

# Scanning electron microscopic observations of antennal sensilla, and the cloning and expression of the *BiOr816* gene of *Bombus ignites* from China

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## Research article

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## Abstract

Odorant receptors play a crucial role in the special recognition of scent molecules in the bumblebee olfactory system. In this study, four types of sensilla were observed on the antennae of worker bees through a scanning electron microscope (SEM), which were mainly distributed with sensillum placodeum sensillum coeloconicum, sensillum trichodeum and sensillum cavity. The odorant receptor 816 gene (*BiOr816*) from *Bombus ignites* was cloned, and its expression profiles were examined during different developmental stages and tissues. The phylogenetic tree was divided into four branches: *Messor orientalis*, *Apis cerana cerana*, *Apis mellifera* and *Apis dorsata*, *Bombus lantschouensis*, *Bombus terrestris* and *Bombus ignites*; and *Drosophila melanogaster*. The cDNA sequence of *BiOr816* was found to be highly similar to *BtOr22c* (98.2%), which encoded a membrane-coupled protein of 385 amino acids. The *BiOr816* protein belongs to the 7-transmembrane\_6 receptor superfamily. Accordingly, the results of the quantitative real-time PCR (qRT-PCR) indicated that *BiOr816* was expressed at high levels in worker bee antennae compared to its head, thorax, abdomen, legs and wings. This demonstrated that the antenna played a vital role in the process of olfactory recognition in bumblebees and served as its most important olfactory organ, which was consistent with the corresponding electron microscope photos showing many sensilla on the antennae.

## Introduction

*Bombus ignites* Smith belongs to the genus *Bombus* in the Apidae family and is an important native powdering-insect in China, distributed within Heilongjiang, Liaoning, Gansu, Hebei, Yunnan, and other provinces. It is vital in maintaining biodiversity and ecosystem balance (Zhang et al. 2016; Xue et al. 2007). Bumblebees have very long mouthparts and have the ability to make contact with stigmas via high-frequencies when visiting flowers. They have demonstrated great resilience in low temperatures, reduced light conditions and highly humid environments; their abilities in adapting to various environmental conditions are significantly higher than that of honeybees (Fiach and Javier 2019; Wang et al. 2017). Unlike honeybees, bumblebees are able to resist harsh environments and undertake extended daily working times as well as single flower visit times, enabling them to better concentrate on pollinating greenhouse crops (David and Caroline 2015). Above all, bumblebees increase the percentage of fertile fruit and yield, improving the quality of fruits and seeds. Moreover, they can avoid hormone pollution caused by plant growth regulators in flowers.

Chemical receptors are critical for bumblebees to communicate with their external environments and with other individuals, which assists bumblebees in detecting chemical signals in the surrounding environment. Olfactory sensation can mediate the corresponding organs to recognize a plethora of scents in order to search for food and mates, identify companions and enemies, and avoid dangers in the external environment (Pask et al. 2015; Janaina et al. 2019; Wang and Yang 2017). Taste sensation identifies soluble hormones, stimulating their innate mating behaviors (Zhang and Dong 2011). The important proteins that participate in chemical signal reorganization include various kinds of differently sized gene-families, of which the chemoreceptor super-families hold particular importance. They are

primarily made up of odorant receptors, gustatory receptors and ionotropic receptors (Silbering et al. 2011; Xue et al. 2009), where odorant receptors serve as critical components. Odorant receptors of insects include common odor receptors (ORs) and odor co-receptors (Orco). ORs are highly differentiated in different insects (Hu et al. 2013), while the Orco of insects are comprised of just one type and are highly conserved (Ma et al. 2014).

Thus far, few reports exist regarding the study of odor receptor genes in *Bombus ignites*. According to past studies, insect odor receptor genes are generally believed to be G-protein-coupled receptors. This study speculates that odor receptors in *Bombus ignites* may have similar biological effects; in addition, this study attempts to confirm that antennae are the main olfactory receptive organs of *Bombus ignites* in regard to electron microscopy of antennae and odor receptor gene expression. Furthermore, the evaluation of antennae olfactory function was completed according to the receptors distributed on the antennae. Consequently, BiOr816 was observed to play a crucial role in the olfactory recognition process of *Bombus ignites*, providing the foundation for further studies pertaining to odorant receptor gene function and mechanisms associated with olfactory recognition for *Bombus ignites*.

## Materials And Methods

### Bumblebee materials

The *Bombus ignites* specimens used in this study were bred at the bee research institute of Eastern Liaoning university (40.13°N, 124.37°E). Each hive had a mature egg-laying queen and five frames, a feeding temperature of  $28^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ , humidity of  $50\% \pm 5\%$  and were kept without light.

This investigation analyzed disease-free worker bees, where worker bees were immediately stored in liquid nitrogen for subsequent cloning for the target gene. Next, worker bees that immediately came out of the hive after eclosion were marked as 1-day-old with non-toxic and tasteless paint and were then marked every five days from sexually immaturity to maturity, putting them back after marking. Samples were selected on the 1st, 5th, 10th, 15th, 20th, 25th and 30th day, respectively. Afterward, the insects were used for the *BiOr816* gene expression analysis (Villar et al. 2015). In each group, 30 worker bees were randomly collected each time, with three biological replicates from different colonies being examined, where one replicate contained five bumblebees. The experiments were conducted in the genetic engineering laboratory of eastern Liaoning University during the spring of 2018, when nectar and pollen were abundant in the natural environment.

### Electron microscopic scanning of antennal receptors

The antennae of the *Bombus ignites* specimens were soaked in 2.5% glutaraldehyde for 24 hours, which were then cleaned using ultrasonic waves and put it into potassium iodide, an electrically conductive liquid. Next, dewatering and drying were carried out using tertiary butyl alcohol after washing the antennae with distilled water (Chen et al. 2013). The samples were then photographed, and the antennae sensilla were scanned using a Hitachi S-4800 electron microscope.

# Total RNA extraction

The antennae, head, thorax, abdomen, legs and wings of the *Bombus ignites* specimens were dissected with ophthalmic forceps and crushed with a mortar and pestle. The tissue homogenates were placed into Eppendorf tubes filled with liquid nitrogen (Zhang et al. 2013), where the powder was then put into another Eppendorf tube with 1.5 ml RNA-free Trizol (4°C). The total RNA was extracted using a Takara insect total RNA isolation kit, and the RNA integrity was detected. The cDNA was synthesized from the total RNA isolated from antennae using the Primer-Script RT reagent kit (Takaka, [www.takara-bio.com](http://www.takara-bio.com)) according to the manufacturer's instructions (Zhao et al. 2015; Liu et al. 2015). The first cDNA strand was synthesized with total RNA as its template and Oligo-dT as its primers, which were stored in a freezer at -80°C until use. All of the above utilized medicine was purchased from Sangon Biotech (Sangon Biotech Shanghai, China Co., Ltd).

