Molecular Characterization and Differential Expression of an Olfactory Receptor Gene Family in the White-Backed Planthopper *Sogatella furcifera* Based on Transcriptome Analysis

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Abstract

The white-backed planthopper, *Sogatella furcifera*, a notorious rice pest in Asia, employs host plant volatiles as cues for host location. In insects, odor detection is mediated by two types of olfactory receptors: odorant receptors (ORs) and ionotropic receptors (IRs). In this study, we identified 63 *SfurORs* and 14 *SfurIRs* in *S. furcifera* based on sequences obtained from the head transcriptome and bioinformatics analysis. The motif-pattern of 130 hemiptera ORs indicated an apparent differentiation in this order. Phylogenetic trees of the ORs and IRs were constructed using neighbor-joining estimates. Most of the ORs had orthologous genes, but a specific OR clade was identified in *S. furcifera*, which suggests that these ORs may have specific olfactory functions in this species. Our results provide a basis for further investigations of how *S. furcifera* coordinates its olfactory receptor genes with its plant hosts, thereby providing a foundation for novel pest management approaches based on these genes.

Introduction

Insects can exploit chemical signals in the environment using their accurate olfactory systems, thereby mediating many important physiological behaviors, such as mate-finding, host location, and sending alarms to conspecifics. The antennae are the major olfactory organs of insects, and they possess various types of sensilla, where peripheral olfactory signal transduction events occur. At the molecular level, three main types of proteins are generally considered to be involved in odorant molecule transduction in the sensillum. First, odorants may diffuse into the sensillar lymph via pores, where odorant-binding proteins (OBPs) recognize and bind them. Second, OBPs act as transporters to transfer odorants across the sensillar lymph to reach...
olfactory receptors, including odorant receptors (ORs) and ionotropic receptors (IRs), which are located on the dendrites of olfactory receptor neurons (ORNs). Finally, distinct odorant-degrading enzymes act as inactivators to degrade odorants and maintain the sensitivity of ORNs [1–4].

Insect ORs were first identified in *Drosophila* using molecular biology and bioinformatics methods [5–7]. Further studies determined that insect ORs also contain seven-transmembrane domains similar to mammalian G protein-coupled receptors, but their topology is inverted with an intracellular N-terminus and extracellular C-terminus [8–10]. The OR family has undergone rapid evolution in a species-specific manner, according to studies of insect species with available genome sequences. Thus, highly variable numbers of ORs have been identified, e.g., 62 ORs in *Drosophila melanogaster* [5–7], 79 in *Anopheles gambiae* [11, 12], 170 in *Apis mellifera* [13], 259 in *Tribolium castaneum* [14], and 66 in *Danaus plexippus* [15, 16]. Only one OR is relatively conserved among species, i.e., ORco, an obligate and universal co-receptor that interacts with other ligand-specific ORs to form an ORx–ORco complex, which functions as a ligand-gated cation channel [17, 18]. Recently, many studies have focused on pheromone receptors, especially in Lepidoptera [19–23], whereas very few studies have investigated ORs in phytophagous insects.

Animal ionotropic glutamate receptors (iGluRs) are well known for their essential roles in synaptic transmission as receptors of the excitatory neurotransmitter glutamate [24]. Recently, IRs were discovered to be a new olfactory receptor family, i.e., a variant iGluR subfamily [25–27]. IRs are involved in odorant reception, as was shown by combined mis-expression experiments and through the subcellular localization of IRs in olfactory organs with chemosensory sensilla [25]. Further research showed that IRs differ from ORs in that they are attuned to carboxylic acids and amines. In addition to *D. melanogaster*, expressed sequenced tag, transcriptome, and genome analyses have identified insect IRs in representative species from multiple orders, such as *Rhagoletis pomonella* (Diptera) [28], *Anopheles gambiae* (Diptera) [29], *Bombbyx mori* [27], *Spodoptera littoralis* (Lepidoptera) [30], *Chilo suppressalis* (Lepidoptera) [31], and *Aphis gossypii* (Hemiptera) [32]. However, no functional data related to IRs have been reported outside *Drosophila*. Croset et al. [27] suggested that IRs can be classified into two distinct subfamilies with different ancestors: the conserved “antennal IRs” and the species-specific “divergent IRs.” The “antennal IRs” may represent the original olfactory receptor family of insects. The “divergent IRs” derived from “antennal IR” ancestors may be involved in gustation. Chemosensory IRs could have been derived from an animal iGluR ancestor.

