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Chemosensory Characteristics of Two *Semanotus bifasciatus* Populations

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Received: 9 July 2019; Accepted: 1 August 2019; Published: 2 August 2019

Abstract: *Semanotus bifasciatus* (Motschulsky) (Cerambycidae: Coleoptera) is a major forest borer in China, and attractants provide a promising method for the control of this pest. Exploration of the chemosensory mechanisms of *S. bifasciatus* is important for the development of efficient attractants for this pest. However, little information is available about the olfactory mechanisms of *S. bifasciatus*. Previous research has indicated that the trapping effects of the same attractant are different between Beijing and Shandong populations of *S. bifasciatus*. To explore the reasons for this, next-generation sequencing was performed to analyze the antennal transcriptome of both sexes of the two *S. bifasciatus* populations, and the olfactory-related genes were identified. Furthermore, the expression levels and single nucleotide polymorphisms (SNPs) of the olfactory-related genes between the two populations were compared. We found that the expression levels of odorant binding proteins (OBPs), odorant receptors (ORs), and sensory neuron membrane proteins (SNMPs) in male *S. bifasciatus* of the Beijing population were obviously lower than those in the Shandong population, and most of the conserved SNPs in OBPs and ORs of the two populations showed more diversity in the Beijing population. Our work provides a foundation for future research of the molecular olfactory mechanisms and pest management of *S. bifasciatus*, as well as other longhorn beetles.

Keywords: antennal transcriptome; chemosensory genes; expression level; SNPs

1. Introduction

Semanotus bifasciatus (Motschulsky) mainly damages *Platycladus orientalis* (L.) Franco, *Sabina chinensis* (L.) Antoine, and other species, and the damage it causes is very serious in some regions [1], which makes this pest a main forest borer species in China. In recent years, the protection of old cypress trees, forest resources, and the ecological environment has been threatened by *S. bifasciatus* in northern China. For example, 67.3% of planted seedlings were damaged by *S. bifasciatus* in Dalian, Liaoning province in 1988 [2]; about 90% of dead cypresses were infested by *S. bifasciatus* in the Lingyan Forest district of Taian, Shandong province in the period 2002–2006; and 97% of the introduced cypress were damaged by *S. bifasciatus* in Xiangshan Mountain park, Beijing in 1979. Thus, the control of *S. bifasciatus* is urgent and important for forest protection.

However, as a boring insect, *S. bifasciatus* remain concealed during egg and larval stages, so it is hard to control this pest. The adult form of *S. bifasciatus* appears in the forest for about half a month only, and at this stage, we can use treatments on them relatively easily. Thus, developing attractants for *S. bifasciatus* based on volatiles or pheromones has attracted the interest of many forest insect scientists, and some volatile lures, using volatiles from certain host plants, have been used to trap this pest [3,4]. The attracting effect of an attractant was tested at two different places—Beijing and Shandong, China—and the results indicated that the responses of two *S. bifasciatus* populations from

these two places differed greatly (unpublished data). This phenomenon indicated that the olfactory sensibilities of the two *S. bifasciatus* populations were different, and this deserves further research. The results of such research may help us to develop accurate attractants for different pest populations.

However, the olfactory response mechanisms of *S. bifasciatus* are unclear, and only fragmented information has been available until now [5]. Thus, our work focuses on the olfactory variety of *S. bifasciatus* from different places.

In insects, environmental odors are mainly detected by olfactory sensory neurons (OSNs) located in sensilla on the antennae [6]. During transformation of chemical volatile signals to electrical nervous impulses, insects rely on at least six olfactory-related gene groups, including two binding protein families, three receptor families, and the sensory neuron membrane proteins (SNMPs) [7,8]. The three receptor families expressed in chemosensory neurons [9–11], include odorant receptors (ORs), ionotropic receptors (IR), and gustatory receptors (GRs) [12,13]. Insect ORs are seven-transmembrane domain proteins, and their topologies are reversed, comparing to vertebrate ORs [14,15]. ORs always appear as dimers, comprised by one specific OR [16] and one conserved gene, called the odorant receptor co-receptor (Orco) [17]. Most GRs are expressed in gustatory receptor neurons, which are involved in contact chemoreception [18]. IRs refer to receptors that are related to ionotropic glutamate receptors (iGluRs) [9]. In addition to the receptor genes, there are two types of binding proteins in olfactory systems that play critical roles in olfaction. Odorant binding proteins (OBP) are small, soluble proteins with six conserved cysteines [19,20]. OBPs (odorant binding proteins) have been proposed to deliver odor molecules to the receptors [19]. Chemosensory proteins (CSPs) are another type of small binding protein, which are more conserved, and they are characterized by two disulfide bridges formed by the four conserved cysteines [21].

