Sensing volatiles throughout the body: Geographic and tissue-specific olfactory receptor expression in the fig wasp *Ceratosolen fusciceps*

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Abstract

An essential adaptive strategy in insects is the evolution of olfactory receptors (ORs) to recognize important volatile environmental chemical cues. Our model species, *Ceratosolen fusciceps*, a specialist wasp pollinator of *Ficus racemosa*, likely possesses an OR repertoire that allows it to distinguish fig-specific volatiles in highly variable environments. Using a newly assembled genome-guided transcriptome, we annotated 63 ORs in the species and reconstructed the phylogeny of *Ceratosolen* ORs in conjunction with other hymenopteran species. Expression analysis showed that though ORs were mainly expressed in the antennae, 20 percent were also expressed in non-antennal tissues such as the head, thorax, abdomen, legs, wings, and ovipositor. Specific upregulated expression was observed in OR30C in the head and OR60C in the wings. We identified OR expression from all major body parts of *C. fusciceps*, suggesting novel roles of ORs throughout the body. Further examination of OR expression of *C. fusciceps* in widely separated geographical locations, i.e., south (urban) and northeast (rural) India, revealed distinct OR expression levels in different locations. This discrepancy likely parallels the observed variation in fig volatiles between these regions and provides new insights into the evolution of insect ORs and their expression across geographical locations and tissues.

Keywords: Fig wasp, RNA-Seq, Genome guided-transcriptome assembly, Olfactory receptors, Ectopic expression, Evolution, Non-antennal tissues, Ovipositor.

Introduction

Chemical interactions between plant volatiles and insect olfactory receptors (ORs) are essential processes for the survival and reproduction of insects. In insects, ORs are expressed in the dendritic membrane of olfactory sensory neurons (OSNs). Insect ORs are 7-transmembrane domain proteins with an intracellular N-terminus and an extracellular C-terminus (Benton et al. 2006; Smart et al. 2008; Missbach et al. 2014). Functional insect ORs consist of heterodimeric complexes with a highly divergent ligand-specific OR and a highly conserved co-receptor (Orco) (Larsson et al. 2004; Sato et al. 2008; Wicher et al. 2008; Butterwick et al. 2018). During the evolution of insects, ORs became a massively diverse gene family as a result of adaptation to complex and changeable chemical environments (McBride 2007; Linz et al. 2013; Schmidt and Benton 2020; Wicher and Miazzi 2021). In the case of specialist insect pollinators, it is reasonable to expect adaptation to the detection of signature host plant chemical signals.

The fig and fig wasp interaction has emerged as an important model in chemical ecology because of its highly specific mutualism (e.g., Bain et al., 2016; Borges et al., 2008; Borges, 2015, 2021; Borges et al., 2011, 2013; Ghara et al., 2011; Hou et al., 2020; Proffit et al., 2007; Ranganathan & Borges, 2009; Wei et al., 2021; Xin et al., 2020; Yadav & Borges, 2017). This interplay also presents these wasps as an attractive model for the study of olfactory adaptations. Agaonid female wasps enter the enclosed globular fig inflorescence called the syconium, oviposit concurrently with pollination, and later die within the syconium. Female wasps get only one chance to enter an oviposition/pollination chamber since they lose their wings and part of their antennae in the process of entering the syconium. Therefore, female fig wasps are selected for high specificity towards the pollination scent composed of volatile

organic compounds (VOCs) emitted by the pollen-receptive host fig. Male wasps eclose in the enclosed syconium, where they die after mating with eclosed females and thus do not have to find a mate over a long distance. Probably due to this highly specialized lifestyle, pheromones and their receptors are unknown in fig wasps. Finding a pollen-receptive fig syconium is the key for the female pollinator for oviposition and reproduction. We used the widely distributed *Ficus racemosa* and its wasp pollinator *Ceratosolen fusciceps* for the molecular characterization of ORs in northeastern and southern India since this particular fig and wasp species pair has received considerable attention regarding chemical signaling (Bain et al. 2016; Xiao et al. 2021). When insects are highly specialized on their host, the rate of OR gene function loss is 9-to-10 times greater than in generalists as seen in highly specialized compared to generalized drosophilid flies (McBride 2007). The extreme reduction of OR gene numbers in *Ceratosolen solmsi*, an obligate and host-specific pollinator of *Ficus hispida* (just 56 compared to about ~100-300 in other solitary parasitic or predatory Apoid wasps) is likely due to its obligate plant host specificity (Obiero et al. 2021), and we expected a similar reduction in *C. fusciceps***.**

It was initially hypothesized that ORs were exclusively expressed in olfactory tissues such as antennae and mouth parts. However, *Culex* mosquitoes (Leal et al. 2013), leaf beetles (Wang et al. 2016), green plant bugs (An et al. 2016), and butterflies (van Schooten et al. 2020) showed expression of some ORs in a variety of tissues other than the antenna, viz. legs, wings, head, thorax, and abdomen. In the tobacco budworm, *Heliothis virescens*, lower levels of pheromone-detecting receptors were expressed in tissues such as the proboscis, abdomen, leg, wing, and thorax (Krieger et al. 2004).

Widmayer et al. (2009) showed expression of pheromone receptors in ovipositor sensilla of female *H. virescens*. The ovipositor of the noctuid moth (Koutroumpa et al. 2021), grass moth (Xia et al. 2015), and tobacco hornworm (Klinner et al. 2016) also expresses ORs. In *Spodoptera littoralis* chemosensory receptors were identified from mouthparts, legs, and ovipositors (Koutroumpa et al. 2021). The olfactory repertoire of another extreme specialist marine insect, *Clunio marinus*, revealed the possibility of OR expression in legs, genitalia and larval body (Missbach et al. 2020). The expression of ORs outside of antenna and palps is always paralleled by the presence of sensilla (Koutroumpa et al. 2021). Furthermore, Yadav and Borges (2017) showed experimentally that sensilla on fig wasp ovipositors fire in response to fig volatiles, and the ovipositor deflects in response to carbon dioxide puffs, showing clearly that the ovipositor possesses chemosensory abilities. Broad expression of ORs in tissues other than antennae, maxillary palps, and ovipositors hints towards general functional significance in chemical sensing over the insect body. However, there are very few studies on potential functional significance of olfactory detection in other tissues, like the proboscis of *Manduca sexta* in flower humidity perception and nectar foraging (Goyret and Raguso 2006; Havercamp et al. 2016).

Furthermore, intraspecific variation in signal recognition may also be influenced by the complexity of changing environments (Renou and Anton 2020), and floral scents can vary geographically (Skogen et al. 2022). However, variation in OR expression across geographical locations is rarely studied. To understand OR gene expression variation, most comparative transcriptome analyses in insects have been performed between closely

related genera or species (Elgar et al. 2018; Guo et al. 2021) or between genders (Athrey et al. 2021; Xu et al. 2021). Examination of intra-specific OR variation is especially important given changing scenarios of signal content, local volatile environments, and ambient conditions.

Here we compare the host-specific variation of OR expression of pollinating fig wasp. We performed a comparative OR gene expression analysis of *C. fusciceps* tissues across two distinct geographical sites (south India and northeast India bordering China) separated by 3000 km (Fig 1). We expected OR variation between these sites as previous work (Kobmoo et al. 2010; Bain et al. 2016) showed that *C. fusciceps* and its host plant *F. racemosa* form a genetically homogeneous population across south-east Asia (including southern China and Thailand), while populations in south India form separate genetic clusters. We identified 63 ORs in which most ORs were expressed in the antennae, while 20% were also expressed in other olfactory tissues. Upon comparison of ORs from the south and northeast Indian wasps, we observed a few ORs that were exclusively expressed in the antennae of wasps in one region. Other ORs showed significant variation in expression levels between regions which might correspond to variation in fig volatile profiles between these regions (Nongbri and Borges, unpub. data). This inter-population variability in OR expression was also found among ORs expressed outside the antennae.

