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Identification and sex-specific expression of chemosensory genes in the antennal transcriptomes of *Pachyrhinus yasumatsui* (Coleoptera: Curculionidae)

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Pachyrhinus yasumatsui Kono et Morimoto is a major pest of Chinese jujube, which is widespread in northern China and causes severe economic losses in the jujube industry. Chemosensory genes play crucial roles in insect behaviors. Currently, little is known about chemosensory genes in *P. yasumatsui*. In the present study, antennal transcriptomes of female and male adult *P. yasumatsui* were annotated. In total, 113 genes involved in chemosensory functions were identified, including 41 odorant receptors, 28 odorant-binding proteins, 16 ionotropic receptors, 15 chemosensory proteins, 9 gustatory receptors, and 4 sensory neuron membrane proteins. Subsequently, the phylogenetic analyses of these olfactory-related proteins in *P. yasumatsui* were conducted using multiple sequence alignment. Furthermore, sex-specific expression levels of 113 genes were analyzed based on fragments per kilobase of transcript per million mapped reads (FPKM). Then, the quantitative real-time PCR (RT-qPCR) was used to quantify gene expression profiles of 28 *P. yasumatsui* OBPs (*PyasOBPs*) and 15 CSPs (*PyasCSPs*). The results revealed that 20 *PyasOBPs* and 13 *PyasCSPs* exhibited significantly higher expression in the antennae than in the bodies, suggesting that they might have functions in olfaction. Moreover, some OBPs and CSPs (*PyasOBP6*, *PyasOBP7*, *PyasOBP16*, *PyasOBP21*, and *PyasCSP4*) exhibited female-biased expression, indicating that they might take part in several female-specific behaviors. This study will promote the understanding of olfactory mechanism in *P. yasumatsui*, and our findings lay the groundwork for developing environmentally friendly pest management measures.

Key words: antennal transcriptome, chemosensory gene, odorant-binding protein, phylogenetic analysis, gene expression profile

Insect olfaction is known to play the essential role in many important behaviors, like locating hosts and foraging, seeking for mating partners, oviposition, and predator avoidance (Leal 2013, Brito et al. 2016, Fleischer et al. 2018). In insect olfactory system, the antennae are crucial olfaction organs with numerous sensilla located on their surfaces. A variety of chemosensory-related genes contribute to the chemosensory system, including olfactory receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), odorant-binding proteins (OBPs), chemosensory proteins (CSPs), and sensory neuron membrane proteins (SNMPs) (Vosshall et al. 1999, De Bruyne and Baker 2008, Sanchez-Gracia et al. 2009). The general olfactory recognition process involves 2 main steps. In the first step, odorant molecules enter the antennal olfactory sensilla through pores, where they are recognized by some soluble olfactory proteins in the lymph, for example, OBPs or CSPs. Then, these proteins will bind odorant molecules to form OBP/CSP-odorant complexes (Leal 2013). Second, these complexes arrive on the dendritic membranes of olfactory sensory neurons (OSNs) and are shifted to some olfactory-related receptors, for example, ORs, GRs, and IRs (Rützler and Zwiebel 2005, Schmidt and Benton 2020). These receptors convert the chemical signals into electrical signals, which are transmitted through the central nervous system (CNS) and ultimately guide insect physiological responses (Fleischer et al. 2018).

In general, OBPs play a critical role in solubilizing and carrying odorants (Pelosi et al. 2014). They might contribute to the sensitivity of olfactory system (Gomez-Diaz et al. 2013). Also, CSPs are hypothesized to act as odorant carriers, which are similar to OBPs (Wen et al. 2018). OBPS and CSPs participate in various nonsensory processes and are not just found in chemosensory tissues (Dippel et al. 2014, Pelosi et al. 2017). ORs are generally 7-transmembrane domain proteins, including many specific ligand-binding ORs and 1 conserved coreceptor known as Orco (Vosshall et al. 1999, Larsson et al. 2004). GRs are mainly expressed in gustatory receptor neurons of taste organs. Various insect chemoreceptors belong to the GR family, including those for CO_2, sugar, fructose, bitter, and other receptors (Scott et al. 2001, Sato et al. 2011). Insect IRs are usually divided into 2 subfamilies: the conserved "antennal IRs" and the species-specific "divergent IRs" (Croset et al. 2010). They are classified as

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a variant subfamily of ionotropic glutamate receptors (iGluRs) and are associated with non-*N*-methyl-D-aspartic acid (non-NMDA) iGluRs (Benton et al. 2009, Silbering et al. 2011). Antennal IRs are mainly expressed in the antennae and are involved in sensing odors, tastes, temperature, and humidity, whereas divergent IRs are mostly located in gustatory neurons of nonantennal tissues, so they have been linked to gustatory sensations (Rytz et al. 2013). SNMPs are membrane proteins involved in chemosensory neurons in insects and play crucial roles in binding and transporting hydrophobic ligands. Most insect species generally have 2 SNMP subfamilies (SNMP1 and SNMP2) (Nichols and Vogt 2008, Vogt et al. 2009).

Identification of the chemosensory genes is an essential requirement for the functional exploration of olfactory genes. Since the characterization of chemosensory genes from the first Coleopteran species *Tribolium castaneum* (Herbst) (Tribolium Genome Sequencing Consortium 2008), chemosensory genes have been identified in numerous Coleopteran species, such as *Dendroctonus ponderosae* Hopkins (Andersson et al. 2013), *Monochamus alternatus* Hope (Wang et al. 2014), *Colaphellus bowringi* Baly (Li et al. 2015), *Rhynchophorus ferrugineus* (Oliver) (Antony et al. 2016), *Eucryptorrhynchus scrobiculatus* Motschulsky, *Eucryptorrhynchus brandti* (Harold) (Wen et al. 2018), *Anoplophora chinensis* (Forster) (Sun et al. 2018), *Agrilus planipennis* Fairmaire and *Anoplophora glabripennis* Motschulsky (Andersson et al. 2019), *Apriona germari* (Hope) (Qian et al., 2020), *Aethina tumida* Murray (Wu et al. 2021), *Aromia bungii* (Faldermann) (Wu et al. 2022), and *Sympiezomias velatus* (Chevrolat) (Li et al. 2022). These olfactory-related genes can be employed as potential targets to help develop novel strategies of pest management (Wang et al. 2010). However, currently little is known about the chemosensory genes in *Pachyrhinus yasumatsui*.

Pachyrhinus yasumatsui (synonym *Scythropus yasumatsui*) (Coleoptera: Curculionidae), known as 'jujube bud weevil', has been one of the most destructive pests of Chinese jujube in recent years (Tang et al. 2013, Hong et al. 2017). This weevil is widespread in northern China and causes severe ecological damage and considerable economic losses (Hong et al. 2019). At present, chemical pesticides are mainly used for controlling *P. yasumatsui*. Pesticide abuse often leaves residues behind, seriously threatening the environment and food security (Zhang et al. 2015, Yang et al. 2019). *Pachyrhinus yasumatsui* prefers to eat the shoots of *Zizyphus* plants (Wang et al. 2017), indicating that it may rely on olfaction to seek for hosts. Previous studies have shown that *P. yasumatsui* was highly attracted to some volatiles in jujube shoots (Yan et al. 2017, 2020). Therefore, the use of host attractants, as an eco-friendly control strategy against this pest, should be developed.

In the present study, we performed antennal transcriptome analysis of *P. yasumatsui* adults and identified olfactory-related genes. Phylogenetic analyses of these genes were carried out in relation to other insect species. Furthermore, sex-specific expression profiles of these chemosensory genes were analyzed. A quantitative real-time PCR (RT-qPCR) was also performed to analyze the expression patterns of OBP and CSP genes. This study promotes the understanding of olfactory mechanism in *P. yasumatsui*, and our findings lay a foundation for developing environmentally friendly pest management measures.

Materials and Methods

Insects Rearing, Tissue Collection, and RNA Extraction

Pachyrhinus yasumatsui pupae samples were obtained in Jiaxian County (110°21.12′ E, 37°59.88′ N, altitude 750.8 m), Shaanxi province, China. The emerged adults were reared on fresh buds of jujube at 25 ± 1 °C, 60% ± 5% relative humidity, and 16 h:8 h (light: dark) photoperiod. Antennae were excised from 5-day-old adults, placed into a 1.5-ml Eppendorf tube in liquid nitrogen, and stored at −80 °C. Three biological replicates of ~100 antennae from each sex (~300 each sex) were used for transcriptome sequencing. Besides, 3 replicates of 10 bodies (without antennae) from each sex (30 each sex) were used for analysis of gene expression profiles. Subsequently, the total RNA was extracted from samples using the Trizol Reagent (Invitrogen, CA, USA) following the manufacturer's instructions. The quantity, purification, and integrity of RNA were quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA) and Agilent 2100 Bioanalyzer System (Agilent, CA, USA).

cDNA Library Construction and Illumina Sequencing

The cDNA library construction and Illumina sequencing of the samples was performed at Biomarker Technologies Co. Ltd. (Beijing, China). The cDNA library was generated by NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, MA, USA). First, mRNAs were enriched from total RNA samples using oligo (dT) magnetic beads and then fragmented randomly into short fragments. Next, the first-strand cDNA was synthesized by random hexamer primers, and the second-strand cDNA was synthesized with dNTPs and DNA polymerase I. Subsequently, double strands of cDNA were purified with AMPure XP beads (Beckman Coulter, CA, USA) and repaired at the ends, and then the cDNA was enriched by PCR. Finally, the cDNA library was tested for quality and sequenced using the Illumina NovaSeq 6000 platform (Illumina, CA, USA).