Insects display a highly specific and sensitive olfactory sense and complex olfactory behavior, and their olfaction-related genes show large sequence diversity [22–25]. Therefore, identification of olfactory-related genes mainly relies on genomic data [26–28]. Recently, studies on antennal transcriptomes have led to the identification of olfactory-related genes in many insects [29–34], which demonstrated the power of transcriptomic data for olfactory gene identification. However, few studies have compared the olfactory recognition mechanisms between different populations of the same insect species.

In this study, to explore the olfactory variety of different *S. bifasciatus* populations, we sequenced the antennal transcriptomes of both sexes from two places, identified the olfactory-related genes of *S. bifasciatus*, and compared the expression quantity and single nucleotide polymorphisms (SNPs) between the two populations.

2. Materials and Methods

2.1. Insects for Antennal Transcriptome Sequencing

Sections of wood containing overwintering *S. bifasciatus* insects were collected from two sites, the Lingyan Forest district of Taian, Shandong province, and the Mentougou district of Beijing, in March 2018, and were placed in 26 °C ± 2 °C temperature with 50% ± 10% relative humidity and a 16 h light, 8 h dark photo-period. The wood cross-sections were sealed with wax. Eclosion was seen about two weeks later. After eclosion, antennae from 15 female and male *S. bifasciatus* from the two sites were cut off at the base, and the samples were frozen in liquid nitrogen immediately. Antenna from five *S. bifasciatus* were collected as one sample, and a total of three biological replicates were obtained.

2.2. RNA-Seq Library Preparation and Sequencing

We extracted the total RNA of *S. bifasciatus* antenna with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), as previously described [8,35], and then treated them with RNase-free DNase I (TaKaRa, Dalian, Liaoning, China). The RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA), concentration with Qubit® 2.0 Fluorometer (Life Technologies,

Carlsbad, CA, USA), and integrity on the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA), respectively.

A total amount of 1 μg RNA per sample was used to synthetize complementary DNA (cDNA) [36,37]. Then, sequencing libraries were prepared with an Illumina TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). An AMPure XP system (Beckman Coulter, Beverly, MA, USA) was used to select cDNA fragments (of 200 bp length, preferentially), and two-end adaptor ligated DNA fragments were enriched with polymerase chain reaction (PCR). Finally, the products were purified and quantified (Agilent Bioanalyzer 2100). The index-coded samples were clustered and sequenced with an Illumina Hiseq 2500, according to the manufacturer's instructions.

2.3. De Novo Assembly of the Sequences

Clean data were obtained from raw sequencing data, using the following treatments: with self-written Perl scripts, we removed reads containing an adapter sequence, uncertain bases (reads with >10% N), and sequences more than 50% bases with error rates >1%. Pollution of the data was ruled out by comparing with the NT database. Assembly was performed with Trinity (version: trinityrnaseq-r20131110) [38], and redundancy was treated with TGICL [39].

2.4. Annotation

All unigenes were aligned with Blast (The Basic Local Alignment Search Tool) against the NR, SWISSPROT, KEGG, and KOG databases (cut-off by 1e-5), and we selected the highest sequence similarity targets as the functional annotation of the transcripts. Then, Gene Ontology (GO) annotation was performed with Blast 2GO [40,41], and the molecular function, biological process, and cellular component of the genes were assigned [42].

We identified the chemosensory genes in *S. bifasciatus* using contig tBLASTx searches with a query sequence of chemosensory genes from other insects, such as *Drosophila melanogaster*, *Bombyx mori*, and other long-horned beetles. Open reading frames (ORFs) of the candidate genes were found, which were further verified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Gene Expression Quantification

FPKM (fragments per kilobase of exon per million fragments mapped) values were used to measure the expression level of the genes [43]. FPKM value and standard error were obtained from the three biological replicates. Differentially expressed genes (DEGs) of the two *S. bifasciatus* populations were selected using DESeq (http://bioconductor.org/packages/release/bioc/html/DESeq.html) [44]. The compatible-hits-norm model was used to normalize the Unigene expression levels and DEGs [45], and the false discovery rate (FDR) method ($p \leq 0.05$) was used to identify DEGs [46].