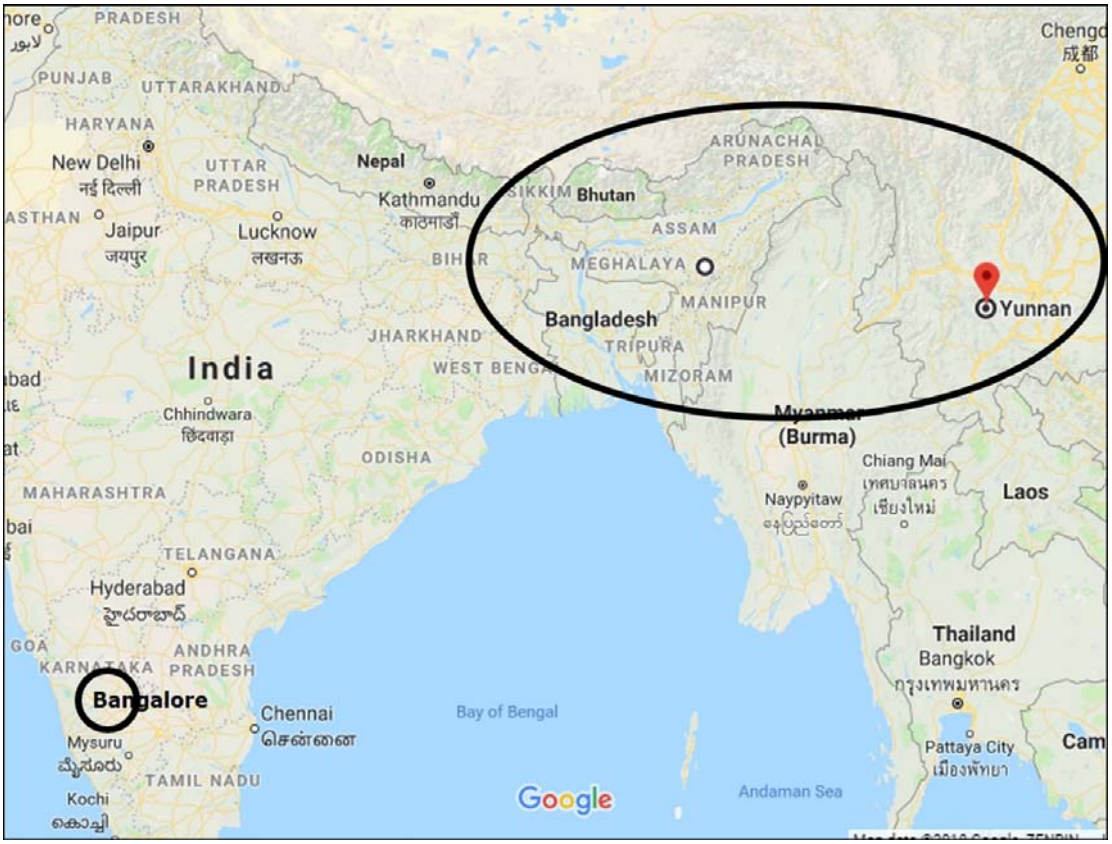


Figure 1. Locations of the study sites in northeastern and southern India. Note that the northeastern site is approximately at the same latitude as the site in Yunnan, China, where earlier data on *F. racemosa* VOCs, plant, and pollinator genetics was established.

Results

Total RNA Sequencing and Genome-Guided Transcriptome Assembly

We performed transcriptome sequencing of 14 samples of 7 tissues (antennae, head, abdomen, thorax, legs, wings, and ovipositor) of the fig pollinator wasps collected from 2 different regions (south and northeast India). An average of 23.9 (south India) and 21.2 million (northeast India) preprocessed reads corresponding to an average of 88% of high-quality data of each tissue sample was retained for the assembly (Additional file: Fig. S1). To get the best quality

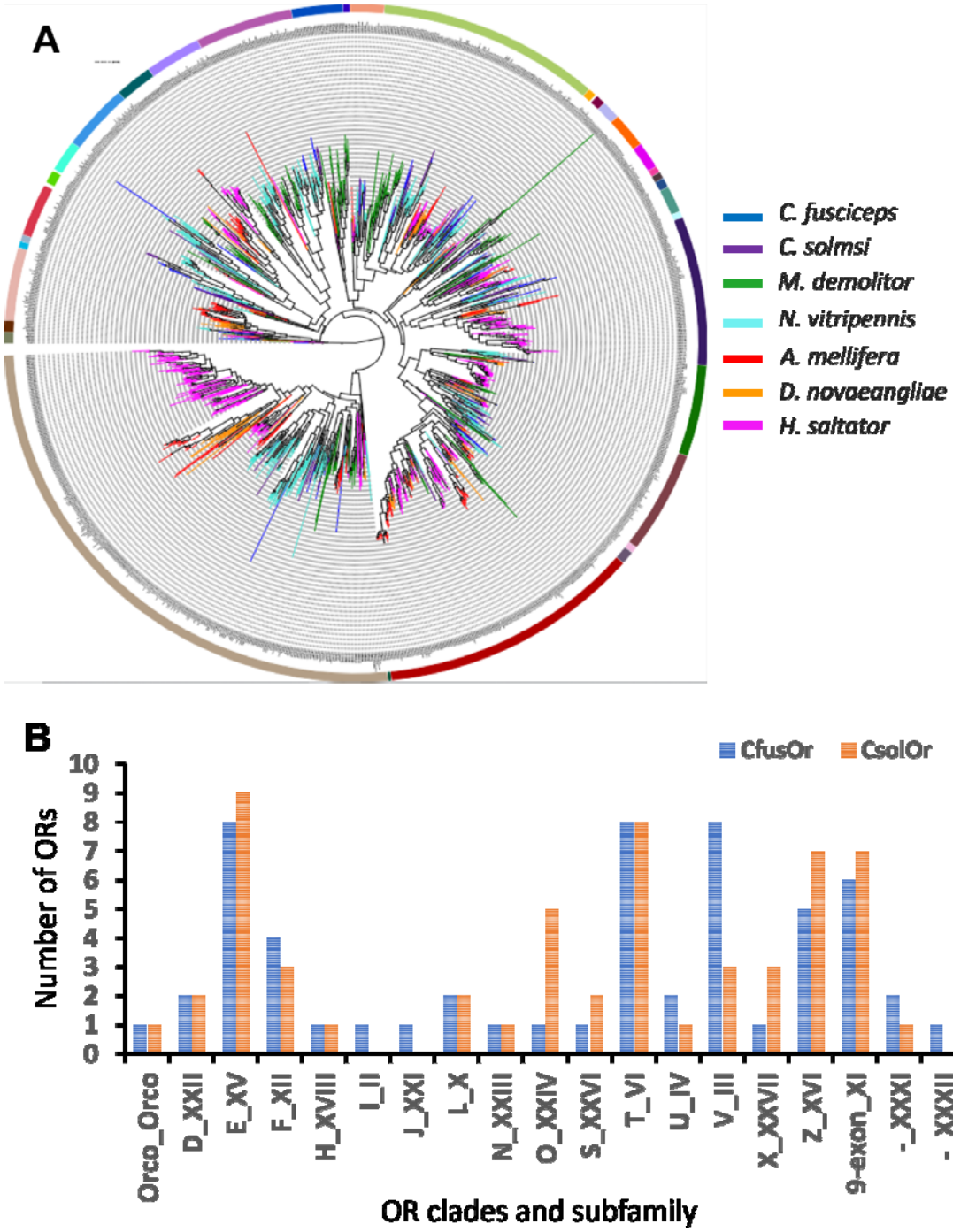
assembly, we assembled the transcriptome sequences by a genome-guided method. For this purpose, we used our newly sequenced and assembled draft genome of *C. fusciceps* (Additional file 1: Table S1). The whole genome of the pollinator wasp was sequenced to get total coverage of 160X using Illumina (100X), Mate pair (40X), and Nanopore (20X) sequencing. We then performed hybrid assembly using MASuRCA (Maryland Super-Read Celera Assembler). As a result, a high-quality genome with a scaffold length of 238 Mb, N50 values of contig 2.2 Mb, and a Scaffold of 4.1 Mb were obtained (Krishnan and Borges, unpublished data). Finally, a good quality transcriptome assembly using Trinity v 2.8.5. was achieved with 58076 transcripts that contained 17746 predicted proteins and 14417 transcripts with known protein domains. BUSCO analysis with Hymenoptera (4415 BUSCOs) and Insecta (1658 BUSCOs) datasets identified 78.2% (3452) and 86.5% (1435) of BUSCOs in our transcriptome assembly (Additional file 1: Table. S2). Based on these results the assembly was considered for further gene annotation.

Annotation of olfactory receptors in *C. fusciceps*

From the genome-guided transcriptome assembly, we found 74 putative OR transcripts (Additional file 2) using hmmscan against the Pfam database. These results were validated using the InsectOR web server (Karpe et al. 2021); further manual curation resulted in 63 ORs. Among these 63 ORs, 48 sequences encoded for proteins of more than 300 amino acids and were annotated as ‘complete’. Of the remaining sequences 15 transcripts encoded for OR proteins, 8 with missing N-terminus, 3 with missing C-terminus, and 4 captured only the middle fragment of the OR protein sequence. Of the 63 OR protein sequences discovered from the curated transcriptome, 56 had more than 200 amino acid sequence lengths. These were further used for

phylogenetic reconstruction of the ORs from two *Ceratosolen* species (*C. solmsi and C. fusciceps*) along with a few other hymenopteran species (Methods). OR sequences containing more than 200 amino acids are generally preferred for phylogenetic analysis (An et al. 2016; Wu et al. 2019; Al-Jalely and Xu 2021; Xu et al. 2021). The inclusion of a diversity of ORs from Hymenoptera also ensured the correct rooting of the tree and its clades. Most of the known hymenopteran OR subfamilies/clades were well-supported in the current reconstruction (Fig 2A, Additional file 1: Table. S3). The ORs of both *Ceratosolen* species had smaller OR repertoires compared to the other hymenopteran species including the two specialized parasitoid wasps, *Microplitis demolitor* (Zhou et al. 2015) and *Nasonia vitripennis* (Robertson et al. 2010). The two *Ceratosolen* species also had the most similar repertoire distribution across OR subfamilies compared to the others.

