or terminate pupal diapause, which is essential for survival under extreme or unfavourable circumstances, whereas PBAN functions in female sex pheromone biosynthesis. The first DH and PBAN proteins were identified and isolated from *Bombyx mori* [6] and *Helicoverpa zea* [7], respectively. Since then, DH-PBAN proteins have been reported for more than 30 insect species spanning four orders (see review by [8]).

The DH-PBAN precursor is cleaved to yield five neuropeptides, namely DH, PBAN, α-, β-, and γ-subesophageal ganglion neuropeptides (SGNP) in lepidopterans, while the α-SGNP is absent from Diptera, Hymenoptera, and Coleoptera DH-PBAN genes [9]. The five neuropeptides of lepidopterans are synthesized mainly in the subesophageal ganglia (SG), and are released into the hemolymph through the corpora cardica (see review by [10]). The full physiological functions of the DH-PBAN neuropeptides have yet to be elucidated, but PBAN/pyrokinin family peptides have been suggested to regulate the diapause and pheromone synthesis in moths, the myotropic function of hindgut in cockroaches [11,12], the cuticular melanization in silkworm larvae [13,14], and accelerate puparium formation in flies [15]. Furthermore, Choi and Vander Meer [16] reported that each PBAN/pyrokinin family peptide stimulates all listed physiological functions in *vivo*, and therefore additional functions are likely to be revealed from this broadly conserved peptide family in Insecta.

DH-PBAN has been widely studied in insects and great emphasis was given to taxa in the lepidopteran superfamilies Noctuoidea and Bombycoidea. There is no previous knowledge of the DH-PBAN genomic structure and gene expression profile in members of the Crambidae, an agriculturally and economically important family of Lepidoptera. The primary objective of the
present study was to molecularly characterize DH-PBAN in a serious lepidopteran pest of leguminous crops, the legume pod borer *Maruca vitrata* Fabricius (= *Maruca testulalis* Geyer). *M. vitrata* is a major agricultural and commercial pest in tropical and subtropical zones and reduces yield in various vegetable and grain legumes by up to 80% [17–20]. The increased and indiscriminate use of chemical insecticides to control *M. vitrata* reported in Asia, Africa, and Latin America has caused severe damage to the environment as well as to human health [21,22]. Therefore it is imperative to develop alternative, environmentally friendly control methods such as pheromone traps to manage *M. vitrata*.

Variations in pheromone composition and preference could be a key to subvert the mating behavior of *M. vitrata* and achieve sustainable pest control. (E,E)-10,12-hexadecadienol, (E,E)-10,12-hexadecadienol, and (E)-10-hexadecenal are the major component and minor components of the *M. vitrata* sex pheromone, respectively [23,24]. Re-examination of *M. vitrata* sex pheromone revealed the presence of two additional components, (E)-10-hexadecanol and (Z,Z,Z,Z,Z)-3,6,9,12,15-tricosapentaene [25]. Variations have been observed in the responses of *M. vitrata* males to synthetic sex pheromone lures over various geographical regions. A synthetic sex pheromone for *M. vitrata* consisting of (E,E)-10,12-hexadecadienol, (E,E)-10,12-hexadecadienol, and (E)-10-hexadecenal was developed and attracted male *M. vitrata* moths in Benin and Ghana, while (E,E)-10,12-hexadecadienol alone was most effective in Burkina Faso [26]. Neither pheromone was effective in Taiwan and Thailand, while a variant blend was effective in India [25]. These observed variations in the responses of male *M. vitrata* moths to synthetic sex pheromone lures over different geographical regions indicated the possibility of the existence of polymorphism in the components of *M. vitrata* sex pheromones. Understanding the role of PBAN in relation to a specific pheromone compound in *M. vitrata* is important because sex pheromone biosynthesis in insects usually is regulated by PBAN. Hence, the first step is to expound the molecular level information of pheromone associated genes, especially the genotypic variation in PBAN among distinct *M. vitrata* populations. In the current study, we identified the structure of the DH-PBAN gene from *M. vitrata* (Marvi-DH-PBAN) and elucidated the genomic organization. The DH-PBAN-based phylogenetic tree was constructed among insect species. We also demonstrated the Marvi-DH-PBAN gene expression profiles at different developmental stages based on quantitative PCR analysis. This information opens the path to study PBAN diversity in *M. vitrata* populations to assess the role of this protein in the regulation of sex pheromone biosynthesis and composition.

**Materials and Methods**

**Ethics Statement**

No specific permits were required for the described field studies, and no specific permissions were required for these locations/activities. We confirm that samples were taken from non-endangered, non-protected species on open, public lands.

**Insects**

*Maruca vitrata* larvae were collected from legume crops in Taiwan. Host legume leaves served as the insects’ diet until pupation. After pupation, each individual pupa was isolated, sexed and maintained in a plastic cup until eclosion. Adults were reared separately in wire-meshed acrylic cylinders (30 cm x 15 cm) and provided with water-soaked sponges and a 10% honey solution. Insect samples were kept at 26°C with a photoperiod of 14:10 (light:dark) commencing with the larval stage until their dissection.

