Antennal transcriptome analysis and expression profiles of odorant binding proteins in two woodwasps, Sirex noctilio and S. nitobei (Hymenoptera: Siricidae)

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Abstract

Background The olfactory system is the foundation of insect behavior. Odorant binding proteins (OBPs) are key components of the insect olfactory system. The woodwasp Sirex noctilio Fabricius is a major quarantine pest worldwide that was first discovered in China in 2013 and mainly harms members of the Pinus genus. S. nitobei Matsumura is a native species in China and is closely related to S. noctilio. To gain insights into the olfactory mechanisms of these two woodwasp species, olfactory genes were identified using antennal transcriptome analysis. We also analyzed the expression profiles of OBPs with RT-qPCR. Results From our transcriptome analysis, 16 OBPs, 7 chemosensory proteins (CSPs), 41 odorant receptors (ORs), 8 gustatory receptors (GRs), 13 ionotropic receptors (IRs), and one sensory neuron membrane protein (SNMP) were identified in S. noctilio, while 15 OBPs, 6 CSPs, 43 ORs, 10 GRs, 16 IRs, and 1 SNMP were identified in S. nitobei. Most of the olfactory genes identified in two species were homologous. However, some species-specific olfactory genes were identified in the antennal transcriptomes, including SnocOBP13, SnocCSP6, SnocOR26, SnitGR9, and SnitIR17. In total, 14 OBPs (7 in S. noctilio and 7 in S. nitobei) were expressed primarily in the antennae of the two woodwasps. SnocOBP11 and SnitOBP11 were highly expressed in antennae and were also clearly expressed in the external genitalia. SnocOBP3 is highly expressed in the genitalia of females, and SnocOBP7 and SnitOBP7 are highly expressed in the genitalia of males. Meanwhile, SnocOBP10 was specifically expressed in male heads. Conclusion In total, 86 olfactory proteins were identified in S. noctilio, and 91 were identified in S. nitobei. Most SnocOBPs and SnitOBPs displayed enriched expression in the antennae, which are involved in odor recognition. A few OBPs were mainly expressed in the external genitals or heads and exhibited an obvious sex bias, which may indicate that the external genitals and heads are able to recognize sex pheromones or plant volatile compounds as a part of normal behaviors such as feeding, mating, or spawning. Our study provides key insights regarding the mechanism of interactions between the insect olfactory system and specific odor molecules.

Background

The woodwasp *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) is native to Europe, Asia, and North Africa, and is attracted to dead or dying pines [1-3]. Due to increased human movement and trade, woodwasps have spread to Oceania, Africa, North America, and South America and have become a globally invasive insect species [4]. Because of the lack of competing species and natural predators, *S. noctilio* has had a major economic impact on various pines in invaded areas [4]. In August 2013, *S. noctilio* was first found in Heilongjiang and then in Liaoning, Jilin, and Inner Mongolia, China [5]. In contrast, *S. nitobei* Matsumura, a species closely related to *S. noctilio*, is native to China, Japan, and North Korea. It has posed a hazard on ancient and debilitated pines such as the *Pinus tabuliformis* in Xiangshan Park, Beijing, China [6] and is a significant threat to other pine species such as *P. armandi*, *P. tabuliformis*, and *Larix* spp. [7]. In China, both species also harm *P. sylvestris* var. *mongolica* Litv. [8].

As wood-boring insects, woodwasps feed on wood during the larval period of their development. Adult woodwasps do not feed and only live for approximately a week [9-10]. Female adults tend to attack

stressed or weakened pines, where they lay eggs and inject toxic mucus and symbiotic fungus into the host [2,11]. The affected pine trees fall into decline and display symptoms such as resinosis, interior blue staining, premature senescence, reduced growth rates, and death [12].

In order to reduce the spread of and damage inflicted by woodwasps, it is important to develop effective detection tools to monitor their populations. Trap trees treated with herbicide or girdling have been used to monitor and survey *S. noctilio* populations [3, 13]. Weak *P. tabuliformis* trees have been harvested for use as trap trees for attracting *S. nitobei* [6]. The trap-tree method has been found to be effective but is expensive and difficult to implement. Kairomone (plant volatiles) lure traps are the most effective in areas where *S. noctilio* populations are large, but the traps are known to be ineffective in areas with small populations, such as newly infested areas in China [14]. Pheromones are also commonly used to develop attractants, and several pheromone compounds for *S. noctilio* have been discovered [15-16]. However, traps with pheromone attractants were found less efficient against *S. noctilio* [17].

Insects use their olfactory systems to sense odors and changes in the environment and thus, to adjust behaviors such as locating hosts for food, mating, and spawning [18]. In this study, we explore the woodwasp olfaction system, particularly, olfactory proteins and their expression profiles in antennae. The antenna is the most important organ for olfactory recognition and sensing of pheromones or plant volatiles. There are multiple olfactory sensilla distributed on insect antennae, which house olfactory sensory neurons (OSNs). Odor molecules pass through pores on the sensilla and enter the sensillum lymph [19-20]. It has been thought that odorant binding proteins (OBPs) and chemosensory proteins (CSPs) in the lymph can recognize, bind, and transport odor molecules. The OBP/CSP-odor molecule complexes then interact with chemosensory receptors, which are located in the dendritic membrane of OSNs [21-22]. These receptors convert the chemical signals into electrophysiological signals and transmit these signals to the central nervous system of insects through axons [23-24]. These signals are integrated in the insect brain to produce behavioral instructions for insects to respond accordingly [25]. At the same time, odor molecules are degraded by odorant-degrading enzymes (ODEs) [26-27].

OBPs are soluble proteins with low molecular weights and are mainly expressed in the lymph of antenna sensilla [28-29]. Some OBPs exhibit specific binding with hydrophobic odorant molecules and can deliver the molecules to membrane-binding receptors [30-33]. Typical OBPs have six conserved cysteine residues, which form three pairs of disulfide bridges [28-29, 34-35]. OBPs can be divided into different subtypes according to the number and arrangement of cysteine residues in its sequence. These subtypes include classic OBPs (6 conserved cysteine residues), Plus-C OBPs (8 conserved cysteine residues and a proline residue), and Minus-C OBPs (only 4 conserved cysteine residues) [36-37]. Within the classic OBP subtype, there are 2 special OBP members, the pheromone binding proteins (PBPs) and the general odorant binding proteins (GOBPs). PBPs have been found to be specific affinity proteins and are involved in sex pheromone recognition [28, 38]. GOBPs achieve their function by binding with general odors such as plant volatiles [39]. CSPs have 4 conserved cysteine residues, fewer than are found in OBPs, and they bind far more odors than OBPs [29, 40]. In most insects, CSPs are widely expressed in both olfactory-

related organs and non-olfactory organs, which may indicate that the function of CSPs is not limited to transport of odor molecules in olfaction but also includes growth and development [41, 42].

Chemosensory receptors are transmembrane proteins and include odorant receptors (ORs), ionotropic receptors (IRs), gustatory receptors (GRs), and sensory neuron membrane proteins (SNMPs). ORs are seven-transmembrane-domain proteins that sense plant volatile odorants [43] as well as pheromones (as pheromone receptors [PRs]) [44-45]. A highly conserved OR, ORco [46], is involved in the localization of ORs to ORN (olfactory receptor neurons) dendrites, enhances odorant responsiveness without altering ligand specificity, and forms complexes with other ORs [23, 47]. GRs are also seven-transmembrane-domain proteins and transport gustatory signals. Multiple GRs have been implicated in the detection of sweet tastes, bitter tastes, and CO2 [48-49]. IRs evolved from ionotropic glutamate receptors (iGluRs) and are conserved in insects [50]. In Drosophila, the majority of IRs have been identified as receptors for amines and acids, whereas ORs are receptors for esters and alcohols [51]. Two slowly evolving receptors, IR8a and IR25a, have been identified as co-receptors that can form complexes with other, more divergent IRs and direct their function as ligand-gated cation channels [52]. SNMPs are membrane proteins of insect olfactory neurons that are homologous with the vertebrate CD36 family and are thought to function as receptors of odorant binding proteins and mediate ligand delivery to chemosensory receptors [53-55].

In this study, we performed a preliminary exploration of the olfactory systems of two species of woodwasps, *S. noctilio* and *S. nitobei*. Ours is the first description of differential expression profiles of OBPs between different tissues and between sexes. We identify genes encoding olfactory proteins via analysis of the antennal transcriptomes of *S. noctilio* and *S. nitobei* and measure the transcript expression of important OBP genes in different tissues of both male and female adults of the two woodwasps using a quantitative real-time PCR method. We found that the olfactory genes of the two woodwasp species are extremely similar, and many are homologous. However, some species-specific olfactory genes were identified in both *S. noctilio* and *S. nitobei*. In total, 14 OBPs (7 in *S. noctilio* and 7 in *S. nitobei*) were primarily expressed in the antennae of two woodwasps. Among them, *SnocOBP11* and *SnitOBP11* not only have high expression in antennae, but also display notable expression in the external genitalia. We also observed several OBPs that are specifically expressed in the external genitals (*SnocOBP3* is expressed mainly in the female genitalia, while *SnocOBP7* and *SnitOBP7* are expressed mainly in male genitalia) or the head (*SnocOBP10* is expressed mainly in the male head). Taken together, our findings reveal molecular mechanisms driving the function of the woodwasps olfactory system.

Results

Transcriptome sequencing and sequence assembly

Using transcriptome sequencing, a total of 174,174,820 and 168,012,792 raw reads were obtained from male and female antennae, respectively, of *S. noctilio*, and a total of 165,394,906 and 164,334,008 raw reads were obtained from male and female antennae, respectively, of *S. nitobei* (Table 1). By removing

low-quality and trimmed reads less than 20 nt in length, 168,575,526 and 164,447,898 clean reads were obtained for male and female *S. noctilio*, respectively, and 161,515,996 and 160,823,260 clean reads were obtained for male and female *S. nitobei*, respectively, to be used for de novo assembly (Table 2). Clean reads from *S. noctilio* were assembled into 47,253 unigenes, with a total length of 61,586,545 base pairs (bp), an average length of 1,303 bp, and a maximum length of 56,024 bp. The sequence length distribution analysis indicated that 16,625 unigenes (35.18%) were longer than 1,000 bp (Table 3). Clean reads from *S. nitobei* were assembled into 46,866 unigenes, with a total length of 55,062,400 bp (Table 4) and an average length of 1,175 bp. Unigenes ranged from 201-39,567 bp, with 13,634 of the unigenes >1,000 bp in length. The raw reads for *S. noctilio* and *S. nitobei* have been deposited in the NCBI SRA database.