Though *C. fusciceps* ORs were well distributed among hymenopteran clades, striking features were observed for a few OR clades (Fig. 2B, Additional file 1: Table. S3).
The highest number of ORs, 8 each, were observed in clade XV (Subfamily E), clade III (Subfamily V), and clade VI (Subfamily T). The next highest number of ORs 6, 5, and 4 were found in clade XI (subfamily 9-exon), clade XVI (subfamily Z), and clade XII (subfamily F) respectively. Equal distribution was present in clade XXII (subfamily D), clade XXXI (subfamily), clade IV (subfamily U), clade X (subfamily L), and clade X rest (clade X includes Xa and Xb subclades and the remaining ORs that do not form a single clade is named as X rest). The remaining 9 clades had only one OR and 21 clades had no *C. fusciceps* ORs, whereas these clades contained ORs from the other hymenopteran species studied here (Fig. 2B, Additional file 1: Table. S3).



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186 **Figure 2.** A. Phylogenetic analysis of hymenopteran ORs. The branches were color-coded as per

187 species. The subfamilies are shown by colored stripes. B. Distribution of *C. fusciceps* (CfusOr)

188 and *C. solmsi* (CsolOr) ORs in different clades and subfamilies.

189 The phylogenetic reconstruction of ORs from the two *Ceratosolen* species (Additional file 1: Fig
190 S2) and the analysis of best-bidirectional BLAST hits demonstrated that although their OR
191 repertoires were similar in size, not all ORs were in a perfect orthologous relationship with *C.*
192 *solmsi* ORs. Considering both approaches, only 19 OR sequences (ORco, OR-5, 6, 7, 8, 12, 15,
193 16, 18, 26, 30, 35, 36, 38, 39, 41, 42, 52, 56) had perfect orthologs across the two species and
194 they belong to 6 different OR clades. There were rare species-specific OR expansions, however,
195 and multiple instances of 1:2 or 1:3 protein homologies were found between the two species of
196 *Ceratosolen*.
197
198 **OR expression analysis and qPCR validation**
199 A heatmap was generated (Fig 3) from the expression matrix containing transcripts per million
200 (TPM) values (Additional file 3). An OR with TPM > = 1 cutoff was considered for its
201 expression and based on this, of the 63 ORs, 54 were expressed in antennae of south Indian
202 wasps and 9 were not expressed; They are OR28_1, 30C, 51, 54, 55N, 60C, 62, 68, 74F. In
203 northeast Indian wasps, 53 were expressed in antennae and 10 were not expressed. These were
204 OR28_2, 30C, 36, 40F, 54 like_1, 55 like_1F, 60C, 65, 66, 73C. Among the 9 ORs which were
205 not expressed in the antennal tissue from south India, except for OR30C and OR60C, all other 7
206 ORs were expressed in the antennae of northeast India. Similarly, among the 10 ORs which were
207 not expressed in the antennal tissue from northeast India, except for OR30C and OR60C, all
208 other 8 ORs were expressed in the antennae of south India. Therefore, OR antennal expression in
209 one site did not parallel antennal expression in the other site. In addition, 20% of the antennal
210 ORs from each region showed ectopic tissue expression.
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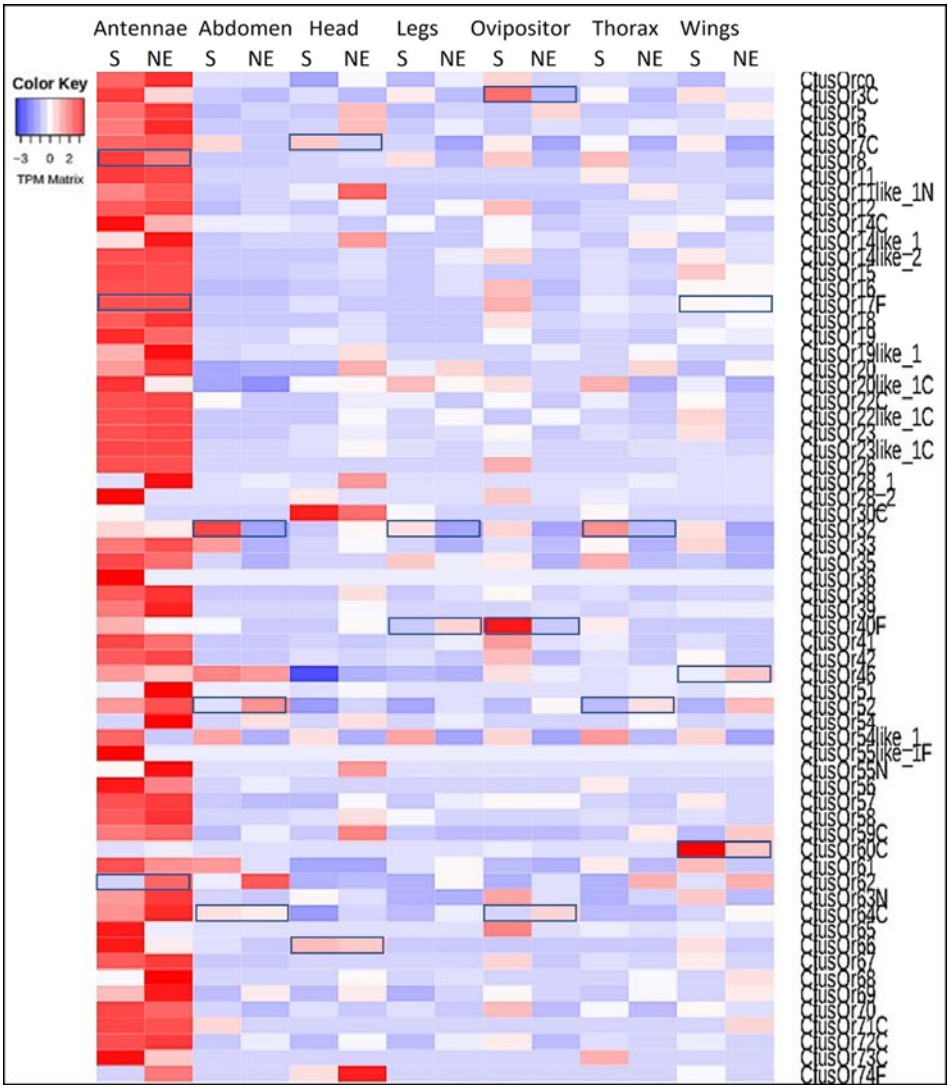

Figure 3. OR expression pattern in *C. fusciceps* tissues across Bangalore (south India) and the northeast Indian region. Heatmap was plotted using heatmap 2 by taking log2 (TPM value +1) and data scaling was done for rows. OR genes indicated in boxes were selected for qPCR analysis and the selection criteria are given in the Methods section.

To confirm the transcription variation observed in the heatmap, we selected a subset of 18 ORs for qPCR analysis (see the justification for selection in Methods, (Fig. 4). They were OR3C, 7C,

8, 12, 17F, 32, 40F, 41, 46, 52, 57, 60C, 62, 64C, 66. These had antennal or non-antennal expressions (Fig. 4).

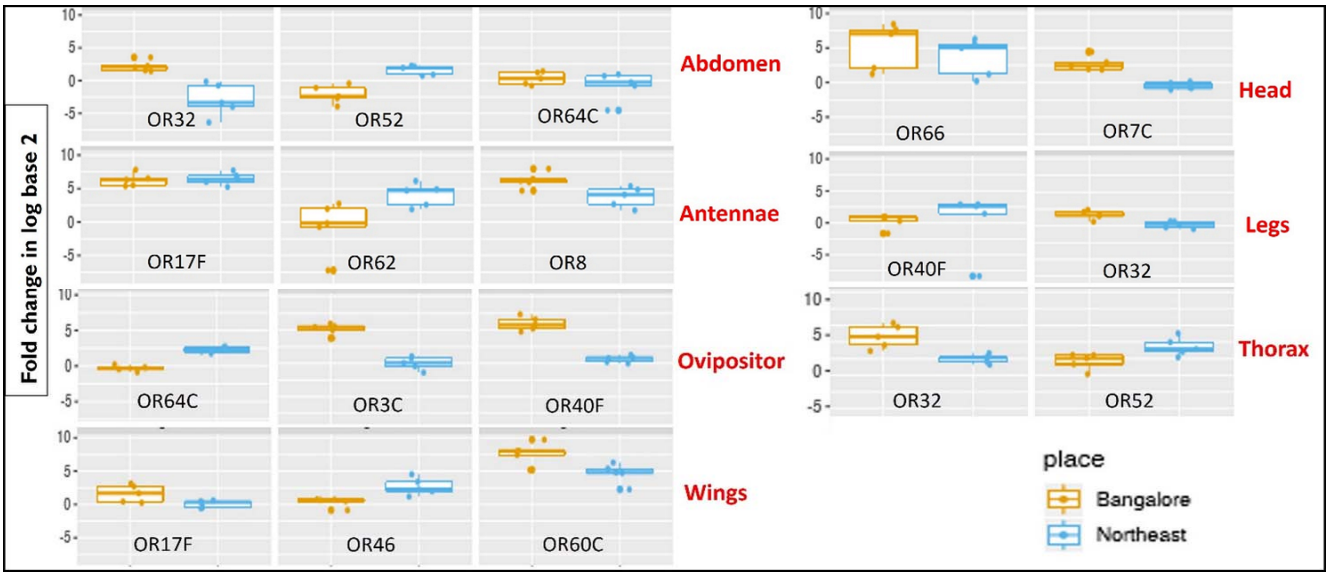

Figure 4. qPCR analysis of *C. fusciceps* ORs from 7 different tissues from south India (Bangalore) and northeast India. Fold-change values in log base 2 values from 5 biological replicates of each tissue were compared across sites by t-tests. P values are provided in Supplemental Table S7. The differential expression of OR genes shown here reproduces the results from the heatmap in Figure 3.

