Identification of neuropeptides and neuropeptide receptor genes in *Phauda flammans* (Walker)

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Neuropeptides and neuropeptide receptors are crucial regulators to insect physiological processes. The 21.0 Gb bases were obtained from Illumina sequencing of two libraries representing the female and male heads of *Phauda flammans* (Walker) (Lepidoptera: Phaudiidae), which is a diurnal defoliator of ficus plants and usually outbreaks in the south and south-east Asia, to identify differentially expressed genes, neuropeptides and neuropeptide receptor whose tissue expressions were also evaluated. In total, 99,386 unigenes were obtained, in which 156 up-regulated and 61 down-regulated genes were detected. Fifteen neuropeptides (i.e., F1b, Ast, NP1, IMF, Y, BbA1, CAP2b, NPLP1, SIF, CCH2, NP28, NP3, PDP3, ARF2 and SNPF) and 66 neuropeptide receptor genes (e.g., A2-1, FRL2, A32-1, A32-2, FRL3, etc.) were identified and well-clustered with other lepidopteron species. This is the first sequencing, identification neuropeptides and neuropeptide receptor genes from *P. flammans* which provides valuable information regarding the molecular basis of *P. flammans*.

Insect neuropeptides as a classic signaling molecule are produced by the neurosecretory cells that are mainly located in the brain and the central nervous system, among others, to reach their distant target organs. They are small proteins with generally about 5–80 amino acid residues, which are one of the structurally most diverse signaling molecules and most diverse group of signaling molecules in multicellular organisms. Most neuropeptide receptors belong to the family of G protein-coupled receptor (GPCR), and most of the neuropeptides act via G protein coupled receptors. It has been widely reported that neuropeptide and their receptors participate in intercellular information transfer from neurotransmission to intrinsic or extrinsic neuromodulation and essential signaling molecules that regulate physiological processes such as growth, development, behavior, reproduction, metabolism and muscle movement in insects.

For now, a plethora of neuropeptides and receptors were investigated in insects, such as myoinhibiting peptides (MIPs) and so forth. Among these, PBAN, galanin and melanocortin are involved in the control of reproduction. NPY is regulating feeding and sleep–wake behavior. Thus, neuropeptides and their receptors could be developed as potential insecticides or targets for a novel generation of pesticides, such as the neuropeptide CCH was proved to be regulates feeding motivation and sensory perception or olfactory behavior and the enteronecroin peptides allatotropin (AT) and GSRYamide having feeding acceleratory effects via controlling intestinal contraction. Therefore, identification and functional characterization of neuropeptides and their receptors from insect pests would enhance our basic understanding of neuropeptide-related signal transduction, and provide important molecular insights for pest management. Up to now, neuropeptide and receptors have been the focus of interest in many species of Lepidoptera, such as *Manduca sexta* and *A. Mylitta*. The diurnal moth *Phauda flammans* (Walker) (Lepidoptera: Phaudiidae) is a serious defoliator which outbreaks that threaten ficus plant seriously, especially *Ficus microcarpa* (Miq.) and *F. benjamina* L. It not only influences the urban landscapes and ecological effects, but also affects normal growth and development of fig plant. This defoliator is abundantly distributed in south and south-east Asia and southern China. At present, most of the researches about *P. flammans* focus on its morphological characteristics. However, the research on neuropeptides and their receptors in *P. flammans* has been limited in comparison to other lepidopteron insects, due to lack of availability of genomic or transcriptomic information.

In this study, we conducted high-throughput sequencing of head, identified members of the neuropeptide and neuropeptide receptor of *P. flammans*, and compared them with those reported transcriptome of other lepidopteron insects.
Materials and methods
Insect rearing and tissue collection for RNA-seq. The mature larvae of *P. flammans* were collected from July to October 2020 in Dinxing County (22°50’10”N, 107°12’27”E), Chongzuo City, Guangxi Province, China, and placed in plastic boxes (diameter = 25.0 cm, height = 15.0 cm) that supplied with fresh ficus leaves per day, at an indoor temperature with 26 ± 2 °C, 70 ± 10% relative humidity (RH) with a photoperiod cycle of 14 h L/10 h D. Differentiate male and female pupae according to their ventral segments and randomly select 1-day-old healthy male and female adults for the experiment after feathering. The tissues head from adult male (n = 90) and female (n = 90) were collected. All samples were immediately frozen in liquid nitrogen and stored at ~ 80 °C until use.