Heads, thoraces, and abdomens from one- to six-day-old pupae and one- to three-day-old adult moths of both sexes were dissected and preserved in RNAlater® solution (Ambion Inc., Austin, TX) for subsequent isolation of total RNA.

**RNA Extraction**

Total RNA was isolated from homogenized heads, thoraces, and abdomens using Total RNA Mini Kit (Tissue) (Geneaid Biotech Ltd., Taipei, Taiwan) according to the manufacturer’s protocol, with in-column DNase I treatment (GMI BioLab Co., Ltd., Taichung, Taiwan). RNA was quantified by absorbance measurement on a spectrophotometer at 260 nm, while the purity of the RNA was determined by the ratio of A<sub>260</sub>/A<sub>280</sub>.

**De novo Transcriptome Sequencing**

The transcriptome sequencing was performed by Genomics BioSci & Tech Co. (Taipei, Taiwan), in order to identify the candidate DH-PBAN gene homologs in *M. vitrata*. In brief, mRNA was enriched from 15 μg total RNA isolated from 3-day-old adult female moths using magnetic oligo (dT) beads. mRNA was fragmented by divalent cations and heat treatment. Random hexamer-primers were used for first-strand cDNA synthesis and the second-strand cDNA was synthesized using RNase H, DNA polymerase I with buffer and dNTPs. Short fragments were purified with a QiAquick PCR extraction kit (Qiagen, Valencia, CA, USA) and further subjected to end-repair and addition of a single (A) base. After that, the short fragments were ligated with sequencing adapters. By gel selection, suitable sizes of cDNA fragments were then PCR amplified as templates. Finally, the library was sequenced using Illumina HiSeq™ 2000 (BGI, Beijing, China).

All dirty raw reads from RNA-seq were filtered, followed by assembly of clean reads into transcript isoforms, using the de novo transcriptome assembler Trinity [27]. Subsequently, a BLASTX search was performed against the databases of non-redundant protein sequences (NR) and SwissProt with the criterion of E-value ≤ 10<sup>-10</sup>. Each unigene was annotated for protein and GO function based on the BLASTX alignment results.

**cDNA Synthesis and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Amplification**

First-strand cDNA synthesis was performed with M-MLV reverse transcriptase (Promega Corp., Madison, WI) and RNase-OUT (Invitrogen, Carlsbad, CA) according to the manufacturers’ protocols, using a mixture of random hexamer and oligo (dT)<sub>20</sub> primers.

The RT-PCR amplification was conducted in a total reaction volume of 15 μl containing 150 ng of first-strand cDNA, 1X Super-Therm Gold Buffer, 0.4 μM of each primer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 1X PCR enhancer CES [28], and 0.04 unit/μl of Super-Therm Gold DNA Polymerase (Bertec Enterprise, Taipei, Taiwan). The gene-specific primers used in the RT-PCR reactions were designed based on sequence information obtained from *de novo* transcriptome sequencing (forward, 5’-CAAGATGGAATGGACAGAGG-3’, and reverse-primer, 5’-GACGTCTGAGCTGAGATAGGTT-3’). RT-PCR amplification was performed as follows: an initial step at 95°C for 10 min and an additional step at 97°C for 1 min; 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The RT-PCR products were visualized after 2% agarose gel electrophoresis and ethidium bromide staining under UV light.
Quantitative PCR (qPCR) Analysis

Reverse transcribed cDNA samples (1:5 dilution) from various developmental stages of *M. vitrata*, including larvae (4th–5th instar), pupae (1–6 days), and adults (1–3 days), were amplified using the Rotor-Gene® SYBR® Green Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s guidelines with the same primer set used for the RT-PCR. The real-time PCR was carried out on a Rotor-Gene 6000 (Corbett Life Sciences, Sydney, Australia) as follows: 5 min at 95°C, 40 cycles of 5 s at 95°C, and 10 s at 60°C, followed by a melting step at 60°C, temperature increasing to 95°C by 1°C every 5 s. β-actin (5′-CATCACCATCGGAAGGAAAG-3′ and 5′-ATACGTGTGCGTGTCAGGTC-3′), which has been reported to be relatively stable in expression across different simulations and developmental stages in insects [29–31], was chosen as an internal control gene to normalize the Marvi-DH-PBAN expression. A serial dilution of the cDNA mixture was used to produce a standard curve and the cDNA of 1-day-old yellow fever mosquito, *Ochlerota* (*Aedes aegypti* and *Anopheles gambiae*) (29.2%). The amino acid sequences of β-SGNPs are 100% identical among all compared lepidopterans, while β-SGNP is absent in *Diptera*, *Hymenoptera*, and Coleoptera. Marvi-β-SGNP is more divergent (20.8–90.5%), and Marvi-γ-SGNP more conserved among the different insect species (55.6–87.5%). The protein sequence of Marvi-PBAN is 75.7% similar to the PBAN protein of *O. fuscidentalis*, but only 21.6% homologous to that of the yellow fever mosquito, *A. aegypti*. Although translational and functional analyses have not yet been completed, we identified the isolated cDNA fragment as the DH-PBAN cDNA with high confidence based on sequence similarities. The lengths of the five neuropetides were compared among the four insect orders, Lepidoptera, *Diptera*, *Hymenoptera*, and Coleoptera. Species of Lepidoptera and Coleoptera apparently have longer DH and PBAN than those of *Diptera* and *Hymenoptera*, while the γ-SGNP lengths of species in different orders were nearly identical, with lepidopterans having the longest β-SGNP (Figure 2C).