The results of the qPCR analysis showed that OR32 had significantly higher expression in south India than in northeast India, whereas OR52 showed lower expression in south India. OR64C had basal level expression in both regions. In the case of the antennae, OR62 showed a significant increase in expression in northeast samples as compared to south India, whereas OR8 showed the opposite expression pattern. OR17F showed equal expressions in both regions. Similarly, OR7C from the head, OR32 from the legs, OR40F and OR64C from the ovipositor,

OR32 and OR52 from the thorax, and OR46, and OR60C from the wings showed significant differences in expression between the two regions. Thus, the qPCR results were in good correspondence with the heatmap derived from TPM values. Therefore, we used the heatmap for further comparison of OR gene expression in different tissues across regions.

OR expressions were specific to antennal and non-antennal ectopic tissues

For variation across south and northeast India, we depicted the heatmap results as tissue-specific to further analyze OR expression in non-antennal tissues. OR expression is depicted in UpSet plots for south and northeast India (Fig. 5) and these regions combined (Fig. 6).

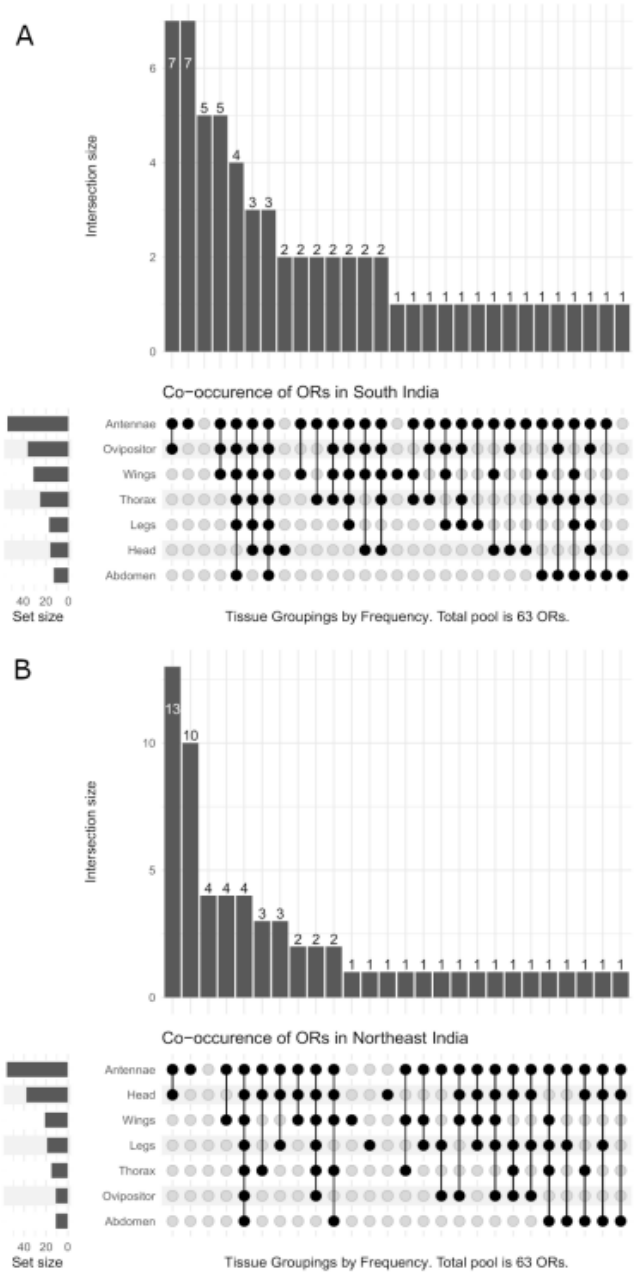

Figure 5.pdf

Figure 5. UpSet plots showing tissue-specific OR expression from south and northeast India. A. OR expression profile of south Indian wasps B. OR expression profile of northeast India.

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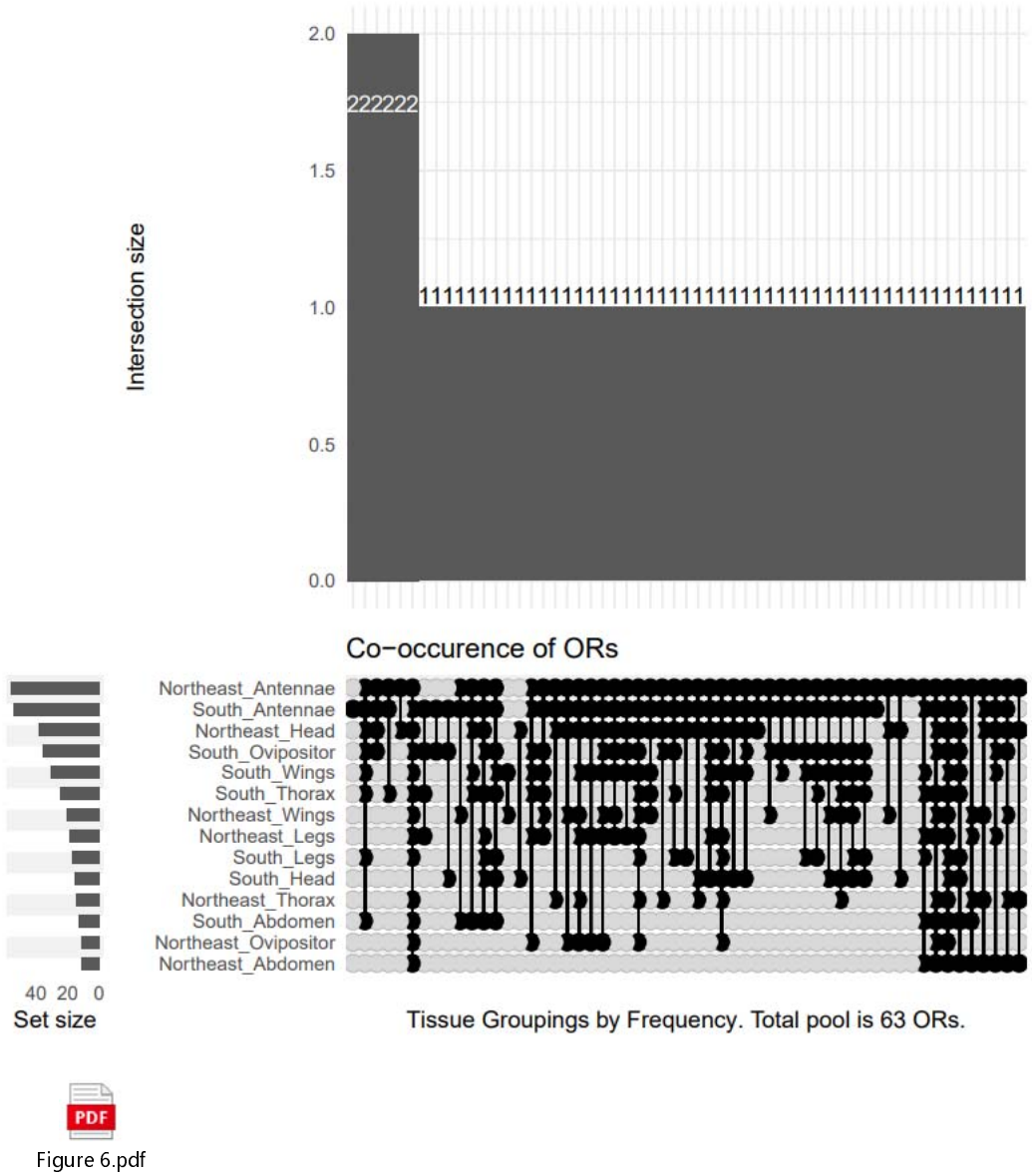

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Figure 6.pdf

250

251 **Figure 6.** Combined upset plots showing tissue- and region-specific OR expression from south

252 and northeast India.

253

254 In the case of south Indian wasps, among 54 antennal OR expressions, 17 ORs were expressed

255 only in the antennae. A total of 10 ORs in the abdomen and legs, 7 ORs in the head, 17 ORs in

the thorax, 22 ORs in the wings; 28 ORs in the ovipositor showed expression. Among all these non-antennal ORs, OR30C (XXVII_X subfamily) and OR60C (9 exon subfamily), were expressed specifically in the head and wings respectively. The remaining ORs were expressed in ≥ 2 tissues. OR46, which belongs to the XXIII_N subfamily, and OR54 like_1, which belongs to OR subfamily IV_U, were expressed in all tissues of south Indian wasps (Fig 3, Fig. 5A).

In the case of northeast Indian wasps, among 53 antennal OR expressions, 21 ORs were expressed only in antennae. A total of 8 ORs in the abdomen and thorax, 27 ORs in the head, 11 ORs in the legs, 15 ORs in the wings, and 9 ORs in the ovipositor showed expression. Like the south Indian wasps, OR30C and OR60C were expressed specifically in the head and wings respectively. The remaining ORs were expressed in ≥ 2 tissues. OR46, which belongs to the XXIII_N subfamily, and OR52, which belongs to XV_E, were expressed in all tissues of northeast Indian wasps (Fig 3, Fig. 5B).