Table 1 Summary of raw reads obtained from *S. noctilio* and *S. nitobei* antennal transcriptomes

Sample_ID	Total_Reads	Total_Bases	Error%	Q20%	Q30%	GC%
*Snoc*1_F	51044156	7707667556	0.0145	96.76	92.3	42.67
*Snoc*2_F	59929292	9049323092	0.0144	96.8	92.36	42.6
*Snoc*3_F	57039344	8612940944	0.0142	96.9	92.63	39.02
*Snoc*1_M	64496020	9738899020	0.0132	97.44	93.67	41.36
*Snoc*2_M	56140478	8477212178	0.0141	96.96	92.64	46.55
*Snoc*3_M	53538322	8084286622	0.0146	96.72	92.21	42.87
*Snit*1_F	65908130	9952127630	0.0143	96.88	92.51	42.13
*Snit*2_F	48942274	7390283374	0.014	96.96	92.79	36.92
*Snit*3_F	49483604	7472024204	0.0142	96.87	92.64	36.44
*Snit*1_M	54558776	8238375176	0.0146	96.73	92.18	42.19
*Snit*2_M	57064726	8616773626	0.0143	96.92	92.57	42.15
*Snit*3_M	53771404	8119482004	0.0143	96.87	92.51	41.91

Table 2 Summary of clean reads obtained from *S. noctilio* and *S. nitobei* antennal transcriptomes

Sample_ID	Total_Reads	Total_Bases	Error%	Q20%	Q30%	GC%
*Snoc*1_F	49891070	7371454411	0.0127	98.18	94.49	42.48
*Snoc*2_F	58669346	8664078283	0.0127	98.18	94.49	42.41
*Snoc*3_F	55887482	8253513321	0.0125	98.25	94.71	38.84
*Snoc*1_M	62192700	9219770317	0.0119	98.52	95.37	41.23
*Snoc*2_M	54045142	7992360451	0.0125	98.25	94.65	46.39
*Snoc*3_M	52337684	7728241956	0.0128	98.16	94.42	42.68
*Snit*1_F	64371826	9532004071	0.0126	98.24	94.63	41.98
*Snit*2_F	47969434	7087270978	0.0124	98.29	94.83	36.73
*Snit*3_F	48482000	7151845379	0.0125	98.26	94.77	36.21
*Snit*1_M	53213400	7872680155	0.0128	98.15	94.39	42.03
*Snit*2_M	55797468	8257697138	0.0126	98.24	94.62	41.99
*Snit*3_M	52505128	7771215028	0.0126	98.25	94.65	41.76

Table 3 Assembly statistics for the *S. noctilio* antennal transcriptome

Type	Total sequences	Total sequence (bp)	GC%	Max length (bp)	Min length (bp)	Average length (bp)	N50	N90
Unigene	47253	61586545	40	56024	201	1303.34	2536	609
Transcript	66977	126429451	40.09	56024	201	1887.65	3937	800

Table 4 Assembly statistics for the *S. nitobei* antennal transcriptome

Type	Total sequences	Total sequence (bp)	GC%	Max length (bp)	Min length (bp)	Average length (bp)	N50	N90
Unigene	46866	55062400	40.71	39567	201	1174.89	2722	406
Transcript	64055	113931759	40.32	39567	201	1778.66	4181	745

Homology analysis and gene ontology annotation

In total, 20,053 unigenes from *S. noctilio* (42.44% of 47,253 unigenes) were annotated in at least one of the databases searched (Nr, Pfam, KOG, COG, Swiss-Prot, KEGG, eggNOG, and GO databases). Homology searches against the Nr database showed that the *S. noctilio* antennal transcriptome shared the greatest homology with sequences from *Apis mellifera* (13%), followed by *Nasonia vitripennis* (11%) and

Harpegnathos saltator (10%). For the *S. nitobei* transcriptome, 25,278 unigenes (53.94% of 46,866 unigenes) were annotated in at least one of the databases. Nr database homology searches showed that the *S. nitobei* antennal transcriptome shared the greatest homology with sequences from *A. mellifera* (9.17%), followed by *N. vitripennis* (8.20%) and *Megachile rotundata* (7.27%).

Among the 47,253 *S. noctilio* and 46,866 *S. nitobei* unigenes, 10,556 (22.3%) and 13,487 (28.8%), respectively, correspond to at least one GO term. GO annotation indicated that the distributions of GO terms in the unigenes sets were highly similar between the two species (Figs. 1 & 2). Within the biological process category, the most abundant terms were 'cellular process' 'single-organism process,' and 'metabolic process.' 'Cell' and 'cell part' were the most commonly represented of the cellular component terms. In the molecular function category, 'binding' and 'catalytic activity' were the most abundant terms.

Identification and analysis of chemosensory-related genes

Odorant binding proteins

We identified 16 and 15 *OBP*s in the *S. noctilio* and *S. nitobei* antennal transcriptomes, respectively (Additional file 1, Table S1). Both *S. noctilio* and *S. nitobei* contained 15 full-length *OBP*s with complete open reading frames (ORFs) of at least 300 bp and a signal peptide (except SnocOBP13). According to the *OBP* classification system, in both species, two *OBP*s (*SnocOBP11* and *SnitOBP11*) were found to be members of the Minus-C *OBP* subclass, members of which are characterized by their lack of two cysteine residues (C2 and C5). No Plus-C *OBP*s were found in either the *S. noctilio* or the *S. nitobei* transcriptome. Two woodwasp *OBP*s (*SnocOBP9* and *SnitOBP9*) were homologous to *PBP*s of *A. mellifera*. Five *S. noctilio OBP*s (*SnocOBP3*, *SnocOBP4*, *SnocOBP10*, *SnocOBP14*, and *SnocOBP15*) and five *S. nitobei OBPs* (*SnitOBP3*, *SnitOBP4*, *SnitOBP10*, *SnitOBP14*, and *SnitOBP15*) exhibited similarity with *GOBP*s of other insects by NCBI BLASTX. The cysteine sequence pattern of the full-length classic OBPs was found to be C1-X26-32-C2-X3-C3-X36-42-C4-X8-12-C5-X8-C6, and the sequence produced by ClustalW displayed 6 conserved cysteine residues (Additional file 3). We found 13 *SnocOBP*s and 14 *SnitOBP*s with expression values greater than 1 FPKM, while 6 *SnocOBP*s and 7 *SnitOBP*s exhibited expression values greater than 100 FPKM, indicating high expression of these OBPs in the antennae.

Construction of a phylogenetic tree was used to compare insect OBP protein sequences from members of the Hymenoptera, Diptera, and Lepidoptera (Fig. 3). According to the OBP phylogenetic tree, most SnocOBP and SnitOBP sequences clustered together. With a 1.00 bootstrap support value, the PBP lineages contained SnocOBP9, SnitOBP9, and other Hymenopteran PBPs, which further confirmed that the two OBPs could be PBPs. OBP4, OBP7, and OBP10 of both woodwasps were clustered in the GOBP lineages with 0.75, 1.00, and 1.00 bootstrap support values, respectively.

Chemosensory proteins

We identified 7 *SnocCSP*s and 6 *SnitCSP*s in the antennal transcriptomes of the two woodwasp species (Additional file 1, Table S2). Among all *CSP*s, 5 *CSP*s in *S. noctilio* and 4 in *S. nitobei* were full-length *CSP*s

with complete ORFs, signal peptides, and a cysteine sequence pattern of C1-X5-8-C2-X18-C3-X2-C4 (Additional file 4). The expression values (FPKM) of 4 *SnocCSP*s and 5 *SnitCSP*s were greater than 1, while 1 *SnocCSP* and 3 *SnitCSP*s displayed expression values greater than 100, indicating that these genes are highly expressed in woodwasp antennae.

As with OBPs, a phylogenetic tree was used to show the evolutionary relationships between insect CSPs (Fig. 4). In the phylogenetic tree, most *SnocCSP*s and *SnitCSP*s clustered with other Hymenopteran CSPs.

Odorant receptors

We identified 41 and 43 ORs in the *S. noctilio* and *S. nitobei* antennal transcriptomes, respectively (Additional file 1, Table S3). Two woodwasp *OR* transcripts were identified as odorant co-receptors, and designated as *SnocORco* and *SnocORco*, respectively.

In total, 28 *SnocOR*s and 30 *SnitOR*s were comprised of greater than 350 amino acids and contained complete ORFs, indicating that they are nearly full-length. Using the TMHMM Server, we predicted the presence and location of transmembrane helices in the protein sequences. We found that 4 full-length *SnocOR*s (*SnocORco*, *SnocOR5*, *SnocOR8*, and *SnocOR30*) and 3 full-length *SnitOR*s (*SnitORco, SnitOR8*, and *SnitOR30*) possess 7 transmembrane helices. No transmembrane helices were predicted in *SnocOR32*, *SnocOR33, SnitOR31*, and *SnitOR32*, which is likely due to short fragments and incomplete reading frames. Four *OR*s (*SnocOR18*, *SnocOR30, SnitOR18* and *SnitOR30*) displayed a >10-fold difference in expression between males and females. The difference in the expression levels between the sexes of these 4 genes suggests that they play a role in identifying gender-related odors.

A phylogenetic tree was used to analyze the evolutionary relationships between insect ORs. Most SnocORs and SnitORs clustered together (Fig. 5), and 2 species-specific lineages were identifed in the tree. The ORco lineage contains SnocORco and SnitORco (1.00 bootstrap support value), which further confirms that these two OBPs are ORcos.

Sensory neuron membrane proteins

We identified one *SNMP* in *S. noctilio* (*SnocSNMP1*) and one in *S. nitobei* (*SnitSNMP1*) (Additional file 1, Table S4). SnocSNMP1 and SnitSNMP1 are predicted to possess 2 transmembrane regions, which may indicate that *SnocSNMP1* and *SnitSNMP1* are full-length genes. The expression values (FPKM) for *SnocSNMP1* and *SnitSNMP1* were both found to be greater than 100, indicating that *Sirex SNMP*s are highly expressed in antennae.

SNMPs are considered to be highly conserved in holometabolous insects, but SNMP1 and SNMP2 which are members of different subfamilies, clustered separately in disparate lineages. In our phylogenetic tree, SnocSNMP1 and SnitSNMP1 clustered in the SNMP1 lineage with a bootstrap support value of 1.00 (Fig.6).

Gustatory receptors

We identified 8 and 10 *GR*s in the *S. noctilio* and *S. nitobei* antennal transcriptomes, respectively (Additional file 1, Table S5). Using BLASTX sequence alignment, we found that 2 and 7 of the *SnocGR*s and *SnitGR*s, respectively, are GRs for sugar taste, and most were found to be trehalose receptors.

In the phylogenetic tree (Fig. 7) of GR sequences, there are two sugar taste lineages and two bitter taste lineages. One SnocGR and 3 SnitGRs clustered in the sugar taste lineages and no *Sirex* GRs clustered in the bitter taste lineages. Most *Sirex* GRs exhibited homology to sugar taste receptors, which may indicate that the function of GRs in the *Sirex* olfactory system is to detect carbohydrates.

Ionotropic receptors

We identified 13 and 16 IRs in the *S. noctilio* and *S. nitobei* antennal transcriptomes, respectively (Additional file 1, Table S6). The expression values (FPKM) of 4 *SnocIR*s and 11 *SnitIR*s were greater than 1. Of these, *SnocIR6* and *SnitIR6* have the greatest expression values in the antennal transcriptome.