Five primer pairs located within exons, each amplifying across a putative intron, were synthesized to retrieve the genomic sequence of Marvi-DH-PBAN based on the full-length cDNA sequence (Supporting Table S1). Potential intron-exon boundaries were predicted by the sequence alignments of the Marvi-DH-PBAN cDNA with homologs of the known DH-PBAN DNA sequences from *Clasteria* (*Agromyza*) (EF614262), *Helicoverpa armigera* (AF492474), and *B. mori* (D16290). The 6,231 bp genomic DNA sequence of Marvi-DH-PBAN (GenBank accession number JX412479) is composed of six exons and five introns (Figure 3). The largest intron is 1,873 bp; the others ranged from 840–1,087 bp. The distribution of intron phases are phase 0, 2, 1, 2, and 1, respectively. All five introns are canonical introns, meaning that they exclusively possess 5′-GT-AG-3′ boundaries. The first two exons encode the Marvi-DH peptide, whereas Marvi-α-SGNP, β-SGNP, and partial PBAN are translated by Exon 4. The remaining Marvi-PBAN and γ-SGNP are encoded in Exon 5.

Phylogenetic Relationship of DH-PBAN

The interspecific phylogenetic relationships based on the DH-PBAN ORFs from *M. vitrata* and the 18 other available insect DH-PBAN peptide sequences derived from GenBank are shown in Figure 4. The phylogram is consistent with the taxonomic classification of insects. *M. vitrata* was clustered together with *O. fuscidentalis* (*Lepidoptera: Crambidae*) with 100% bootstrap support. The members of the order Lepidoptera form a separate clade from the *Diptera*, Coleoptera and Hymenoptera of compared insects.

Expression of Maruca Vitrata DH-PBAN Gene

The spatial distribution of Marvi-DH-PBAN transcripts was examined in head and thoracic complex and abdomen tissues from three-day-old adult males and females, which were previously reported to have the highest mating frequency [42].
RT-PCR amplicons of anticipated size of Marvi-DH-PBAN cDNA were observed in the head and thoraces from *M. vitrata* adults. However, no transcriptional signal was found in abdominal tissues of either sex, or else the expression level—thus amplified copy number—was too low to be detected by agarose gel electrophoresis.

A real-time PCR analysis was performed to measure Marvi-DH-PBAN expression in different developmental stages of *M. vitrata*. Figure 6 shows that the relative number of transcripts was low in the 4th and 5th instar larvae. Over the pupal developmental stage, the DH-PBAN mRNA copy number was relatively low during the first three days, with higher expressions in males than in females, and then increased dramatically and reached its peak on day 4. From day 4 through day 6, expression of the gene gradually decreased while female pupae dominated male pupae in the expression level. The gene expression of Marvi-DH-PBAN decreased immediately after the adult emergence in males, but was elevated promptly from day 2 until day 3. On the contrary, DH-PBAN expression in female moths remained at a consistent level with that of the late pupal stage and subsequently decreased on day 2 and day 3.

**Discussion**

Sex pheromone traps play an important role in pest management as an environmentally safe bio-control means, and the genes involved in the biosynthesis of sex pheromone in insect pests have been of great interest to entomologists. Although the structural and functional properties of DH, PBAN and the other PBAN/pyrokinin family members have been studied extensively in insects, the data in the present study are the first in which the genomic organization and phylogenetic characterization of DH-PBAN have been documented for an insect species in the family Crambidae. We have successfully isolated a full-length DH-PBAN cDNA from *M. vitrata*. The 677 bp transcript includes an ORF that encodes the DH-PBAN protein of 197 amino acids. The structural organization of the DH-PBAN polyprotein is highly conserved among lepidopterans. Moreover, the phylogenetic analysis of DH-PBAN genes also matches the phylogeny and evolutionary diversity in insects.