Wasp OR expressions were variable across the region

Among 63 ORs, 46 ORs showed antennal expressions in both south and northeast India; 15 ORs were expressed in only one region; 2 ORs (OR30C and 60C) were not expressed in antennae from any region (Fig 3, Fig 6). Among the 46 antennal OR expressions, 15 ORs showed nearly equal expression levels across regions, whereas the remaining 31 ORs showed moderate to highly variable expressions (Fig 3). This kind of expression variation was also observed in non-antennal tissues such as the abdomen, head, legs, ovipositor, thorax, and wings (Fig 3, Fig. 6). In the abdomen, among the 5 ORs expressed in both regions, 3 ORs showed variable expression. In

the head, among 4 ORs expressed in both regions, 2 were variable across regions. In the legs, 5 ORs were expressed in both regions in which 3 ORs were variable. In the ovipositor, 5 ORs were expressed in both the regions in which 4 ORs were variable. In the case of the thorax, 3 ORs were expressed in both regions, and 1 OR was variable in its expression across regions. In the wings, 31 ORs were expressed in both regions and 25 showed variable expressions across regions. OR 46 was expressed in all tissues from both regions.

In both regions, the highest percentage (37% in south India and 43% in northeast India) of ORs were expressed in the antennae (Fig 7). In south India, the next highest level of OR expression was observed from the ovipositor (19%) followed by wings (16%), thorax (12%), legs (6%); the abdomen and head showed the least (5%) OR expression. In northeast India, the next highest level of OR expression was observed from the head (20%) followed by the wings (12%), legs (8%), and abdomen (6%); the thorax, and ovipositor showed the least (5%) OR expression.

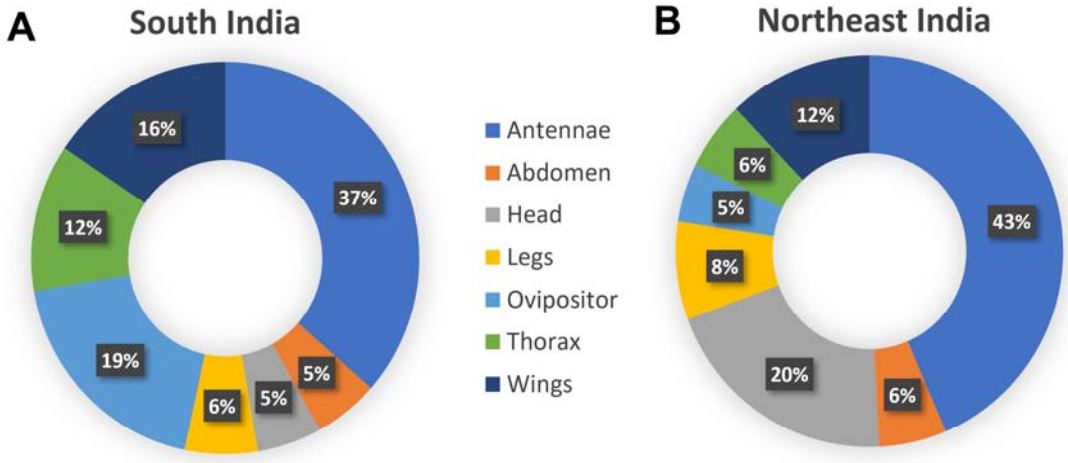

Figure 7. Distribution of OR genes expressed in different fig wasp tissues. A. South India B. Northeast India. The values within the colored sectors are the percentage of ORs expressed in the tissues.

Discussion

Investigation of OR expression in non-model organisms has unique challenges as well as opportunities. In the present study, we annotated 63 ORs using the newly assembled genome of the non-model organism *C. fusciceps* in India (Krishnan and Borges, unpublished data) and examined differential expression patterns in most tissues of this pollinating wasp. We also compared intraspecific non-chemosensory tissue OR expression patterns across widely separated geographic areas, i.e., south and northeast India. Meanwhile, Xiao et al. (2021) annotated 60 OR transcripts, using the *C. fusciceps* genome of Chinese fig wasp populations that had a scaffold length of 235 Mb, and scaffold N50 of 7.1Mb. The number of genes annotated was also comparable (this study: 12,363; Xiao et al. (2021): 12,171). We were unable to compare the OR sequences from this study with that of the Chinese wasp population because annotated OR sequences for *C. fusciceps* were not provided in Xiao et al. (2021).

The total number of ORs found was comparable to a closely related fig wasp species *C. solmsi* with 56 ORs (Xiao et al. 2013; Zhou et al. 2015). Within the Hymenoptera, the bees and ants investigated for their ORs were mostly generalists; the number of reported ORs is more than 100 for bees (Smith et al. 2011; Karpe et al. 2016, 2017) and more than 200 for ants (Nygaard et al. 2011; Kocher et al. 2015). In wasps, generalist parasitoid wasps such as *N. vitripennis*

(Robertson et al. 2010), *Aenasius bambawale* (Nie et al. 2017), *M. demolitor*, and *Cotesia vestalis* have more than 100 ORs. In specialist fig wasps, less than 100 ORs were recorded (Zhou et al. 2015; Xiao et al. 2021) (Additional file 1: Table S4). This OR reduction may be attributed to the obligate plant host specificity of these wasp species. This hypothesis is further strengthened by the finding that *Drosophila sechellia,* a plant host specialist, is losing ORs 10 times faster than its generalist sibling *D. simulans* (McBride 2007).

Phylogenetic reconstruction shines a light on the differences in OR evolution across the hymenopteran ORs that were compared: i.e., two pollinator fig wasp species *(C. fusciceps and C. solmsi),* two parasitoid wasps (*N. vitripennis* and *M. demolitor*), a generalist and eusocial honeybee (*Apis mellifera*), a solitary bee that forages specifically (*Dufourea novaeangliae*) and an ant species which is primitively eusocial (*Harpegnathos saltator*) (Fig 2A, Additional file 1: Table S3). Clade XXX (Subfamily Zb) contained expanded ORs from only the two parasitoid wasps and could be important for these two species. Clade XXIII (subfamily N) and clade XXIV (subfamily O) contained one or few ORs per wasp species but none for the other bees and the ant, indicating a probable involvement in a crucial function for the four wasp species but not for the others. Clade IX (subfamily K) was uniquely absent only in the two *Ceratosolen* species but present in low numbers (1 or 2) in the others. Clade XI (subfamily 9-exon) has several cuticular hydrocarbon (CHC)-sensing ORs in the ant species (however, it is not the only CHC sensing OR subfamily) and this was heavily expanded in almost all ant species. Given the CHC-based nest-mate recognition observed in ants (Lorenzi and D'Ettorre 2020), it was predicted that the few ORs from other species belonging to this clade might also recognize CHCs. It was interesting to note that these ORs were expanded to a greater extent in other species compared to the two

Ceratosolen species. If indeed these receptors are important for CHC detection in all the species, their reduction in *Ceratosolen* could be attributed to the specialization of the two *Ceratosolen* species to specific fig species and the closed chemical environment within which CHC recognition for processes such as mating occurs within the fig syconium. The clades VII (subfamily M) and VIII (subfamily P) were absent in any fig wasp ORs. Subgroup a of clade X (subfamily L) contains the 9-ODA receptor, a receptor for one of the major components of honeybee queen mandibular pheromone (Wanner et al. 2007) and previously identified to be expanded in two eusocial honeybees while almost absent in two solitary bees (Karpe et al. 2017). Therefore, the other ORs from these clades are likely involved in the recognition of the other major components of the honeybee queen mandibular pheromone. As expected, the two *Ceratosolen* species did not display any representative ORs belonging to subgroups Xa and Xb.

Our phylogenetic analysis of ORs from *C. fusciceps* and *C. solmsi* showed that only 19 OR sequences had perfect orthologs across the two species. This was unlike another example of two evolutionarily closely related hymenopteran species of *Apis*, which showed that around 70% of the ORs from the two species had perfect bidirectional best Blast hits as well as phylogenetic clustering (Karpe et al. 2016). Some differences could be associated with the difference in the approach used between this study and Xiao et al. (2013) to annotate the ORs across the two *Ceratosolen* species (from transcriptome vs genome). However, as our transcriptome curation was guided by a high-quality draft genome assembly (Additional file 1: Table S1) and 98% of the transcripts were aligned to the genome, this is an unlikely possibility. The observations of subtle differences in OR evolution across the two species could result from the difference in the volatile profile of their host (*Ficus racemosa* vs *Ficus hispida*) or other environmental forces.

Understanding variation in the insect olfactory system based on variation in the ecologically relevant chemical environment via OR tuning and changes in antennal morphology are already well-established fields. However, possible variation in non-antennal ectopic OR expression and its functional significance is an emerging field of research. OR expression in non-antennal tissues was observed in other insects, e.g. 13 ORs showed expression in non-antennal tissues of the hemipteran *Apolygus lucorum* with 8 ORs exhibiting high expression in heads, legs, and wings (An et al. 2016). OR22 was highly expressed in non-antennal tissues in the coleopteran *Holotrichia oblita* (Li et al. 2017). Expression of the OR2 gene in ectopic tissues of female fig wasps associated with *F. hispida* including the pollinator, *C. solmsi*, strongly indicates the presence of cryptic olfactory or other sensory inputs in these tissues (Lu et al. 2009). In previous studies, ORs showing high expression in non-antennal tissues were expressed in antennae as well (Leal et al. 2013; van Schooten et al. 2020). An et al. (2016) identified highly expressed OR genes in *A. lucorum* non-antennal tissues, in which AlucOR20 showed high expression in wings and AlucOR27 in the abdomen, while expression of these two ORs in antennae was not detected. Similarly, Koutroumpa (2021) showed that 5 SlitORs were expressed only in the leg/palp of *S. littoralis*. We observed that OR30C expression from the head and OR60C from the wings was highly tissue-specific and this was not expressed in the antennae. OR60C is within the 9-exon subfamily that generally detects cuticular hydrocarbons (Pask et al. 2017), and we speculate that it performs a similar function in *C. fusciceps*.