De Novo Assembly and Functional Annotation

High-quality reads were obtained from raw reads by removing adaptors, low-quality reads, and repeat reads. These reads were de novo assembled into transcripts first, then into unigenes using Trinity v2.5.1 (Grabherr et al. 2011). All assembled unigenes were annotated based on nucleotide sequence using BLASTx search with an *E*-value cutoff of 10^{-5} against the following databases: NCBI Non-Redundant protein (NR, ftp://ftp.ncbi.nih.gov/blast/db), Swiss-Prot (https://www.uniprot.org/), Gene Ontology (GO, http://geneontology.org/), Protein family (Pfam, http://pfam.xfam.org/), Cluster of Orthologous Groups/euKaryotic Orthologous Groups (COG/KOG, https://www.ncbi.nlm.nih.gov/COG/), eggNOG (http://eggnog5.embl.de/), and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/).

All putative unigenes involved in *P. yasumatsui* olfaction were identified based on functional annotation results and then checked by NCBI BLASTx searches. The open reading frames (ORFs) of candidate genes were identified by the ORF Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/) and confirmed by comparing the predicted protein sequences to NR database using BLASTp. All the protein sequences were predicted using the Pfam web server (Mistry et al. 2021). For OBPs and CSPs, SignalP 6.0 (https://services.healthtech.dtu.dk/service.php?SignalP) was performed to identify putative signal peptides (Teufel et al. 2022). For some chemoreceptor genes (e.g., ORs, GRs, IRs) and SNMP genes, TMHMM 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) was performed to predict the transmembrane domains.

Phylogenetic Analyses

Amino acid sequences of these chemosensory genes in *P. yasumatsui* were aligned using MAFFT v7.037 with the FFT-NS-2

algorithm, BLOSUM62 scoring matrix and other default parameters (Katoh and Standley 2013). FastTree v2.1.11 was used to generate maximum-likelihood (ML) phylogenetic trees with 1,000 bootstrap replicates (Price et al. 2010). The analyses included chemosensory-related sequences from *P. yasumatsui* and other Coleopteran species, such as *T. castaneum* (Engsontia et al. 2008, Nichols and Vogt 2008), *C. Bowringi* (Li et al. 2015), *Cylas formicarius* (Fabricius) (Bin et al. 2017), *S. velatus* (Li et al. 2022), *Sitophilus zeamais* Motschulsky (Tang et al. 2019), *Sitophilus oryzae* (L.), *Megacyllene caryae* (Gahan) (Mitchell et al. 2012), *Dendroctonus adjunctus* Blandford (Torres-Huerta et al. 2020), *Rhynchophorus palmarum* Linnaeus (Gonzalez et al. 2021), *A. tumida* (Wu et al. 2021), *D. ponderosae*, and *A. glabripennis* (Andersson et al. 2019). Lastly, FigTree v1.4.3 was used to visualize and edit the phylogenetic trees.

Sex-Specific Gene Expression Profiles

Expression levels of chemosensory genes in the antennae of male and female *P. yasumatsui* adults were evaluated according to fragments per kilobase of transcript per million mapped reads (FPKM) (Trapnell et al. 2010). Differentially expressed genes (DEG) were identified between male and female antennae using DESeq2 v1.6.3 with |log2(foldchange)| > 1 and *P*-value < 0.05 as the threshold (Love et al. 2014). Subsequently, the relative expression levels of OBP and CSP genes were validated by RT-qPCR. Total RNA was extracted from antennae and bodies (without antennae) of both male and female adults, and then the cDNA was synthesized. The gene-specific primers for RT-qPCR were designed by Primer Premier 5.0 (Supplementary Table S1). RT-qPCR reactions were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) with TB Green Premix Ex Taq II (TaKaRa). Reaction programs were set to 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. The *EF1-α* gene (GenBank no. OK105108) and *β-actin* (GenBank no. OK322363) gene were selected as reference genes to normalize expression profiles of these OBP and CSP genes. The relative expression levels of target genes were measured with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All of the experiments were conducted with 3 replicates, and the significant differences were evaluated by one-way ANOVA and Tukey's multiple comparisons ($P < 0.05$). All data were analyzed by SPSS 26.0.

Results

Transcriptome Sequencing and Sequence Assembly

A total of 23,572,425 and 26,618,778 reads were obtained from male and female antennal transcriptomic data of *P. yasumatsui* after filtering out all low-quality reads, respectively. After a combined transcriptome assembly, 51,590 unigenes were obtained, and their average length and N50 length were 2,068 and 3,447 bp, respectively. The unigene length distribution showed that 39,152 unigenes were >500 bp in size, accounting for 75.89% of all unigenes (Supplementary Fig. S1). The raw transcriptome data in the present study were submitted to the NCBI Sequence Read Archive (SRA) database.

Functional Annotation of Unigenes and Overview of Chemosensory Genes

In total, 30,111 unigenes (58.37%) against other insect species were annotated in at least one database: 28,954 unigenes (56.12%) were annotated by NR database, 24,979 (48.42%) by Swiss-Prot, 14,489 (28.08%) by GO, 14,458 (28.02%) by Pfam, 5,110 (9.91%) and 11,423 (22.14%) by the COG/KOG database, 13,397 (27.13%) by eggNOG, and 5,186 (10.05%) by KEGG (Supplementary Fig. S2).

Homology searches of 28,954 unigenes against the NR database showed that *P. yasumatsui* antennal transcriptomes shared the greatest homology with sequences from *S. oryzae* (39.0%), followed by *D. ponderosae* (31.2%), *A. glabripennis* (4.3%), and *Callosobruchus maculatus* Fabr (3.7%) (Supplementary Fig. S3). Based on their GO annotation, a total of 14,489 unigenes were classified into 3 functional categories: "biological process," "cellular component," and "molecular function" (Fig. 1). In the category of

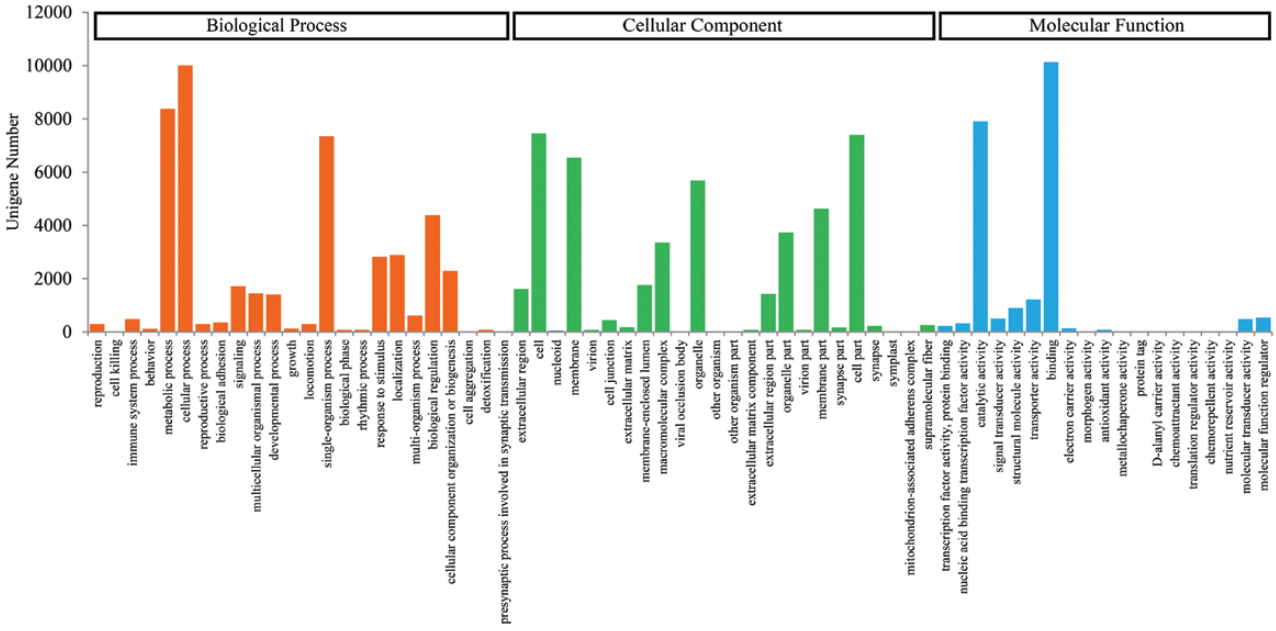

Fig. 1. GO classification for the unigenes in *P. yasumatsui* antennal transcriptome.