2.6. SNP Calling

We used the Genome Analysis Toolkit (GATK2) software to carry out SNP calling [47]; the GATK standard filter method and other parameters were used to filter the Raw vcffiles (cluster Window Size: 35; MQ0 ≥ 4; and (MQ0/(1.0 × DP)) > 0.1, QUAL < 10, QUAL < 30.0, or QD < 5.0, or HRun > 5) and retain the SNPs with distance >5. The SNPs that showed stable diversity between the two *S. bifasciatus* populations or between sexes were selected as conserved SNPs.

2.7. Data Analysis

The expression levels of the identified chemosensory genes were compared according to the FPKM values and based on the three biological replicates. Significant difference between the two populations were tested using ANOVA test (in SPSS 18, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Antennal Transcriptomes Assembly and Annotation

The transcriptomic sequence data were generated using an antenna cDNA library and Illumina HiSeq TM2000/MiSeq technology. About 6 Gbp of clean sequence data were obtained for each sample. Assembly resulted in 55,355 contigs, with an N50 of 2813 bp. The number of contigs longer than 1 kb was 29,791. Gene annotation to several databases obtained gene annotation information for 35,188 genes.

3.2. Chemosensory Gene Identification of S. bifasciatus

We focused on olfactory gene families to perform a detailed analysis of the transcriptomes. In total, 18 OBPs, 21 CSPs, 66 ORs, 24 GRs, 14 IRs, and 4 SNMPs in *S. bifasciatus* were identified (Supplementary Table S1).

As the trapping effects of the attractant differed obviously between Shandong and Beijing, we further analyzed the possible reasons for this situation. As the expression levels and gene sequence variations were two factors that may have been related to the trapping effects, we further analyzed the differences of these aspects in the two *S. bifasciatus* populations.

3.3. Chemosensory Gene Expression Characteristics of Two S. bifasciatus Populations

Chemosensory gene expressions of the two *S. bifasciatus* populations in both sexes were analyzed. Two types of binding proteins (OBPs and CSPs) related to chemosensory were compared between the two populations firstly. Among the 18 identified OBPs, 4 OBPs (OBP3, OBP4, OBP11, and OBP15) were expressed significantly differently between females of the two populations (Figure 1A), while 8 (OBP2, OBP5, OBP7, OBP9, OBP12, OBP13, OBP15, and OBP17) were expressed significantly differently between males of the two populations (Figure 1B). These results indicate that more gene expression varieties were found in males than females of the two populations. A different situation was detected in CSPs, another binding protein of the olfactory system (Figure S1), which means that only a few genes (2 between females and 3 between males of the two populations) were significantly expressed between the two populations.

Among the three type of receptor genes related to chemosensory, we found that the ORs possessed most gene expression differences. Similar to OBPs, varieties of males were more than that of females between the two populations: 10 ORs (OR14, OE16, OR19, OR38, OR41, OR43, OR44, OR47, OR59, and OR63) were expressed significantly differently between females of the two populations (Figure 2A), while 20 ORs (OR2, OR3, OR7, OR9, OR11, OR13, OR14, OR18, OR19, OR22, OR34, OR36, OR40, OR46, OR48, OR49, OR50, OR51, OR56, and OR63) were expressed significantly differently between males of the two populations (Figure 2B). The expression differences of GRs and IRs in the two populations were also compared (Figures S2 and S3), and we found that the differently expressed genes of these two types of receptor were less than that of ORs. Three (in females) and 6 (in males) GRs were differently expressed between the two populations (Figure S2), and 2 (in females) and 4 (in males) IRs were expressed differently between the two populations (Figure S3). It is worth mentioning that SNMPs expressed obviously different in male antenna from the two populations, which means that all the four identified SNMPs expressed significantly differently between males from the two populations (Figure 3). These differently expressed genes may have mediated the olfactory differences of the two *S. bifasciatus* populations.