The expression of ORs in non-antennal tissues implies that fig wasps may expand their chemosensory system for precise identification of their specific host fig species. Though ORs in

384 the antennae act primarily in olfaction, the ORs in non-antennal tissues may have an additional
385 role in chemical sensing. Alternatively, since the *Drosophila* wing was known to be a taste
386 organ for many years (He et al. 2019) and during the evolution of flying insects from
387 terrestrial organisms, the OR family evolved from the gustatory receptor (GR) family
388 (Robertson et al. 2003; Wicher and Miazzi 2021), the remnants of GR sensing may be the
389 manifestation of OR expression in non-antennal tissues. Later there may have been a great
390 expansion of OR genes (Missbach et al. 2014; Brand et al. 2018; Thoma et al. 2019). He et al.
391 (2019) reported that in *Drosophila* a candidate ionotropic pheromone receptor (IR52a) is
392 expressed in the chemosensory sensilla of the wing. They also found that the sensilla on
393 the wing margin express many genes including ionotropic receptors (IRs), GRs, and
394 olfactory binding proteins (OBPs) associated with pheromone and general odor
395 perception. Non-antennal IRs were found in the labella, pharynx, legs, and wings of
396 *Drosophila* (Joseph and Carlson 2015; Sánchez-Alcañiz et al. 2018).
397 Considering the extremely short life span of the fig wasp (1–2 days), the extra-antennal
398 expression of OR receptors might help the tiny fig wasp to detect host-specific odors
399 quickly while flying in highly variable odorscapes.
400
401 Antennal morphology is a result of various selection pressure (Elgar et al. 2018), where the size
402 and structure of the antennae often correlates with increased sensitivity to olfactory stimuli.
403 Antennal morphology may also reflect constraints imposed by the physical environment
404 (Hansson and Stensmyr 2011). In the case of the fig pollinator, an increase in the size of the
405 antennae would affect the entry of the wasp into the fig microcosm through the tiny ostiole;
406 antennae break off and get damaged during this entry process. Hence, to compensate for the tiny

size of the fig pollinator antennae, non-antennal tissues may extend their role in the precise sensing of the scents. Consequently, non-antennal tissues such as the abdomen or ovipositor may guide the wasps towards appropriate egg-laying or pollination sites within the dark fig microcosm interior even in the absence of antennae. Also, due to the tiny size of the wasp, non-antennal tissue expression may expand the surface area of tissues bearing ORs to increase olfactory sensitivity both at long and short distances.

Upon comparison of south and northeast Indian wasps regarding OR expression patterns, we observed a significant variation between these regions, not only in antennal ORs but also in all non-antennal ORs. In parallel with the OR variation, we observed variation in the volatile profile of the corresponding *F. racemosa* samples between south and northeast India during the pollen receptive phase (Additional file 1: Table S5) (Nongbri and Borges, unpublished data). A total of 65 compounds under three main groups of volatile chemicals were identified out of which 34 were found to be in common between the two regions (Nongbri and Borges, unpublished data). Around 15 volatiles were specific to south India and 16 were specific to the northeast region. This region-specific variation in the volatile profile (Additional file 1: Table S4) could be the reason for region-specific variation in OR gene expression. This question can be definitively answered only by deorphanizing the ORs.

Insects show high variation in their chemosensory genes to support rapid adaptation of odorant detection capacities (Andersson et al. 2015). Plants may also modify their VOC profiles according to the biotic and abiotic changes of the habitat (Holopainen and Gershenzon 2010;

Ninkovic et al. 2020; Picazo-Aragonés et al. 2020). These joint forces may explain region-specific variation in VOC profiles and OR expression within plant–insect interactions

Advances in multi-omics approaches provide a better understanding of how gene expression is regulated in response to different environmental conditions, both over short-term and long evolutionary timescales. Our results illustrate that OR evolution may lead to prominent differences in olfactory sensing at the population level within species. Persistent changes in gene expression occurring at short evolutionary scales can support cellular adaptation to environmental changes and might also trigger longer-term adaptations (López-Maury et al. 2008). These kinds of studies will also help in important future research focused on unraveling the role of insect olfactory plasticity in response to changing odorscapes since insects also show good plasticity in terms of adjusting to the rapid changes in the environment through learning (Conchou et al. 2019). Investigations with non-model insects will give insight into unexplored modes of plasticity in their olfactory receptor systems and physiological responses. Our study has also provided evidence of non-antennal expression of ORs and adds to the burgeoning data on such ectopic expressions whose function is not yet deciphered.

Materials and Methods:

Fig pollinator collection and dissection

Ficus racemosa trees located in and around the Indian Institute of Science campus, Bangalore, south India, and Shillong, northeast India, were used to collect pollinator

wasps. The fig bunches were enclosed with nylon mesh bags in their pre-pollination phase to prevent unregulated oviposition by fig wasps. Pollinating fig wasps, *Ceratosolen fusciceps* were introduced singly into figs during the pollen receptive stage of the syconium. The figs were allowed to mature, and the female pollinator offspring from a single foundress female were collected and immediately placed in RNA-later solution and stored at –20°C. The fig wasps stored in RNA-later were dissected to separate their tissues such as the head, thorax, abdomen, antennae, ovipositor, legs, and wings. The dissections were carried out under the microscope for the precise separation of tissues in the presence of RNA-later. A total of 14 samples of 7 tissues each for south and northeast India were taken for RNA extraction.

Total RNA extraction

RNA-later was removed from the dissected tissues and the tissues were quickly washed with 1X PBS. The washed tissues were frozen under liquid nitrogen, mixed with cold TRIzol (Life Technologies), and homogenized using a TissueLyser II (Qiagen). The lysate was then purified using the DirectZol kit (Zymoresearch) following the manufacturer's protocol. Additional on-column DNAseI treatment was given to remove any traces of genomic DNA according to the manufacturer's guidelines. Total RNA bound to the membrane was eluted in RNase-free water. The quality and quantity of isolated RNA were analyzed using a nanophotometer (IMPLEN). The integrity of total RNA was assessed in a bio-analyzer (Agilent 2000, Agilent Technologies, USA) using an RNA 6000 Nano Lab Chip (Agilent Technologies, USA). The RNA integrity (RIN) was

473 calculated by considering 18s and 28s ribosomal RNA ratios and baseline correction
474 factors. The samples with >7 RIN values were considered for further analysis.

475

476 **cDNA library prep**

477 Good quality RNA at the required concentration was used to synthesize a cDNA library
478 using NEB Next Ultra Directional RNA library prep. RNA (1 µg) was taken for mRNA
479 isolation, fragmentation, and priming. The fragmented and primed mRNA was further
480 subjected to first-strand synthesis in the presence of Actinomycin D (Gibco, Life
481 Technologies, CA, USA) followed by second-strand synthesis. The double-stranded
482 cDNA was purified using HighPrep magnetic beads (Magbio Genomics Inc, USA). The
483 purified cDNA was end-repaired, adenylated, and ligated to Illumina multiplex barcode
484 adapters according to the NEB Next Ultra Directional RNA Library Prep Kit protocol.
485 The adapters used in the study were the Illumina Universal Adapter:

486 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC
487 GATCT-3' and Index Adapter:

488 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC[INDEX]
489 ATCTCGTATGCCGTCTTCTGCTTG-3'.

490 The adapter-ligated cDNA was purified using HighPrep beads and was subjected to 15
491 cycles of Indexing-PCR (37˚C for 15 mins followed by denaturation at 98˚C for 30 secs),
492 and cycling (98˚C for 10 sec, 65˚C for 75 sec and 65˚C for 5 mins) to enrich the adapter-
493 ligated fragments. The final PCR product (sequencing library) was purified with

494 HighPrep beads, followed by a library-quality control check. The Illumina-compatible
495 sequencing library was initially quantified by Qubit fluorometer (Thermo Fisher
496 Scientific, MA, USA) and its fragment size distribution was analyzed on Agilent 2200
497 TapeStation.