"biological process," the unigenes expressed in antennae were mostly related to "cellular process" (10,011), "metabolic process" (8,370), and "single-organism process" (7,349). In the category of "cellular component," the unigenes expressed in the antennae were mostly related to "cell" (7,456), "cell part" (7,405), "membrane" (6,543), and "organelle" (5,689). Meanwhile, "binding" (10,133) and "catalytic activity" (7,905) were the most abundant groups among the category of "molecular function."

In total, 5,186 unigenes were annotated and classified into 5 pathway categories described in the KEGG database: organismal systems (1,200), metabolism (1,507), genetic information processing (1,027), environmental information processing (674), and cellular processes (778) (Fig. 2). Most genes were involved in signal transduction (577), followed by translation (416); folding, sorting, and degradation (330); transport and catabolism (329); and the endocrine system (307).

After annotation, 113 chemosensory genes were identified from antennal transcriptomic data in *P. yasumatsui*, including 28 OBPs, 15 CSPs, 41 ORs, 9 GRs, 16 IRs, and 4 SNMPs. Information of candidate OBPs, CSPs, ORs, GRs, IRs, and SNMPs, such as gene name, predicted protein lengths, the annotation in NR database, and protein domains, are listed in Supplementary Tables S2 and S3. In addition, predicted protein sequences and unigene sequences of 113 candidate genes are listed in Supplementary Materials S1 and S2.

Identification of Candidate OBPs

A total of 28 OBP-encoding unigenes (*PyasOBP1-28*) were identified in *P. yasumatsui* antennal transcriptomes. All unigenes represented full-length ORFs and encoded 126–258 amino acids. Among these OBP sequences, all OBPs contained an N-terminal signal peptide of 15–29 amino acids (Supplementary Table S2). Multiple sequence alignment results showed that 12 PyasOBPs belonged to Classic OBPs (with 6 conserved cysteines) and 15 PyasOBPs belonged to Minus-C OBPs (with 4 conserved cysteines). In addition, 1 Plus-C OBP (PyasOBP26) was found in *P. yasumatsui* (Supplementary Figs. S4–S6), similar to other Coleopterans (Andersson et al. 2019, Wu et al. 2021).

A phylogenetic tree was produced with 216 OBPs of *P. yasumatsui* and other insects (Fig. 3), and the results showed that 28 PyasOBPs were clustered into 3 groups—Minus-C OBPs (15), Plus-C OBPs (1), and Classic OBPs (12)—which was consistent with the multiple sequence alignment results. Except for PyasOBP12, all the PyasOBPs formed a cluster with 1–3 orthologs from other Coleopterans. For example, PyasOBP1 formed a cluster with DponOBP39 and DadjOBP24, while PyasOBP2 formed a cluster with SvelOBP37. Three-fourths (21 of 28) of PyasOBPs were closely related to the orthologs of the great gray weevil, *S. velatus*. The remaining PyasOBPs were closely related to the orthologs of *D. ponderosae* and *D. adjunctus*. Additionally, one subfamily of classic OBPs, which also has 6 conserved cysteine residues, is further classified as "antennal binding protein II" (ABPII) (Dippel et al. 2014). The phylogenetic tree demonstrated that parts of ABPII subfamily members clustered together with some well-studied pheromone-binding proteins such as PjapPBP and AosaPBP (Wojtasek et al. 1998), and PyasOBP2, 3, 4, 23, 24, and 25 were clustered into ABPII clades.

Identification of Candidate CSPs

Fifteen unigenes encoding putative CSPs (*PyasCSP1-15*) were identified, and all these unigenes had full-length ORFs. Among these CSPs, all contained a signal peptide with 17–26 amino acids

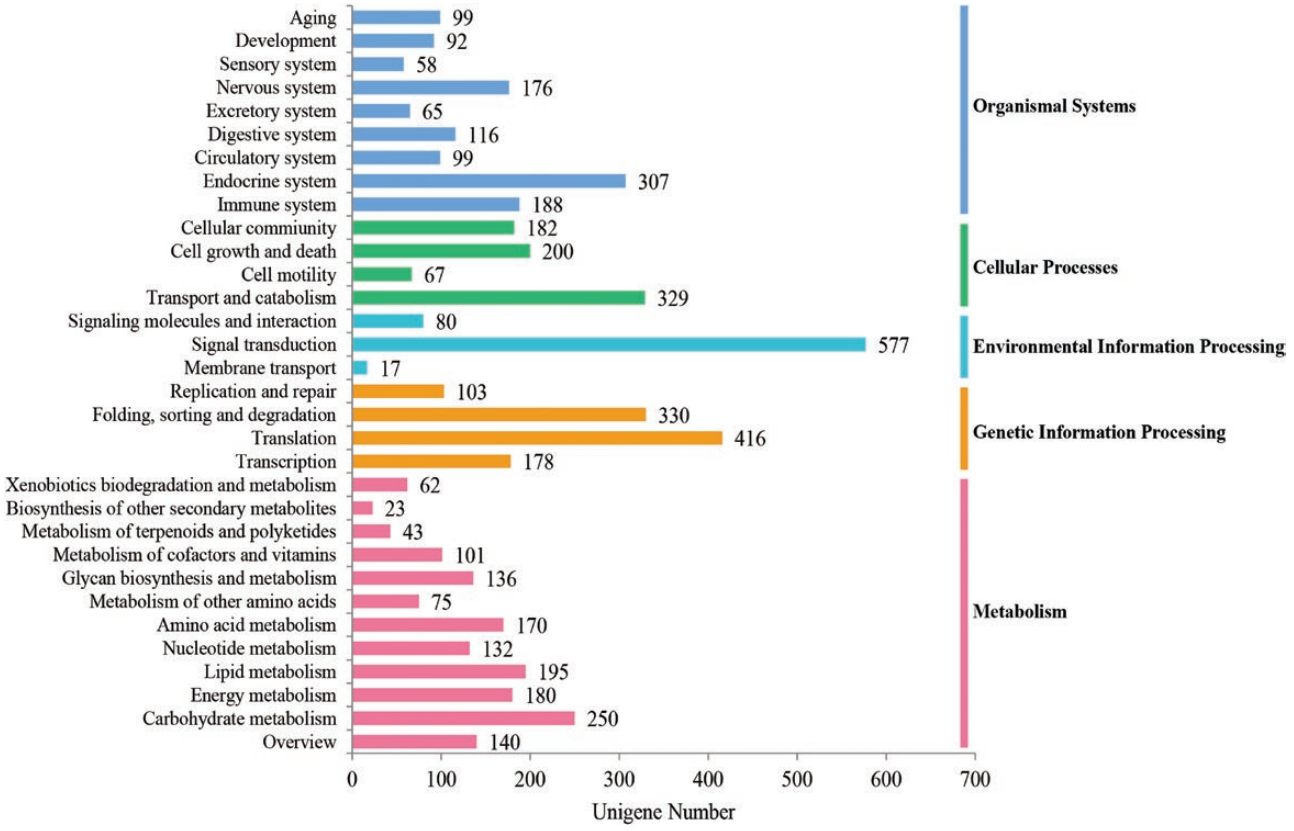

Fig. 2. KEGG classification for the unigenes in *P. yasumatsui* antennal transcriptome.