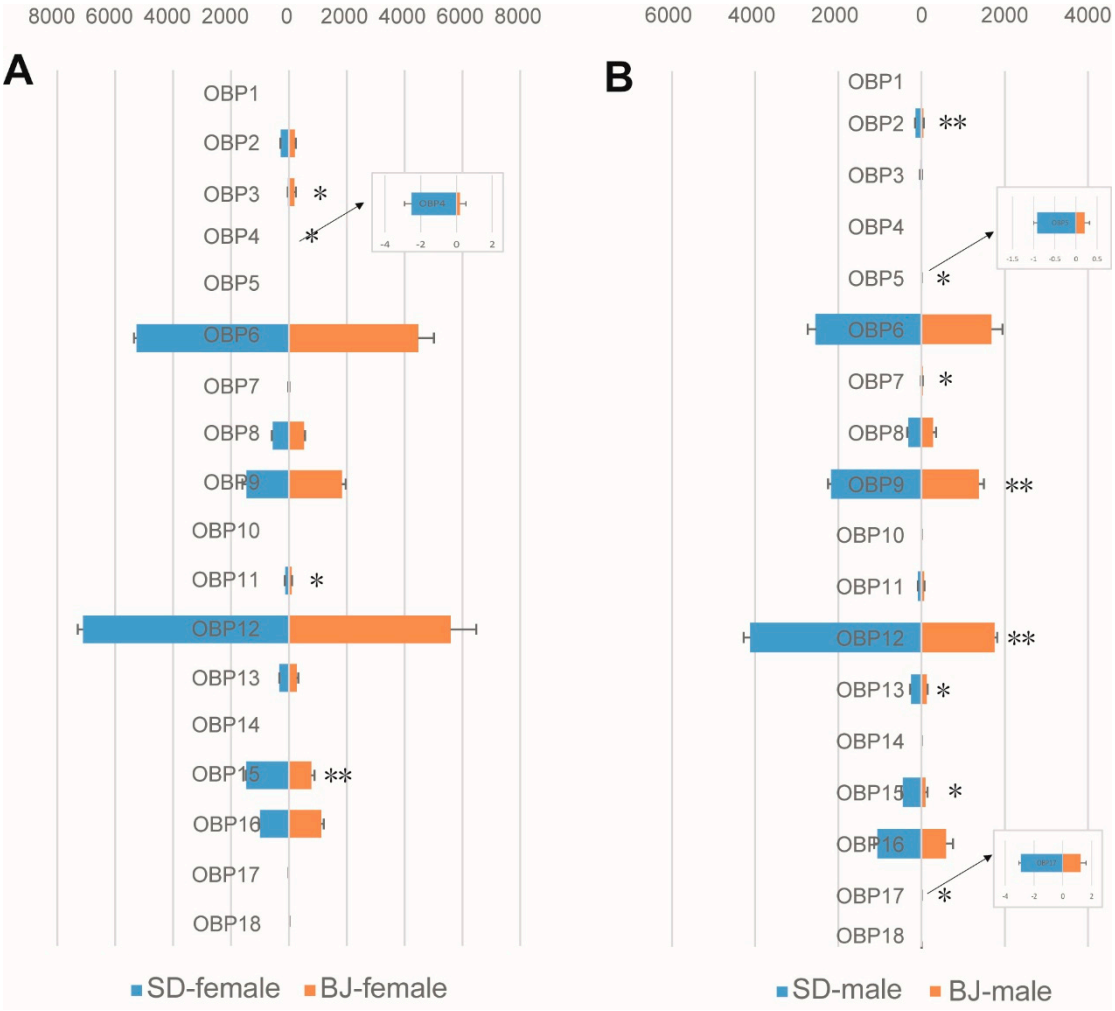

Figure 1. Comparison of odorant binding protein (OBP) genes expression levels in both sexes of the two *S. bifasciatus* populations. The horizontal axis refers to the expression level of the genes (FPKM values, mean ± SE, $n = 3$). (**A**): OBPs expression level comparison between females of the two population; (**B**): OBPs expression level comparison between males of the two population. BJ, Beijing population; SD, Shandong population. * means the difference is significant at the 0.05 level; ** means the difference is significant at the 0.01 level.

Figure 2. Comparison of odorant receptor (OR) genes expression levels in both sexes of the two *S. bifasciatus* populations. The horizontal axis refers to the expression level of the genes (FPKM values, mean ± SE, n = 3). (**A**): ORs expression level comparison between females of the two population; (**B**): ORs expression level comparison between males of the two population. BJ, Beijing population; SD, Shandong population. * means the difference is significant at the 0.05 level; ** means the difference is significant at the 0.01 level.