Previous studies have indicated that IR8a and IR25a are the co-receptors of IRs. In our phylogenetic tree (Fig. 8), SnocGR6 and SnitGR6 clustered in the IR8a lineages with a bootstrap support value of 1.00, while SnocGR4 and SnitGR4 clustered in the IR25a lineages with a bootstrap support value of 1.00. IR6 and IR4 exhibit high expression in *Sirex* transcriptomes, which may indicate that the IRs are co-receptors for IRs. Additionally, two pairs of NMDA receptors are found in the phylogenetic tree.

Homologous olfactory system genes in *S. noctilio* and *S. nitobei*

Among the olfactory genes identified in the transcriptome, we observed that some of the same types of olfactory genes are located on the same transcripts, such as *SnocOBP10* and *SnocOBP16*, and *SnitCSP3* and *SnitCSP4*. The distances between them are nearly 1,000 bp, which may explain their evolutionary relationship.

We used the olfactory genes of the two woodwasp species to build multiple phylogenetic trees (Fig. 9). In our phylogenetic tree, most of the *S. noctilio* and *S. nitobei* olfactory genes clustered together. Additionally, we found that most of the *S. noctilio* and *S. nitobei* olfactory genes are homologous, supporting the close evolutionary relationship between the two species.
Heatmap analysis demonstrates the differential expression between homologous genes of *S. noctilio* and *S. nitobei*, such as *SnocOR1* and *SnitOR1*, *SnocOR3* and *SnitOR3*, *SnocOR5* and *SnitOR5*, *SnocOR17* and *SnitOR17*, and *SnocOR20* and *SnitOR20*. These differentially expressed genes may denote molecular mechanisms underlying the reproductive isolation between the two species.

Fluorescent quantitative real-time PCR

To verify *OBP* expression in antennae and characterize the expression profiles of *OBP*s in 4 chemosensory tissues (antennae, legs, heads, and externalia), 10 *SnocOBP*s and 10 *SnitOBP*s with high FPKM values were selected for fluorescent quantitative real-time PCR (Fig. 10 & Fig. 11). Primers for *OBP*s and for an internal reference gene (*β-tubulin*) are listed in the Additional file 2. Most *OBP*s were

expressed mainly in the antennae of the two woodwasps. The observed high expression in the antennae indicated that the OBPs may play a role in binding and transporting odor signals in antennae.

For both *OBP*s that were identified as *PBP* homologues, *SnocOBP9* and *SnitOBP9*, we observed high expression in antennae but no expression in the other organs. The expression profiles indicate that the primary expression of these two OBPs occurs in the antennae, and both OBPs show a weak expression bias between the two sexes. *SnocOBP11* and *SnitOBP11* not only display high expression in the antennae, but also have obvious expression in the external genitalia. Additionally, *SnocOBP11* and *SnitOBP11* are more highly expressed in the female external genitalia and male antennae when compared to the male external genitalia and female antennae.

High expression of *SnocOBP7* and *SnitOBP7* was detected in male externalia, and *SnocOBP3* was primarily expressed in the genitalia of female *S. noctilio*. *SnocOBP10* was mainly expressed in male heads, while *SnitOBP3* and *SnitOBP10* did not show obvious tissue bias due to low expression levels.

Significant sex-biased expression was observed for many OBP genes, including *SnocOBP3*, *SnocOBP4*, *SnocOBP7*, *SnocOBP10*, *SnocOBP15, SnitOBP4*, *SnitOBP7*, and *SnitOBP15*. This sex bias may denote different functions of these OBPs between males and females, such as the perception of the opposite sex or oviposition behavior. We found that some homologous genes differ greatly in their expression profiles between the two species, especially those not expressed in the antennae. The differential expression of these homologous genes may indicate that they strengthened or lost their original function during species differentiation, resulting in olfactory differences between the two species.

Discussion

The number of OBPs varies greatly among different species

Evolution of OBPs is mainly driven by lineage-specific amplification, with few distinct homologues in non-relative species [56-58]. Most OBPs in *S. noctilio* and *S. nitobei* are orthologous and display strong homology with other Hymenopteran species, further illustrating the evolutionary relationship of OBPs between these species.

Compared with the OBPs of model species, the numbers of OBPs encoded in the genomes of *Sirex* spp. were less than those in *Drosophila melanogaster* (51 OBPs), *Anopheles gambiae* (57 OBPs), *Bombyx mori* (44 OBPs), *Apis mellifera* (21 OBPs), and *Nasonia vitripennis* (82 OBPs) [56, 58-61]. In Hymenoptera, there are species with a large number of OBPs gained from transcriptomes, such as those of *Aenasius bambawalei* (54 OBPs), while there are also species with few OBPs, such as *Osmia cornuta* (6 OBPs), *Ceratosolen solmsi* (7 OBPs), and *Microplitis mediator* (9 OBPs) [62-65]. It is generally believed that more singular and closed environments are associated with less lineage-specific evolution of olfactory function. Consequently, these insects have fewer olfactory genes as a result of positive selection pressure [64, 66]. *S. noctilio* and *S. nitobei* live in pure forests or mixed coniferous forests and thus are more likely

to receive plant volatiles or interspecies pheromone substances with a more concentrated population density, so the number of OBPs in *Sirex* spp. is less.

Special subfamilies of OBPs in *Sirex*

We did not uncover any Plus-C OBPs in the transcriptomes of *S. noctilio* and *S. nitobei*. Plus-C OBPs were found in Lepidoptera (*B. mori*), Diptera (*D. melanogaster*), Coleoptera (*Anoplophora chinensis*), but were not found in existing Hymenoptera genomes or transcriptomes [67]. For example, Plus-C OBPs were not found in the genomes of Hymenoptera such as *A. mellifera* and *N. vitripennis*. This finding suggests that the Plus-C subtype is rare or even absent in Hymenoptera and has had a weak influence on the evolution of olfactory recognition in Hymenoptera.

Although the Plus-C subtype has not been discovered in Hymenoptera, the Minus-C subtype has been widely observed in Hymenopterans. For example, *AmelOBP14-21* and *NvitOBP27, -38, -56, and -58–62* are Minus-C OBPs. We identified one Minus-C OBP in each *S. noctilio* and *S. nitobei*. These two Minus-C OBPs are not homologous with the Minus-C OBPs of *A. mellifera* and *N. vitripennis*, but are homologous to a Classic OBP of *Meteorus pulchricornis*, *MpulOBP1* [68]. In *A. mellifera*, due to sequence similarity and evolutionary tree topology, the eight Minus-C OBPs and *AmelOBP13* are speculated to share a common evolutionary ancestor. It can be inferred that the Minus-C OBP originated from a classic OBP that lost two cysteine residues during evolution, rather than having evolved from a Minus-C OBP of another species. The expression values of the two Minus-C OBPs in the *Sirex* transcriptomes are extremely high, indicating that these two OBPs may play special and important roles in the olfactory system of woodwasps.

An olfactory gene in the venom gland of *S. noctilio*

In a previous report, four *OBP*s and five *CSP*s were found in the venom gland proteome of *S. noctilio* [69]. Through sequence alignment, it was found that the *OBP*s identified in the venom gland were *SnocOBP2*, *SnocOBP6*, *SnocOBP9*, and *SnocOBP11*, and the *CSP* identified was *SnocCSP2-5*, indicating that these *OBP*s and *CSP*s may play special roles in transport or recognition of chemical signals in both antennae and venom glands. *CSP5,* which was detected in the venom gland, was not detected in our antennal transcriptomes.

Olfactory genes colocalized on the same transcript

For various olfactory genes, we have found that two or more genes are colocalized on the same transcript. In previous studies, it was found that olfactory gene families expand through gene duplication and subsequent evolution [70]. The colocalization of olfactory genes on the same transcript indicates that these genes share a common ancestor gene and supports the gene duplication model of olfactory gene family expansion.

Characteristics and function of ORco and ORs with sex-biased expression

It has been shown that some ORs bind a single odor signal, while other ORs are able to respond to a low concentration of a single compound as well as higher concentrations of other substances [22]. ORs play

an important role in the selectivity of the insect olfactory system, but other olfactory proteins may contribute to its overall specificity [22]. ORco has been observed in Diptera, Lepidoptera, Coleoptera, and Hymenoptera. Compared to traditional odor receptors, ORco is highly conserved, and its homology among insects can reach 50%-99%. Amino acid sequence analysis revealed a highly conserved region at the end of the ORco sequence [71].

There are four OR genes (*SnocOR18*, S*nocOR30*, *SnitOR18*, *and SnitOR30*) in the two *Sirex* species, and their expression levels in the antennae display 10-fold differences between the sexes. Some of these OR genes exhibit high male-biased expression, suggesting that these four OR genes may function as sex pheromone receptors. *SnocOR30* and *SnitOR30* display high homology with *AmelOR170*. *AmelOR170* has shown a biased expression pattern in drone antennae, but the receptor does not bind 9-oxo-2-decenoic acid [72]. *MmedOR9*, which is homologous to *SnocOR18* and *SnitOR18*, is also highly expressed specifically in males, suggesting that these genes may be pheromone receptors that interact with each other in Hymenoptera [73].

Expression of trehalose receptors in *Sirex* spp.

We identified 2 and 7 sweet receptors in the transcriptomes of *S. noctilio* and *S. nitobei*, respectively. Most of these sweet receptors were found to be trehalose receptors. Trehalose is a non-reducing sugar composed of two α-glucose molecules joined by an 1,1-glycosidic bond. The sugar is chemically stable and protects plants, plant cells, and plant proteins from freezing and drying. It is a stress-resistant protection mechanism. Trehalose is also present in the body fluid of insects and can be used as a energy source for flying. As an important blood sugar in insects, trehalose is present in almost all tissues and organs of insects [74]. Trehalose can influence insects' choice for food via recognition by GRs. Thus, trehalose receptors are important for vital biological processes in insects.

The expression pattern of OBPs in *Sirex* spp.

SnocOBP9 and *SnitOBP9*, which are *PBP* homologues, are mainly expressed in antennae and thus could be speculated to play a key role in identification of pheromone components. The weak sex bias and high levels of tissue expression may indicate that both males and females need these OBPs to sense sex pheromones or aggregation pheromones.

SnocOBP11 and *SnitOBP11* display significant sex-biased expression in the female external genitalia and male antennae. This may indicate that OBP11 can detect and control the number of sex pheromones released by females, and that male woodwasps receive sex pheromones through OBP11. This expression profile is distinct from OBPs of other insects, further indicating that OBP11 may be specialized proteins that receive sex pheromones in woodwasps.

A substantial number of OBPs exhibit a highly specific expression pattern in the male and female genitalia, which may indicate that the external genitalia can recognize sex pheromones or plant volatile components, and help guide normal behaviors such as feeding, mating, or spawning.