The white-backed planthopper, *Sogatella furcifera* Horváth (Hemiptera: Delphacidae), is a notorious rice pest that migrates among many Asian countries each year, where its sap-sucking feeding style and transmission of the southern rice black-streaked dwarf virus causes severe losses in rice crops. In addition to rice crops, *S. furcifera* also damages many other plants, including other Gramineae, such as *Echinochloa crus-galli*, *Zea mays*, and *Paspalum distichum* [33, 34]. Depending on population survival, *S. furcifera* naturally occurs in two phenotypically distinct forms, i.e., short- and long-winged. The short-winged adults lay more eggs when the host plant abundance is sufficiently rich, whereas the long-winged adults migrate to find rice plants with higher nutritional quality. These behaviors of *S. furcifera* suggest a crucial role for chemosensation. However, the precise physiological mechanisms that mediate these important behaviors remain unknown at present.

Three decades ago, Obata et al. [35, 36] found that volatiles and extracts from rice appeared to attract three related Delphacidae rice planthopper species (*S. furcifera*, *Nilaparvata lugens*, and *Laodelphax striatellus*), but not when they lacked antennae in dark conditions. However, except for our previous reports on OBPs functional research [37, 38, 39], the molecular mechanisms of olfaction in Delphacidae are largely unknown. ORs bind odorants more specifically

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**Competing Interests:** The authors have declared that no competing interests exist.
than OBPs, and a single OR is sufficient to change insect behavior, whereas a specific OBP is not needed to invoke behavioral change [37]. Thus, in the present study, we sequenced and analyzed the head transcriptome of *S. furcifera* adults using next generation sequencing, where we identified 63 OR and 14 IR transcripts in this pest insect species. We also conducted transcriptome sequencing and gene ontology (GO) annotation, as well as scanned sequences for motif-patterns and examined phylogenetic relationships.

**Results**

**Transcriptome sequencing and sequence assembly**

The *S. furcifera* head transcriptome was sequenced using the Illumina HiSeq™ 2000 platform and assembled with Trinity (v2012-10-05) (Table 1 and Fig 1). In total, about 163 million reads were obtained. After filtering, 142 million clean reads were generated, which comprised 14.2 gigabases (Gb), with a longest length of 28,290 nt and a median length of 456 nt. These reads were assembled into 89,810 transcripts and 43,712 unigenes, with N50 lengths of 3,014 and 2,217 nt, respectively (Table 1). In addition, the unigenes with a sequence length >1000 nt accounted for 29.63% of the transcriptome assembly (Fig 1). The transcriptome raw reads have been deposited with the NCBI SRA database (accession number: SRR2068690).

**Homology analysis and GO annotation**

BLASTx homology searches of all 43,712 unigenes showed that 14,430 (33.1%) had homologous genes in the non-redundant (NR) protein database with a cut-off E-value of $10^{-5}$. The best match percentage (14.3%) was with *Tribolium castaneum* sequences, followed by sequences from *Acyrthosiphon pisum* (13.2%), *Pediculus humanus* (8.6%), *Nasonia vitripennis* (4.7%), and *Megachile rotundata* (4.6%) (Fig 2A).

GO annotations for all the unigenes were obtained using the Blast2GO pipeline according to the BLASTx search against NR. The GO annotations were used to classify the transcripts into functional groups according to specific GO categories. Among the 43,712 unigenes, 13,265 (30.3%) could be assigned to various GO terms. In the molecular function category, the genes expressed in the head were mostly enriched for binding (e.g., nucleotide, ion, and odorant binding) and catalytic activity (e.g., hydrolase and oxidoreductase). In the biological process category, the most common were cellular and metabolic processes. In the cellular component terms, the most abundant were cell and organelle (Fig 2B).

**Table 1. Summary of *S. furcifera* transcriptome assembly.**

|                          | 14.2 Gb          |
|--------------------------|------------------|
| Total size               | 139043608        |
| Number of transcripts    | 89810            |
| Total unigene count      | 43712            |
| Genes with homologs in NR| 14430            |
| Total transcript nucleotides | 3014 nt        |
| N50 transcript length    | 2217 nt          |
| Longest unigene length   | 28920 nt         |
| Median unigene length    | 456 nt           |
| GC content               | 42.85%           |

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Identification OR/IR genes

The unigenes related to candidate olfactory receptors (ORs/IRs) were identified based on keyword searches of the BLASTx annotations. The predicted protein unigene protein sequences were also analyzed using PSI-BLASTp with known aphid olfactory receptors [32, 38]. In total, we identified 77 unigenes that belonged to the olfactory receptor family in the head transcriptome of *S. furcifera*, including 63 ORs and 14 IRs, all of which shared similarity with other insect OR and IR genes. Among these, 27 OR and 3 IR genes encoded putative, complete opening reading frames. Further information for the OR and IR genes including the unigene references, lengths, and best BLASTx his are listed in Tables 2 and 3. To validate the reliability of the transcriptome assembly, we randomly chose 32 full-length ORs for RT-PCR validation. To cover a sequence that was as long as possible, the primers were designed to span the ORF, the primer sequences are listed in S1 Table. As a result, all 32 ORs were successfully amplified by RT-PCR (S1 Fig). The PCR results were confirmed by sequencing. All of the OR and IR sequences in this study are listed in S1 File.