The five FXPRL neuropeptides released from the Marvi-DH-PBAN precursor were cleaved at six potential endoproteolytic cleavage sites that, except for the K-K site, share a conserved sequence of G-(K/R)-R (Figure 1). Whereas the basic arginine residue functions as a canonical signal preceding the cleavage sites [43], the presence of the glycine residue indicates amidation of the C-terminus of neuropeptides in Marvi-DH-PBAN, which is pivotal for the biological activity of peptide hormones [44]. The cleavage motif of basic K-K residues has been reported to occur less frequently in neuropeptide precursors; however, it was observed being cleaved in 64% of its occurrence in...
Figure 2. Comparative analysis of the homology of the five DH-PBAN neuropeptides. (A) Alignment of the FXPRLamide neuropeptide sequences of insect DH-PBAN genes. The five putative neuropeptides from the Marvi-DH-PBAN were compared with those of 15 other lepidopterans [Ornhia fuscidentalis (AFL87384), Manduca sexta (AA018192), Antherea pernyi (AAR7699), Samia cynthia ricini (AAP41132), Bombyx mori (AA243327), Spodoptera exigua (AAAT4424), Helicoverpa assulta (AACC5293), Helicoverpa zea (AAAU20661), Aonagis ipsil (CA0A8774), Orgia thyellina (BAE94185)], Clanera asamamensis (ABR04903), Ascotsis selenaia cretacea (BFF45458), Plutella xylostella (AAX92220), Aedaphyphys sp. (A5729260), Danaus plexippus (EHJ67284)], two dipteras [Dedes aegypti (Q16880), Anopheles gambiae (Q7PP12)], two hymenopterans [Solenopsis geminata (AD898478), Apis mellifera (ABCL69)] and a coleopteran [Tribolium castaneum (EFA11568)]. Highly conserved C-terminal residues are shaded in black. (B) A line chart comparing the amino acid sequence similarities of the five FXPRL peptides. Percentages on the Y-axis are the homologies to Maruca vitrata. (C) Comparison of average lengths of DH-PBAN peptides of species of four orders in Insecta.
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Apis mellifera and Drosophila melanogaster [41]; thus it was not surprising to find it present in the Marvi-DH-PBAN.

We have confirmed the molecular structure similarity of known DH-PBAN genes among species in the order Lepidoptera (Figure 2A, B). Within Lepidoptera, the full protein sequence of Marvi-DH-PBAN is most homologous to DH-PBAN from O. fuscidentalis of the family Crambidae (83%), and least similar to that of Ascotsis selenaia cretacea in the Geometridae (63.9%). Figure 2B shows that neuropeptide homology decreased dramatically when comparing Marvi-DH, β-SGNP, PBAN, and γ-SGNP with those from members of the Diptera, Hymenoptera, and Coleoptera. The DHH neuropeptide was observed to have the most conserved C-terminal motif, WFGPRL, of the DH-PBAN polyprotein throughout insect species (Figure 2A), and it has been reported to be functional in two opposing effects: inducing embryonic diapause in B. mori [6,45,46], and causing pupal diapause in moths from Noctuidae [47–51]. The function of Marvi-DH remains unrevealed; however, it is noteworthy that DH peptides from Crambidae (Pyraloidea) members lack a histidine between residues A35-A36 which is exclusively conserved in closely related species from Bombycoidea and Noctuoidea, something that has not been previously reported. A further comparison among organisms of the three super-families may advance our understanding of the structural and functional variations of DH neuropeptide.

α-SGNP, missing in Diptera, Hymenoptera, and Coleoptera, has a peptide sequence similarity to leukopyrokinin, which stimulates the hindgut muscle contraction in Leucophaea maderae [52]. Although it is not clear whether Marvi-α-SGNP performs a similar function, as α-SGNP is identically conserved across lepidopteran species (suggesting essential function within this peptide), it is plausible that the vital functional domain had been excised from the original locus, and was subsequently incorporated into the DH-PBAN gene sequence through gene shuffling [53,54]. Alternatively, we may hypothesize that the novel primordial α-SGNP gene fragment was horizontally transferred into and has been fixed in ancestral strains of the relatively young order Lepidoptera (arising in the Eocene epoch; see review by [55]), and passed to all descended lineages, under positive selection over the insect evolutionary history (see review in analogous issue by [56]). Choi and Vander Meer [16] proposed another theory in which the α-SGNP domain was fused with β-SGNP resulting in a single neuropeptide in fire ants. These hypotheses require more evidence for support from future studies.

β-SGNP was shown to be the most divergent among the five peptides deduced from DH-PBAN genes (Figure 2B). It is not uncommon for peptide sequences to have evolved either gain or loss functional domains to adapt to environmental changes. This is known, for example, among closely related taxa [57,58]. The sequence of Marvi-γ-SGNP provided the highest similarity to those from the Pyraloidea, Bombycoidea, and Noctuoidea (87.5%), excluding Manduca sexta (62.5%), which is one amino acid shorter, and C. anastomosis (75%), which possesses a variant C-terminal pentapeptide sequence, LTPRLamide.

In the PBAN protein, the YFSRPL motif is highly conserved within lepidopteran species (Figure 2A). Intriguingly, the third residue of this domain (serine) was substituted in M. vitrata with asparagine, each of which possesses polar neutral side-chains but with distinct hydrophobicity. Up to now, this substitution has been found only in M. vitrata in the present study, Lassance et al. [59] reported different sex pheromone blend ratios as a result of the
presence of multiple allele forms at a fatty acid reductase gene in the European corn borer, *Ostrinia nubilalis*, which led to the formation of reproductively isolated races. Because the PBAN protein is commonly known to associate with the stimulation of pheromone synthesis, we posited that such a substitution in the Marvi-PBAN gene may have some impact on pheromone production, and play a role in the evolution of races within *M. vitrata*. However, further validation of the correlation between the residue substitution in PBAN and *M. vitrata* race evolution awaits phylogenetic analysis of large populations.