Conclusions

By examining their antennal transcriptomes, we analyzed 86 and 91 olfactory genes from *S. noctilio* and *S. nitobei*, respectively. Additionally, we verified the expression of 10 *SnocOBP*s and 10 *SnitOBP*s in male and female antennae, respectively, thereby confirming the accuracy of our transcriptome data. The high expression of OBPs in antennae supports the function of OBPs in the antennal recognition process. A few OBPs that display biased expression in other organs suggest the presence of olfactory binding function in external genitals and heads.

The olfactory proteins of *Sirex* spp. were obtained through antennal transcriptome sequencing. However, in order to explore the olfactory mechanisms of *Sirex* spp., it is necessary to perform protein expression, fluorescence competition, and molecular docking studies. Substances should be screened for efficacy and timing for control of woodwasps in order to obtain substances that are active against *Sirex* spp. Then, behavior research and attachment development can be coordinated to regulate and control woodwasp behavior in order to specify directions for more effective prevention and control of woodwasps.

Materials And Methods

Sample collection and preservation

S. noctilio adults emerge in early July over a period of 2 months and *S. nitobei* adults emerge in early September, but adult woodwasps only live for 5-12 days [8, 10]. The *S. noctilio* and *S. nitobei* woodwasps used in these experiments were collected from Tongliao, Inner Mongolia. Injured wood that exhibited premature aging and teardrop-like flow gum points were selected. The selected woods were cut into 1-m long sections and placed in a net cage while waiting for the wasps to emerge. The wasps were caught immediately after eclosion and time, status, and sex information were recorded. We separated the antennae from the adults and put them in RNA later buffer solution (Invitrogen, Carlsbad, CA, USA). The antennae were stored at 4°C for 24 h and then stored at -20°C. The antennae were placed at -80°C for long-term storage at the Forest Conservation Laboratory.

RNA extraction and Illumina transcriptome sequencing

Total RNA was extracted from adult antennae of both sexes separately (20 antennae each from males and females) using the QIAGEN RNeasy Mini Kit (No. 74134; Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA concentration was quantified using a NanoDrop 8000 spectrophotometer (Thermo, Waltham, MA, USA). RNA quality was verified using a 2100 Bioanalyzer RNA Nanochip (Agilent, Santa Clara, CA, USA). The high-quality RNA samples (OD260/280=1.8-2.2, OD260/230≥2.0, RIN≥6.5, 28S:18S≥1.0, >10 μg) were placed at -80°C and were used to generate cDNA libraries.

The cDNA library construction and Illumina sequencing on the HiSeq 4000 platform were performed at Majorbio Corporation (Shanghai, China). RNA-seq transcriptome libraries were prepared using 5 μg of total RNA using the TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA, USA). First,

messenger RNA (mRNA) was isolated by oligo(dT) beads according to the poly-A selection method, and then the mRNA was fragmented using fragmentation buffer. Second, double-stranded cDNA was synthesized using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) with random hexamer primers (Illumina). Next, the synthesized cDNA was subjected to end-repair, phosphorylation, and 'A' base addition according to the Illumina library construction protocol. Libraries were size-selected for cDNA target fragments of 200-300 bp on 2% Low Range Ultra Agarose followed by PCR amplification using Phusion DNA polymerase (New England Biolabs) for 15 cycles. After quantification using a TBS380 mini fluorometer, the paired-end RNA-seq libraries were sequenced using an Illumina HiSeq 4000 to generate 150-bp paired-end reads.

Assembly and function annotation

Trimming and quality control of the raw paired-end reads was performed using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) to generate clean, high-quality reads. De novo transcriptome assembly was carried out with the short read assembly program Trinity (http://trinityrnaseq.sourceforge.net/) [75]. The consensus cluster sequences and singletons comprise the UniGene dataset. The annotation of unigenes was performed by NCBI BLASTX against a pooled database of non-redundant (Nr) String, KEGG, and SwissProt protein sequences with an E-value threshold <1e-5. The BLAST results were then processed with the Blast2GO program (http://www.blast2go.com/b2ghome) for gene ontology (GO) annotation, which described the function of unigenes, such as their biological processes, molecular functions, and cellular components [76]. The longest ORF for each unigene was determined by the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Expression levels are displayed as FPKM values (fragments per kilobase per million reads), which were calculated by RSEM (RNA-Seq by Expectation-Maximization) v1.2.6.

Identification of chemosensory-related genes

The annotation of olfactory unigenes, OBP, CSP, OR, GR, IR, and SNMP, was performed by NCBI BLASTX searches against the Nr protein database, with an E-value threshold of 1e-5. The available protein sequences from Insecta species were used to identify candidate olfaction unigenes in *S. noctilio* and *S. nitobei*. The candidate OBPs and PBPs were searched for the presence of N-terminal signal peptides using Signal P4.0 (http://www.cbs.dtu.dk/services/SignalP/), and the transmembrane domains of candidate ORs, IRs, GRs, and SNMPs were predicted using the TMHMM server v3.0 (http://www.cbs.dtu.dk/services/TMHMM/). Amino acid sequence alignment was performed using the Muscle method implemented in the MEGA v6.0 software package. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with the P-distances model and pairwise deletion of gaps in the MEGA v6.0 software package. The reliability of the tree structure and node support was evaluated by bootstrap analysis with 1,000 replicates. Only bootstrap values ≥0.5 were shown at the corresponding nodes. The lineages with bootstrap values ≥0.9 were labelled with red spots, and bootstrap values

between 0.7 and 0.9 were marked with blue spots. The phylogenetic trees were modified with ITOL (https://itol.embl.de/).

Expression analysis by quantitative real-time PCR

Fluorescent quantitative real-time PCR (RT-qPCR) was performed to verify the expression of candidate chemosensory genes. RT-qPCR experiments in *S. noctilio* and *S. nitobei* were performed separately. Antennae, feet (including the propodium, mesopodium, and metapedes), heads, and external genitals were collected from 10 male and 10 female adult woodwasps for each sample. Total RNA was extracted following the methods described above and used as template for cDNA synthesis using the PrimeScript RT Reagent Kit with gDNA Eraser (No. RR047A; Takara, Shiga, Japan). Gene-specific primers were designed using Primer3Plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) (see Additional file 2). β-Tubulin was identified from the *S. noctilio* antennal transcriptome and used as a reference gene. PCR analysis was conducted using the Bio-Rad CFX Connect PCR System (Hercules, CA, USA). SYBR Premix Ex Taq™ II (No. RR820A; Takara) was used for the PCR reaction. Each 25 μl PCR reaction contained 12.5 μl of SYBR Premix Ex Taq II, 1 μl of each primer (10 mM), 2 μl of sample cDNA (2.5 ng of RNA), and 8.5 μl of dH2O (sterile distilled water). The RT-qPCR cycling parameters were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and finally, 65°C to 95°C in increments of 0.5°C for 5 s to generate melting curves. To examine reproducibility, each qPCR reaction for each tissue was performed using 3 technical replicates. Negative controls without either template were included in each experiment. Bio-Rad CFX Manager (version 3.1.1517.0823) was used to normalize expression based on ΔΔCT values, with the male external genitals samples as control samples. The 2−ΔΔCT method was used to generate relative expression values.

Abbreviations

OSNs, olfactory sensory neurons; GO, Gene ontology; ORFs, Open reading frame; FPKM, fragments per kilobase per million reads; NJ, neighbor-joining; RT-qPCR, Fluorescent quantitative real-time PCR; OBPs, Odorant binding proteins; CSPs, Chemosensory proteins; ORs, Odorant receptors; Orco, Odorant receptor co-receptor; SNMPs, Sensory neuron membrane proteins; GRs, Gustatory receptors; IRs, Ionotropic receptors; ODEs, odorant-degrading enzymes;

Declarations

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Availability of data and materials

All supporting data is included within the article and its additional files. And the transcriptome data were submitted to NCBI, the accession number of *S. noctilio* are from SAMN11338151 to SAMN11338156 and the accession number of *S. nitobei* are from SAMN11338569 to SAMN11338574. All of the olfactory protein gene sequences were submitted to Genbank, accession number are MK425751- MK425766, MK674426- MK674453 and MK748989- MK749121.

Authors' contributions

Bing Guo collected almost all samples, carried out the molecular genetic studies, performed the sequence alignment, experiments and drafted the manuscript. Jing-Zhen Wang participated in some experiments and helped to draft the manuscript. Hai-Li Qiao and Wei-Wei Wu participated in all experiments. Jing-jiang Zhou participated in some experiments and helped to revise the manuscript. Peng-Fei Lu designed and conceived of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The woodwasps, *S. noctilio* and *S. nitobei* (Hymenoptera: Siricidae) are forestry pests in China, which collections were made with the direct permission of Tongliao forestry bureau. They are not included in the "List of Endangered and Protected Animals in China". All operations were performed according to ethical guidelines in order to minimize pain and discomfort to the insects.

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Supplemental File Legend

Additional file 1: Best blastX hits for putative odorant binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), sensory neuron membrane proteins (SNMPs), ionotropic receptors (IRs), and gustatory receptors (GRs) of *S. noctilio* and *S. nitobei*. (Table S1, Table S2, Table S3, Table S4, Table S5 and Table S6) (PDF 394 kb)

Table S1. Sequence information and best blasts match information of odorant binding proteins (OBPs).

Table S2. Sequence information and best blasts match information of chemosensory proteins (CSPs).

Table S3. Sequence information and best blasts match information of odorant receptors (ORs).

Table S4. Sequence information and best blasts match information of sensory neuron membrane proteins (SNMPs).

Table S5. Sequence information and best blasts match information of gustatory receptors (GRs).

Table S6. Sequence information and best blasts match information of ionotropic receptors (IRs).