Motif-pattern and phylogenetic trees analysis

Conserved motifs are important elements of functional domains. We used the MEME server to identify conserved motifs in 130 hemiptera ORs. Parameters used in this and all other motif predictions of this study were: minimum width = 6, maximum = 10, maximum number of motif to find = 8. As a result, eight motifs (Most case occur: Motif-1, ALYSNCNWTDM; Motif-2, LLTMQMNNAN; Motif-3, PTKIVNLEMF; Motif-4, QLFMYCYIFD, Motif-5, DLKSIKDHQ; Motif-6, GHYQIIDPET, Motif-7, TYNAYYIFY; Motif-8, CYTVSVVLLN) were found for hemipteran ORs (Fig 3). Most motif amino acid residues locate in intramembrane domain, not in transmembrane domain. Motif 1, 4, 5 were the top three motifs present.
in these ORs, the ratio were 44.6%, 32.3% and 33.1%, respectively. We also carried out a motif-pattern analysis of hemipteran ORs. It was quite different between species with the exception of the ORco sequences, which exhibited the same "4–1" motif-pattern. The "6-7-5-4-1-2-3-8" pattern was the most common motif in aphids with 25 ORs in A. pisum and 10 ORs in A. gossypii exhibiting the pattern. The most prevalent motif pattern in S. furcifera was the "5–1" motif, which was found in 8 SfurORs.

To distinguish putative OR or IR functions, we also constructed two phylogenetic trees using 57 ORs (>150 aa), 7 IRs, and 3 iGluRs (>120 aa) from S. furcifera, as well as known hemipteran ORs (Fig 4) and other insect IRs (Fig 5). In the OR tree, the co-receptor ortholog of S. furcifera SfurORco was easily assigned because it shared high similarity with the conserved hemipteran co-receptors. Other ORs were assigned to various clades, which indicated their distinct functions. Most of the other SfurORs had orthologous genes, such as SfurOR21/28/29/ ApOR29, SfurOR59/ApOR5/AgoOR5, SfurOR3/RproOR-trT1H999, SfurOR18/56/RproOR-
Table 2. Unigenes of candidate odorant receptors.