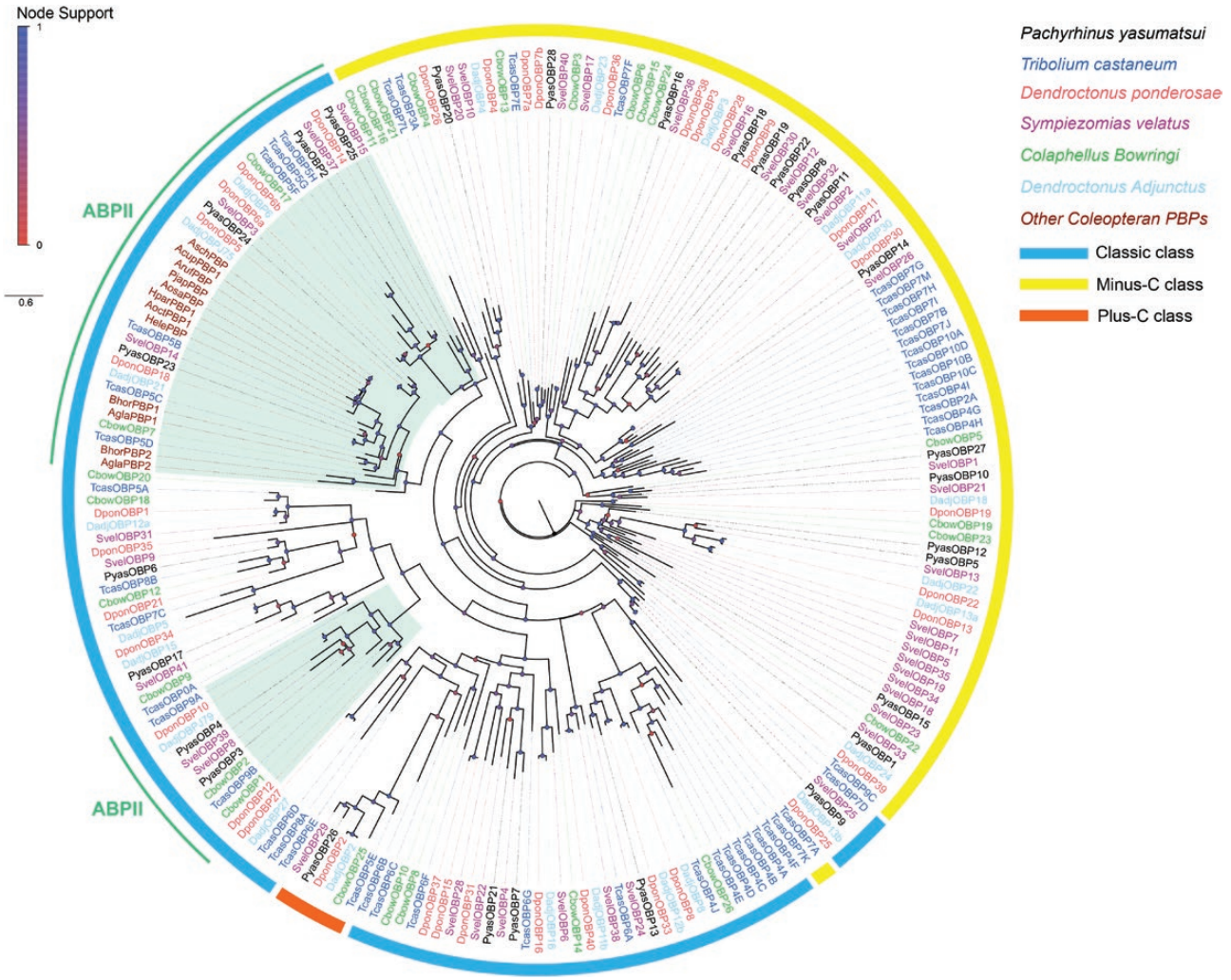

Fig. 3. Phylogenetic tree of 216 OBPs. These sequences were obtained from 16 Coleopteran species (28 from *Pachyrhinus yasumatsui*, 50 from *Tribolium castaneum*, 36 from *Dendroctonus ponderosae*, 41 from *Sympiezomias velatus*, 26 from *Colaphellus bowringi*, 23 from *Dendroctonus adjunctus*, and 12 from 10 other Coleopterans). The node support values are indicated by colored circles. The sources of sequences were detailed in Supplementary Table S5 (the same below).

(Supplementary Table S2) and followed the classic cysteine pattern (C1-$X_{6\text{-}8}$-C2-X_{18}-C3-X_2-C4, where X represents any amino acid) in Coleopterans (Xu et al. 2009) except PyasCSP1, which possessed 19 amino acids between the second and third cysteines (Supplementary Fig. S7). The phylogenetic tree showed that more than one-half (8 of 15) of PyasCSPs were closely related to orthologs of *S. velatus* (Fig. 4). Additionally, similar to what has been observed in *A. tumida* (Wu et al. 2021), a species-specific expansion of CSPs (PyasCSP3/7/8 and PyasCSP10/12) was found in *P. yasumatsui*.

Identification of Candidate ORs

A total of 41 unigenes for candidate ORs, including *PyasOrco* and *PyasOR1-40*, were identified in *P. yasumatsui*. Of these unigenes, 31 encoded the full-length proteins of 335–482 amino acids with 4–7 transmembrane domains (TMDs), and the remaining were partial sequences (Supplementary Table S3). Apart from 6 ORs less than 200 amino acids, 35 PyasORs were used to construct the phylogenetic tree with ORs from other beetle species. The phylogenetic tree clustered Coleopteran ORs into 9 subgroups (1, 2A, 2B, 3, 4, 5A, 5B, 6, and 7) (Mitchell et al. 2020) and Orco subgroup (Fig. 5). Thirty-one PyasORs were divided into subgroups 1, 2A, 2B, and 7. Among those, 16 PyasORs are present within subgroup 7, followed by subgroup 1 (7 PyasORs) and 2A (6 PyasORs). Furthermore, 21 PyasORs were closely related to the orthologs of *S. velatus*. Remarkably, 3 PyasORs (PyasOR28/29/31) clustered together as a single clade, which suggested that a species-specific expansion occurred in *P. yasumatsui*. However, they were not divided into any subgroup of Coleopteran ORs.

Identification of Candidate GRs

We identified 9 candidate GRs (*PyasGR1-9*) in the *P. yasumatsui* antennal transcriptome. Among these GR genes, 7 contained the full-length sequences, which encoded proteins of 276–478 amino acids with 4–7 TMDs, and 2 PyasGRs (PyasGR7/9) were partial sequences (Supplementary Table S3). Eight PyasGRs more than 200 amino acids were used to construct a phylogenetic tree (Fig. 6). The phylogenetic analysis revealed that PyasGR1 was clustered into the carbon dioxide (CO_2) receptor clade and was the ortholog of DponGR1 and SvelGR1 (Scott et al. 2001). Furthermore, 3 PyasGRs (PyasGR2/5/6) were clustered into the sugar receptor clade, while PyasGR3 was clustered into the fructose receptor clade, indicating

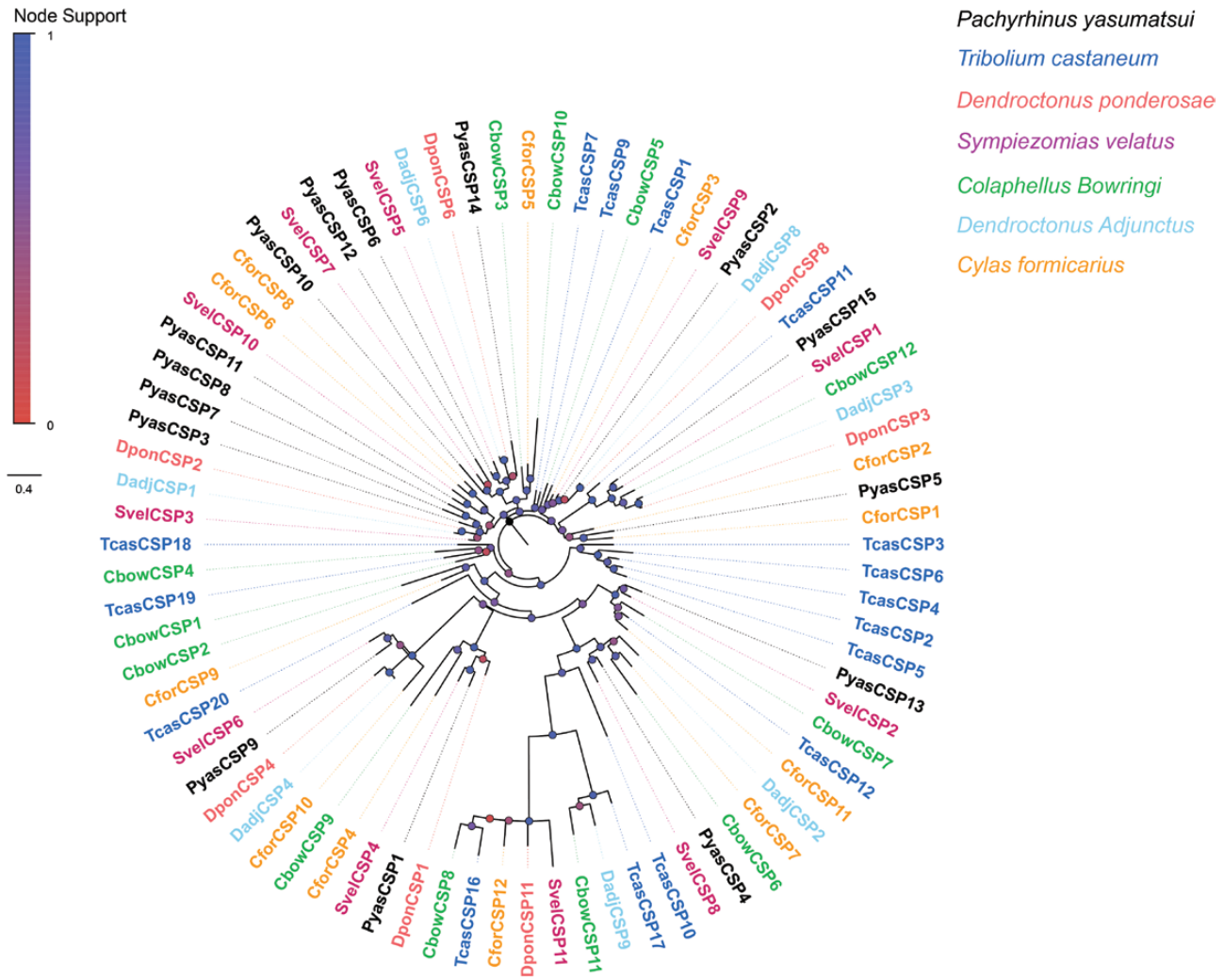

Fig. 4. Phylogenetic tree of 80 CSPs. These sequences were obtained from 7 Coleopteran species (15 from *Pachyrhinus yasumatsui*, 16 from *Tribolium castaneum*, 7 from *Dendroctonus ponderosae*, 11 from *Sympiezomias velatus*, 12 from *Colaphellus bowringi*, 7 from *Dendroctonus adjunctus*, and 12 from *Cylas formicarius*). The node support values are indicated by colored circles.

that they might possess similar functions with orthologs of sugar and fructose receptors in other Coleopterans (Li et al. 2022).