It is interesting to note that in Figure 2C the lengths of the PBAN/pyrokinin family of peptides in Lepidoptera are generally longer than those of the compared orders. Since Lepidoptera is the most diverse order of insects [60] and DH-PBAN is closely associated with sexual behavior and reproduction in moths, neuropeptide length variation among insect orders may partially reflect insect diversity. Because peptide length is expected to be positively proportional to the number of functional motifs or sites and to the mutation rate [61,62], which is a key source of evolution and speciation, it is plausible that lengthy DH-PBAN neuropeptides in lepidopterans resulted in their abundant speciation events. This inference is further supported by the species richness of the compared orders. Whereas Diptera, with the shortest DH-PBAN peptides, has only 125,000 species, there are approximate 350,000 species in Coleoptera, 160,000 species in Lepidoptera, and 150,000 species in Hymenoptera [63,64]. In particular, there is a statistically significant relationship of mean peptide length of both DH and PBAN when compared to the biodiversity of the four orders (i.e., Coleoptera>Lepidoptera>Hymenoptera>Diptera; $\chi^2 = 9.928$, $df = 3$, $P < 0.05$ and $\chi^2 = 8.969$, $df = 3$, $P < 0.05$, respectively, Kruskal-Wallis test). It could be postulated that the PBAN/pyrokinin neuropeptide size may therefore be correlated with species diversity in Insecta but further investigation is necessary to confirm this relationship.

The genomic locus of Marvi-DH-PBAN contains five introns, all spliced following the "GT-AG" rule (Figure 3), consistent with the genomic structure reported in *B. mori* [65], *H. armigera* [66], and *C. anastomosis* [67]. In addition, all the Marvi-DH-PBAN introns share the same intron positions and phases as those observed in *B. mori*, *H. armigera*, and *C. anastomosis*. These results support previous studies that indicate introns maintain their...
position in most lineages over long evolutionary periods (see review by [68]). Previous studies have indicated that coding regions conserved the general junction sequences of 5’-A/CG | G-3’ (where the annotation “|” refers to the intron position) of the intron-exon boundaries in eukaryotes [69,70]. Of all six exons in the Marvi-DH-PBAN gene, Exon 1, 2, 3 and 5 match the pattern perfectly, whereas Exon 4 and 6 exhibit a variety of the exon sequences that may be ascribed to the evolutionary process.

The phylogenetic construction, consisting of the majority of known DH-PBAN protein sequences across four orders (Lepidoptera, Diptera, Coleoptera and Hymenoptera), was carried out in the present study using the neighbor-joining method (Figure 4). Examined species from the same genus, Helicoverpa assulta and H. zeae are monophyletic. Without exception, members from the same family formed monophyletic clades (Noctuidae, Saturniidae, and Crambidae). The families Noctuidae, Lymantriidae and Noto-dontidae form a monophyletic clade comprising the superfamily Noctuoidea. The sister group to the Noctuoidea is the Bombycoidea, to which the families Saturniidae, Sphingidae and Bomby- cidae belong. The Geometroidea (Ascotis selenaria cretacea) and the Pyraloidea form a trichotomy with the Notuuoidea plus Bombycoidea clade. The sister taxon to the Noctuoidea is the Bombycoidea, to which the families Saturniidae, Sphingidae and Bomby- cidae belong. The Geometroidea (Ascotis selenaria cretacea) and the Pyraloidea form a trichotomy with the Notuuoidea plus Bombycoidea clade. The sister taxon to the above clade is the Yponomeutoidea plus Tortricoidea and the basal clade of the Lepidoptera is the Papilionoidea. Successive out-groups to the Lepidoptera are the Diptera (Aedes aegypti), the Coleoptera (T. castaneum), and the Hymenoptera (Apis mellifera). On the whole, the DH-PBAN protein sequence similarity is correlated with the basic taxonomic relationships among the species and infers the feasibility and sensitivity of the DH-PBAN gene sequences as a phylogenetic marker in class Insecta. The placement of the Papilionoidea (Dananus plexippus) at the most basal group of the Lepidoptera, and thus a sister group to the other super-families of the order, is unexpected [71] and poses some questions, raising the likelihood that the Lepidoptera might be polyphyletic.

Thus far, the DH-PBAN neuropeptides have been known to be primarily synthesized in and released from the subesophageal ganglion (SG) in moth species. In the present study, the DH-PBAN transcripts were exclusively located in the head and thorax of adult male and female M. vitrata, but no expression was detected in the abdomen of adults (Figure 5). Our results do not conflict with published results that the DH-PBAN gene is also feebly expressed in thoracic ganglia of H. zeae [72], H. virescens [50], Samia cynthia ricini [73], M. sexta [74], Plutella xylostella [75], Ascotis selenaria cretacea [76], Antheraea pernyi [77], and Solenopsis invicta [78]. Association between DH-PBAN expression and abdominal tissue distribution has been reported only in the FXPRLamide-like immunoreactivity assays in abdominal ganglions [73,77–79]. Hence, further investigation using immunostaining techniques seems necessary not only to confirm the DH-PBAN peptide location but also to shed light on the PBAN transport path from the SG to the pheromone gland in female moths.