| Gene name | Gene id | Gene Length (bp) | Amino acid length | Full-length | NR ID | NR E value | NR Description |
|-----------|---------|------------------|-------------------|-------------|--------|------------|----------------|
| Orco      | comp60837_c0 | 3496 | 473 | yes | KC526964.1 | 5.29E-08 | Tribolium castaneum or16 gene for olfactory receptor 16 |
| OR1       | comp61288_c0 | 1571 | 416 | yes | ABQ84982.1 | 5.12E-09 | putative chemosensory receptor 12 [Spodoptera littoralis] |
| OR2       | comp61986_c0 | 1785 | 432 | yes | XP_002067278.1 | 7.75E-09 | PREDICTED: putative odorant receptor 94b-like [Megachile rotundata] |
| OR3       | comp58780_c0 | 1772 | 427 | yes | EEZ99241.1 | 3.50E-11 | odorant receptor 28 [Tribolium castaneum] |
| OR4       | comp57076_c0 | 1537 | 415 | yes | EFN89949.1 | 2.86E-06 | putative odorant receptor 85d [Harpegnathos saltator] |
| OR5       | comp63016_c0 | 1445 | 407 | yes | EFA09246.1 | 4.16E-07 | odorant receptor 15 [Tribolium castaneum] |
| OR6       | comp61037_c0 | 1459 | 402 | yes | ACX53766.1 | 4.45E-06 | chemosensory receptor [Heliothis virescens] |
| OR7       | comp58541_c0 | 1676 | 473 | yes | XP_556129.1 | 5.22E-07 | PREDICTED: putative odorant receptor 9a-like [Apis florea] |
| OR8       | comp32401_c0 | 1490 | 399 | yes | XP_001354859.2 | 2.81E-08 | GL14885 [Drosophila persimilis] >gi|194107678|gb|EDW29721.1|GL14885 [Drosophila persimilis] |
| OR9       | comp63582_c0 | 1640 | 402 | yes | XP_002067278.1 | 4.00E-09 | GI14807 [Drosophila mojavensis] >gi|193906881|gb|EDW07548.1|GI14807 [Drosophila mojavensis] |
| OR10      | comp60973_c0 | 1673 | 412 | yes | ABQ84982.1 | 1.16E-06 | putative chemosensory receptor 12 [Spodoptera littoralis] |
| OR11      | comp52202_c0 | 1709 | 219 | 5’ exon lost | NP_001177607.1 | 6.96E-07 | odorant receptor 267 [Nasonia vitripennis] |
| OR12      | comp57173_c0 | 1347 | 425 | yes | XP_966790.1 | 5.82E-08 | PREDICTED: similar to Odorant receptor 85d, putative [Tribolium castaneum] >gi|226334912|gb|CAM84018.1|olfactory receptor 20 [Tribolium castaneum] |
| OR13      | comp59765_c0 | 1520 | 432 | yes | NP_001177518.1 | 6.19E-06 | odorant receptor 99 [Nasonia vitripennis] |
| OR14      | comp59929_c0 | 1565 | 426 | yes | NP_001166620.1 | 4.20E-12 | odorant receptor 101 [Nasonia vitripennis] |
| OR15      | comp50637_c0 | 798  | 225 | 5’ exon lost | EAT45323.2 | 1.10E-10 | olfactory receptor 9 [Helicoverpa armigera] |
| OR16      | comp62723_c0 | 1674 | 466 | yes | AFC91748.1 | 4.87E-10 | putative odorant receptor OR40, partial [Cydia pomonella] |
| OR17      | comp61628_c0 | 1478 | 434 | yes | XP_003694963.1 | 4.87E-07 | PREDICTED: putative odorant receptor 9a-like [Apis florea] |
| OR18      | comp37650_c0 | 1561 | 398 | yes | EFN71826.1 | 1.85E-14 | Putative odorant receptor 24a [Camponotus floridanus] |
| OR19      | comp59170_c0 | 1853 | 416 | yes | NP_001164457.1 | 2.87E-06 | odorant receptor 265 [Nasonia vitripennis] |
| OR20      | comp64230_c0 | 2420 | 512 | yes | EFZ21798.1 | 3.69E-11 | Putative odorant receptor 13a [Harpegnathos saltator] |
| OR21      | comp60687_c0 | 1451 | 277 | 5’ exon lost | EU67075.1 | 3.14E-07 | putative chemosensory receptor 10 [Danaus plexippus] |
| OR22      | comp56083_c0 | 1281 | 323 | yes | EAT37621.2 | 4.86E-11 | Odorant receptor 9a, putative [Aedes aegypti] |
| OR23      | comp147392_c0 | 1523 | 399 | yes | EEZ99373.1 | 2.34E-06 | Odorant receptor 2a [Camponotus floridanus] |
| OR24      | comp60798_c0 | 2098 | 423 | yes | EFN67925.1 | 8.65E-09 | Putative odorant receptor 9a [Camponotus floridanus] |
| OR25      | comp55722_c0 | 1603 | 396 | yes | XP_002067278.1 | 7.97E-09 | olfactory receptor, putative [Aedes aegypti] >gi|108873398|gb|EAT37623.1|AAEL010426-PA [Aedes aegypti] |
| OR26      | comp57440_c0 | 1843 | 438 | yes | EEZ99406.1 | 8.15E-12 | odorant receptor 32 [Tribolium castaneum] |
| OR27      | comp57217_c0 | 1952 | 493 | yes | XP_003246096.1 | 3.79E-30 | odorant receptor 57 [Tribolium castaneum] |
| OR28      | comp49303_c0 | 765  | 193 | 5’ exon lost | EFA09294.1 | 1.95E-08 | odorant receptor 10 [Tribolium castaneum] |
| OR29      | comp44516_c0 | 1543 | 412 | yes | ACC63238.1 | 2.85E-08 | GK16337 [Drosophila willistoni] >gi|194163363|gb|EDW72624.1|GK16337 [Drosophila willistoni] |
| OR30      | comp48809_c0 | 592  | 164 | 5’ exon lost | XP_002067278.1 | 1.65E-14 | PREDICTED: odorant receptor 46a, isoform A-like [Apis mellifera] |
| OR31      | comp56791_c0 | 1503 | 385 | yes | EFN67929.1 | 4.68E-13 | Odorant receptor 49b [Camponotus floridanus] |
| OR32      | comp43248_c0 | 1409 | 434 | 3’ exon lost | AFL3413.1 | 3.47E-09 | odorant receptor 2 [Locusta migratoria] |
| OR33      | comp51860_c0 | 728  | 221 | 5’ exon lost | EFA09294.1 | 7.12E-16 | odorant receptor 10 [Tribolium castaneum] |
| OR34      | comp42791_c0 | 400  | 111 | 5’ exon lost | XP_002015587.1 | 2.16E-09 | Or43a [Drosophila pseudoobscura pseudoobscura] >gi|198135482|gb|EAL24773.2|Or43a [Drosophila pseudoobscura pseudoobscura] |

(Continued)
In the IR tree, all 7 SfurIRs and 3 SfuriGluRs were assigned to known insect IR clades, i.e., SfurIR3 (IR64a clade), SfurIR4 (IR8a clade), SfuriGluR1/2/3 (ionotropic glutamate receptor clade), SfurIR9 (IR25a clade), SfurIR10 (unidentified clade), SfurIR11 (IR40a clade), SfurIR14 (IR76b clade), and SfurIR15 (IR93a clade).