Identification of Candidate IRs

In total, 16 candidate IR-encoding unigenes were identified. Among these IR genes, 14 contained full-length ORFs, which encoded proteins of 574–923 amino acids, and 2 unigenes (*PyasIR21a* and *PyasIR75c*) were partial sequences (Supplementary Table S3). In this study, 113 iGluRs/IRs from 7 Coleopteran species were clustered into 4 subgroups (antennal IRs, divergent IRs, NMDA iGluRs, and non-NMDA iGluRs) in the phylogenetic tree (Fig. 7). Nine PyasIRs, including 3 IR co-receptors (PyasIR8a/25a/76b) and 6 conserved IRs (PyasIR21a/64a/75b/75c/75s/93a), were clustered into the antennal IR subgroup. However, we did not identify any divergent IRs (e.g., *IR60a/100a*) from the *P. yasumatsui* antennal transcriptome. Additionally, 1 NMDA iGluR (PyasNmdar1) and 6 non-NMDA iGluRs (PyasGluR1-6) were found in the phylogenetic tree, and they were most closely related to orthologs from *S. oryzae*. The functions of non-NMDA iGluRs should be further studied.

Identification of Candidate SNMPs

Four unigenes encoding putative SNMPs were identified and included 2 *SNMP1* subfamily members (*PyasSNMP1a* and *PyasSNMP1b*) and 2 *SNMP2* subfamily members (*PyasSNMP2a* and *PyasSNMP2b*). All 4 unigenes contained full-length ORFs encoding proteins of 506–561 amino acids with 2 TMDs (Supplementary Table S3). The phylogenetic tree demonstrated that PyasSNMP1a and PyasSNMP1b were divided into the SNMP1 clade; PyasSNMP2a and PyasSNMP2b were divided into the SNMP2 clade (Fig. 8). Of those, PyasSNMP1a, PyasSNMP1b, and PyasSNMP2b were most closely related to orthologs from *S. velatus*.

Gene Expression Profiles

According to an average FPKM value of 3 replicates, $\log_{10}$ (FPKM + 1) was calculated to process expression patterns of 113 chemosensory genes from the antennae of female and male adult *P. yasumatsui* (Fig. 9, Supplementary Table S4). In general, *PyasOBPs* exhibited significantly higher expression levels (FPKM > 2000) than other chemosensory gene families. *PyasOBPs* (*PyasOBP2/3/4/23/24/25*) belonging to the ABPII subfamily, as well as *PyasOBP1*, *5*, *6*, *10*, and *27*, were greatly expressed in the antennae of both sexes (FPKM > 1,000). Among these genes, *PyasOBP2* exhibited the highest expression level (the average FPKM value = 28,318.56), followed by *PyasOBP24* (10,485.39), *PyasOBP5* (9,717.01), and *PyasOBP25* (6,240.42). Three *PyasCSPs* (*PyasCSP3/4/15*) were greatly expressed in the antennae of both sexes (FPKM > 1,000).

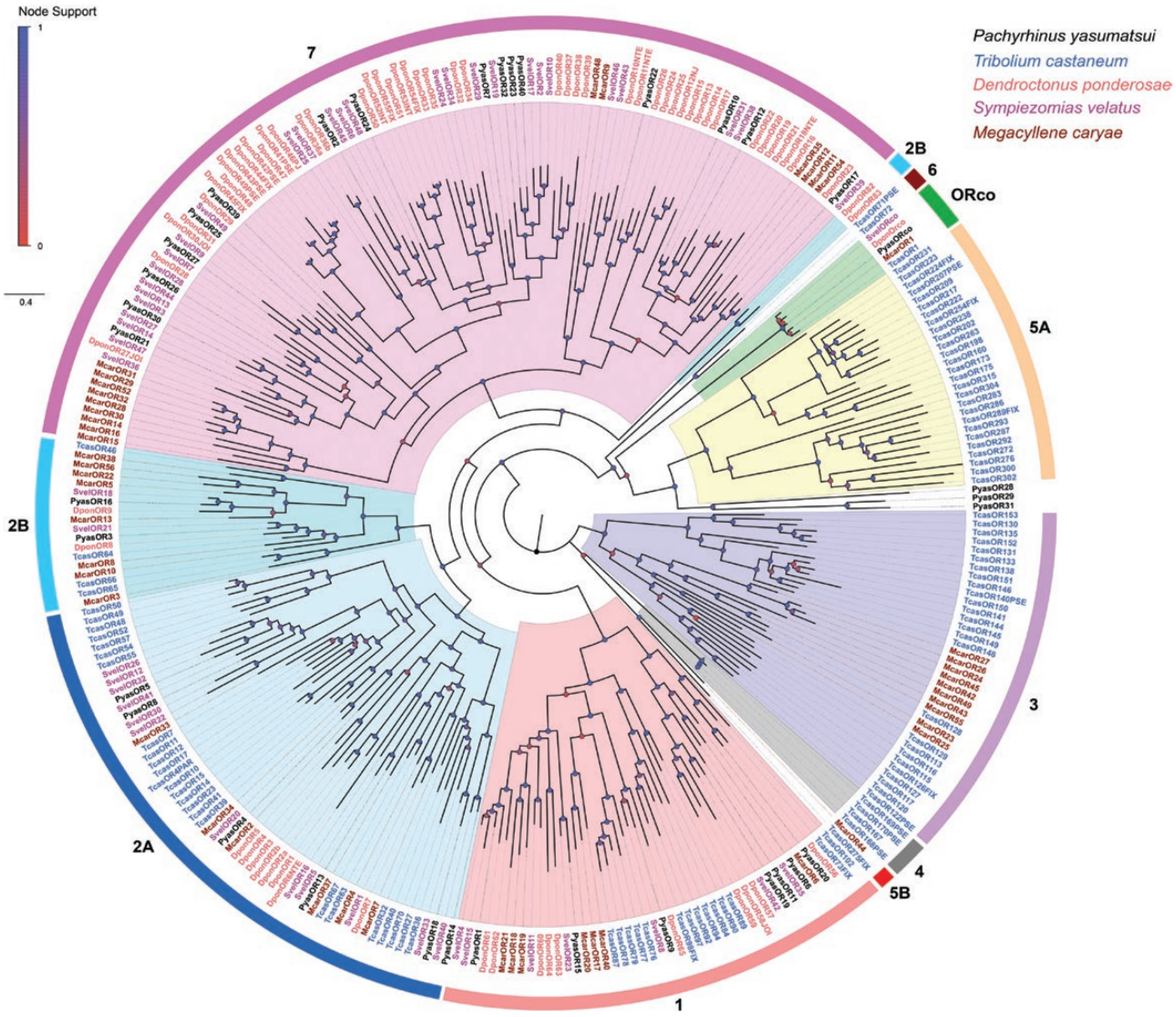

Fig. 5. Phylogenetic tree of 307 ORs. These sequences were obtained from 5 Coleopteran species (35 from *Pachyrhinus yasumatsui*, 104 from *Tribolium castaneum*, 70 from *Dendroctonus ponderosae*, 50 from *Sympiezomias velatus*, and 48 from *Megacyllene caryae*). The node support values are indicated by colored circles.

Among these genes, *PyasCSP3* exhibited the highest expression level (FPKM = 9,886.79). Among all *PyasORs*, *PyasORco*, *PyasOR1*, *16*, and *30* exhibited higher expression levels (FPKM > 30). Compared with other olfactory-related gene families, *PyasGRs* showed the lowest expression levels (the average FPKM value < 10), and among all *PyasGRs*, *PyasGR3* and *PyasGR4* exhibited higher expression levels (FPKM > 15). Among the *IRs/iGluRs*, *PyasIR93a* had the highest expression (FPKM = 115.32), followed by *PyasIR76b* (64.58). Among the 4 *SNMPs* from *P. yasumatsui*, *PyasSNMP1a* and *1b* (the average FPKM value > 50) displayed significantly higher expression than *PyasSNMP2a* and *2b* (FPKM < 5). In terms of sex-specific expression of chemosensory receptor genes, the expression levels of 12 *PyasORs* (including *PyasOrco*) in male antennae were significantly higher than in female antennae, whereas 3 *PyasORs* (*PyasOR1/15/38*) were female-biased. Additionally, among 29 IRs, GRs and SNMPs from *P. yasumatsui*, other than 5 *PyasIRs*, *PyasGR4* and *PyasSNMP1a*, all other genes did not exhibit sex-biased expression (Fig. 9).