The temporal gene expression profile of DH-PBAN over different developmental stages of insects in the family Crambidae has not been analyzed previously. Here we report, for the first time, the gene expression changes of DH-PBAN during M. vitrata development (Figure 6). Based on the results of qPCR analysis, the

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**Figure 6. Developmental expression of the Marvi-DH-PBAN gene.** The results are shown as the expression level relative to the female pupal (1-d pupae) DH-PBAN gene expression. The experiment was conducted for three times. doi:10.1371/journal.pone.0084916.g006
Marvi-DH-PBAN mRNA is expressed at a relatively low level during late larval stages. Much higher expressions of the Marvi-DH-PBAN gene were observed during the mid- and late-pupal phase, similar to the mode observed for S. cynthia ricini [73] and non-diapausing A. pernyi [77], but contrary to those that have been reported in C. anisomorpha [67], diapausing M. sexta [74] and diapausing A. pernyi [77]. As it is commonly known that growth and differentiation occur within the chrysalis, the gene expression pattern at this stage suggests that the Marvi-DH-PBAN protein may be involved in the stimulation and regulation of adult development in pupae, which is consistent with the physiological function of DH-PBAN in stimulating adult development and formation in H. virescens and H. armigera pupae [48–51].

In adult M. vitrata females, the Marvi-DH-PBAN mRNA expression is greater on the first day than on day 2 and 3, which coincides with the timing of the activation of sex pheromone synthesis for mating attraction. It has already been affirmed that the pheromone production is significantly higher in virgin moths than mated moths [80,81]. When mated Heliothis subflexa and H. virescens females injected with PBAN, they produced pheromone at levels and ratios comparable to those produced by virgin females [82], which confirmed the higher expression of PBAN in newly emerged female moths. Interestingly, the Marvi-DH-PBAN expression was moderately decreased in day 2 and 3 in adult samples from female cDNA, whereas the level of DH-PBAN mRNA in male moths increased dramatically from day 2 to day 3, an event that has never been before reported in the literature. As the pheromonotropic function of PBAN might not be expected in male moths, and that M. vitrata does not undergo diapausis [83], FXPRL peptides may also participate in many other bioactivities in addition to the stimulation of pheromone biosynthesis (see reviews by [8,10]). Our finding here may in turn imply the extra need for male M. vitrata to exploit various physiological effects, modulated by FXPRLamides, to pinpoint their mating targets and/or for other mating behaviors in the field shortly after the release of sex pheromone from females. For instance, in H. armigera, PBAN was demonstrated to up-regulate fatty-acid and alcohol components, correlating with chemical communication systems and behavioral responses, in hair-pencil-aedagous complexes. This is a male-specific tissue structurally homologous to the female pheromone gland, and the timing of regulation was coincident with female pheromone production [84]. Due to the fact that the full DH-PBAN gene undergoes transcription even if only one or a few of the five neuropeptides are present, a striking and dominant expression level of DH-PBAN transcript in male moths of M. vitrata is evident. Moreover, the dominant expression level of DH-PBAN in M. vitrata females from day 4 of the pupal stage may to some extent be correlated with the regulatory mechanism of PBAN in order for rapid stimulation and synthesis of the sex pheromone soon after adult eclosion. Additional study of Marvi-DH-PBAN at pre- and post-translational levels is required to validate this hypothesis and to understand its biological functions.

Conclusions

The present study provides several new clues about DH-PBAN. The genomic DNA sequence of DH-PBAN was for the first time elucidated in a Crambidae species, M. vitrata, along with its transcript cDNA sequence encoding putative protein. A single residue substitution of the C-terminal FXPRLamide in the PBAN peptide was found only in M. vitrata, which could be a valuable phylogenetic marker for differentiating M. vitrata from other insects. As insect metamorphosis and pheromontropic activity are believed to be regulated by DH-PBAN, it is anticipated that developmental and reproductive behavior in M. vitrata could be disrupted by interference with DH-PBAN at the DNA or protein level, which in turn hindered the stimulating physiological responses. For example, the RNA interference (RNAi) pathway was shown to be capable of inducing larval lethality in coleopterans through intake of dsRNA expressed in the host plants [85], and this technique may be introduced to silence the DH-PBAN gene expression in M. vitrata and achieve more environmentally friendly methods of pest management. Furthermore, the existence of population variation in sex pheromone component responses observed in M. vitrata has increased the difficulty of pest control. We are hopeful that the fundamentals of Marvi-PBAN indicated in this study can be employed in future studies to clarify the relation of PBAN to specific pheromone blends in various geographical regions and relevant biosynthesis pathways.

Supporting Information

Table S1 List of primer sequences. Primer pairs used in the current study for amplification of intronic sequences in the Marvi-DH-PBAN gene.

(DOCX)

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Author Contributions

Conceived and designed the experiments: JC SR. Performed the experiments: JC. Analyzed the data: JC. Contributed reagents/materials/analysis tools: SR. Wrote the paper: JC SR.