### Table 2. (Continued)

| Gene name | Gene id | Gene Length (bp) | Amino acid length | Full-length | NR ID   | NR E value | NR Description |
|-----------|---------|------------------|-------------------|-------------|---------|------------|----------------|
| OR35      | comp52124_c0 | 1463             | 408               | yes         | XP_003704145.1 | 1.15E-18   | PREDICTED: odorant receptor Or2-like [Megachile rotundata] |
| OR36      | comp58617_c0 | 1753             | 429               | yes         | EEU9404.1    | 4.86E-09   | odorant receptor 29 [Tribolium castaneum] |
| OR37      | comp39740_c0 | 588              | 185               | 5’ exon lost | ACX53766.1  | 7.12E-06   | chemosensory receptor [Heliothis virescens] |
| OR38      | comp50926_c0 | 766              | 191               | 5’ exon lost | EFA02940.1  | 7.88E-14   | odorant receptor 47 [Tribolium castaneum] |
| OR39      | comp57366_c0 | 1396             | 411               | yes         | XP_001850048.1 | 1.94E-06   | Odorant receptor 9a [Culex quinquefasciatus] >gi|167867973|gb|EDS31356.1| Odorant receptor 9a [Culex quinquefasciatus] |
| OR40      | comp572797_c0 | 203              | 68                | 5’3’ exons lost | XP_003699516.1 | 4.03E-08   | odorant receptor 9 [Tribolium castaneum] |
| OR41      | comp51830_c0 | 847              | 273               | 5’ exon lost | XP_003402693.1 | 3.83E-09   | PREDICTED: odorant receptor 2a-like [Bombus terrestris] |
| OR42      | comp53845_c0 | 1238             | 398               | yes         | NP_001177520.1 | 8.20E-12   | odorant receptor 101 [Nasonia vitripennis] |
| OR43      | comp47056_c0 | 436              | 145               | 5’ exon lost | ACX53766.1  | 5.24E-09   | chemosensory receptor [Heliothis virescens] |
| OR44      | comp662599_c0 | 271              | 78                | 5’ exon lost | NP_001164458.1 | 3.79E-06   | odorant receptor 98 [Nasonia vitripennis] |
| OR45      | comp49469_c0 | 1298             | 398               | yes         | EEU9404.1    | 3.09E-14   | putative chemosensory receptor 10 [Danaus plexippus] |
| OR46      | comp49409_c0 | 781              | 234               | 5’ exon lost | XP_002056218.1 | 1.32E-09   | Odorant receptor 9a, putative [Aedes aegypti] |
| OR47      | comp59794_c0 | 2202             | 431               | yes         | EEU9404.1    | 2.05E-15   | odorant receptor 44 [Tribolium castaneum] |
| OR48      | comp53841_c0 | 1605             | 418               | yes         | XP_001651754.1 | 1.58E-09   | odorant receptor [Aedes aegypti] >gi|108878225|gb|EAT42450.1| AAEL006005-PA [Aedes aegypti] >gi|197322752|gb|ACH69140.1| odorant receptor 9 [Aedes aegypti] |
| OR49      | comp47025_c1 | 753              | 166               | 5’ exon lost | EEU9404.1    | 1.02E-09   | odorant receptor 60 [Tribolium castaneum] |
| OR50      | comp50910_c0 | 1518             | 425               | yes         | ADK48356.1  | 1.19E-06   | odorant receptor 43a [Drosophila melanogaster] >gi|301032209|gb|ADK48416.1| odorant receptor 43a [Drosophila melanogaster] |
| OR51      | comp43499_c0 | 1525             | 425               | yes         | CAD31949.1  | 1.70E-06   | putative chemosensory receptor 8 [Heliothis virescens] |
| OR52      | comp53588_c0 | 1613             | 464               | yes         | CAG8118.1    | 4.06E-23   | putative chemosensory receptor 17 [Heliothis virescens] |
| OR53      | comp33086_c0 | 667              | 196               | 5’ exon lost | NP_001177509.1 | 3.62E-13   | odorant receptor 69 [Nasonia vitripennis] |
| OR54      | comp48912_c0 | 1539             | 462               | yes         | XP_003246096.1 | 1.42E-29   | olfactory receptor, putative [Aedes aegypti] |
| OR55      | comp47360_c0 | 1427             | 418               | yes         | NP_001177515.1 | 2.40E-16   | odorant receptor 89 [Nasonia vitripennis] |
| OR56      | comp36388_c0 | 570              | 166               | 5’ exon lost | EEU9404.1    | 1.18E-11   | odorant receptor 36 [Tribolium castaneum] |
| OR57      | comp26089_c0 | 689              | 229               | 5’3’ exons lost | EEU9404.1  | 2.23E-08   | odorant receptor 29 [Tribolium castaneum] |
| OR58      | comp524680_c0 | 323              | 72                | 5’3’ exons lost | DAA05996.1 | 3.43E-06   | TPA: odorant receptor 40 [Bombus mori] |
| OR59      | comp971_c0  | 1022             | 340               | 5’3’ exons lost | NP_001091787.1 | 6.97E-11   | olfactory receptor 10, partial [Helicoverpa armigera] |
| OR60      | comp31797_c0 | 328              | 94                | 5’ exon lost | NP_001116810.1 | 3.03E-10   | olfactory receptor 46 [Bombus mori] |
| OR61      | comp3354_c0  | 1128             | 359               | 3’ exon lost | EEU9404.1    | 1.93E-07   | odorant receptor 28 [Tribolium castaneum] |
| OR62      | comp50731_c0 | 988              | 329               | 5’3’ exons lost | EFA01342.1 | 5.84E-07   | odorant receptor 171 [Tribolium castaneum] |