RNA-seq. Total RNA of *P. flammans* was extracted by TRIzol (Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s instructions. The integrity of the RNA was determined with an Agilent 2100 bioanalyzer through agarose gel electrophoresis. The Nanodrop micro-spectrophotometer (Thermo Fisher, USA) was determined the purity and concentration of the RNA. After total RNA extraction, transcriptome sequencing was performed on an Illumina NovaSeq 6000 by Gene Denovo Biotechnology Co. (Guangzhou, China). To obtain high quality clean reads, reads were further filtered with fastp (version 0.18.0), mainly by removing reads containing adapters, removing reads containing more than 10% unknown nucleotides (N), and removing low quality reads with > 50% low quality reads (q value ≤ 20). Reads were then mapped to the ribosomal RNA (rRNA) database using the short reads matching tool Bowtie2 (version 2.2.8). The mapped rRNA reads were removed, and the remaining clean reads were assembled by the short read assembly program Trinity v3.030 to obtain the total unigene. The transcriptomic data were submitted to the National Center for Biotechnology Information (NCBI, USA) (http://www.ncbi.nlm.nih.gov/) with accession number of PRJNA702893.

Transcriptome data analysis. The unigene expression was calculated and normalized to RPKM (Reads Per kb per Million reads) and the relative expression of differential expressed genes were viewed by volcano plot.

Sequence analysis and phylogenetic tree analysis. Transmembrane domains (TMDs) were calculated using the TMHMM 2.0 prediction software (http://www.cbs.dtu.dk/services/TMHMM/). The presence of signal peptide was predicted using SignalP software version 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The splice sites were predicted using the Known Motif and Insect Models methods of NeuroPred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) and were corrected based on the processing procedures of known insect neuropeptide precursors. These sequence alignments were done using CLUSTALW, the result were implemented in MEGA7.034 and GeneDoc software. With tBLASTn, the available sequences proteins from lepidoptera species were used as queries to identify candidate unigene involved in neuropeptides and neuropeptide receptor genes in *P. flammans*. To construct an evolutionary tree of neuropeptides and receptors, the amino acid sequences of the *Atrijuglans hetoaei, Bombyx mori, Chilo suppressalis, H. armigera, Grapholita molesta, Ostrinia furnacalis, Papilio machaon* and *Pl. xylostella* were downloaded from the NCBI database and performed in MEGA7 and the tree was constructed using the Neighbor-Joining method with 1000 bootstraps.

Tissue expression profile via quantitative PCR. The head (without antennae), thoraxes (without legs), abdomens were dissected from 15 virgin 1-day-old of females or males, respectively. These tissues were immediately transferred into 1.5 mL RNA-free tube, super-cooled via liquid nitrogen, and then stored at ~ 80 °C freezer. These tissues were used for RNA extraction with RNAiso Plus (TAKARA, 9109, Dalian, China) and then cDNA synthesis with A Prime Script RT reagent Kit with gDNA Eraser (TAKARA, RR047, Dalian, China). The quantitative PCR reactions were conducted on an ABI QuantStudioTM 6 Flex system (Thermo Fisher Scientific, Massachusetts, USA). The PCR reaction was performed with each reaction was performed with Green Premix Ex Taq II Kit (TAKARA, RR820A, Dalian, China) and prepared as introduced. The expression level of target gene was normalized with reference gene *TUB1* (a-tubulin) and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) via 2^−ΔΔCT method according to our previous works. The primers used in this research were listed in the Table S1.

Statistical analysis. The normality and homoscedasticity of data on tissue expression of neuropeptides in female and male *P. flammans* adults were tested prior to analysis using Kolmogorov–Smirnov and Levene’s tests, respectively. And, they were further analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) multiple test (P < 0.05). Data analysis was performed using SPSS 25.0 (IBM Corp., Armonk, New York, USA).
Results

Overview of cephalic transcriptomes. The cDNA libraries were constructed from *P. flammans* tissue samples of male and female heads to next-generation sequencing analysis by using Illumina HiSeq (TM) 4000 platform. A total of 21.0 G of clean bases were obtained, Q20 and Q30 values were all >93%, and GC content was 39.82~40.87%. The combined unigene of *P. flammans* were functionally annotated by BLASTx according to six functional public databases: NCBI non-redundant protein (Nr), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, Swiss-Prot, Cluster of Orthologous Groups (COG) and gene ontology (GO) (e value < 0.00001). A total of 99,386 unigene (average length 911 bp) were obtained with 37,602, 28,494, 17,458, 19,910 annotations to the Nr, KEGG, KOG, SwissProt databases, respectively. A total of 40,131 annotations, account for 40.38% of the total unigene (Table 1).