Given the essential roles of OBPs and CSPs in insect olfaction, we analyzed expression levels of all 43 genes (28 *PyasOBPs* and 15 *PyasCSPs*) in the antennae and bodies (without antennae) of both sexes from *P. yasumatsui* by RT-qPCR. Among these *PyasOBPs*, 20 candidate genes were expressed significantly higher in the antennae than in the bodies ($P < 0.05$). Moreover, sex-biased expression of *PyasOBPs* was found in antennae; the expression levels of 11 *PyasOBPs* (*PyasOBP2/3/4/5/13/15/17/23/24/25/26*) in male antennae were higher than in female antennae. Four *PyasOBPs* (*PyasOBP6/7/16/21*) were female-biased (Supplementary Fig. S8A), indicating that these genes might take part in several female-specific behaviors, such as mating and locating oviposition sites (Liu et al. 2022). Of 15 *PyasCSPs*, 13 were expressed at significantly higher levels in the antennae than in the bodies ($P < 0.05$). Seven *PyasCSPs* (*PyasCSP1/3/5/6/7/11/15*) had male-biased expression in the antennae, whereas *PyasCSP4* showed female-biased expression (Supplementary Fig. S8B). These results were highly in agreement with FPKM values (Supplementary Table S4), indicating that the antennal transcriptome results were dependable.

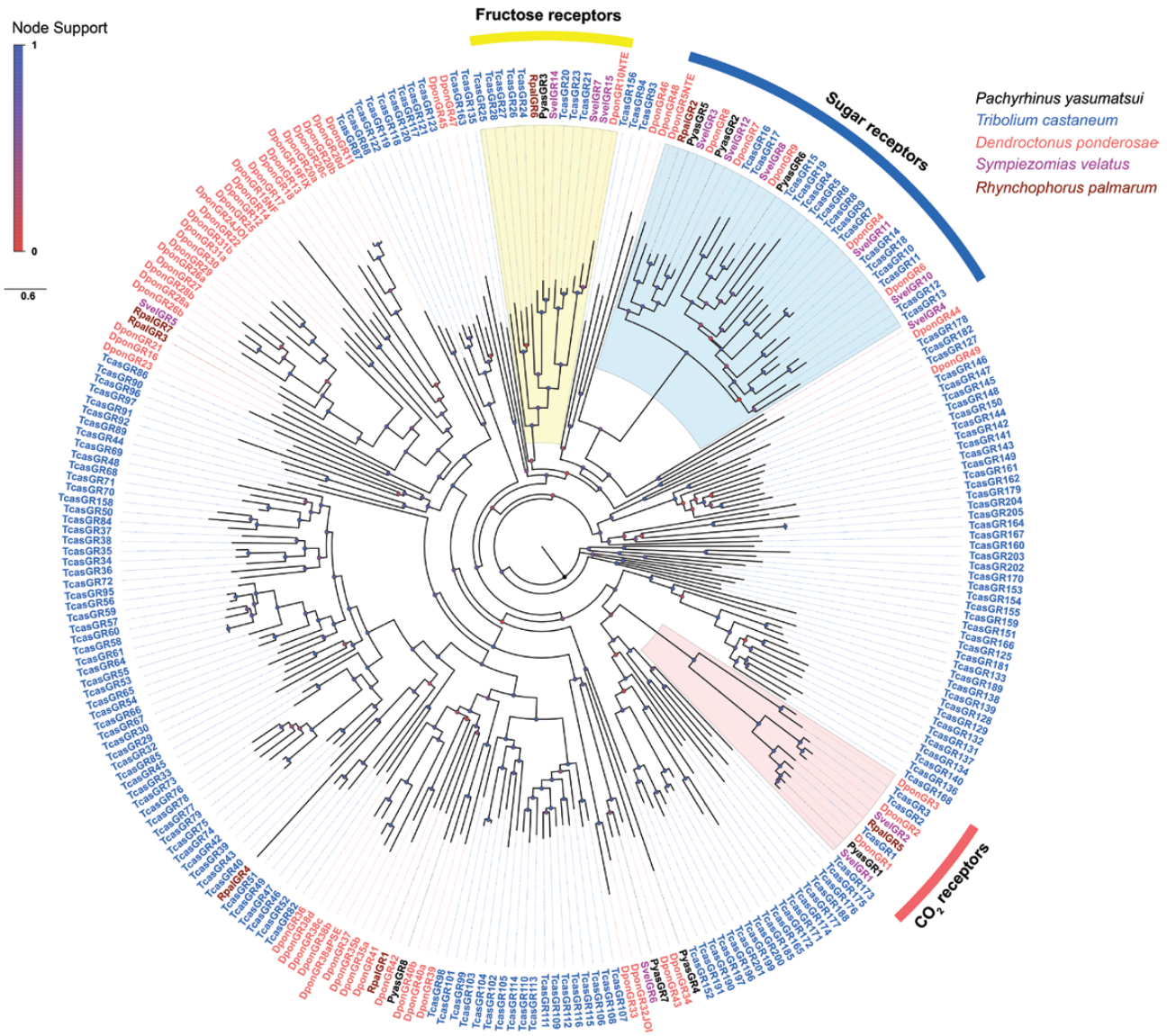

Fig. 6. Phylogenetic tree of 269 GRs. These sequences were obtained from 5 Coleopteran species (8 from *Pachyrhinus yasumatsui,* 181 from *Tribolium castaneum,* 60 from *Dendroctonus ponderosae,* 13 from *Sympiezomias velatus,* and 7 from *Rhynchophorus palmarum*). The node support values are indicated by colored circles.

Discussion

Compared to Lepidopterans and Dipterans, the molecular mechanism of chemoreception in Coleopterans, particularly in the family Curculionidae, is relatively poorly understood. In the present study, we sequenced, assembled, and analyzed the antennal transcriptomes of *Pachyrhinus yasumatsui* (Coleoptera: Curculionidae) for the first time. In total, 51,590 unigenes were assembled from the antennal transcriptome, and 30,111 unigenes were annotated against 8 public databases. Subsequently, 113 novel chemosensory genes (28 OBPs, 15 CSPs, 41 ORs, 9 GRs, 16 IRs, and 4 SNMPs) were identified from annotated unigenes, and the phylogenetic trees were generated to examine the similarities in related genes. The results enrich the gene database of the family Curculionidae and provide a genetic basis for the management of jujube pests. However, this study is based on transcriptome analysis, and the number of the chemosensory genes in the genome is likely to be greater (Engsontia et al. 2008, Andersson et al. 2019).

In total, we identified 28 *OBPs* from the antennal transcriptome of *P. yasumatsui*. The number of *PyasOBPs* was similar to that found in *C. bowringi* (26) and *E. brandti* (28), but less than in *S. velatus* (41) and *D. ponderosae* (36). The phylogenetic analysis showed that the majority of PyasOBPs (21) clustered with the OBPs of the great gray weevil, *S. velatus* (Fig. 3), indicating that OBPs with a high degree of sequence homology among closely related species may also possess similar functions (Xu et al. 2019). Additionally, 6 classic OBP-encoding genes (*PyasOBP2/3/4/23/24/25*) were identified as members of the ABPII family and were also highly expressed in male antennae (Fig. 3 and Supplementary Fig. S8A), suggesting that these genes might have crucial roles in sex pheromone perception. As shown by RT-qPCR analysis, the majority of *PyasOBPs* (20 of 28 OBPs) were expressed significantly higher in antennae than in bodies (without antennae), which was accordance with other Coleopterans, such as *Pagiophloeus tsushimanus* Morimoto (Chen et al. 2021) and *Plagiodera versicolora* (Laicharting) (Liu et al. 2022). In addition,