## EST-SSR primers development and screening

All of the available *Bombus ignites* EST sequences up to March 26, 2018 were downloaded from the GenBank/NCBI EST database (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). To develop the EST-SSR primers, the following steps were performed. Using Perl software ([www.perl.org](http://www.perl.org)), all 5068 bumblebee EST sequences were assembled in FASTA format. The Blastclust software ([www.Ncbi.nlm.nih.gov](http://www.Ncbi.nlm.nih.gov)) was then used to search for redundant sequences in all EST sequences. Then, using the CD-HIT software (<http://weizhongli-lab.org/cd-hit/>), the short (< 100 bp) and long sequences (> 700 bp) were removed, along with the 5'-hat and 3'-poly tail (A or T) of mRNA using the EST-TRIMMER software. The MISA.PL software was used to search, identify and locate SSR sites, and primer 3.0 was used to develop the EST-SSR primers. The designed parameters for the EST-SSR primers were as follows: length of 20–26 bp, annealing temperature (Tm) of 55–65 °C, content of G + C of 30–70%, and length of PCR amplification product larger than 150 bp. Finally, BLAST was used to test the developed primers. The primers were synthesized by Sangon Biotech (Sangon Biotech Shanghai, China Co., Ltd) (Table 1).

The developed EST-SSR primers were used to PCR amplify the DNA samples of the bumblebees. The amplified fragments were separated and analyzed using 6% polyacrylamide gel electrophoresis for 1.5 h (constant power 55 W, DNA marker GsDL0501 from 50 bp to 500 bp). The primers were tested and screened three times; of the screened primers, those that were amplified as stable, polymorphic and clear bands were chosen to be in the final study.

## Cloning of *BiOr816*

Since odorant receptors are distributed mainly in the antennae, and their expression patterns are especially enriched in bumblebee antennae, the antennae were used for cloning (Brockmann et al. 2001). The primers used to amplify *BiOr816* (Table 2) were designed using the primer premier 5.0 software (Premier Biosoft International Co., Palo, CA) using the input mRNA sequence of the odorant receptor gene *BtOr22c* (Accession number: XM\_012312228.1), which was already reported in GenBank.

The PCR thermocycling conditions were: 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 59°C for 45 s, 72°C for 3 min, and a final extension at 72°C for 10 min. PCR reactions: 2.5 µL 10 × reaction buffer, 1.5 µL 2.5 mmol·L<sup>-1</sup> of each dNTPs, 0.5 µL 200U mL<sup>-1</sup> Taq DNA polymerase, 1 µL 10 µmol·L<sup>-1</sup> each of forward and reverse primers, 50–100 ng tissue homogenate, and 1.5 µL 1.5 mmol L<sup>-1</sup> MgCl<sub>2</sub>. Double-distilled water was then added to achieve a total of 20 µL reaction volume. The PCR products were then resolved using electrophoresis on a 1% agarose gel and purified using a Gel Extraction Kit (Cwbiotech, [www.cwbiotech.bioon.com.cn](http://www.cwbiotech.bioon.com.cn)). Next, the purified products were ligated into a pEASY-T3 Clone Vector and were subsequently transformed into a Trans 5a Chemically Competent Cell (TransGen Biotech) (Zhang et al. 2012). Positive clones were screened and sequenced by Sangon Biotech.

## Sequence analysis

After sequencing, the cDNA sequence of *BiOr816* was obtained by assembling the forward and reverse sequencing reads using the SeqMan program in the DNASTAR 5.0 software (Lynnon Biosoft, Quebec, Canada). The online software ORF Finder (<http://www.ncbi.nlm.gov/gorf/gorf.html>) was used to search for the open reading frame (ORF) of the sequence. The amino acid sequence was then translated using the DNAMAN software. Similarity searches were conducted using the BlastP program on the NCBI website (<http://blast.Ncbi.nlm.nih.gov/Blast.cgi>). The isoelectric point (pl) and molecular weights (MW) were computed using Compute pl/MW ([http://www.expasy.ch/tools/pi\\_tool.html](http://www.expasy.ch/tools/pi_tool.html)). The transmembrane helix (TMH) was predicted using the TMHMM Server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM>). Alignments of multiple sequences were carried out using the ClustalX program (Thompson, Higgins, & Gibson, 1994). The phylogenetic tree was constructed using MEGA 7.0 (<http://www.megasoftware.net/index.php>) with the neighbor-joining method. A portion of sequences for known Ors of bumblebees were acquired from GenBank.

## Expression of *BiOr816*

Total RNA was isolated from the antennae, heads, thoraces, abdomens, and legs of adult worker bees to determine the expression of *BiOr816*. The quantitative Real-Time PCR (qRT-PCR, ABI 7500 instrument) was performed using the SYBR Premix Ex Taq kit (Takara) in a total reaction volume of 20 µL. The reaction mixture was prepared adhering to the following parameters: SYBR Premix Ex Taq II(2×)10 µL, 0.8 µL 10 µmol·L<sup>-1</sup> each of forward and reverse primers, 0.5 µL ROX Reference Dye (50×), and 50–100 ng cDNA, followed by the addition of double-distilled water to reach a total of 20 µl reaction volume. The primers (Table 2) for qRT-PCR were designed using the primer premier 5.0 software, and the actin gene was used as the internal control. qRT-PCR was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles at 94°C for 30 s, and 72°C for 40 s. A melting curve analysis was conducted to verify the specificity of amplification. The relative expression patterns of *BiOr816* mRNA were calculated using the 2<sup>-ΔΔ</sup>Ct comparative CT method (Schmittgen and Livak 2008). In order to ensure the accuracy and stability of the qRT-PCR results, under the same conditions, three parallel experiments should be done as biological replicates, with one replicate containing 10 bumblebees.

## Statistical analysis

Differences in the relative expressions of the *BiOr816* gene were determined using a *t*-test analysis in SPASS 17.0 (IBM, Armonk, NY).  $P \leq 0.05$  was considered to be statistically significant in all treatments.

## Results

# Scanning electron microscope photographs of the antennal receptors of *Bombus ignites*

Four types of sensilla were observed on the antennae of worker bees through a scanning electron microscope (SEM), which are regarded as the main olfactory organ of bumblebees and were mainly distributed with sensillum placodeum and sensillum coeloconicum, though sensillum trichodeum and sensillum cavity were still present (Fig. 1). Sensillum placodeum has a disk-like shape, and many differently sized protrusions exist on its surface, which are closely distributed on the flagellum of the antennae. Around the sensillum placodeum, many sensillum coeloconicum were observed having short-nail-like features born in a mortar-like socket. The top of the sensillum trichodeum was slightly curved, and its base extended out from the sheath-like shape of the antennae surface, growing to the top. The sensillum cavity was sparsely distributed over the antennae, which depressed inward from the antennae surface. Here, many irregularly saw-tooth structures were present in the middle of the depressions.

## Cloning and sequence analysis of *BiOr816*

To explore the molecular functions of *Bombus ignites* odorant receptor 816 (*BiOr816*), the cDNA sequence of *BiOr816* containing the complete coding region was cloned. Its amino acids were predicted using the DNAMAN program, and the *BiOr816* cDNA was found to possess a 5'-terminal untranslated region (UTR) of 39 bp, a 3'-terminal UTR of 67 bp, and an open reading frame (ORF) of 1158 bp encoding a polypeptide of 385 amino acids (Fig. 2). Through the ProtParam program (<http://www.expasy.org/tools/protparam.html>) the molecular mass of the deduced *BiOr816* protein was predicted to be 48.17 kDa, its molecular formula was  $C_{3592}H_{6021}N_{1165}O_{1502}S_{235}$ , and the isoelectric point (pI) was calculated to be 5.06, which was acidic and uncharged. The hydrophilic coefficient of the *BiOr816* protein was 0.769, and its instability index was computed to be 50.31, indicating that the protein was unstable and hydrophobic. Moreover, the *BiOr816* protein belongs to the 7-transmembrane\_6 receptors superfamily, which consists of 7 trans-membrane domains: TM- $\alpha$ (28–54), TM- $\alpha$ (95–116), TM- $\alpha$ (126–148), TM- $\alpha$ (187–206), TM- $\alpha$ (245–265), and TM- $\alpha$ (301–320). Furthermore, the amino terminus of the protein was located in the membranes, with three phosphorylation sites (Fig. 2 and Fig. 3).