References

1. Nasell DR (2002) Neuropeptides in the nervous system of Drosophila and other insects: multiple roles as neuromodulators and neuropehones. Prog Neurobiol 68: 1–84.
2. Choi MY, Rafaeli A, Jurenka RA (2001) Pyrokinin/PBAN-like peptides in the central nervous system of Drosophila melanogaster. Cell Tissue Res 306: 459–465.
3. Raine AK, Kempe TG (1990) A pentapeptide of the C-terminal sequence of PBAN with pheromonotropic activity. Insect Biochemistry 20: 499–511.
4. Fonagy A, Schoofs L, Matsunoto S, De Loof A, Minaei T (1992) Functional cross-reactivities of some locustamlytinopsins and Bombyx pheromone biosynthesis activating neuropeptide, Journal of Insect Physiology 38: 651–657.
5. Raine AK, G. Kempe T (1992) Structure activity studies of PBAN of Helicoverpa armigera (Lepidoptera:Noctuidae). Insect Biochemistry and Molecular Biology 22: 221–225.
6. Imai K, Konno T, Nakazawa Y, Komiya T, Lobe M, et al. (1991) Isolation and structure of diapause hormone of the silkworm, Bombyx mori. Proceedings of the Japan Academy, Series B67: 98–101.
7. Raine AK, Jaffe H, Kempe TG, Keim P, Blacher RW, et al. (1989) Identification of a neuuropeptide hormone that regulates sex pheromone production in female moths. Science 244: 796–798.
8. Choi MY, Vander Meer RK (2012) Molecular structure and diversity of PBAN/ pyrokinin family Peptides in Ants. Front Endocrinol (Lausanne) 3: 32.
9. Choi MY, Vander Meer RK, Valles SM (2010) Molecular diversity of PBAN family peptides from fire ants. Arch Insect Biochem Physiol 74: 67–80.
10. Jurenka R, Rafaeli A (2011) Regulatory role of PBAN in sex pheromone biosynthesis of Heliothis moths. Front Endocrinol (Lausanne) 2: 46.
11. Nachman RJ, Holman GM, Cook BJ (1986) Active fragments and analogs of the insect neuropeptide leucopyrokinin: structure-function studies. Biochem Biophys Res Commun 137: 936–942.
12. Predel R, Nachman RJ (2001) Efficacy of native FXPRlMamides (pyrrokinins) and synthetic analogs on visceral muscles of the American cockroach. J Insect Physiol 47: 207–293.

13. Matsumoto S, Kitamura A, Nagasawa H, Kataoka H, Orikasa C, et al. (1990) Functional dissection of a neuropeptide produced by the suboesophageal ganglion: molecular identity of melanization and reddish colouration hormone and pheromone biosynthesis activating neuropeptide. J Insect Physiol 36: 427–432.

14. Hassan MN (2007) Re-investigation of the female sex pheromone of the black-back prominent moth, <i>Clostrera anastomosis</i> (Lep., Pyralidae). Pan-Pacific Entomologist 83: 226–234.

15. Li B, Predel R, Neupert S, Hauser F, Tanaka Y, et al. (2008) Genomics, transcriptions, and peptidomics of neuropeptides and protein hormones in the red flour beetle <i>Tribolium castaneum</i>. Genome Res 18: 113–122.

16. Horowitz DS, Krainer AR (1994) Mechanisms for selecting 5′ splice sites in mammalian pre-mRNA splicing. Trends Genet 10: 100–106.

17. Predel R, Nachman RJ (2000) Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. Arch Insect Biochem Physiol 43: 49–63.

18. Southey BR, Sweeney JL, Rodriguez-Zas SL (2000) Prediction of neuropeptide cleavage sites in insects. Bioinformatics 24: 815–825.

19. Lu P, Qiao HL, Wang SY (2007) The emergence and mating rhythms of the legume pod borer, <i>Macaia vitrata</i> Fabricius, 1787 (<i>Lepidoptera: Pyralidae</i>). Pan-Pacific Entomologist 83: 226–234.

20. Rashid A SH, Akhtar LH, Siddiqi SZ, Arshad M (2003) Comparative efficacy of two commercially available enhancer mixes for PCR. Biochem Biophys Res Commun 30: 427–429.

21. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

22. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29: 64–70.

23. Subramanian S, Kumar S (2004) Gene Expression Intensity Shapes Evolutionary Responses in Drosophila melanogaster. Genome Research 13: 2260–2264.

24. Brooks DR, Aagaard KR (2000) Developmental regulation of FXPRLamide neuropeptides. Regul Pept 116: 163–171.

25. Bock R (2010) The give-and-take of DNA: horizontal gene transfer in plants. Genetica 136: 549–554.

26. Mouginis-Mark LE (2004) Application for the control of cowpea pests. Crop Protection 10: 363–370.

27. Grulke MS, Lonsdorf JE, Miles KA, Schmitt TJ, Tatem RW, et al. (2010) Molecular characterization of the silkworm diapause hormone-pheromone biosynthesis activating neuropeptide gene of <i>Helicoverpa armigera</i> encodes multiple peptides that break, rather than induce, diapause. J Insect Physiol 56: 547–554.