Additional file 2: Primers of odorant binding proteins and reference gene for quantitative real-time PCR. (PDF 54.5 kb)

Additional file 3: The sequence of OBPs with alignment were shown six conserved cysteine residues. (PDF 67.2 kb)

Additional file 4: The sequence of CSPs with alignment were shown four conserved cysteine residues. (PDF 57 kb)

Additional file 5: The OBPs, CSPs, ORs, SNMPs, GRs and IRs of Hymenoptera, Diptera, Lepidoptera, Coleoptera, Blattaria and Orthoptera used in building neighbor-joining phylogenetic trees. (PDF 150 kb)

Figures

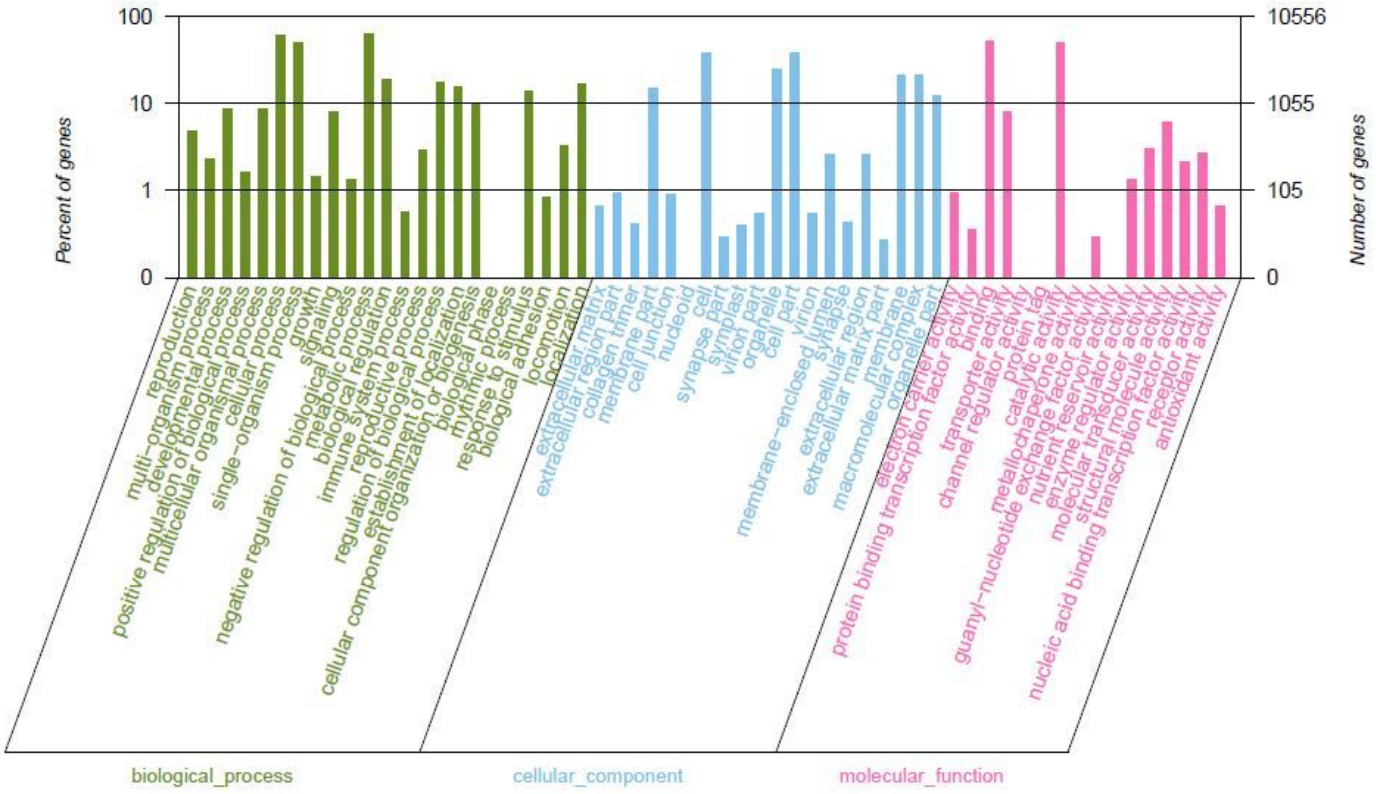

Figure 1

Gene ontology (GO) classification of S. noctilio unigenes obtained using the Blast2GO program

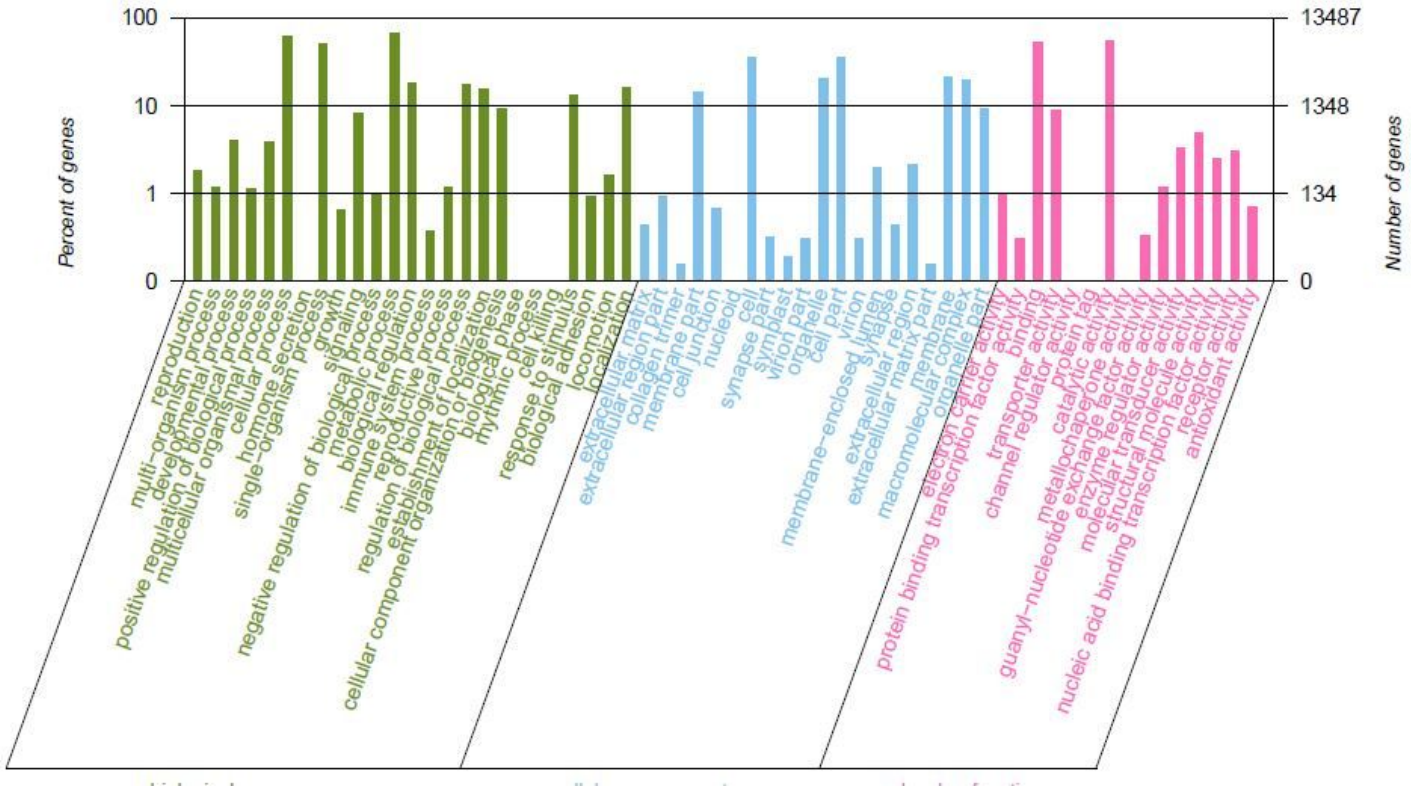

Figure 2

Gene ontology (GO) classification of S.nitobei unigenes obtained using the Blast2GO program

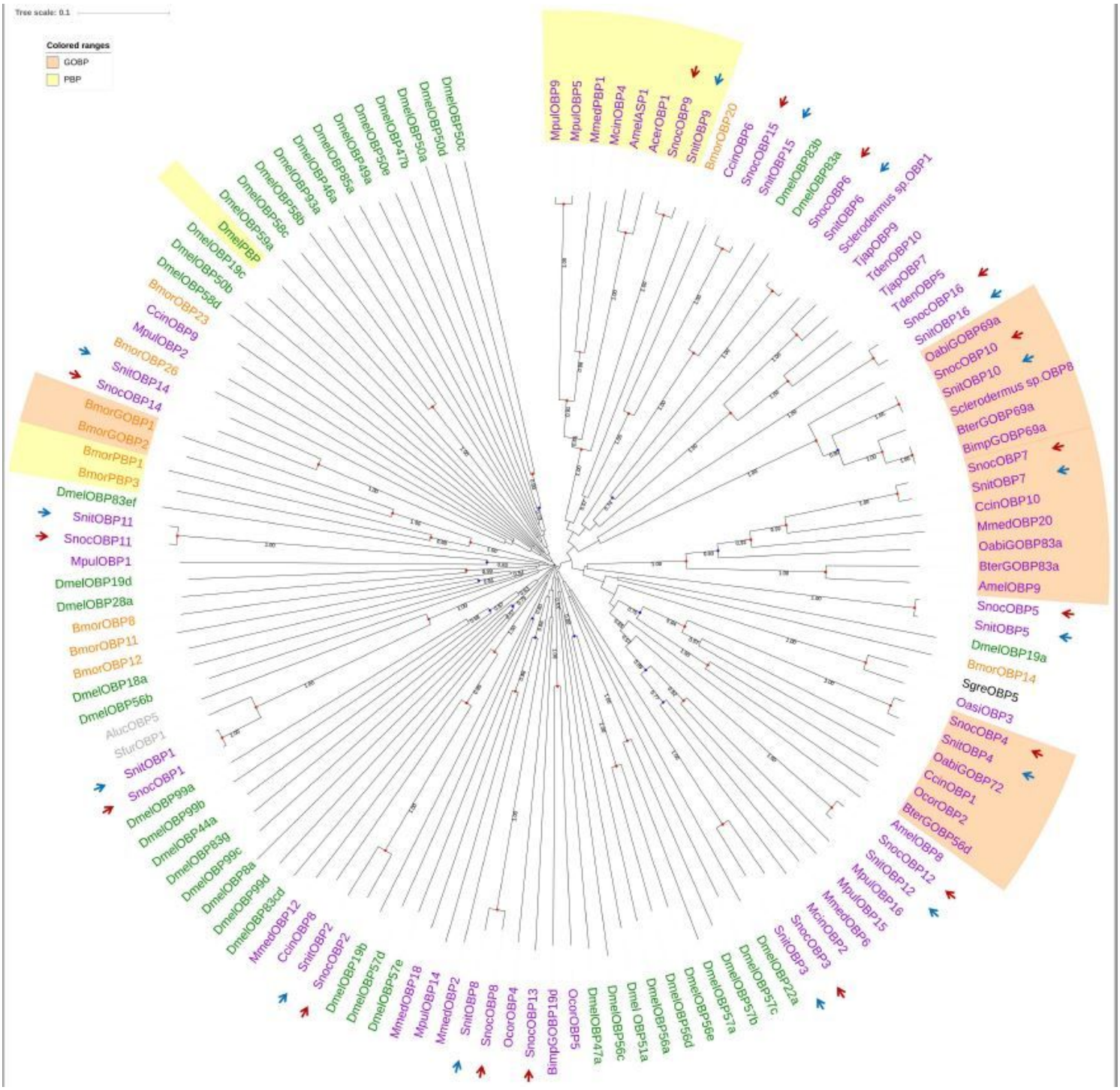

Figure 3

Candidate odorant binding proteins (OBPs) of Hymenoptera (purple), Diptera (green), Hemiptera (gray), Orthoptera (black) and Lepidoptera (orange) were included in a neighbor-joining phylogenetic tree. The PBP and GOBP lineages are labelled in yellow and orange, respectively. SnocOBPs and SnitOBPs are indicated with red and blue arrows, respectively.