## Amino acid sequence alignment and phylogenetic analysis

To determine the evolutionary relationship of *BiOr816* with *Bombus ignites* and other species, a blast protein alignment and neighbor-joining tree were constructed for further identifying the relationships between the *BiOr816* and other related protein sequences from 11plants already obtained (Fig. 4 and Fig. 5). Comparison of the deduced amino acid sequence of *BiOr816* to that of *BtOr22c*

(XM\_012312228.1) revealed a high degree of identity of 98.2%. Similarly, alignments with the Ors of other insects exhibited high homology with *BtOr9a* (NC\_015772.1, identity 95.1%) and *BtOr13a* (XM\_0123091122, identity 92.6%). Using the deduced amino acid sequence of *BiOr816*, a phylogenetic tree was constructed using the MEGA 7.0 software. The corresponding phylogenetic analysis demonstrated that *BiOr816* and *BtOr22c* belong to a single genus and is embodied by a high degree of identity. Alignments with the Ors of other insects homologous with *BiOr816* are *Formica*, *Apis* and *Bombus*; the homology of its amino acid is between 35% and 96%. The phylogenetic tree was divided into four branches, with the first branch belonging to *Messor orientalis*, the second to *Apis cerana ceran*, *Apis mellifera* and *Apis dorsat*, the third to *Bombus lantschouensis*, *Bombus terrestris* and *Bombus ignites*, and the fourth to *Drosophila melanogaster*. Accordingly, the *Bombus ignites BiOr816* sequence was found to be closely related to the *Bombus terrestris* odorant receptor *BtOr22c* sequence and possesses a high level of homology, indicating that the *BiOr816* sequence cloned in this study is the *Bombus ignites* odorant receptor gene and the *BiOr816* protein had similar structure and likely shared some gene functions.

## Analysis of *BiOr816* expression via qRT-PCR

The expression profiles of *BiOr816* were characterized across different developmental stages and tissues of *Bombus ignites* using qRT-PCR. The expression of *BiOr816* located in the head of 10-day-old worker bees was utilized as the control, and its expression was then analyzed in different tissues of 1, 5, 10, 15, 20, 25 and 30-day-old specimens. The results showed that (Fig. 6) the expression of *BiOr816* changed significantly according to increasing age among different tissues, and its expression in the antennae was obviously higher than that of other tissues within 1–30 days ( $P < 0.01$ ). Moreover, the fluctuations in antennal expression among the analyzed days were not found to be significant. Specifically, antennae had lower expressions at 1-day-old, then slowly increased; however, it suddenly decreased at 15-day-old, rebounding to its peak value at 25-day-old, which declined afterward. While the abdomen possessed the lowest levels of expression in all tissues, antennae expression was observed to be about 299–759 times higher than that in the abdomen at the same age. In contrast, in terms of the feet and wings, no significant differences were present, though their expressions were slightly higher than that of the thorax. Additionally, the expression of *BiOr816* in all tissues decreased significantly at the age of 15 days, which may be because day 15 was the transition stage from house bees to field bees, resulting in lower levels of expression of this odorant receptor. The expression level of each tissue also decreased significantly after 30 days, indicating that the expression level of odorant receptor proteins would gradually decrease when the bumblebees had entered the aging stage. Overall, the results demonstrated that antennae played a vital role in the process of olfactory recognition in bumblebees, which was the most important olfactory organ in bumblebees.

## Discussion

The proteins of general odorant receptors are highly diverse in insects, which also holds true for the conservative co-receptor family (Gao and Chess 1999). Bumblebees are able to feel the volatility of the

surrounding environment through their sense of smell. The antennae of the bumblebees possess a large number of olfactory receptors, which are particularly important for foraging, coupling, reproduction, and avoiding dangerous enemies (Hu et al. 2013). In *Bombus ignites*, a number of olfactory neurons express sex pheromone receptors in order to increase their sensitivity in responding to pheromones. The process entailing bumblebees' recognition of external scent molecules is very complex, and it is comprised of numerous types of proteins, namely, odorant binding protein (OBPs), chemosensory proteins (CSPs), sensory neuron membrane protein (SNMPs) and odorant degrading enzymes (ODEs). Moreover, each olfactory receptor has many olfactory receptive neurons (Foret and Maleszka 2006), and external scent molecules were initially observed to form a complex with the odor-binding protein, subsequently passing through the lymph to reach the odor receptors. The odor receptors are stimulated to produce action potentials to generate the olfactory signals, which are then transmitted to the antennal lobe of the bumblebee's brains via olfactory neurons. Accordingly, abstract information pertaining to the scent molecules were finally obtained, which guided the various behaviors of the bumblebees. Receptor proteins predicted to be expressed by the *BiOr816* odorant gene were also found to possess the structural characteristics of G protein-coupled receptors (GPCR), such as 7 lipophilic transmembrane regions, one glycosylation site and three phosphorylation sites. In addition, its N-terminal was intracellularly located, while its C-terminal was located outside the cell, consistent with the results of *PxyOr18* in *Plutella xylostella* (Kong et al. 2015) as well as *AcerOr1* and *AcerOr3* in *Apis cerana* (Zhao et al. 2014), which is a structure mosaic found in the phospholipid bilayer of olfactory receptor neuron cell membranes. The *BiOr816* gene may have activated the G protein after producing cAMP with a slow and long ion conduction duration under cAMP stimulation, thereby promoting olfactory signal transduction. However, unlike vertebrate G protein-coupled receptor topology, the extracellular second loop of the *BiOr816* protein does not contain two cysteines at the end of the fifth transmembrane region. Generally, a high degree of variation is present in the third, fourth, and fifth transmembrane regions. The different domains of insect odorant receptors in vertebrates may be due to the formation of different olfactory systems during evolution, making proteins very different in structure.

In the present study, a putative odorant receptor gene *BiOr816* in *Bombus ignites* was identified. The *BiOr816* amino acid sequence was observed to share many similar characteristics with that of the *Bombus terrestris* *BtOr22c* orthologue. Interestingly, homologs of *BiOr816* were not observed in other insects outside of *Bombus*. In general, ORs exhibited a high sequence divergence among insects including classical model insects (Hill et al. 2002). These observations were consistent with the ancient origin of the OR family. As demonstrated by this study's constructed phylogenetic tree, *BiOr816* was found to be more closely related to *BtOr22c*, *BtOr9a* and *BtOr13a* of *Bombus terrestris*. However, it showed less homologous homology with the genus of *Formica* or other genera of *Hymenoptera*. Accordingly, different insects may be observed to have different olfactory signals to the same scent molecules, leading to a high degree of variation in their odor receptor genes (Vikas et al. 2006). This result molecularly confirms the traditional phylogenetic classes of bees (Robertson and Wanner 2006), suggesting that *BiOr816* belongs to a typical odorant-receptor protein family in *Bombus ignites*.