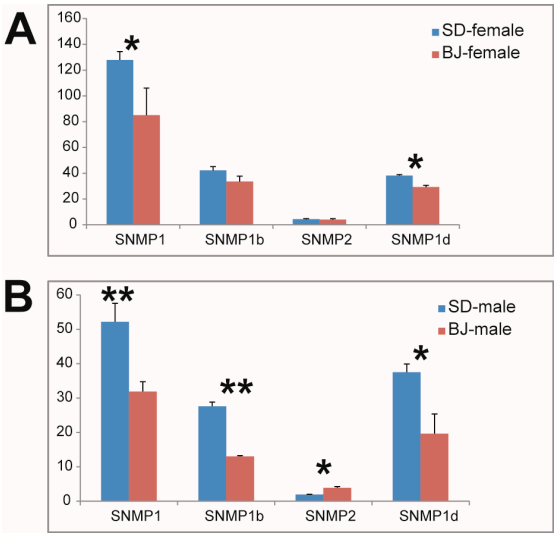

Figure 3. Comparison of sensory neuron membrane protein (SNMP) genes expression levels in both sexes of the two *S. bifasciatus* populations. The vertical axis refers to the expression level of the genes (FPKM values, mean ± SE, $n = 3$). (**A**): SNMPs expression level comparison between females of the two population; (**B**): SNMOs expression level comparison between males of the two population. BJ, Beijing population; SD, Shandong population. * means the difference is significant at the 0.05 level; ** means the difference is significant at the 0.01 level.

3.4. SNP Analysis of Chemosensory Genes in Two S. bifasciatus Populations

We collected the conserved SNP sites of the chemosensory genes of both sexes in the two *S. bifasciatus* populations, where 2 SNPs in OBPs, 2 SNPs in CSPs, 63 SNPs in ORs, 12 SNPs in GR, 16 SNPs in IRs, and 11 SNPs in SNMPs were obtained (Supplementary Table S2). Then we analyzed the SNP sites of each gene. If the acid bases at the SNP site were the same in the three repeats of Shandong population but different in that of the Beijing population, we record this SNP site as Beijing diversity SNP site; reversely, if the acid bases at the SNP site were the same in the three repeats of the Beijing population but different in that of the Shandong population, it was recorded as Shandong diversity SNP site. Similar rules were used in the SNP site recording related to sexes. Figure 4 illustrates the total SNP site numbers that we found as Beijing diversity, Shandong diversity, and diversity between sexes in each chemosensory gene type. Interestingly, most of the SNPs in OBPs and ORs were Beijing diversity ones, while fewer showed diversity in the Shandong population and only several SNPs were correlated to the sexes of *S. bifasciatus*; however, the Beijing diversity and Shandong diversity SNPs number were similar in CSPs, GRs, IRs, and SNMPs.

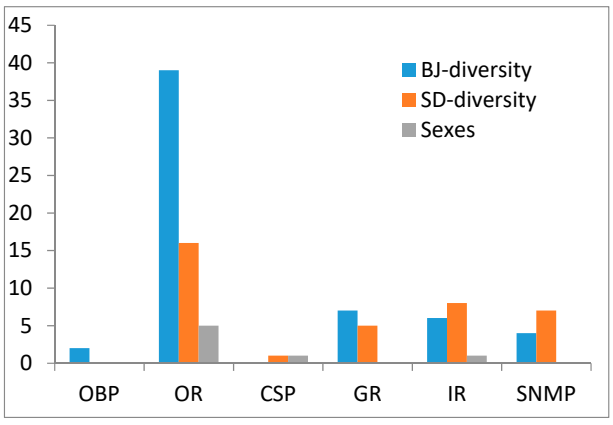

Figure 4. Three categories of collected conserved SNPs. BJ, Beijing; SD, Shandong.

4. Discussion

Little is known about the olfactory mechanisms of *S. bifasciatus*, which are very serious cypress pests [3,4]. At the same time, the olfactory response of *S. bifasciatus* populations from different places to the same attractant are varied (unpublished data). The genetic basis related to physiological differences of insects may be gene expression level differences or sequences variation [48–50]. We found that in males of *S. bifasciatus*, the expression levels of OBPs, ORs, and SNMPs of the Beijing population were obviously lower than those of the Shandong population, and most conserved SNPs of OBPs and ORs from the two populations were the ones that showed more diversity in the Beijing population.