The Nr databases comprise all non-redundant protein sequences in GenBank, EMBL, DDBJ and PDB that belong to phylogenies of more than 70,000 species. Based on Nr annotation, unigene sequences of *P. flammans* can be mapped with sequences from 10 top species (Fig. 1). The number of homologous sequences sorted from most to least is *Eumeta japonica* (7.56%), *B. mori* (5.37%), *Galleria mellonella* (4.34%), *O. furnacalis* (4.15%), *Hyposmocoma kahamanao* (3.65%), *Amyelois transitella* (3.32%), *Helicoverpa armigera* (3.26%), *Papilio machaon* (3.05%), and *Pa. xuthus* (2.98%).

Differentially expressed genes (DEGs) between female and male heads. The results of differential expression analysis of genes in the heads of male and female adult *P. flammans* showed that a total of 217 differentially expressed genes were screened, with 156 genes up-regulated and 61 genes down-regulated, using FDR < 0.05 and \(|\log_{2} FC|> 1\) as screening criteria (Fig. 2). The detailed information about these DGEs were listed in the supporting information 1.

| Details | Number |
|---------|--------|
| Clean reads from all samples (Gb) | 21.00 |
| Q20 (%) | 97.72 ~ 97.93 |
| Q30 (%) | 93.18 ~ 93.00 |
| GC content (%) | 39.82 ~ 40.87 |
| Total unigene | 99,386 |
| Average length of total unigene (bp) | 911 |
| N50 of unigene (nt) | 11,923 |
| Unigene with homolog in Nr | 37,602 |
| Unigene with homolog in KEGG | 28,494 |
| Unigene with homolog in Swiss-Prot | 19,910 |
| Unigene with homolog in KOG | 17,458 |
| Total number of annotation genes | 40,131 |

Table 1. The four major databases annotate the statistics of *P. flammans*.
Identification of neuropeptides and their receptors. The neuropeptides in *P. flammans* were identified (Table 2). The neuropeptides F1b, Ast, NP1, IMF, Y, BbA1, CAP2b, NPLP1, SIF, CCH2, NP28, NP3, PDP3, ARF2, and SNPF were identified from the data sets with the length between 331 and 2947 bp. Except for NP1 and BbA1 have 3′ non-coding regions, and the others had complete open reading coding frames (ORFs), includ-
Phylogenetic analyses. Neuropeptide sequences of *P. flammans* were used to construct maximum likelihood phylogenetic trees with 137 published neuropeptide sequences from lepidoptera including *A. hetaohei*, *B. mori*, *C. suppressalis*, *H. armigera*, *G. molesta*, *Pa. machaon* and *O. farnacalis* (Fig. 4). Among all neuropeptides, F1b, Ast, NP1, IMF, Y, BbA1, CAP2b, NPLP1, SIF, CCH2, NP2, NP3, PDP3, ARF2, and SNPF were clustered together with the orthologs from other lepidoptera insects in the same clade. On the contrary, ARF2 and NP28 were individually clustered together. A21-1, A21-2, A21-3 and A21-4 were individually clustered together, and it's the same with CCHIR-1, CC1R1, CPR2 and CPR3. It showed that neuropeptide receptor emerged highly differentiation in *P. flammans*. The remaining receptors were clustered together with the orthologs from other lepidopteran insects in the same clade.

Tissue expression profile in female and male adults. The expression profiles of 12 neuropeptides of *P. flammans* in heads, thorax, abdomens of male and female adults were showed in Fig. 6. The expression of CA, LM, Ast, F1b, and NPLP1 were significantly higher in heads than other two body parts in both female and male. While the expression level of AR, DP3, and NP28 showed no significant difference in these three body parts in both sexes. All these neuropeptides showed no difference in female and male heads except for CCH2.