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**Olfactory Receptor Genes in Sogatella furcifera**

trT1H8H6, and SfurOR20/RproOR-trT1H805. In the IR tree, all 7 SfurIRs and 3 SfuriGluRs were assigned to known insect IR clades, i.e., SfurIR3 (IR64a clade), SfurIR4 (IR8a clade), SfuriGluR1/2/3 (ionotropic glutamate receptor clade), SfurIR9 (IR25a clade), SfurIR10 (unidentified clade), SfurIR11 (IR40a clade), SfurIR14 (IR76b clade), and SfurIR15 (IR93a clade).
OR/IR transcript expression levels

Among the 63 ORs, SfurORco had the highest expression level in the transcriptome data (fragments per kilobase per million mapped reads, FPKM = 68.96), followed by SfurOR2 (FPKM = 22.21), SfurOR3 (FPKM = 6.54), SfurOR4 (FPKM = 5.73), and SfurOR5 (FPKM = 5.72) (Fig 6A). For the 16 SfurIRs, SfurIR3 had the highest expression level (FPKM = 12.46), followed by SfurIR9 (FPKM = 4.06), and SfurIR7 (FPKM = 2.73) (Fig 6B). SfurIR11 and SfurIR14 had very low expression levels in our transcriptome dataset.
Fig 3. Motif analysis of ORs in the Hemipera. Parameters used for motif discovery were: minimum width = 6, maximum width = 10, maximum number of motif to find = 8. The upper parts listed the eight motifs discovered in the 130 ORs using MEME (version 4.9.1) on line server (http://meme.nbcr.net/meme/). The lower parts of different colors indicate approximate locations of each motif on the predicted protein sequence. The numbers in the boxes correspond to the numbered motifs in the upper part of the figure, where small...
Discussion

In this study, we determined the repertoire of olfactory receptor superfamilies (ORs and IRs) in *S. furcifera* due to their potential significance as target genes for developing new pest control strategies, as well as for elucidating the molecular mechanisms that underlie insect-host plant interactions. In total, 14.2 Gb of *S. furcifera* head transcriptome data were sequenced, which is higher than that processed in most other studies [31, 32, 39–41]. After extensive sequencing and assembly using Trinity RNA-Seq, we identified 63 ORs and 14 IRs in *S. furcifera*. The number of ORs lies between that of two hemipteran aphids, *A. gossypii* (45 ORs) [32] and *A. pisum* (73 ORs) [38, 42], with sequenced genomes, and it is similar to the 62 ORs found in *D.*
and the 79 ORs in A. gambiae [11, 12], but much lower than those in T. castaneum (259 ORs) [14] and A. mellifera (170 ORs) [13]. The number of IRs was similar to the 14 IRs found in A. gossypii [32], 18 in D. melanogaster [44], and 22 in A. gambiae [29], but slightly higher than those in T. castaneum (10 IRs) (these data were obtained from GenBank) and A. mellifera (nine IRs) (these data were obtained from GenBank). These findings suggest that the adaptation of distinct species to their plant hosts has led to the diversification of ORs and IRs during their evolution.

We conducted a MEME motif analysis using multiple hemiptera ORs to investigate differences among various species. Unlike insect OBPs [45], hemiptera ORs exhibit more differences, likely because ORs are more specific for odorant substrates than OBPs. In support of this, a single silkmoth pheromone receptor was activated by tis ligand to trigger sexual behaviors without the need of a specific OBP [37]. Furthermore, among various suborders of hemiptera the
respective hosts are quite different, for example *R. prolixus* utilize blood meals and *S. furcifera* is an oligophagous pest that feeds only on few plants such as rice, maize. Thus we propose that they locate different hosts via volatiles based on their specific ORs. The exception, ORco, is more highly conserved than other ORs, which reflects its functional role in interacting with specific ORs to form the ligand—gated ion channel [17, 18]. mong the *Sfur*ORs, the *Sfur*ORco gene had the highest mRNA abundance, which is similar to *Ago*ORco in *A. gossypii* [32]. In insects, the ORco gene is a co-receptor that forms a functional heteromer with specific ORs [17, 18]. In addition to *Sfur*ORco, the *Sfur*OR1 gene had higher expression levels than the other *Sfur*ORs, thereby suggesting that it may bind key plant host volatiles in *S. furcifera*, although further functional research is required to confirm this suggestion. The phylogenetic analysis of hemipteran ORs suggested that the *Sfur*ORs have undergone functional differentiation due to their scattered distribution. One specific *Sfur*OR sub-clade, which included *Sfur*OR16, 23, 33, 35, 37, and 55, had no counterparts in other species in this analysis, thereby suggesting that these six ORs may be activated by the specific host plant volatiles of *S. furcifera*.