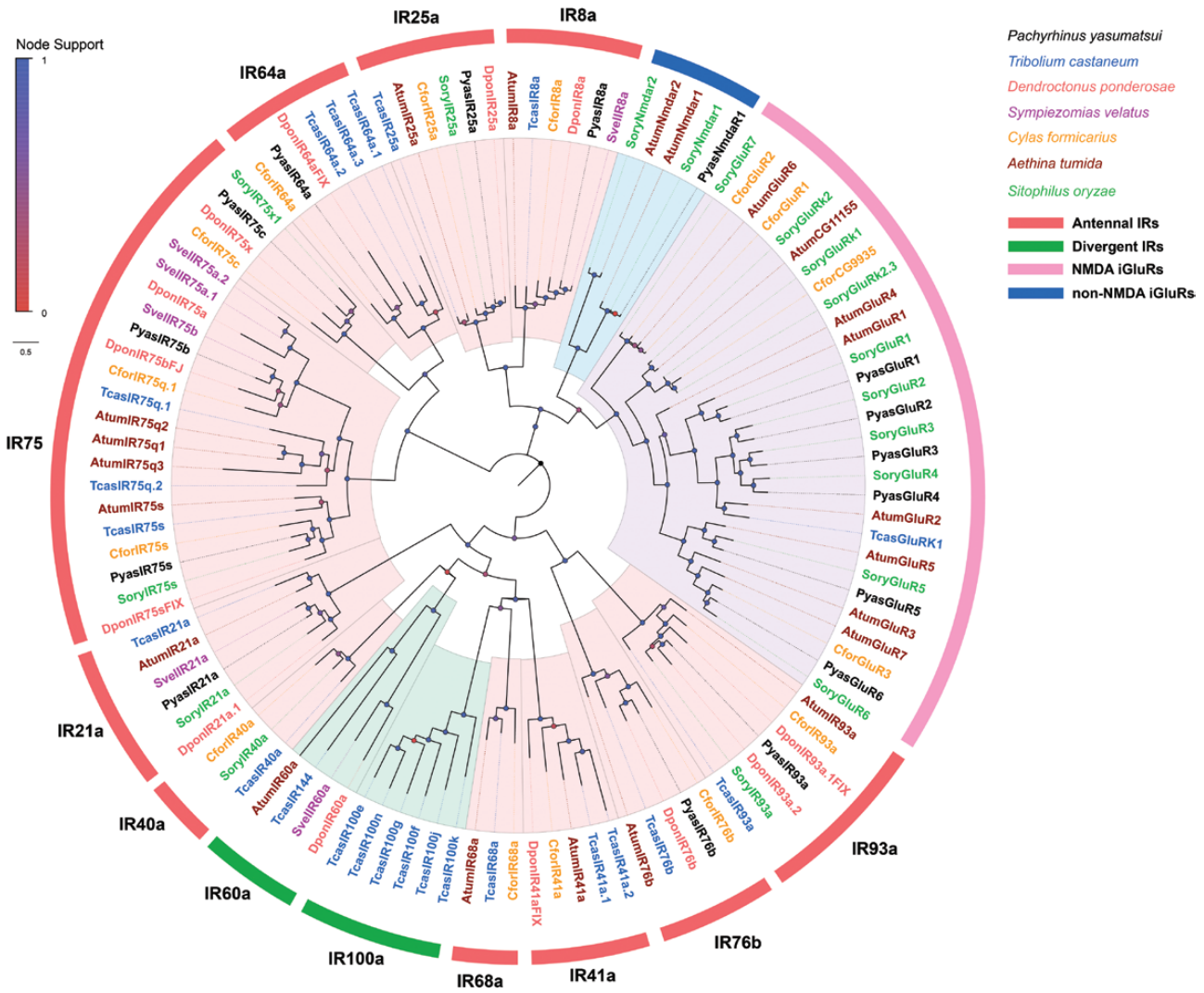

Fig. 7. Phylogenetic tree of 113 IRs. These sequences were obtained from 7 Coleopteran species (16 from *Pachyrhinus yasumatsui*, 23 from *Tribolium castaneum*, 13 from *Dendroctonus ponderosae*, 6 from *Sympiezomias velatus*, 15 from *Cylas formicarius*, 22 from *Aethina tumida* and 18 from *Sitophilus oryzae*). The node support values are indicated by colored circles.

PyasOBPs showed gender bias. Antennal expression levels of 11 *PyasOBPs* were significantly higher in females than those in males, whereas 4 *PyasOBPs* were male-biased, suggesting that OBPs could bind different semiochemicals and might be due to some physiological behaviors, such as aggregation and mating between sexes (Plettner et al. 2000, Liu et al. 2022). In this study, the number of identified *PyasCSPs* (15) was close to that in *A. planipennis* (14) and *A. glabripennis* (17), but less than *A. tumida* (22) and *T. castaneum* (20). Among these *PyasCSPs*, 8 PyasCSPs clustered with the CSPs of *S. velatus* in the phylogenetic tree. qRT-PCR results indicated that the majority of *PyasCSPs* (13 of 15) were expressed higher in antennae than in bodies. Antennal expression levels of 7 *PyasCSPs* were male-biased, whereas *PyasCSP4* showed female-biased expression. In addition, 2 CSP-encoding genes (*PyasCSP5* and *PyasCSP15*) were present exclusively in the antennae (Supplementary Fig. S8B), suggesting they might be involved in olfaction.

Compared with other olfactory receptors, ORs have been deeply studied in Coleopteran species (Mitchell et al. 2020). In total, we identified 41 *PyasORs* in *P. yasumatsui*, similar to the number in *C. bowringi* (43) and *P. versicolora* (40) (Liu et al. 2022). In the phylogenetic tree, PyasORco was clustered into the Orco subgroup (Fig. 5), indicating that Orco is highly conserved within Coleopteran species and could be used as potential interference target genes for integrated pest management (Butterwick et al. 2018, Wu et al. 2022). Usually, Orco is expressed at a much higher level than the other ORs (Antony et al. 2016, Bin et al. 2017, Wu et al. 2021). It is interesting that *PyasOrco* exhibited significantly higher expression than the majority of PyasORs, but exhibited a similar expression level relative to a few *PyasORs* (*PyasOR1*, *PyasOR16*, *PyasOR30*, etc.). The same result was reported in *SvelORs* from *Sympiezomias velatus*, a species closely related to *P. yasumatsui*, both belonging to the subfamily Entiminae. Expression level of a few *SvelORs* (*SvelOR4*, *SvelOR18*, *SvelOR28*, *SvelOR53*, etc.) was similar to that of *SvelOrco* (Li et al. 2022). Therefore, we speculated that the high expression level of a few odorant receptors might be specific to species in the subfamily Entiminae (broad-nosed weevils). In further studies, we will focus on the functions of these PyasORs. Other than that, ORs from 5 Coleopteran species were divided into 10 subgroups, and the majority of PyasORs were clustered into subgroups 1, 2A, 2B, and 7 (Fig. 5), which was consistent with the observations in some species of the family Curculionidae

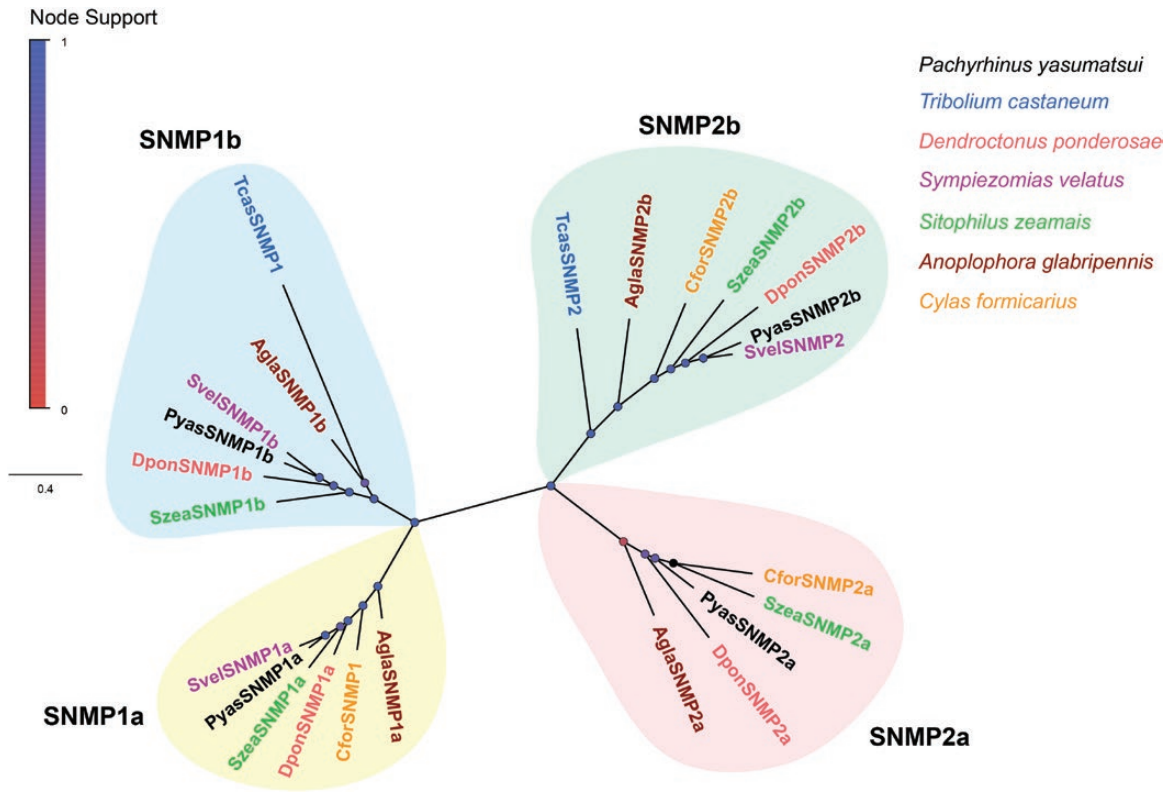

Fig. 8. Phylogenetic tree of 24 SNMPs. These sequences were obtained from 7 Coleopteran species (4 from *Pachyrhinus yasumatsui*, 2 from *Tribolium castaneum*, 4 from *Dendroctonus ponderosae*, 3 from *Sympiezomias velatus*, 4 from *Sitophilus zeamais*, 4 from *Anoplophora glabripennis*, and 3 from *Cylas formicarius*). The node support values are indicated by colored circles.