Discussion
Neuropeptides and receptors regulate a wide range of physiological processes in insects. Transcriptome sequencing is fundamental to dentification of genes, and identification of neuropeptides and their receptors is the first and foremost step of deep function depth studies in physiological processes. However, the types and expression of neuropeptides and their receptors in *P. flammans* are unavailable. Therefore, a sequencing analysis was performed of head in *P. flammans*. After high-throughput sequencing, among the 99,386 unigene acquired by the assembly program Trinity, 40.38% could be annotated through NR, KEGG, Swiss-Prot, KOG and GO databases, implies that not all unigene contain annotated genes. Some unigene may be non-coding which do not BLAST with the non-redundant protein/nucleotide database. Compared with the transcriptome data of head in *P. flammans*, the number of homologous sequences most with *E. japonica*, *B. mori*, *G. mellorella*, which all
| Gene name | Unigene ID | ORF (aa) | Complete ORF |
|-----------|------------|----------|--------------|
| CPRLIX3   | Unigene0087541 | 56 | YES | Neuropeptides capa receptor-like isoform X3 |
| CPRL      | Unigene0087541 | 56 | YES | Neuropeptides capa receptor-like isoform X3 |
| CPR1      | Unigene0087541 | 56 | YES | Neuropeptides capa receptor-like isoform X3 |
| CPR2      | Unigene0087541 | 56 | YES | Neuropeptides capa receptor-like isoform X3 |
| CPR3      | Unigene0087541 | 56 | YES | Neuropeptides capa receptor-like isoform X3 |
| CPR4      | Unigene0087541 | 56 | YES | Neuropeptides capa receptor-like isoform X3 |

continued
order of Lepidoptera, suggesting that the transcriptome was commendably sequenced and annotated. Overall, the assembly quality of transcriptome was adequate.

Basically, the number of achieved target gene should be closely related to the sample resource and expression abundance in addition to sequencing depth with species specificity. The same was true for neuropeptides and neuropeptide receptors in *P. flammans*. Totally, 15 neuropeptides and 66 neuropeptide receptors were identified from head of adult *P. flammans*, which was different with other lepidopteran species and should partly be relevant with their differences in sample physiological status. For example, in *B. mori*, 32 neuropeptide genes and 6 neuropeptide-like precursor genes were identified from larval and pupal brain. In *C. suppressalis*, 43 neuropeptide precursors and 51 putative neuropeptide G protein-coupled receptors were identified the fifth instar larval central nervous system including brain, suboesophageal ganglion, thoracic ganglion, and the abdominal ganglion. In *H. armigera*, 34 neuropeptides and peptide hormones, 17 neurotransmitter precursor processing enzymes, and 58 neurotransmitter receptors were identified from mixed pupa and adult head. It seems that more sophisticated sampling would yield a larger number of neuropeptides and receptor genes. In addition, the number of identified genes might also have species specificity. The number of identified neuropeptides of *P. flammans* was less than the number of some other lepidopteran species such as from the transcriptome data of head, such as *A. hetaohei* and *B. mori*.

There are several factors that may account for the difference in the number of identified genes of specific functions which has been discussed. Firstly, the head used as the sequenced samples did not cover complete individual and all stages of life cycle. Secondly, some genes with small expression levels made it impossible to quantitatively measure the gene expressions in samples presented a not expression state, or they may not have been expressed at all. And then, due to does not involve the modification of corresponding protein-coding regions, many genes lack strong sequence conservation, their clear orthologs could not be found in *P. flammans* based on homology searches. The neuropeptides may truly present with small quantity in *P. flammans* because of highly species specificity which needs further investigation.

In this analysis, female and male head transcriptome in *P. flammans* was performed with focus on the feeding behavior regulation and sexual difference. Only a total of 217 differentially expressed genes were screened, with 156 genes up-regulated and 61 genes down-regulated. Approximately 12% of these DGEs were olfactory associated genes (Supporting information 1), while no neuropeptide or neuropeptide receptor were found. Moreover, some neuropeptide and neuropeptide receptors have reported to induce sex pheromone biosynthesis and feeding behaviors. Therefore, the small number of neuropeptides and neuropeptide receptors from head in *P. flammans* might lead to those gene tightly to olfactory regulation and reduce workload in targeting behavior regulation gene. For instance, there were 19 unigenes which located in the Ko00981, the insect hormone biosynthesis pathway, where only unigene0063695 and unigene0024395 were significantly differential expressed and annotated as gene cytochrome P450 18a1 (CYP18A1) and farnesol dehydrogenase-like (FoLDH), respectively (Fig. S1). CYP18A1 played a controlling role in 20-hydroxycycayne inactivation in *B. mori* and were reported to function in development, especially to regulate dimorphic metamorphosis via insect hormones. FoLDH could induce oxidation of farnesol to farnesal and produce the second branch of JH III in *Pl. xylostella*. In
addition, DGE Unigene0010507 was annotated as juvenile hormone binding-like protein (Supporting information 1) and how the relationship between it and insect hormone biosynthesis pathway attracted our attention. Therefore, the functions of these DGEs require further analysis and validation in *P. flammans*.