*Bombus ignites* are social insects, where worker bees take on different aspects of work according to their physiological age. They served as house bees before 18-day-old and were responsible for various tasks like incubation, secreting beeswax, making bee's spleen, cleaning, and feeding larvae and the queen bee. After 18-day-old worker bees become more mature, they gradually start gathering nectar and water outside of their hive. The above theoretical analysis aligns with that of the proposed experiment regarding *BiOr816* gene expression. The obtained results demonstrated that the expression of *BiOr816* suddenly dropped at 15-days of age, which was presumably due to its transitional stage when house bees become field bees, as they are influenced by changes in the environment, hormones as well as other factors, leading to the odorant receptors with low levels of expression. However, its levels of expression also illustrated a declining trend from 25-days of age, which may be due to field bees taking on more work than their 10-day-old counterparts, a decline in learning ability, the accompanying oxidative damage from brain proteins and lipids, and the decrease in synapses and neurons, which increase worker bee mortality (Dukas 2008; Munch et al. 2010). Olfaction is the process in which bumblebees sense volatiles in their surroundings. There are four kinds of receptors on the antennae of *Bombus ignites* worker bees: plate sensilla, piercing sensilla, hairy sensilla and cavity sensilla, which are particularly important for foraging, courtship, reproduction, and avoiding hostile confrontations. Numerous studies have shown that sensilla trichodea and sensilla basiconca possess olfactory functions, of which sensilla trichodea are mainly sensitive to external hormones, while sensilla basiconca are mainly sensitive to mechanical sensation, liquid chemical sensation and external common odors such as plant odors and odors emitted by natural enemies and other stimuli (Almaas and Mustaparta 1991; Gnatzy et al. 1984; Ljungberg et al. 1993; Steinbrecht 1970; Steinbrecht and Gnatzy 1984). Sensilla coeloclnica sense water vapor, carbon dioxide, humidity changes, and plant odors, and large cavity cones may also have olfactory functions (McIver 1982). Furthermore, sensilla placodea may also possess functions in olfaction (Steinbrecht et al. 1992; Steinbrecht et al. 1995). The expression of *BiOr816* changed significantly in different tissues, and expression in the antennae was observed to be the highest in each worker bee and significantly higher than that in other tissues, indicating that antennae are the main olfactory organs of *Bombus ignites*.

## Conclusion

In the paper, we firstly confirmed the structure of the odor receptors in *Bombus ignites*, and its gene expression, which tested that antennae are the main olfactory receptive organs of *Bombus ignites* in regard to electron microscopy of antennae and odor receptor gene expression. Furthermore, the evaluation of antennae olfactory function was completed according to the receptors distributed on the antennae. Consequently, *BiOr816* was observed to play a crucial role in the olfactory recognition process of *Bombus ignites*, providing the foundation for further studies pertaining to odorant receptor gene function and mechanisms associated with olfactory recognition for *Bombus ignites*. According to the physical and chemical properties of the protein, we believed that *BiOr816* was the G-protein-coupled receptors have similar biological effects like other insects.

## Declarations

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**Author's contributions:** Dan-Dan Wang conducted the experiments, interpreted the data and prepared the figures and tables, then wrote the paper. Xiao-Hong Li and Xing Wang carried out the collections, helped interpret the data and gave their helpful discussion and comments on the manuscript. All authors read and approved the final manuscript.

**Competing interests:** The authors declare that they have no competing interests to declare.

**Availability of data and materials:** All data generated or used during the study appear as stated in the article.

**Consent for publication:** Not applicable.

**Ethics approval consent to participate:** Not applicable.

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## Tables

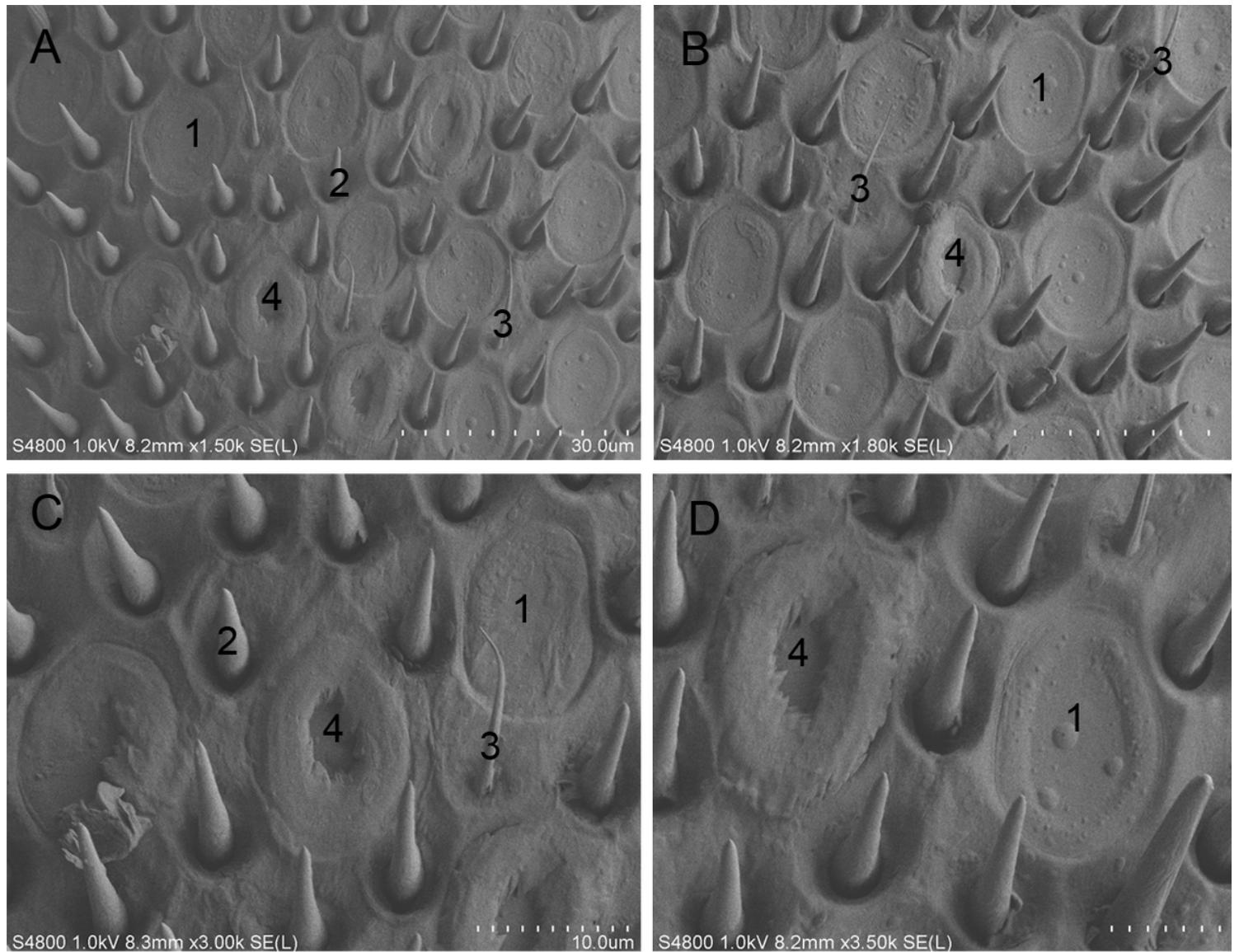
**Table 1** The information of EST-SSR primers