28. Zhang YT, Sun JS, Zhang QP, Xu J, Jiang RJ, et al. (2004) The diapause hormone-pheromone biosynthesis activating neuropeptide gene of <i>Helicoverpa armigera</i> encodes multiple peptides that break, rather than induce, diapause. J Insect Physiol 50: 257–263.

29. Sato Y, Oguchi M, Menjo N, Imai K, Saito H, et al. (1993) Precursor polypeptides for multiple neuropeptides secreted from the suboesophageal ganglion of the silkworm <i>Rotundana mus</i> characterization of the cDNA encoding the diapause hormone precursor and identification of additional peptides. Proc Natl Acad Sci U S A 90: 3251–3255.

30. Zhao JY, Xu WH, Kang L (2003) Functional analysis of the SGNP I in the pupal diapause of the oriental tobacco budworm, <i>Helicoverpa assulta</i> (Lepidoptera: Noctuidae). Regul Pept 118: 25–31.

31. Rashid A SH, Akhtar LH, Siddiqi SZ, Arshad M (2003) Comparative efficacy of two commercially available enhancer mixes for PCR. Biochem Biophys Res Commun 30: 427–429.

32. Sun WJ (2000) Relative potential of fruits for reverse transcription-qPCR studies of physiological responses in <i>Drosophila melanogaster</i>. J Insect Physiol 46: 830–835.

33. Zhao S, Fernald RD (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 12: 1047–1064.

34. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment. Nucleic Acids Res 25: 4876–4879.

35. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.
70. Long M, de Souza SJ, Rosenberg C, Gilbert W (1998) Relationship between "proto-splice sites" and intron phases: evidence from dicodon analysis. Proc Natl Acad Sci U S A 95: 219–223.

71. Cranston PS, Gullan PJ (2009) Phylogeny of insects. In: Rech VH, Carde RT, editors. Encyclopedia of Insects. 2 ed. San Diego: Academic Press. 780–793.

72. Ma PWK, Roelofs WL, Jurenka RA (1996) Characterization of PBAN and PBAN-encoding gene neuropeptides in the central nervous system of the corn earworm moth, Helicoverpa zea. J Insect Physiol 42: 257–266.

73. Wei ZJ, Zhang FY, Sun JS, Xu YJ, Xu WH, et al. (2004) Molecular cloning, developmental expression, and tissue distribution of the gene encoding DH, PBAN and other FXPR1 peptides in Samia cynthia ricini. J Insect Physiol 50: 1151–1161.

74. Xu WH, Denlinger DL (2004) Identification of a cDNA encoding DH, PBAN and other FXPR1 neuropeptides from the tobacco hornworm, Manduca sexta, and expression associated with pupal diapause. Peptides 25: 1099–1106.

75. Lee DW, Boo KS (2005) Molecular characterization of pheromone biosynthesis activating neuropeptide from the diamondback moth, Plutella xylostella (L.). Peptides 26: 2404–2411.

76. Kawai T, Ohnishi A, Suzuki MG, Fujii T, Matsuoka K, et al. (2007) Identification of a unique pheromonotropic neuropeptide including double FXPR1 motifs from a geometrid species, Ascotis selenaria cretacea, which produces an epoxyalkenyl sex pheromone. Insect Biochem Mol Biol 37: 330–337.

77. Wei ZJ, Hong GY, Jiang ST, Tong ZX, Lu C (2008) Characters and expression of the gene encoding DH, PBAN and other FXPR1amide family neuropeptides in Antheraea pernyi. Journal of Applied Entomology 132: 59–67.

78. Choi MY, Meer RK, Shoemaker D, Valles SM (2013) PBAN gene architecture and expression in the fire ant, Solenopsis invicta. J Insect Physiol 59: 161–165.

79. Choi MY, Lee JM, Han KS, Boo KS (2004) Identification of a new member of PBAN family and immunoreactivity in the central nervous system from Adoxophyes op. (Lepidoptera: Tortricidae). Insect Biochem Mol Biol 34: 927–935.

80. Foster SP (1993) Neural inactivation of sex pheromone production in mated lighthouse apple moths, Epiphyas postvittana (Walker). Journal of Insect Physiology 39: 267–273.

81. Foster SP, Roelofs WL (1994) Regulation of pheromone production in virgin and mated females of two tortricid moths. Archives of Insect Biochemistry and Physiology 25: 271–285.

82. Groot A, Fan Y, Browne C, Jurenka R, Gould F, et al. (2005) Effect of PBAN on pheromone production by mated Heliothis zea and Heliothis subflexa females. Journal of Chemical Ecology 31: 263–268.

83. Okeyo-Owuor JB, Ochieng RS (1981) Studies on the legume pod-borer, Maruca testulalis (Geyer)-I: life cycle and behaviour. International Journal of Tropical Insect Science 1: 263–268.

84. Bober R, Rafaeli A (2010) Gene-silencing reveals the functional significance of pheromone biosynthesis activating neuropeptide receptor (PBAN-R) in a male moth. Proc Natl Acad Sci U S A 107: 16858–16862.

85. Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, et al. (2007) Control of coleopteran insect pests through RNA interference. Nat Biotechnol 25: 1322–1326.