Chemosensory mechanism studies of longhorn beetles have been few, up to now, and chemosensory genes from only a few species have been identified [51]. We identified 18 OBPs, 21 CSPs, 66 ORs, 24 GRs, 14 IRs, and 4 SNMPs in *S. bifasciatus*, and these numbers were compared to those of *Anoplophora glabripennis* (42 OBPs, 12 CSPs, 37 ORs, 11 GRs, 4 IRs, and 2 SNMPs), a longhorn beetle whose genome has been sequenced [52]. Thus, our results applied the chemosensory genes of *S. bifasciatus* relatively completely, and this will facilitate the chemosensory study of *S. bifasciatus* and longhorn beetles.

Previous trapping variation in *S. bifasciatus* from two different sites indicated that the olfactory systems of these two populations were different (unpublished data). The reasons could be quantitative or qualitative diversities of their chemosensory genes [48–50]. Thus, to explore the quantitative differences in the olfactory systems of these two *S. bifasciatus* populations, we analyzed the expression levels of *S. bifasciatus* from the two sites. Interestingly, both of these aspects were different in these two *S. bifasciatus* populations. Many OBP, OR, and SNMP genes expressions were lower in males of *S. bifasciatus* from Beijing than those from Shandong. However, the attracting effect of the lure in the Shandong population was worse than in the Beijing population (unpublished data). We can deduce that either the olfactory levels were not the main reason for the attraction differences, or that the expression level was negatively related to the attraction effects of the lure we used.

To analyze the qualitative differences between the olfactory systems of the two *S. bifasciatus* populations, olfactory gene SNP analysis was performed. For OBPs, ORs, the SNP sites referring to the diversity of the Beijing population were more than that of the Shandong population, which may explain the fact that our lure could attract the Beijing population better, as the diversity of the chemosensory genes may contribute to the recognition of lures [53]. Few studies related to the SNP variations between populations of forest pests have been carried out [53,54], and our work has applied a new sight-to-reason analysis of the population olfactory differences of the same forest pests. However, further analysis of OBP and OR gene functions is necessary to explain the chemosensory recognition diversity of the two *S. bifasciatus* populations.

5. Conclusions

To explore the olfactory variety of *S. bifasciatus*, we used next-generation sequencing to analyze the antennal transcriptome of both sexes of the two populations. The olfactory-related genes were identified. Furthermore, expression levels and SNPs of the olfactory-related genes between the two populations were analyzed. We found that in males, the expression levels of many OBP, OR, and SNMP genes of *S. bifasciatus* from Beijing were obviously lower than those from Shandong, and most conserved SNPs in OBPs and ORs from the two populations showed more diversity in the Beijing population. This work provides a foundation for future research into the molecular olfactory mechanisms of *S. bifasciatus*, as well as other longhorn beetles, and a basis for the development of new and accurate attractants for the management of this forest pest.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/8/655/s1, Table S1: Identified chemosensory genes in *S. bifasciatus* and their unigene ID, Table S2: Collected conserved SNPs in two *S. bifasciatus* populations, Figure S1: Comparison of chemosensory protein (CSP) genes expression levels in both sexes of the two *S. bifasciatus* populations. The horizontal axis refers to the expression level of the genes (FPKM values, mean ± SE, $n = 3$), Figure S2: Comparison of gustatory receptor (GR) genes expression levels in both sexes of the two *S. bifasciatus* populations. The horizontal axis refers to the expression level of the genes (FPKM values, mean ± SE, $n = 3$), Figure S3: Comparison of ionotropic receptor (IR) genes expression levels in both sexes of the two *S. bifasciatus* populations. The vertical axis refers to the expression level of the genes (FPKM values, mean ± SE, $n = 3$).

Author Contributions: S.Z. (Sufang Zhang) designed the experiments and performed the bioinformatic analysis, as well as drafting the manuscript; S.S. assisted in identification of the chemosensory genes; S.Z. (Shiyu Zhang), X.K., H.W., and F.L. collected the insects.; Z.Z. designed the experiments and modified the manuscript.

Funding: This research was funded by The National Key Research and Development Program of China, grant number 2018YFC1200400 and 2017YFD0600102, The Fundamental Research Funds for the Central Non-profit Research Institution of CAF, grant number CAFYBB2018SZ006 and CAFYBB2017QB003, and The National Nature Science Foundation of China, grant number 31670657.

Acknowledgments: We thank the BioMarker Company for transcriptome sequencing.

Conflicts of Interest: The authors declare no conflicts of interest.

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