Primer	GenBank ID	Sequence 5' → 3'	Temperature Tm(°C)	Product (bp)	Percentage of polymorphic bands (PPB %)
BI02	BI517258	ATAGAGGGATGAAAAGGGGG TTCCTGCCTCAACTCTTT	60	270—280	100.00
BI05	BI517189	AAAGAAGCGTGAAAGGGGAT CGATTACGTGTGCATTACG	61	250—260	86.91
BI06	BI517175	GATTGTTTCTCGCTGCTCC CTCGATGATATCGCTCGTCA	60	210—230	92.87
BI07	BI517174	GCATCCCTTCCTGTTCCCT TCCAATGATCTCGGTGGT	61	180—210	89.31
BI09	BI517090	CTCTGATTAACACGCCACGA TCACTGTATTGCGGTGTGGT	61	210—230	79.68
BI15	BI516965	CATCTCGCTCTCGCTCTT CTTATCCTGTCGTGATGTCG	59	270—280	100.00
BI16	BI51696	TGGGTGTTGTCCTGTATGT GGGCTCGCTTCACGTAAT	60	270—280	96.66
BI17	BI516954	TCACCAGGTGCAGAAAGAAA TCGTTGAAATGGAAACGTGA	60	240—250	81.97
BI18	BI516952	CATCCGGTCTTCTATCCGTC AAGTAAATCGGCCCTGTGTG	60	250—260	100.00
BI19	BI516965	GTGAAGGATCGAACATCGAGGA ACGTGTTGCGAGCGTTTAT	61	210—230	100.00
BI20	BI516965	CATCTCGCTCTCGCTCTT CTTATCCTGTCGTGATGTCG	59	270—280	100.00
BI21	BI516961	TGGGTGTTGTCCTGTATGT GGGCTCGCTTCACGTAAT	60	270—280	87.36
BI22	BI516954	TCACCAGGTGCAGAAAGAAA TCGTTGAAATGGAAACGTGA	60	240—250	91.58
BI23	BI516952	CATCCGGTCTTCTATCCGTC AAGTAAATCGGCCCTGTGTG	60	250—260	100.00
BI24	BI516935	TTCAATGTATGTGAGAGAGCGA CATTGAAAACATTTTATTCACT	59	230—240	91.67
BI25	BI516934	ATCGATACGACGCCAGATT CGAACAAAGAGAATTTCGGC	60	240—250	87.61
BI26	BI516927	CGTAACCTGGGACAAGGGAA CGAATAAGATAACGAACGGTG	60	260—270	75.97
BI27	BI516913	GGGATACTTGCACCGATCAT CATTGTACGCCCTCAAAGTGA	60	260—270	100.00
BI28	BI516816	CGAGATTCGGAACGATTCA GATTCACACTCGATTGCCAC	61	260—270	99.67
BI29	BI516791	TGCATTCAACTGTTATGCA ACGCACGCACGTATATGCT	60	260—270	88.93
BI30	BI516780	TGCGGTTGTATGTTCGTGT ACCGACTTGAAACAAACCTG	61	250—270	100.00
BI31	BI516778	TTGGTAAAGAAGGCAGGGAA CTCGATATTGTTGCAGCGAG	60	260—270	100.00
BI32	BI516725	TTGGCACATCCAAGAATTGA ATTCAAGCCGACTTAGCAA	60	200—210	73.82
BI33	BI516616	TAATCGGTGTCTCCACAGA CGACGAAGCAGCAAGTGT	60	260—270	78.66
BI34	BI516593	TGCGTTTGATTGCGATGTC TTCGCGATAGAATCGACAG	60	280—300	92.87
BI35	BI516426	TCGAGCCTATCACAGACG CTCGTTGAGGTGTTCGAT	59	190—210	99.62
BI36	BI516434	GATCGGGCGAAACTTAGCTA CACCTATCGTGTGCATGTG	60	260—280	90.87
BI37	BI516419	CGACACGAAGAGGGAGAGT TTAAAGCGTCATTGTGTGC	60	200—210	100.00
BI38	BI516410	AATTTCGCGAACTCCCTCAAAT CGATACGTGCATTCTAACTA	59	280—300	100.00
BI39	BI516361	ACCGTTGACGTGCGATTCTC TAGATGTCGCCAAATAACGC	60	200—210	100.00

Table 2 Primers used to *BiOr816* gene clone and qRT-PCR

Gene	Sequence (5' → 3')
<i>BiOr816</i>	F: CGCCGATCGTCATCGACTAA R: GCACAATGTTGGGTAGCACG
Quantitative Real-time PCR primers:	
<i>BiOr816</i>	F: CCTAACGCACTACTTGCCTA R CAGCGAACGATTAGTCCAA
reference gene: <i>β-actin</i>	F: GCGCGACATTAAGGAGAAC R: CCATACCCAGGAAGGAAGGT

## Figures



**Figure 1**

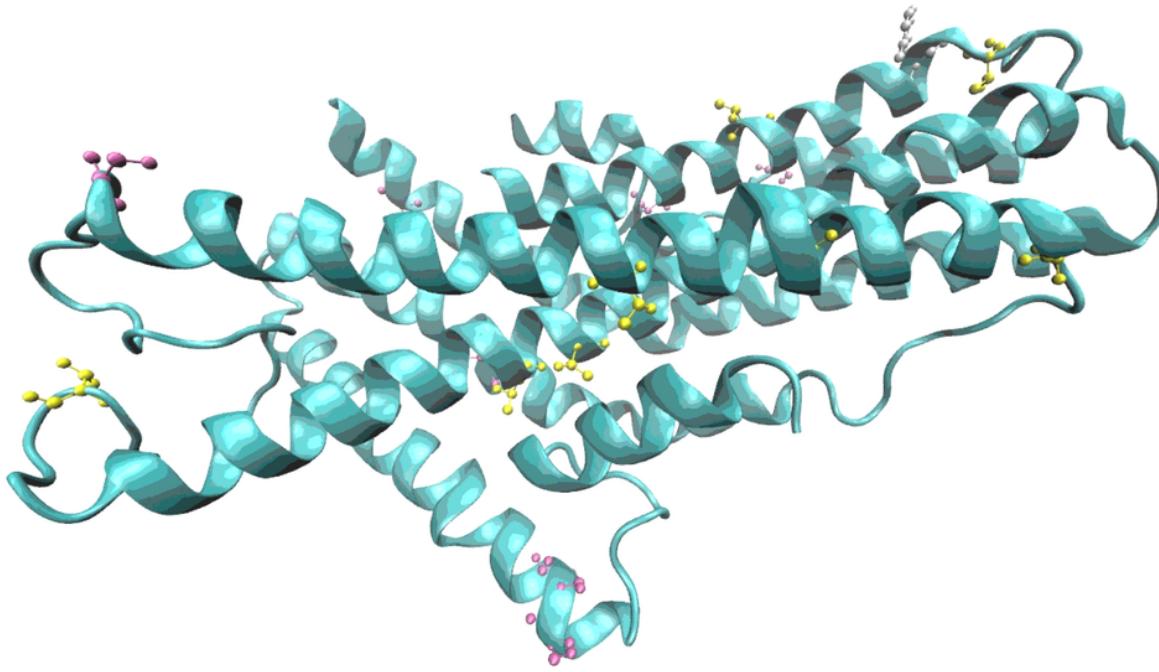
The SEM photographs of antennal sensilla of *Bombus ignites* Note: 1: sensillum placodeum; 2: sensillum coeloconicum; 3: sensillum trichodeum; 4: sensillum cavity