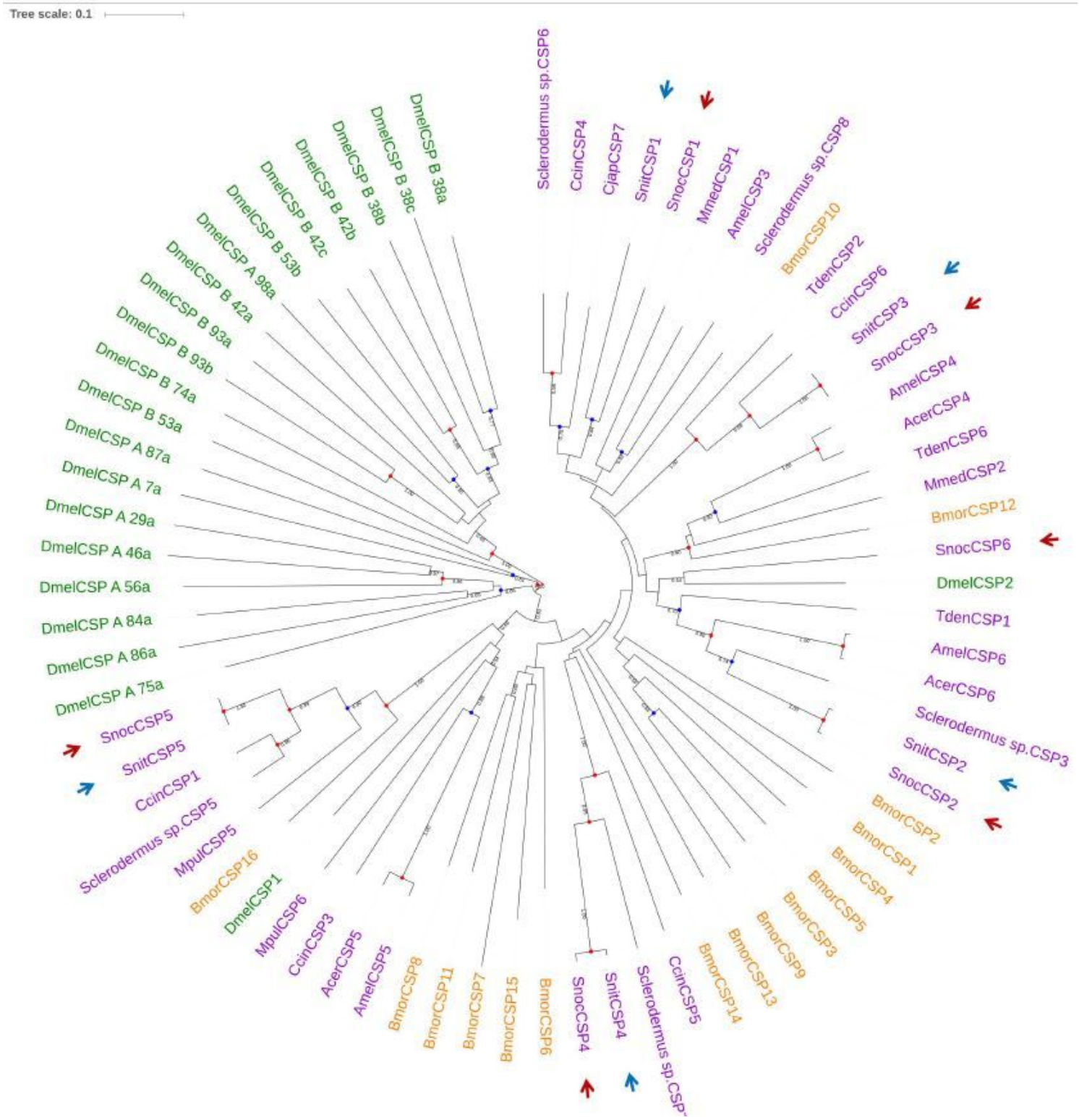

Figure 4

Candidate chemosensory proteins (CSPs) of Hymenoptera (purple), Diptera (green), and Lepidoptera (orange) are displayed in a neighbor-joining phylogenetic tree. SnocCSPs and SnitCSPs are marked with red and blue arrows, respectively.

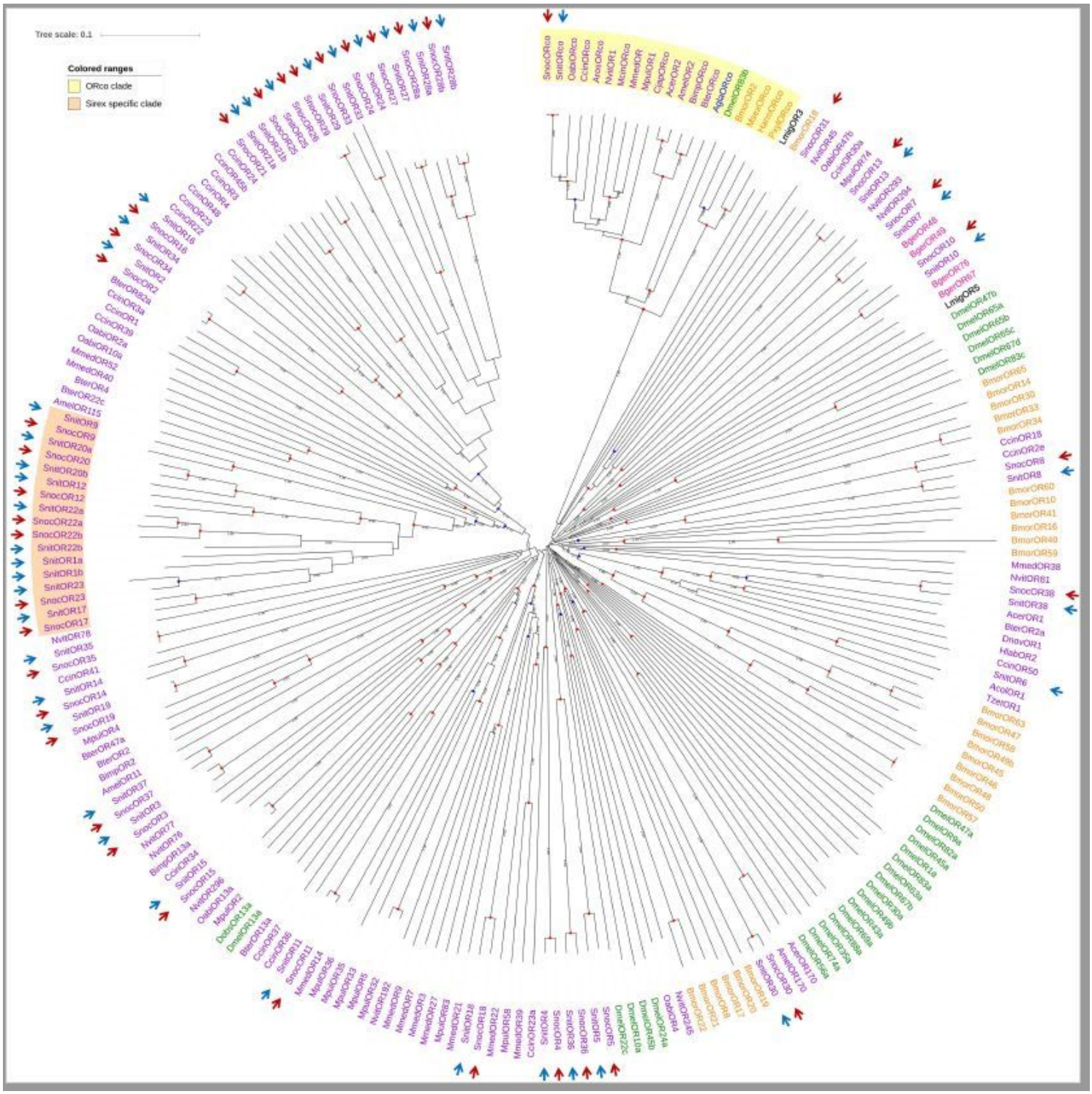

Figure 5

Candidate odorant receptor (ORs) of Hymenoptera (purple), Diptera (green), Lepidoptera (orange), Coleoptera (blue), Orthoptera (black), and Blattaria (pink) are displayed in a neighbor-joining phylogenetic tree. The ORco lineage and Sirex-specific lineages are labelled. SnocORs and SnitORs are marked with red and blue arrows, respectively.

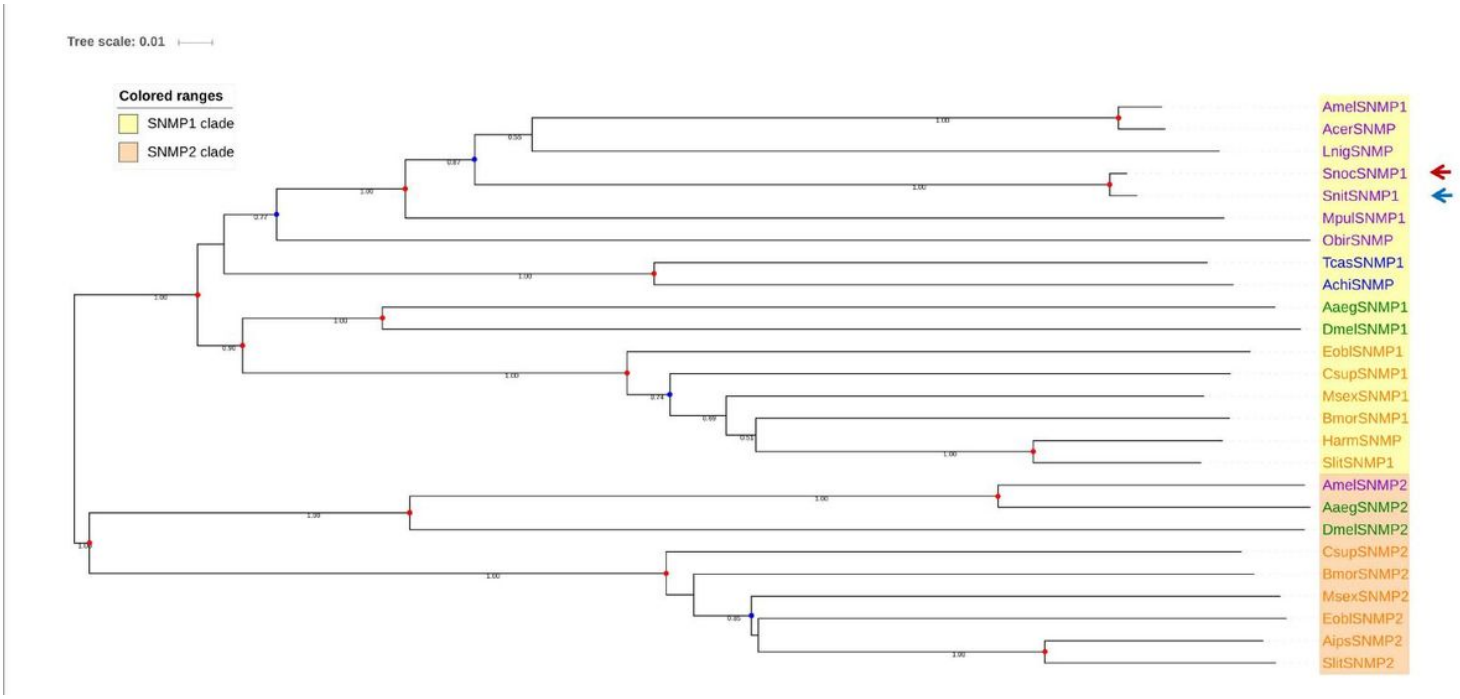

Figure 6

Candidate sensory neuron membrane proteins (SNMPs) of Hymenoptera (purple), Diptera (green), Lepidoptera (orange), and Coleoptera (blue) are displayed in a neighbor-joining phylogenetic tree. SnocSNMP1 and SnitSNMP1 are marked with red and blue arrows, respectively.