498 **RNA-Sequencing and preprocessing**

499 The cDNA library fragment size ranged from 300 bp to 700 bp. As the combined adapter
500 size is approximately 120 bp, the effective insert size is 180 bp to 580 bp. Thus, the
501 resultant cDNA library had enough concentration and was suitable for paired-end (150*2)
502 sequencing using an Illumina HiSeq 4000 platform to get the desired amount of
503 sequencing data. The raw sequencing data were checked for quality using FastQC
504 (Andrews 2010) and were pre-processed, which included removing adapter sequences and
505 low-quality bases. Raw reads were processed by “Cut Adapt” for adapters (Martin 2011)
506 and low-quality bases trimming towards 3'-end using Trimmomatic from Trinity. An
507 average of 23.87 (South India) and 21.23 million (Northeast India) preprocessed reads were
508 used for downstream analysis.
509
510 **Genome-guided transcriptome assembly**
511 Pre-processed RNA-seq reads from seven fig wasp tissues across two regions were mapped
512 separately to the *C. fusciceps* genome with mapping rates of 73% (south India) and 60%
513 (northeast India) for the pooled tissues of each region. STAR (2.7.0a) (Dobin et al. 2013). These
514 two mapping files were used to assemble genome-guided Trinity assemblies (Haas et al. 2013)
515 for the two regions (v 2.8.5) The initial number of transcripts obtained were 262904 and 255260

for south and northeast India with contig N50 of 1323 and 1227 base pairs, respectively. These two sets of transcripts were combined and the transcripts with 100% sequence identity were removed which resulted in 334449 transcripts with contig N50 of 1420 base pairs. To avoid the removal of highly similar paralogs, as these were often found in olfactory receptors, a lower redundancy cutoff was not chosen. Instead, further filtering was done based on the alignment of these transcripts to the high-quality genome and the annotation of these transcripts. Mainly longer, protein-coding and known protein-domain containing transcripts were prioritized over other transcripts. 'Transdecoder' (Gotoh 2000) was used to translate the predicted transcripts and hmmscan (Mistry et al. 2013) was used to detect the presence of any known protein domains from the Pfam protein family database (Sonnhammer et al. 1997; Finn et al. 2015). All these 3,34,449 transcripts were aligned to the *C. fusciceps* draft genome (Supplemental Table S1). using SPLAN 2.3.2a (Iwata and Gotoh 2012). This aligner predicts approximate 'gene' regions/clusters where multiple transcripts align. Each such 'gene' region was examined individually, and preference was given to the longest transcripts and the remaining transcripts were compared with the longer transcript. The following criteria were used to select one or few representative transcripts per gene cluster: If one transcript was found nested within another transcript (according to the alignment boundaries taken from the alignments with the genome), the shorter one was removed if the alignment score was lower and or no protein domain was found within. In the case of partial overlap amongst transcripts with a minimum of 50% overlap to the longest transcript, the one without the protein domain was removed. In case both or neither of the partially overlapping transcripts coded for a valid protein domain, then the one with the lesser alignment score was removed. In this way, few alternatively spliced transcripts that were sufficiently different from the longest transcripts per gene were also retained. The number of

finally retained transcripts was 58,076. Out of these 17,746 were protein-coding and 14,417 contained known protein domains. RSEM with Bowtie2 (Langmead and Salzberg 2012) was used for mapping reads to the transcriptome assembly and further quantification of expression. Around one thousand transcripts without any read mapping were removed. The mapping was used to create an expression matrix for all 14 tissues from 2 different regions containing FPKM/TPM values. On average South Indian reads had 62.2% mapping and Northeast Indian reads had 52.1% mapping. Approximately an average of 10% of reads were not mapped per tissue per location.

Gene Annotation

UniProt (Bateman et al. 2020) and KAAS (Moriya et al. 2007) were used for functional annotation of the transcripts. Clustered transcripts were annotated using the homology approach to assign functional annotation using BLAST (Camacho et al. 2009) against "Insecta" data from the Uniprot database containing 2,883,368 protein sequences. Transcripts were assigned with a homolog protein from other organisms if the match was found at an e-value less than e-5 and a minimum identity of greater than 30%.

OR annotation

The putative OR transcripts with the 7tm_6 domain were identified as potential Olfactory Receptors with the help of hmmscan against the Pfam database. This resulted in 74 putative OR transcripts. To corroborate the results, the InsectOR web server was used on the combined transcriptome containing 3,34,449 unique transcripts. InsectOR performs OR gene prediction directly from the genome/transcriptome without prior prediction of proteins from another tool

and is more sensitive than a few other genome annotation tools (Karpe et al. 2021). This resulted in the identification of 514 protein sequences with the 7tm_6 domain. These were further filtered based on the presence of the identified transcripts in the final non-redundant transcript assembly containing the 58K chosen transcripts. This resulted in 74 ORs as before. These 74 transcripts were manually studied, 3 transcripts were found to produce identical amino acid sequences to another sequence and a few others were found to be arising from the same genomic location with minor differences. Finally, 63 manually curated high-quality OR transcripts were used for further studies (Additional file 2).

Phylogenetic analysis

Well-curated OR protein sequences from *C. fusciceps* (this study), *C. solmsi* (Zhou et al. 2015), *M. demolitor* (Zhou et al. 2015), *N. vitripennis* (Robertson et al. 2010), *A. mellifera* (Robertson and Wanner 2006), *D. novaeanglie* (Robertson and Wanner 2006) and *H. saltator* (McKenzie et al. 2014) were collected and partial sequences (<200 amino acids) were removed. Chosen sequences were aligned using MAFFT (v7.123b, E-INS-i strategy, JTT200 matrix, 1000 iterations). The alignment was trimmed using trimAl ('automated1' option). A maximum likelihood (ML) based phylogenetic tree was reconstructed using RAxML (v7.4.2, PROTCATJTTF matrix, 100 rapid bootstraps, seven olfactory receptor-coreceptor (Orco) sequences as outgroup). This tree was used as a guide for the second iteration of the alignment by MAFFT (Katoh et al. 2002; Katoh and Standley 2013). The second alignment was trimmed using the trimAl option 'gappyout' (Capella-Gutierrez et al. 2009). The refined phylogenetic tree was reconstructed again with RAxML (Stamatakis 2006) with similar parameters. (iTOL) v3 (Letunic and Bork 2007, 2016) was used for tree visualization. The

tree was annotated and divided into subfamilies /clades with the help of an existing hymenopteran OR tree.

OR expression matrix generation

To find the expression profile of 63 ORs, RNA-seq reads were mapped to the transcriptome using Bowtie2 (Langmead and Salzberg 2012) and RSEM (Li and Dewey 2011). An average of 62.2% and 52.1% mapping rates were obtained for two regions because the transcriptome was assembled with 60–70% of the total reads that mapped to the genome. Also, an average of 10% of reads was not mapped per tissue per region. The mapped transcripts were used to create an OR expression matrix for all 14 tissues from the 2 regions containing TPM (transcripts per million) values (Additional file 3) and plotted in the form of a heatmap for easy comparison.

qPCR Analysis

RNA was isolated as explained previously. According to the manufacturer's protocol, the total RNA was converted into cDNA using Prime Script RT Reagents (Takara). In brief, approximately 500 ng of RNA from each sample was taken for cDNA synthesis and the first strand of cDNA was synthesized using universal oligo dT primers. The synthesized cDNA was stored at -20°C. The primers were designed (Additional file 1: Table S6) for selected genes using Primer 3 Plus online primer design software considering the exon and coding regions of the transcripts. The designed primers were analyzed for their specificity by In-Silico PCR in UCSC *In-silico* PCR online bioinformatics tool and the primer characteristics were analyzed in a multiple primer analyzer (Thermo Scientific, USA) for the possibility of primer dimer formation. The primer sequences that passed all the quality criteria were processed for synthesis on a 10 nm

scale and purified by HPLC. The final set of primer sequences is listed in (Additional file 1: Table S6). The synthesized primers were validated for their specificity using pooled cDNA from all the tissues of the south and northeast regions. In brief, the 1µl quantities of cDNA from each sample were pooled and diluted to the final concentration of 10 $ng^{-\mu L}$ and 1 µL was used for each qPCR reaction with picomols (pM) of primer concentration. The primers which showed a good amplification curve with a single melt curve and desired specific product size on agarose gel were used for further relative quantification by qPCR. The expression levels of selected genes were analyzed using SYBR Green chemistry (Brilliant II SYBR Green qPCR master mix (Agilent Technologies, USA) in the Stratagene mx3005P instrument (Agilent Technologies, USA). The amplification cycling conditions were as follows: initial denaturation for 95°C for 10min followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec. The dissociation curve analysis was performed after amplification for primer specificity; the conditions were as follows: 95°C for 1min, 55°C, for 30 sec, and 0.2°C/sec increment up to 95°C (continuous fluorescence collection from 55–95°C). The mean Ct value of technical replicates was used to calculate the relative expression level of genes. The relative quantification of genes was analyzed using standard $2^{-\Delta\Delta Ct}$ as described by Pfaffl (2001). Fold-change values in log base 2 values from 5 biological replicates of each tissue were compared across sites by t-tests. P values are provided in Additional file 1: Table S7. Beta-actin was used as a reference gene to normalize the qPCR experiment after comparing it with RPS18. The subsets of OR genes for qPCR analysis were selected in such a way that each selected OR showed upregulated expression in one region (south or northeast India) and downregulated expression in another region (south or northeast India). In addition, ORs were selected that showed equal levels of expression. Accordingly, the following ORs were selected (showing opposite patterns of expression at the two sites): OR32 and the

OR52 from the abdomen, OR8 and OR62 from the antenna, OR3C, OR40F, and OR64C from the ovipositor, OR46 and OR60C from wings, OR7C from the head, OR32 and OR40F from legs, OR32 and OR52 from the thorax, and the following showing nearly equal expression at the two sites: OR64C from the abdomen, OR17F from antennae and wings, OR66 from the head.