Total amino acid number: 385

GGCAACCACG TTATATAGTT ATGTTGAAAC AAATAACAC  
1 ATGTGTGTACAACGTGTTCTATATTATGGCATGGGACAAGAACAAACCGAGATGACA  
1 M C V Q L F L Y Y G M G Q E F T T E M T  
61 GAAATAGCTAACACAGCCTATAATACCAAGTGGTACAATTACCTCCCAAAAATGCAAGA  
21 E I A N T A Y N T K W Y N L P P K N A R  
121 TGGCTTGTAAATTATTATGTGTCGTGCCAGGTCTTCACCCCTACAAATTACAGCTGGCAGA  
41 W L V I I M C R A R S S P L Q I T A G R  
181 TTTTGTTCCTTACAAACCGCCTCTATTGTCAGCTACTACCACACATATCAGCAAAGGTT  
61 F C S F T N A L Y C Q L L P H I S A K V  
241 TACACAAAAGTATGTGGTTGCTGTGACAGCAGTCAGTTTGAAACGTAGAACAGGTGGT  
81 Y T K Y V V C C D S S Q F L N V R T G G  
301 TCAATTACGAGAAATAAGAGCCCTATGGATATCCAACGAGGCAGCAGTATATGTGTACGGT  
101 S I T R I K S P M D I Q R G A I Y V Y G  
361 TGGAACCTACAATATGCTGAAGTTTGGGGTATTTGCCGGAAAGAGAGGAAATGGAATCGA  
121 W N Y N M L K F W G I W P E E R K W N R  
421 TCTTCCAGTTACTTCATTCTGTTACCGTGTATCCACACGGTGTGCTTCATTGTGCTCCA  
141 S S S Y F I L L P C I H T V C F I C A P  
481 CAAACTATTAATCTTCCGATAATCGCGGGTGA C T G A T C T G G T G A T C G A G A A T C T A T C G  
161 Q T I N L P I I A G D S D L V I E N L S  
541 ACTAACATTACTATCACGATTCTCTTATGAAGACTATGGCTGTTGGATCAAAGGAAA  
181 T N I T I T I S L M K T M A V W I K G K  
601 CCATTAAGTCTTGCTGAAATGCATGGCAATGACTGGGATACAGTGACAAACAAACGCA  
201 P L K S L L K C M A N D W D T V T N N A  
661 GATCGAGAAAAAAATGGTGACCATCGCAGGGATCACTAAAAAGATTACGATAAAAGTATA  
221 D R E K M V T I A R I T K K I T I K S I  
721 CTGCTGGCCAACATCGTAGTTGCAGCTTTTGCCCTGCACGATTGTCTAGCATGATATA  
241 L L A N I V V A A F L P A R L S S M I Y  
781 AACGACAAGGAACGTGTTACCGTGGTTATTACCCCTACAATACTACGATCAGTCCAAAT  
261 N D K E L F Y R G Y Y P Y N T T I S P N  
841 CTGATGCGACCAACTAACGGTCAATTGATGGCTGCTTTGTGACTACAAACATATA  
281 C L M R P L I G Q L M A A L L R L Q T Y T  
901 ACGGTAGATACTTTGTCGTCTGTTGATTCTAGCACTACGTTGACAACTTCGATTCTG  
301 T V D T F V V L L I L A L R G Q L S I L  
961 AGAGACGATCCTCTCATCTCATTGATGACAAAAATGTAGAAATGAAATTACAG  
321 R D D P L S S H S Y D D K N V E M K L Q  
1021 AAGATCGTGCCTAGAGAGAGATACTTAATAGGTTCGCAGAAAAGATAAGAGAAATCTTC  
341 K I V P R E R Y I N R F A E T I E K S F  
1081 AACATGATGCTACTGTTCAAATGCTTGGTTGCACCAACTCAGCTATGCTCTCAGACTTAT  
361 N M M L L F Q M L G C T T Q L C S Q T Y  
1141 CAAGTTATTATGTCATAAGATACGCGGAAGAAGTGTAAATAGTTTCGATTTGGTAC  
381 Q V I M S \*  
1181 TGTATGCC ATTGGAGTAT TTGCACT

Figure 2

Nucleotide and translated amino acid sequences of BiOr816 cDNA of *Bombus ignites* Note: The red letters were 7 trans-membrane domains