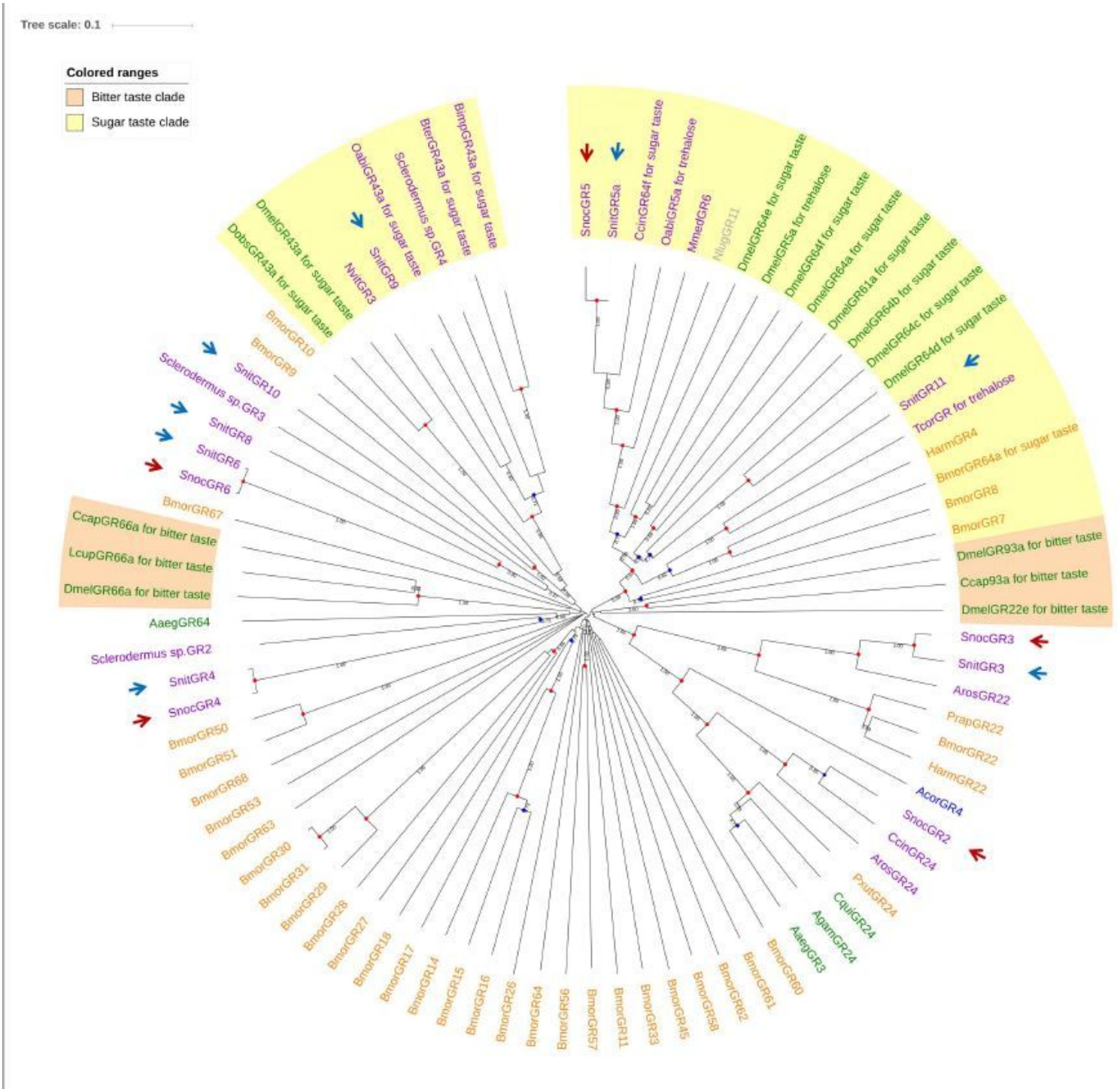

Figure 7

Candidate gustatory receptors (GRs) of Hymenoptera (purple), Diptera (green), Lepidoptera (orange), Coleoptera (blue), and Hemiptera (gray) are displayed in a neighbor-joining phylogenetic tree. The sugar taste lineages and bitter taste lineages have been labelled. SnocGRs and SnitGRs are marked with red and blue arrows, respectively.

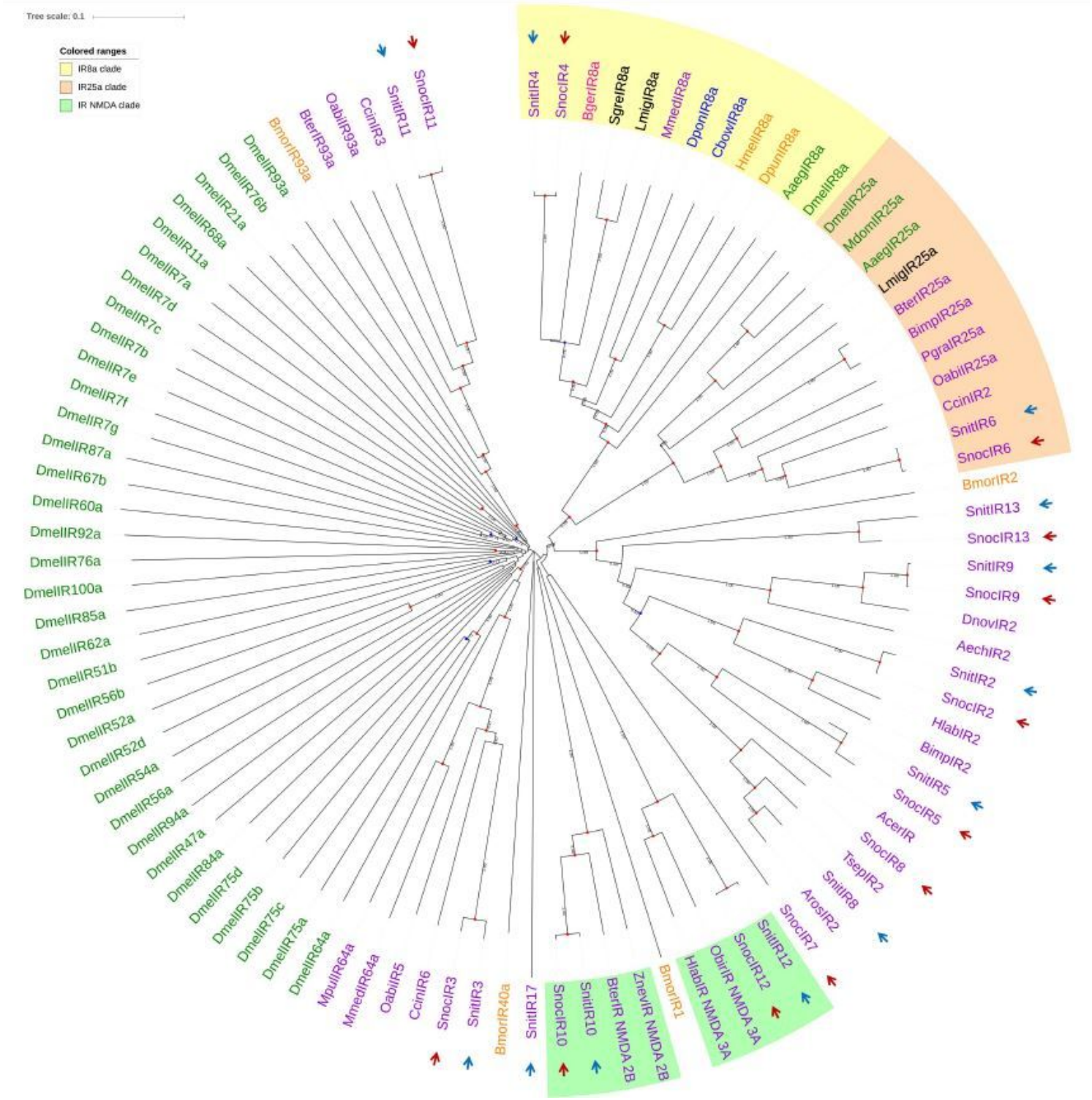

Figure 8

Candidate ionotropic receptors (IRs) of Hymenoptera (purple), Diptera (green), Lepidoptera (orange), Coleoptera (blue), Blattaria (pink) and Orthoptera (black) are displayed in a neighbor-joining phylogenetic tree. The IR8a lineage, IR25a lineage, and NMDA lineages have been labelled. SnocIRs and SnitIRs are marked with red and blue arrows, respectively.