To further distinguish putative IRs from iGluRs, the *Sfur*IRs were aligned with IR orthologs from other insect species and some DmeliGluRs for BLASTx and phylogenetic analysis. We demonstrated that there were obvious differences in the distributions of DmeliGluRs and insect IRs. Like the ORco gene, the IR8a and IR25a genes are thought to act as co-receptors because of their co-expression with other IRs [46]. Our expression profiles were consistent with this hypothesis because IR3 (IR8), IR9 (IR40a.1), and IR7 (IR25a) were the top three genes among
the 14 SfurIRs, This result also agrees with the higher expression levels of AgoIR8a and AgoIR25a in *A. gossypii* [32].

In conclusion, based on analyses of head transcriptomic data, we identified 63 ORs and 14 IRs in the insect species *S. furcifera*. Our method was successful in identifying chemosensory receptor genes with low expression levels and our results provide a valuable resource for investigating and elucidating the mechanisms of olfaction in *S. furcifera*. As a crucial first step toward understanding their functions, we also conducted a comprehensive examination of the expression patterns of these olfactory receptor genes, which demonstrated that most of these OR and IR genes were expressed in chemosensory organs. Our findings provide the foundation for future research into the olfactory system of *S. furcifera* and for further investigations of classic behaviors, such as migration, as well as large numbers of potential target genes for controlling this pest.

**Materials and Methods**

**Insect rearing and tissue collection**

*S. furcifera* were collected from rice fields with the permission of the agricultural bureau in Libo county (25° 21’ N; 107° 49’ E), Guizhou province, China. The field studies did not involve endangered or protected species and no specific permissions were required for these insects. Collected insects were reared in the laboratory on rice seedlings at 26 ± 1°C, with a 16 h light: 8 h dark cycle. We collected 1000 heads of 1- to 3-day-old long-winged adults (male/female = 1/1) for transcriptome sequencing. We dissected various tissues (approximately 300 antennae, 150 mouthparts, 150 heads, 500 legs, and 50 bodies for each replicate) from long-winged adults under a microscope and we collected three replicates for each tissue type. The tissue samples were stored in RNAlater reagent (Qiagen, Valencia, CA, USA) at 4°C until further use.

**cDNA library construction and Illumina sequencing**

Total RNA was extracted using TRIzol reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer’s protocol. The cDNA library construction and Illumina sequencing of the samples were performed by Novogene Bioinformatics Technology Co. Ltd, Beijing, China. The mRNA was purified from 10 μg of total RNA from *S. furcifera* heads using NEBNext oligo (dT)25 magnetic beads (NEB Next™ Poly(A) mRNA Magnetic Isolation Module, NEB, Beverly, MA, U.S.A.). NEBNext™ mRNA Library Prep Master Mix Set for Illumina® (NEB, Beverly, MA, U.S.A.) was used for further library construction, mRNAs were fragmented into short sequences in the presence of RNA Fragmentation Reaction Buffer at 94°C for 5 min. Next, the first-strand cDNA was generated using Random Primer reverse transcription by using ProtoScript II Reverse Transcriptase (NEB, Beverly, MA, U.S.A.) at 25°C for 10 min, then 42°C for 15 min, and inactivation by heating at 70°C for 15 min, Second-strand cDNA using Second Strand Synthesis Enzyme Mix (NEB, Beverly, MA, U.S.A.) at 16°C for 2.5 hour with heated lid set at 40°C. Then NEBNext End Repair Enzyme Mix (NEB, Beverly, MA, U.S.A.) was used to perform end repair of the cDNA library at 30 minutes at 20°C. NEBNext dA-Tailing Reaction Buffer and Klenow Fragment (3’→5’ exo−) (NEB, Beverly, MA, U.S.A.) were used to dA-tail of cDNA Library at 37°C for 30 minutes. After end repair and dA-tailing, NEBNext Adaptor and USER™ enzyme, (NEB, Beverly, MA, U.S.A.) were used to ligate library DNA at 37°C for 15 minutes. After end repair and ligation of the adaptors, the products were amplified by PCR and purified using a QIAquick PCR Purification Kit to create a cDNA library, which was sequenced using the HisSeq™ 2000 platform.
**De novo assembly of short reads and gene annotation**

After removing the adaptor sequences, low-quality reads, and reads where N ≥ 0.1%, the remaining reads were treated as clean reads. De novo transcriptome assembly was performed using the short reads assembly program Trinity (v2012-10-05) [47]. The overlap settings used for the assembly were 30 bp and 80% similarity, and all of the other parameters were set to their default values.