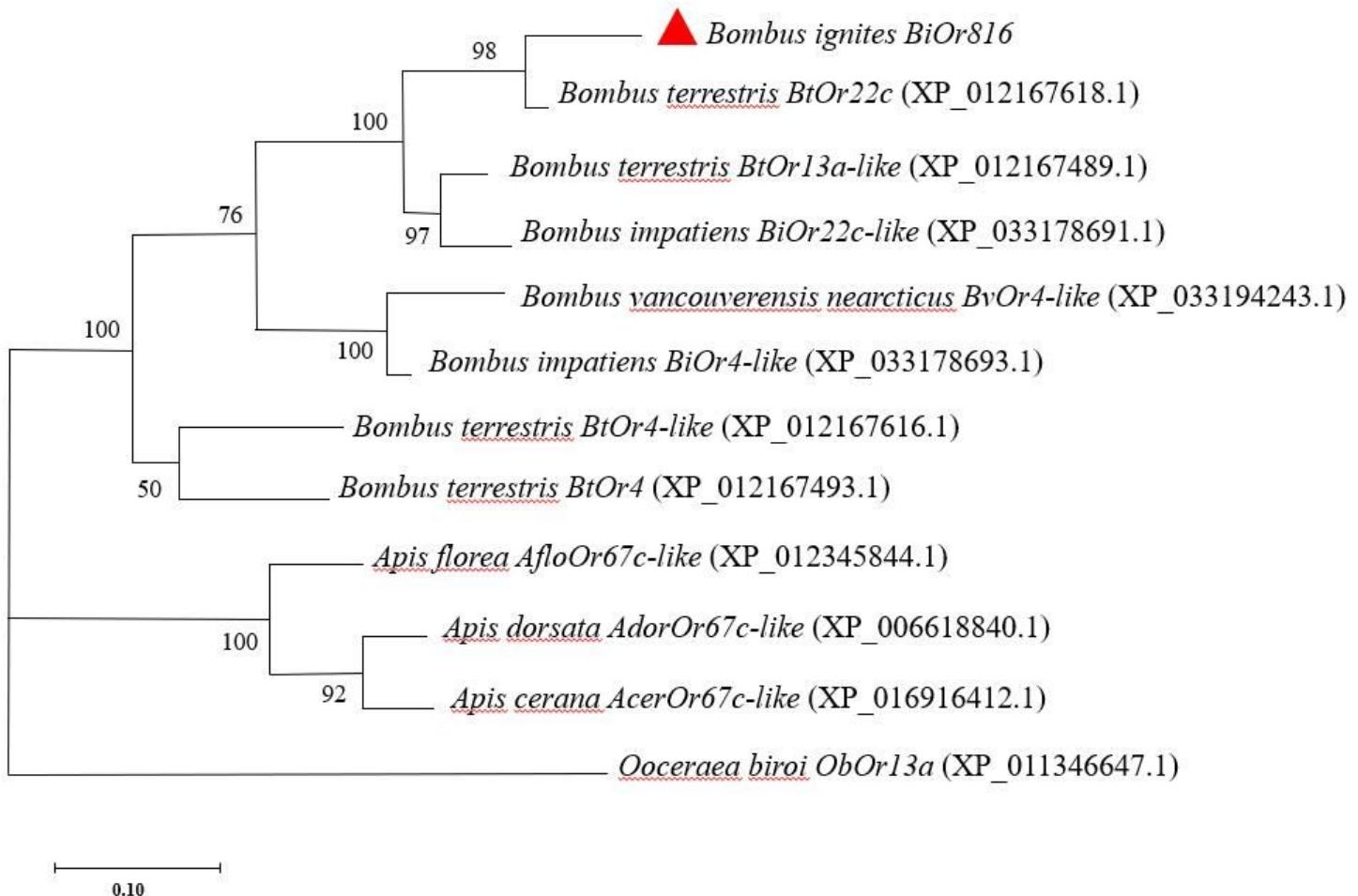
**Figure 3**

Three-dimensional structure of BiOr816 protein Note: Purple is the phosphorylation site of serine; yellow is the phosphorylation site of threonine; white is the phosphorylation site of tyrosine

BiOr816	MCVQLFLLYGMGQEFTTEMTEIANIANTKWKYNLLEPPNARWLVIIMCRARSSPLCITAGRFCSTFLNALYCQLLPHISAKVYTKYVVCCSSQFLNVRTGGSITRKSPMDIQRGAIIVYGVNNYNMLKEWGWFPEPKWNRSSSVFILIPC	150
BtOr22c	MRLQLFILIVCMGQEFTTESTEIANIANTKWKNLPPNARWLVIIMCRARSSPLCITAGRFCSTFLNALYCQLLPHISAKVYTKYVVCCSSQFLNVRTGGSITRKSPMDIQRGDLINVVGWNINMLKEIGWFPEPKWNRSSSVFILIPC	150
AcerOr67c-like	.....	.....
AdorOr67c-like	.....	.....
AfloOr67c-like	.....	.....
BtOr4-like	.....	.....
ObOr13a	.....	.....
BtOr4	.....	.....
BtOr22c-like	.....	.....
BtOr13a-like	.....	.....
BtOr4-like	.....	.....
Consensus	.....	.....
BiOr816	MTIIVPICAPOINIPSIAGESLSLVENLSTNITIIISIPLTMWIKGRPSLSLKGMANCWDIVINNAADREAVVIAIRIKRITISILLANDVAAFPLPARLSMSIYNDKEELFPGYFPNTTISIENLMRELICQIMAAL..LRIQ	297
BtOr22c	IVMVPSICAPOINIPSIAGESLSLVENLSTNITIIISIPLTMWIKGRPSLSLKGMANCWDIVINNAADREAVVIAIRIKRITISILLANDVAAFPLPARLSMSIYNDKEELFPGYFPNTTISIENMFELILICLMAAL..YTAI	297
AcerOr67c-like	.....	.....
AdorOr67c-like	.....	.....
AfloOr67c-like	.....	.....
BtOr4-like	.....	.....
ObOr13a	.....	.....
BtOr4	.....	.....
BtOr22c-like	.....	.....
BtOr13a-like	.....	.....
BtOr4-like	.....	.....
Consensus	.....	.....
BiOr816	mtdiq dl y ygwny mlkfmqiwpeerkwnr say vllpc	22
BtOr22c	mtdiq dl y ygwny mlkfmqiwpeerkwnr say vllpc	297
AcerOr67c-like	.....	.....
AdorOr67c-like	.....	.....
AfloOr67c-like	.....	.....
BtOr4-like	.....	.....
ObOr13a	.....	.....
BtOr4	.....	.....
BtOr22c-like	.....	.....
BtOr13a-like	.....	.....
BtOr4-like	.....	.....
Consensus	.....	.....
BiOr816	ITIIVPICAPOINIPSIAGESLSLVENLSTNITIIISIPLTMWIKGRPSLSLKGMANCWDIVINNAADREAVVIAIRIKRITISILLANDVAAFPLPARLSMSIYNDKEELFPGYFPNTTISIENLMRELICQIMAAL..LRIQ	297
BtOr22c	IVMVPSICAPOINIPSIAGESLSLVENLSTNITIIISIPLTMWIKGRPSLSLKGMANCWDIVINNAADREAVVIAIRIKRITISILLANDVAAFPLPARLSMSIYNDKEELFPGYFPNTTISIENMFELILICLMAAL..YTAI	297
AcerOr67c-like	.....	.....
AdorOr67c-like	.....	.....
AfloOr67c-like	.....	.....
BtOr4-like	.....	.....
ObOr13a	.....	.....
BtOr4	.....	.....
BtOr22c-like	.....	.....
BtOr13a-like	.....	.....
BtOr4-like	.....	.....
Consensus	.....	.....
BiOr816	imnccfcapqnti iagdddlvienst ntitislnkt awmkglpkksll cmndw tvt asezetmv iaritstir tirstim nivv afvparslssm ynd lf rgypynttis elt iqq aal yaa	192
BtOr22c	imnccfcapqnti iagdddlvienst ntitislnkt awmkglpkksll cmndw tvt asezetmv iaritstir tirstim nivv afvparslssm ynd lf rgypynttis elt iqq aal yaa	193
AcerOr67c-like	.....	.....
AdorOr67c-like	.....	.....
AfloOr67c-like	.....	.....
BtOr4-like	.....	.....
ObOr13a	.....	.....
BtOr4	.....	.....
BtOr22c-like	.....	.....
BtOr13a-like	.....	.....
BtOr4-like	.....	.....
Consensus	.....	.....
BiOr816	TYITIDDEEVVLILAIERGQLSILRDRDPLSSHSYIDRNNEVKLQHIVPRPFRINRFAETIEIKSFNMLLFCMGCOTICHCCTCYVIMS.....	385
BtOr22c	TYITIDDEEVVLIFHVVGQLSGIKNEISLRLPTYDKKDLTRKEDVQRHEVYNNRERIEIENCFNMLLFCMGCOTICHCCTCYVIMS.....	446
AcerOr67c-like	SYTAIDTEVAMILHLHVVGQLSGIKNEISLRLPTYDKKDLTRKEDVQRHEVYNNRERIEIENCFNMLLFCMGCOTICHCCTCYVIMS.....	349
AdorOr67c-like	SYTAIDTEVAMILHLHVVGQLSGIKNEISLRLPTYDKKDLTRKEDVQRHEVYNNRERIEIENCFNMLLFCMGCOTICHCCTCYVIMS.....	349
AfloOr67c-like	SYTAIDTEVAMILHLHVVGQLSGIKNEISLRLPTYDKKDLTRKEDVQRHEVYNNRERIEIENCFNMLLFCMGCOTICHCCTCYVIMS.....	349
BtOr4-like	TYIAIDTEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSVESMEIANAYNTETWNLPERIT	349
ObOr13a	TYIAIDTEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSVESMEIAKAINQTWTLPERIT	349
BtOr4	TYIAIDTEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSVESMEIAKAINQTWTLPERIT	349
BtOr22c-like	TYIAIDTEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSVESMEIAKAINQTWTLPERIT	344
BtOr13a-like	TYIAIDTEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSVESMEIAKAINQTWTLPERIT	344
BtOr4-like	TYIAIDTEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSVESMEIAKAINQTWTLPERIT	344
Consensus	TYIAIDTEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSVESMEIAKAINQTWTLPERIT	340
BiOr816	TYITIDDEEVVLILAIERGQLSILRDRDPLSSHSYIDRNNEVKLQHIVPRPFRINRFAETIEIKSFNMLLFCMGCOTICHCCTCYVIMS.....	339
BtOr22c	TYITIDDEEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSSESAEIATYAAEWNLPERIT	339
AcerOr67c-like	TYITIDDEEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSSESAEIATYAAEWNLPERIT	341
AdorOr67c-like	TYITIDDEEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSSESAEIATYAAEWNLPERIT	342
AfloOr67c-like	TYITIDDEEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSSESAEIATYAAEWNLPERIT	342
BtOr4-like	TYITIDDEEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSSESAEIATYAAEWNLPERIT	318
Consensus	TYITIDDEEVVLIFHVVGQLSLNRLDRDLRKHITYSKRDEKTLRKEVTCVOCFCGFSGEACEQYLYFCMLFLFIVYFYVMLQLYLYCIVGERLSSESAEIATYAAEWNLPERIT	318
BiOr816	RNWVIMCRARSSPLCITAGRCFCFTIVLIVYSGTCGNVVRKSRSKRNVIIQKKGFEENEELKYMISHIYINIYRAIFSLCGSLKNLDGSNEIKVVRKIRHCG	385
BtOr22c	RNWVIMCRARSSPLCITAGRCFCFTIVLIVYSGTCGNVVRKSRSKRNVIIQKKGFEENEELKYMISHIYINIYRAIFSLCGSLKNLDGSNEIKVVRKIRHCG	549
AcerOr67c-like	KMLIIMCRAKSPLIIITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	401
AdorOr67c-like	KMLIIMCRAKSPLIIITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	401
AfloOr67c-like	KMLIIMCRAKTPLIIITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	401
BtOr4-like	KMLIIMCRAKTPLIIITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	404
ObOr13a	RSLIIIIYRARSPLIIITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	394
BtOr4	RNLIIIMCRARSSPLKIKATGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	377
BtOr22c-like	RNLIIIMCRARSSPLKIKATGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	391
BtOr13a-like	RNLIIIMCRARSSPLCITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	392
BtOr4-like	RNLIIIMCRARSSPLCITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	356
Consensus	RNLIIIMCRARSSPLCITAGRCFCFTIVLIVYSEVLKTSMSRILSVIAYAKUKIKH.....	356