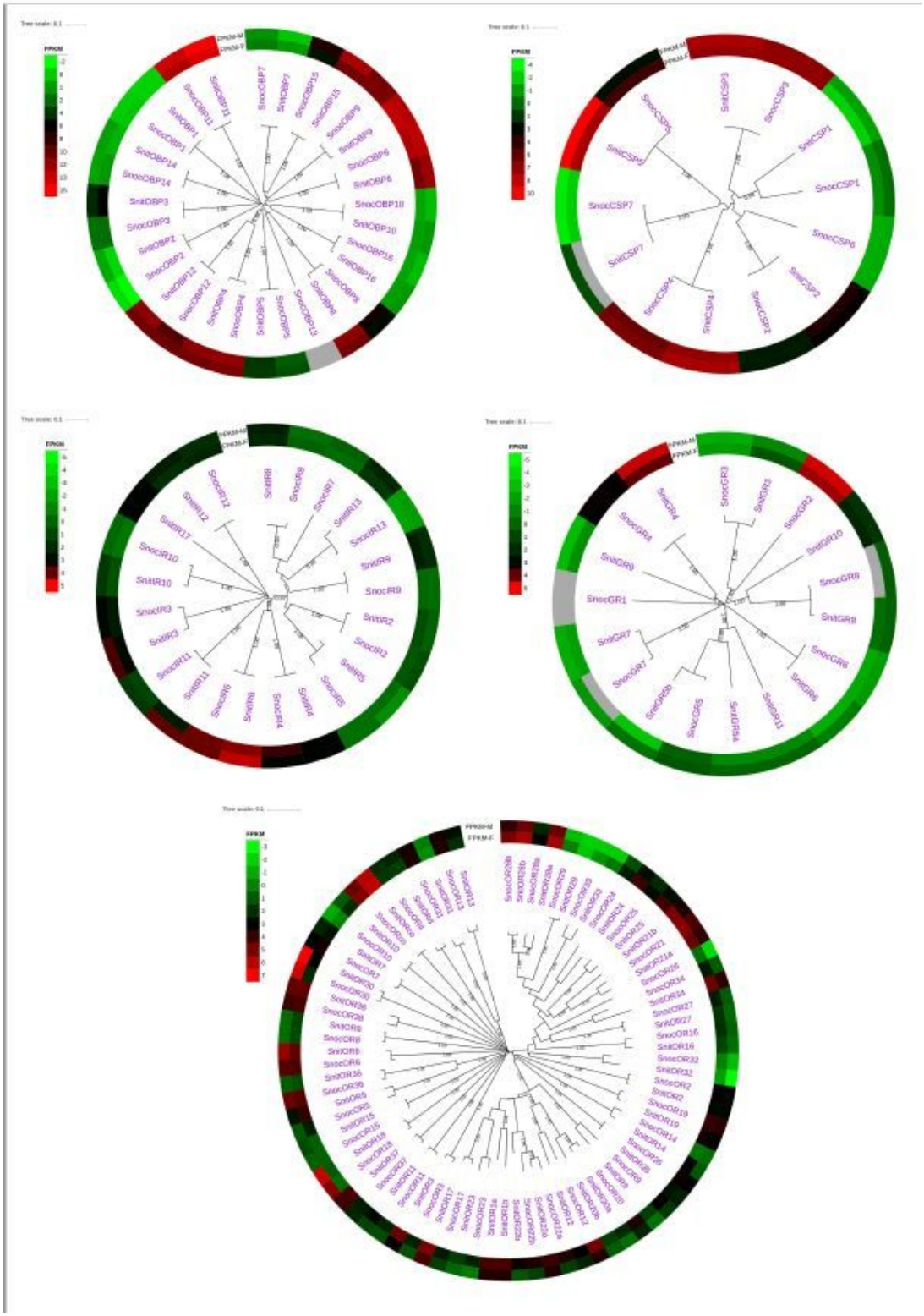

Figure 9

Candidate ionotropic receptors (IRs) of Hymenoptera (purple), Diptera (green), Lepidoptera (orange), Coleoptera (blue), Blattaria (pink) and Orthoptera (black) are displayed in a neighbor-joining phylogenetic tree. The IR8a lineage, IR25a lineage, and NMDA lineages have been labelled. SnocIRs and SnitIRs are marked with red and blue arrows, respectively.

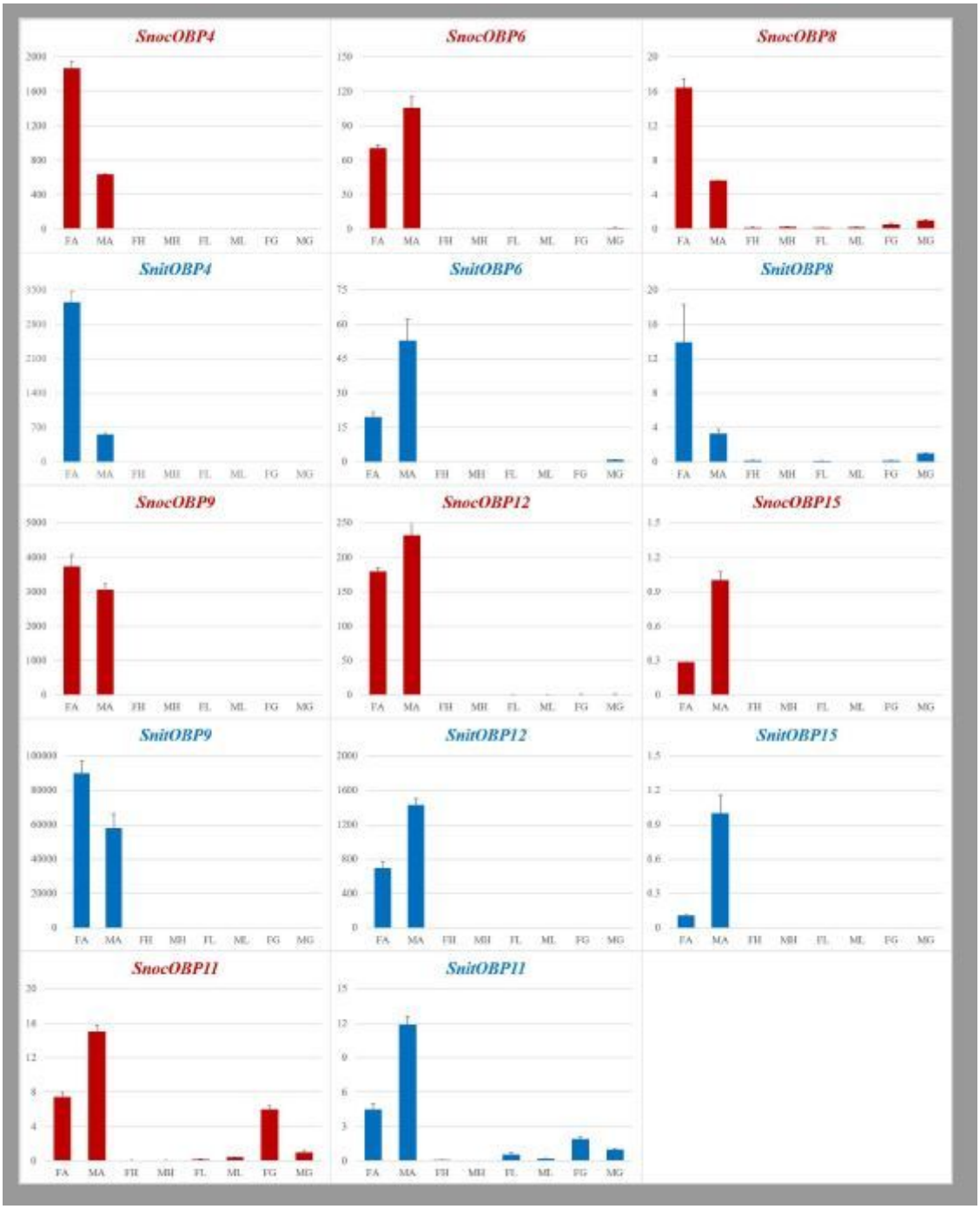

Figure 10

Odorant binding protein (OBP) transcript levels with antennae-biased expression in 4 tissues of male and female Sirex. S. noctilio data is colored red and S. nitobei data is colored blue. The expression level of the male genitalia was set to 1 in order to obtain the relative expression in each tissue. The expression of SnitOBP15 in male antennae was set to 1 because the expression of male genitalia was too low to display.

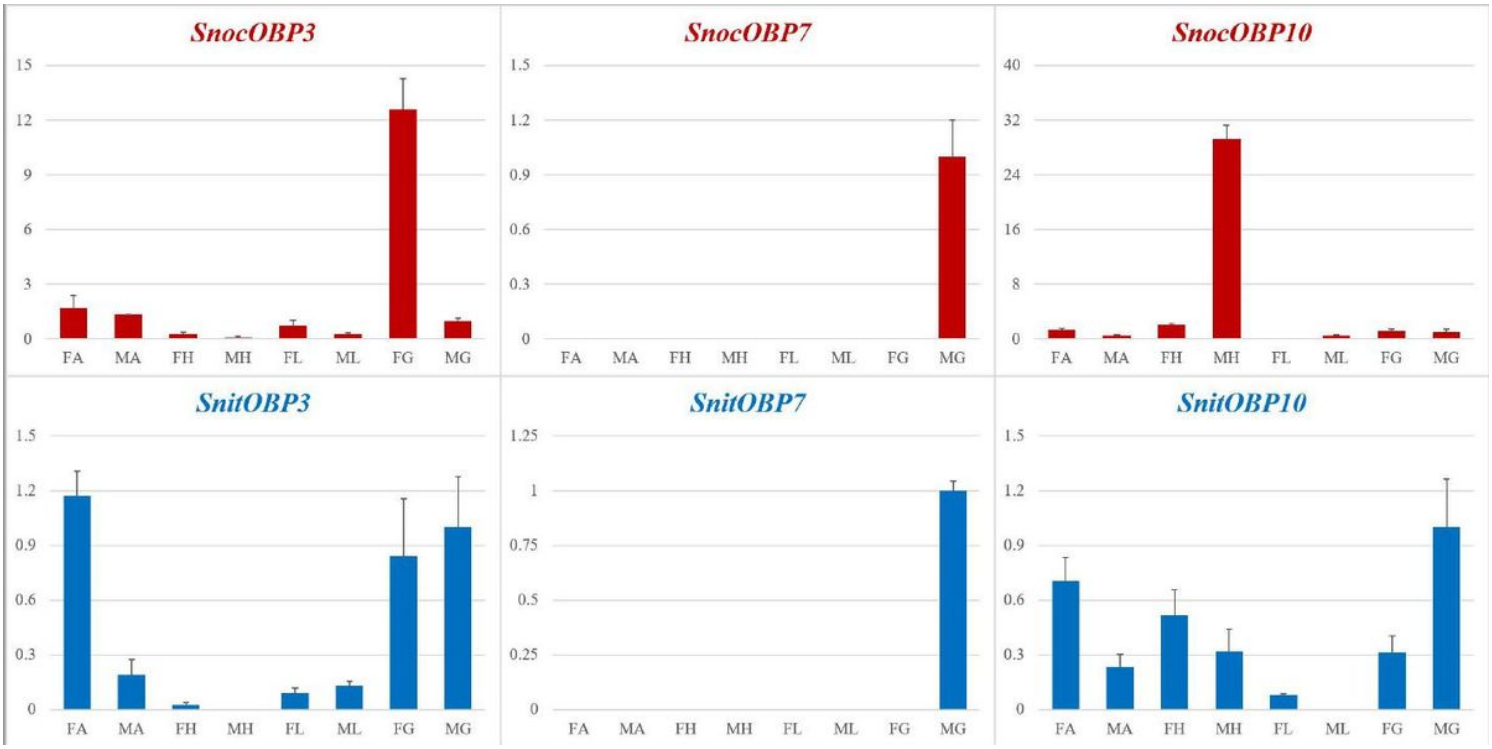

Figure 11

Odorant binding protein (OBP) transcript levels with head-biased, genital-biased or unbiased expression in 4 tissues of male and female Sirex spp. S. noctilio data is colored red and S. nitobei data is colored blue. The expression level in male genitalia was set to 1 in order to display the relative expression in each tissue.

Supplementary Files

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