Neuropeptides and neuropeptide receptors identified from the head of *P. flammans* showed no significant difference between male and female adults, however, they are crucial in regulating a range of physiological functions, including development, reproduction and feeding\(^\text{56}\). Therefore, identification and analysis neuropeptides and their receptors are still necessary and meaningful. In the aspects of feeding behavior, for example, short neuropeptide F peptide is expressed in the nervous system and it regulates food intake and body size by over-expression of SNPF with regulate expression of insulin-like peptides in *Drosophila*\(^\text{57}\). Another example, NPF as a pleiotropic factor, is well known for its role in the regulation of feeding\(^\text{58}\), through activating neuropeptide G protein-coupled receptor to regulate feeding and growth in *B. mori*\(^\text{59,60}\), which is also a daily oligophagous species.

![Figure 4. Phyllogenetic analysis of lepidopterous neuropeptides.](https://www.nature.com/scientificreports/)

Ah: *A. hetaohei*; Bm: *B. mori*; Cs: *C. suppressalis*; Ha: *H. armigera*; Gm: *G. molesta*; Pf: *P. flammans*, Pm: *Pa. machaon*; Of: *O. furnacalis*. The *P. flammans* neuropeptide are labeled with red, and the colors of other species are shown in the icon. The tree was conducted with MEGA 7.0, using the Maximum-Likelihood method and the bootstrap analysis with 1000 replicates.
that might provide some references for *P. flammans*. In the aspects of sexual difference, the release of SIFamide in the brain could inhibit sexual behavior until the flies encounter the right physiological conditions\(^6\), which might also function in sexual differences. All these deductions need further confirmation far and away via quantitative PCR, tissue localization, function inhibition and so on.

Neuropeptides were less abundant in this study and easier to target their expression in different tissues. From a general view, all the measured neuropeptides were expressed highly or moderately in heads where they were identified from transcriptome (Fig. 6). As mentioned above, the neuropeptide *CCH2* and the neuropeptide receptor *CCH1R-1* could be identified, but them were no significantly different expressed in the head of females and males (Supporting information 1), while quantitative PCR results showed a slightly significant difference in *CHH2* (Fig. 6C). Similar results were also found in *CCHamide 1* and *CCHamide 2* which were significantly different expressed in the head of females and males of *A. hetaohei*\(^7\). Fold changes in *CHH2* expression in female and male heads by QPCR was minor, and therefore the conflicting point shall result from the sensitiveness of QPCR and RNA-Seq methods. In addition, SIFamide a highly conserved neuropeptide and has been reported to

**Figure 5.** Phylogenetic tree analysis of lepidopterous neuropeptide receptors. Bm: *B. mori*; Cs: *C. suppressalis*; Ha: *H. armigera*; Gm: *G. molesta*; Pf: *P. flammans*, Px: *P. xylostella*. The *P. flammans* neuropeptide receptors are labeled with red, and the colors of other species are shown in the icon. The tree was conducted with MEGA 7.0, using the Maximum-Likelihood method and the bootstrap analysis with 1000 replicates.
modulate courtship behavior differently in female and male *Drosophila*[^61][^62], which making SIF a gene of interest in *P. flammans*.

The drawbacks of the adopted second generation sequencing were undoubted. However, we did obtain a mass of valuable genetic data for *P. flammans* with a tight fund, especially in neuropeptides and their receptors. Novel neuropeptides could be supplemented via Genomics- and peptidomics-based discovery in the future[^63]. Moreover, association of multiple omics, such as full-length transcriptome, proteome and metabolome might be needed[^64], which would contribute to the feeding and sexual behavior regulation researches in this diurnal moth *P. flammans* by outlining a chain with cascaded neuropeptide, neuropeptide receptor, pheromone metabolism and behavior.

**Conclusion**

In this study, 15 neuropeptides and 66 neuropeptide receptors were identified from *P. flammans*, and the genes exhibited no significantly different expression in head between female and male. Phylogenetic analyses tree with neuropeptides and receptors of other lepidopteran species illustrated clear interspecies relationships and contributed to further function understanding. Our findings enriched neuropeptides and neuropeptide receptor gene database, which provide a theoretical support for pest management strategies and physiological and biochemical researches in *P. flammans*.

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