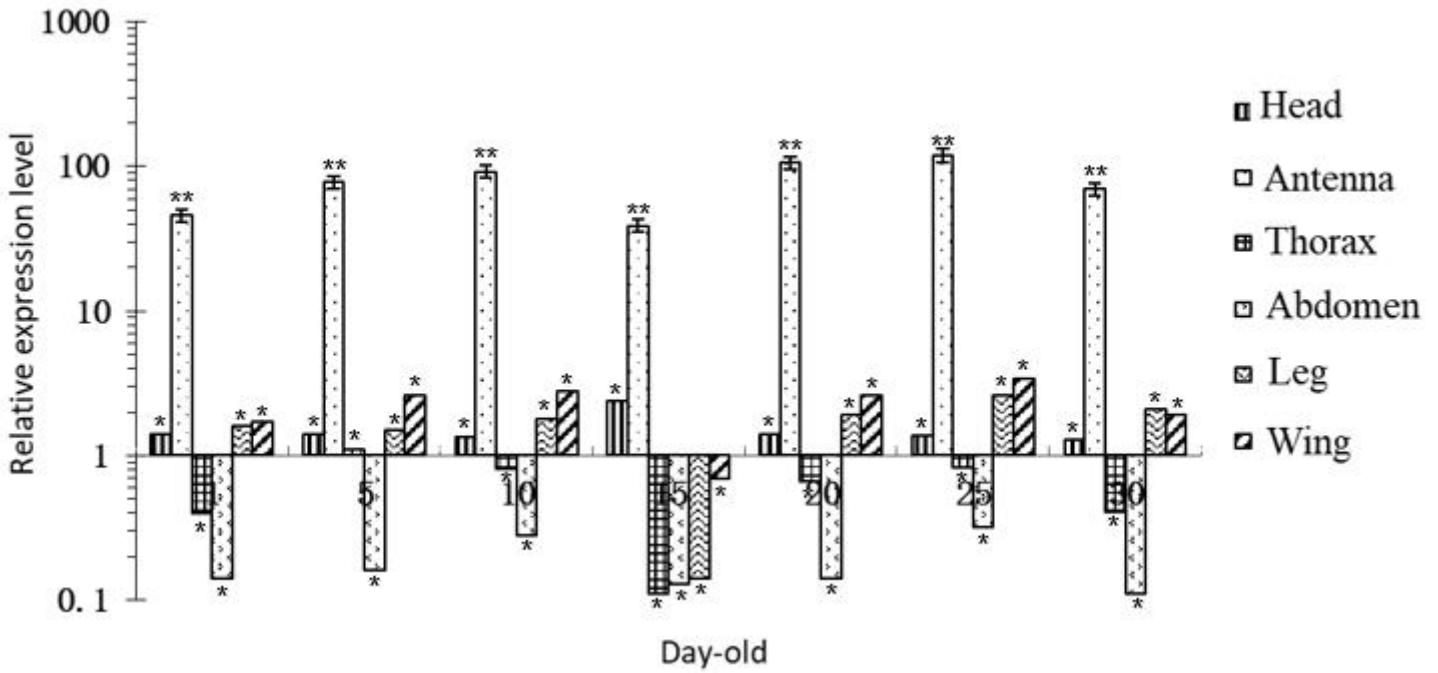
**Figure 4**

Homology comparison of amino acid sequence of *Bombus ignites* BiOr816 and other species Note: *Bombus ignites* BiOr816; *Bombus terrestris* BtOr22c (XP\_012167618.1); *Bombus terrestris* BtOr13a-like (XP\_012167489.1); *Bombus impatiens* BiOr22c-like (XP\_033178691.1); *Bombus vancouverensis* nearcticus BvOr4-like (XP\_033194243.1); *Bombus impatiens* BiOr4-like (XP\_033178693.1); *Bombus terrestris* BtOr4-like (XP\_012167616.1); *Bombus terrestris* BtOr4 (XP\_012167493.1); *Apis florea* AfloOr67c-like (XP\_012345844.1); *Apis dorsata* AdorOr67c-like (XP\_006618840.1); *Apis cerana* AcerOr67c-like (XP\_016916412.1); *Ooceraea biroi* ObOr13a (XP\_011346647.1)



**Figure 5**

Phylogenetic tree of Ors based on amino acid sequence of BiOr816 with neighbor-joining method



**Figure 6**

Relative expression of BiOr816 in different stages and different tissues of *Bombus ignites* Note: The expression of BiOr816 in the head of worker bees at 10-days-old as the control quantity. The data was the expression of BiOr816 at different ages and tissues of *Bombus ignites* (mean $\pm$ SE). “\*\*” the difference was statistically very significant ( $P\leq 0.01$ ), “\*” the difference was statistically significant ( $P\leq 0.05$ ), the following figures was the same as.