Unigenes >150 bp were aligned by BLASTx with protein databases, including Nr, Swiss-Prot, KEGG, and COG (e-value < 10^-5), to identify protein with high sequence similarity and assign putative functional annotations. Next, we used the Blast2GO program [48] to obtain GO annotations of the unigenes and we obtained the GO functional classifications using WEGO software [49].

**Expression level analysis for the unigenes**

The expression levels (abundances) of the unigenes were calculated with the FPKM method [50] using the formula: FPKM (A) = (10,000,000 × C × 1,000)/(N × L), where FPKM (A) is the expression level of gene A, C is the number of reads uniquely aligned to gene A, N is the total number of reads uniquely aligned to all genes, and L is the number of bases in gene A. The FPKM method can eliminate the influence of different gene lengths and sequencing discrepancies when calculating the abundance of expression.

**RNA extraction and cDNA synthesis**

The approximately 300 *S. furcifera* heads were dissected and used for RNA extraction. The collected tissues were fast-frozen in liquid nitrogen and kept at -70°C for further use. Total RNA was extracted using a MiniBEST Universal RNA Extraction Kit (TaKaRa, Liaoning, Dalian, China) following the manufacturer’s instructions. The cDNA template was synthesized with Oligo(dT)18 primer as anchor primers, using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Liaoning, Dalian, China) at 42°C for 1 hr. The reaction was terminated by heating at 70°C for 15 min.

**PCR validation**

Gene specific primers across ORF of selected OR genes were designed using "Primer Premier 5.0" for RT-PCR validation. The sequences of these primers are listed in Table A1. PCR experiments were carried out using a C-1000 thermocycler (Bio-Rad, Waltham, MA, USA), and Touchdown PCR reactions were performed under the following conditions: 94°C for 3 min; 20 cycles at 94°C for 50 sec, 60°C for 30 s, and 72°C for 2 min, with a decrease of the annealing temperature of 0.5°C per cycle. This was followed by 15 cycles at 94°C for 50 sec, 50°C for 30s, and 72°C for 2 min, and final incubation for 10 min at 72°C. The reactions were performed in 25 μl with 100 ng of single-stranded cDNA of *S. furcifera* heads, 2.0 mM MgCl2, 0.5 mM dNTP, 0.4μM for each primer and 1.25 U Taq polymerase or EX-Taq polymerase (TaKaRa, Liaoning, Dalian, China). PCR products were analyzed by electrophoresis on 1.5% w/v agarose gel in TAE buffer (40 mmol/L Tris—acetate, 2 mmol/L Na2EDTA-H2O) and the resulting bands visualized with ethidium bromide. DNA purification was performed using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa, Liaoning, Dalian, China). Purified products were sub-cloned into a T/A plasmid using the pEASY-T3 vector system (Trans-Gen Biotech, Beijing, China) following the manufacturer’s instructions. The plasmid DNAs was transformed into competent Trans1-T1 cells, positive clones were checked by PCR, and then sequenced by Sangon Biotech (Shanghai, China).
Motif-pattern analysis

A total of 130 of hemipteran ORs were used for motif discovery and pattern analysis. The MEME (version 4.9.1) online server (http://meme.nbcr.net/meme/), which has been widely used for the discovery of DNA and protein motifs. The parameters used for motif discovery were as follows: minimum width = 6, maximum width = 10, and the maximum number of motifs to find = 8.

OR/IR phylogenetic trees

MEGA 6.0 Beta [51] was used to construct two phylogenetic trees using 156 hemiptera ORs and 209 insect IR sequences (which is referred to IR phylogenetic tree in Liu, et al 2015 [52]) respectively with the neighbor-joining method. We also performed a bootstrap analysis of 1000 replicates to evaluate the branch strength in the phylogenetic tree. The OR dataset comprised ORs in from available databases: *A. gossypii* [32], *Acyrthosiphon pisum* [38, 42], and *Aplysus lucorum* ORco [53]. The IR dataset comprised IRs from: hemipteran species, i.e., *Aphis gossypii* [32], and *A. pisum* [38, 42]; lepidopteran species, i.e., *B. mori* [27], *C. pomonella* [54], *D. plexippus* [16], *M. sexta* [40], *S. littoralis* [55], and *S. nonagrioides* [41]; as well as IRs and iGluRs from the model insect *D. melanogaster* [27].

Supporting Information

S1 Fig. Agarose gel electrophoresis of RT-PCR verification. (DOCX)

S1 File. SfurOR and SfurIR sequences. (FASTA)

S1 Table. The RT-PCR primers. (DOC)

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Author Contributions

Conceived and designed the experiments: PH. Performed the experiments: PH MH. Analyzed the data: PH MH. Contributed reagents/materials/analysis tools: PH. Wrote the paper: PH MH Y-NZ.

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