(e.g., *D. ponderosae* and *S. velatus*) (Anderssion et al. 2019, Li et al. 2022). Previous results indicated that 3 ORs (McarOR3/5/20) from *M. caryae* were characterized as pheromone receptors (PRs), which were closely associated with the perception of aggregation pheromones in *M. caryae* (Mitchell et al. 2012). It is worth noting that PyasOR16 was the ortholog of SvelOR18, and these 2 ORs clustered together with McarOR5 in the same clade of the phylogenetic tree (Fig. 5). Consequently, further studies are needed to confirm whether PyasOR16 has similar functions to McarOR5.

Nine *GRs* were found in *P. yasumatsui* in the present study, and the number of *PyasGRs* is less than in *E. brandti* (17), *R. ferrugineus* (15), and *S. velatus* (15) of Curculionidae. This may be due to some putative PyasGRs being expressed at very low levels and overlooked in our antennal transcriptome. According to previous studies, 3 CO_2 receptor-encoding genes are usually found in insects (Xu et al. 2012, Lu et al. 2007). However, only one CO_2 receptor gene, *PyasGR1*, which belongs to the GR1 subfamily, was identified in *P. yasumatsui* (Fig. 6). In addition to CO_2 receptors, 3 PyasGRs (PyasGR2/5/6) and PyasGR3 were identified as putative sugar-taste receptors and fructose-sensing receptor, respectively (Fig. 6). Since GRs are primarily expressed in the mouthparts (such as proboscises and labial palps of adults) rather than in the antennae (Clyne et al. 2000, Sparks et al. 2013), antennal expression levels of all the *PyasGRs* were the lowest among various gene families. Therefore, more GRs should be identified from transcriptomes of other adult tissues in *P. yasumatsui* in subsequent studies.

In this study, we identified 16 *IRs/iGluRs* from the antennal transcriptome of *P. yasumatsui*, which was similar to that in *D. ponderosae* (15) and *E. scrobiculatus* (17). Phylogenetic analysis indicated *PyasIR8a* and *PyasIR25a* were clustered into the IR8a/IR25a group and were the orthologs of *SvelIR8a* and *DponIR25a*, respectively (Fig. 7), suggesting that they may act as co-receptors and participate in many physiological processes in insects (Rytz et al. 2013). Furthermore, 7 *IRs* (*PyasIR21a /64a/75b/75c/75s/93a/76b*) were also identified as antennal IRs. As a result of their high similarity (57.1–73.0% identity) to those in other insects (Supplementary Table S3), these IR orthologs in *P. yasumatsui* may perform a similar function in sensory perception. Among these antennal IRs, *PyasIR93a* and *PyasIR76b* were expressed at relatively higher levels, similar to *C. formicarius* (Bin et al. 2017) and *A. tumida* (Wu et al. 2021). Notably, one non-NMDA iGluR (PyasNmdar1) was identified by phylogenetic analysis in this study, which is rare in other Curculionidae species (Gu et al. 2015, Wen et al. 2018, Li et al. 2022).

Based on phylogenetic analyses, we identified 2 orthologs for SNMP1 (*PyasSNMP1a/1b*), as well as 2 orthologs for SNMP2 (*PyasSNMP2a/2b*) from *P. yasumatsui* (Fig. 8), which are in agreement with the observations in *D. ponderosae*, *S. zeamais*, and *A. glabripennis*. Additionally, FPKM analysis showed that antennal expression levels of *PyasSNMP1* were significantly higher than those of *PyasSNMP2* (Supplementary Table S4), and identical results were seen with *C. formicarius* (Bin et al. 2017) and *S. velatus* (Li et al. 2022), indicating that SNMP1 and SNMP2 may have distinct functions in the olfactory system (Forstner et al. 2008, Nichols and Vogt 2008). However, there is still a lack of information regarding the detailed molecular mechanisms of SNMPs in insects. As a result, some further studies are needed to explore their functions.

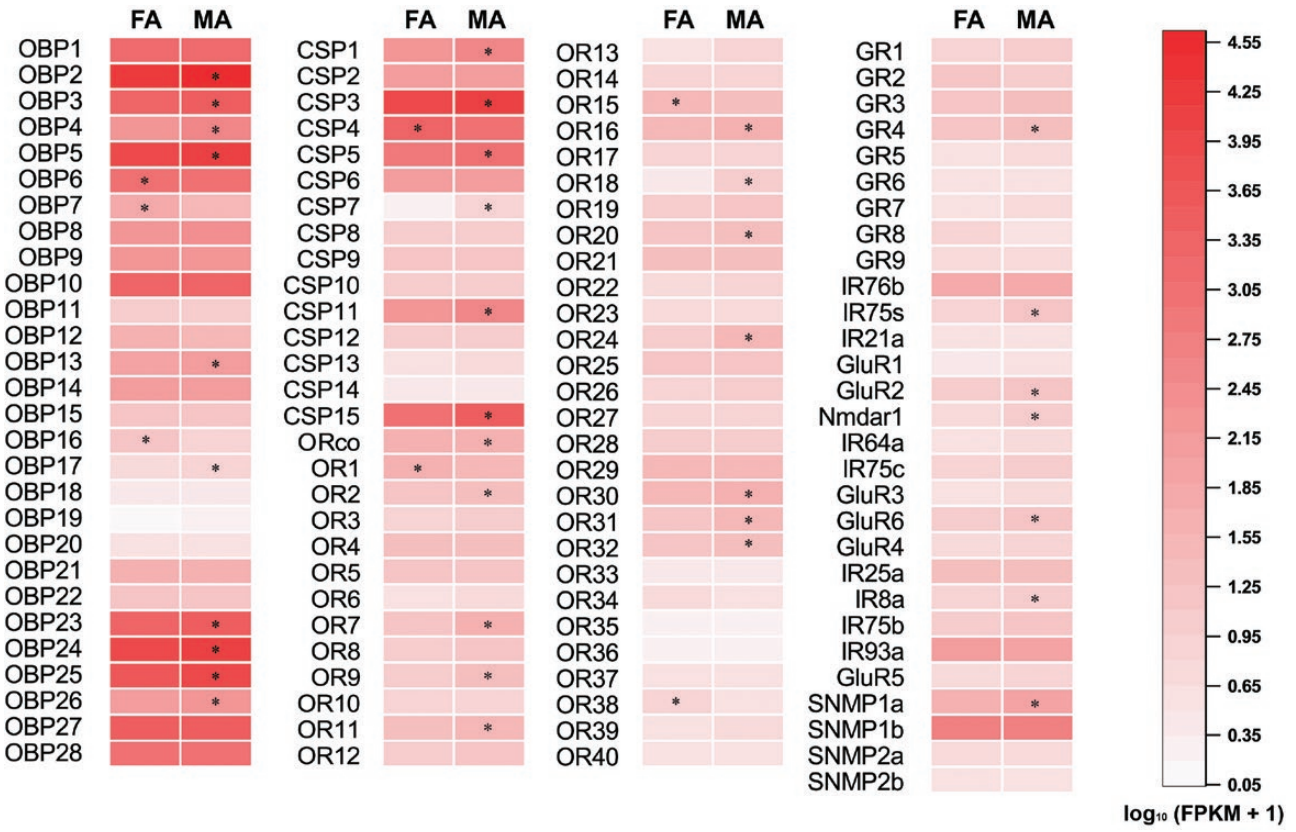

Fig. 9. Expression profiles (displayed as $\log_{10}$ (FPKM + 1)) of 113 chemosensory genes from *P. yasumatsui* according to average FPKM values. The asterisk represents a significant difference between female antennae (FA) and male antennae (MA) at the 0.05 level.

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Author Contributions

Bo Hong (Conceptualization-Equal, Data curation-Equal, Formal analysis-Lead, Investigation-Equal, Methodology-Lead, Writing – original draft-Lead, Writing – review & editing-Equal), Yingyan Zhai (Data curation-Equal, Investigation-Equal), Yiwei Yang (Data curation-Equal, Investigation-Equal), Qing Chang (Formal analysis-Supporting), Guangwei Li (Resources-Equal), Feng Zhang (Conceptualization-Equal, Writing – review & editing-Equal)

Supplementary Material

Supplementary material is available at *Journal of Insect Science* online.

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