Declaration

Ethics approval and consent to participate.

Ethics approvals are not applicable to this study.

Competing interests

The authors declare no competing interests.

Data Availability

The data generated and analyzed in this study are included within the manuscript and supplementary data. All raw sequencing data generated in this study have been submitted to the NCBI SRA (https://submit.ncbi.nlm.nih.gov/subs/sra/SUB11600929).

The raw RNA-seq reads data generated in this study have been submitted to the NCBI Bio Project (PRJNA853513) database under accession number SUB11600929.

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947

948

949 **Authors contribution**

950 Conception, R.M.B., and S.K.; Sample collection, S.K. and L.B.N.; Dissection and RNA
951 isolation, S.K.; Transcriptomics, S.K. H.K and E.G.; Genome assembly, H.K.; Genome guided
952 transcriptome assembly, S.D.K.; OR annotation, Phylogeny, Expression analysis, S.K., S.D.K.
953 and E.G.; qPCR analysis, S.K.; Writing, S.K., S.D.K., E.G., B.S.H., and R.M.B.; Supervision,
954 S.R., B.S.H., E.G. and R.M.B.. The authors read and approved the final manuscript.

955

956 **Figure Legends**

957 **Figure 1.** Locations of the study sites in northeastern and southern India. Note that the
958 northeastern site is approximately at the same latitude as the site in Yunnan, China,
959 where earlier data on *F. racemosa* VOCs, plant, and pollinator genetics was established.
960 **Figure 2.** A. Phylogenetic analysis of hymenopteran ORs. The branches were color-coded as per
961 species. The subfamilies are shown by colored stripes. B. Distribution of *C. fusciceps* (CfusOr)
962 and *C. solmsi* (CsolOr) ORs in different clades and subfamilies.
963 **Figure 3.** OR expression pattern in *C. fusciceps* tissues across Bangalore (south India) and the
964 northeast Indian region. Heatmap was plotted using heatmap 2 by taking log2 (TPM value +1)
965 and data scaling was done for rows. OR genes indicated in boxes were selected for qPCR
966 analysis and the selection criteria are given in the Methods section.

967 **Figure 4.** qPCR analysis of *C. fusciceps* ORs from 7 different tissues from south India
968 (Bangalore) and northeast India. Fold-change values in log base 2 values from 5 biological
969 replicates of each tissue were compared across sites by t-tests. P values are provided in

Supplemental Table S7. The differential expression of OR genes shown here reproduces the results from the heatmap in Figure 3.

Figure 5. UpSet plots showing tissue-specific OR expression from south and northeast India. A. OR expression profile of south Indian wasps B. OR expression profile of northeast India.

Figure 6. Combined upset plots showing tissue- and region-specific OR expression from south and northeast India.

Figure 7. Distribution of OR genes expressed in different fig wasp tissues. A. South India B. Northeast India. The values within the colored sectors are the percentage of ORs expressed in the tissues.

Supplementary Information

Supplementary information is available in the additional file.

Additional File 1

Figure. S1. Bar chart indicating raw and processed reads obtained from fig wasp transcriptome sequencing. Illumina Hi-Seq, 150 X 2 paired-end sequencing was used to sequence 7 tissues each from South India (S) and Northeast India (NE).

Figure S2. Phylogenetic tree reconstruction with *C. fuscicpes* and *C. solmsi* olfactory receptors The branches are color coded as per species - *C. fusciceps* - Blue, *C. solmsi* – Purple.

Table S1. Genome Hybrid assembly statistics

Table S2. BUSCO analysis of transcriptome assembly

Table S3. Detailed information about the hymenopteran OR phylogenetic tree

Table S4. Number of annotated ORs in specialist and generalist Hymenoptera

992 **Table S5**. Proportional abundance (%) of VOC's from receptive (B-phase) of *Ficus*
993 *racemosa* trees (Means ± SD, n=19 for SI, n=17 for NE; RI=Retention Index) from North-east
994 (Meghalaya) and South India (Bangalore)
995 **Table. S6.** Primer sequences for OR genes
996 **Table S7.** Statistical analysis of qPCR analysis of Cfus ORs
997
998 **Additional File 2**
999 Annotation of *C.fusciceps* olfactory receptors
1000
1001 **Additional File 3**
1002 Expression matrix containing transcripts per million (TPM) values for *C.fusciceps* olfactory
1003 receptors
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Additional File 1

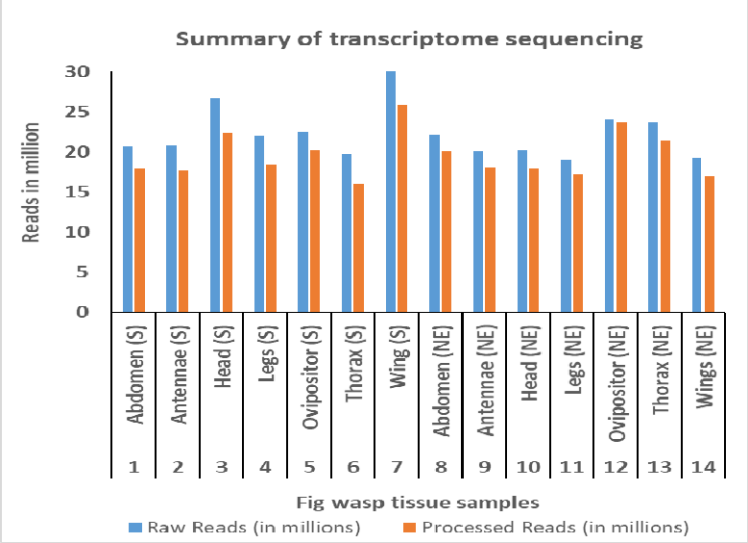
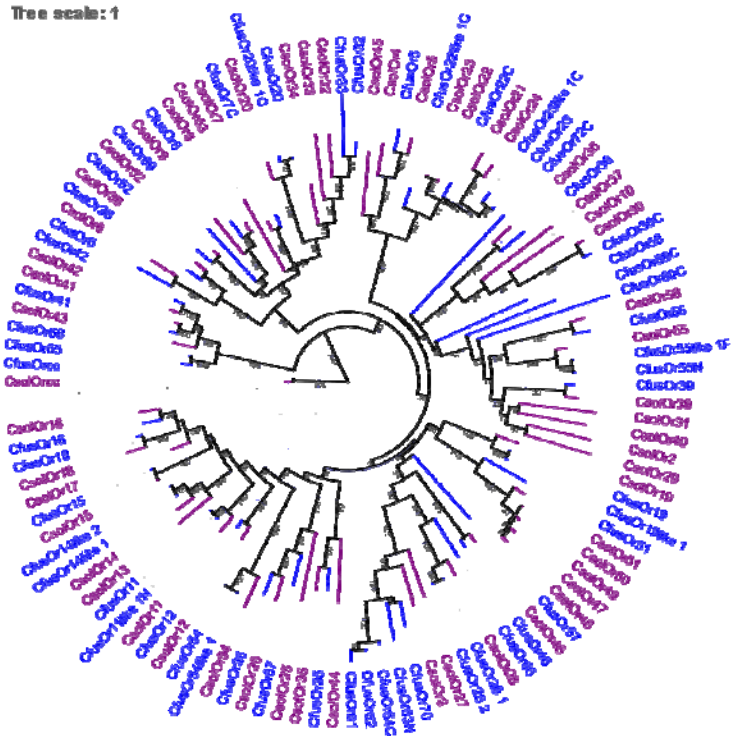


Figure. S1. Bar chart indicating raw and processed reads obtained from fig wasp transcriptome sequencing. Illumina Hi-Seq, 150 X 2 paired-end sequencing was used to sequence 7 tissues each from South India (S) and Northeast India (NE).



1020

1021 Figure S2. Phylogenetic tree reconstruction with *C. fuscicpes* and *C. solmsi* olfactory receptors

1022 The branches are color coded as per species - *C. fusciceps* - Blue, *C. solmsi* – Purple.

1023

1024 Table S1. Genome Hybrid assembly statistics

Scaffolds		**Contigs**	
Scaffolds Generated:	1286	Contigs Generated:	1497
Maximum Scaffold Length (bp):	1,11,87,473	Maximum Contig Length (bp):	59,78,652
Minimum Scaffold Length (bp):	1001	Minimum Contig Length (bp):	165
Average Scaffold Length (bp):	185043	Average Contig Length (bp):	158841

Median Scaffold Length (bp):	4129	Median Contig Length (bp):	1013
Total Scaffolds Length (bp):	**23,79,66,267**	**Total Contigs Length (bp):**	**23,77,85,791**
Scaffolds >= 100 bp:	1286	Contigs >= 100 bp:	1497
Scaffolds >= 200 bp:	1286	Contigs >= 200 bp:	1496
Scaffolds >= 500 bp:	1286	Contigs >= 500 bp:	1492
Scaffolds >= 1 Kbp:	1286	Contigs >= 1 Kbp:	1490
Scaffolds >= 10 Kbp:	232	Contigs >= 10 Kbp:	346
Scaffolds >= 1 Mbp:	57	Contigs >= 1 Mbp:	72
N50 value:	**41,21,315**	**N50 value:**	**22,03,451**

1025

1026 Table S2. BUSCO analysis of transcriptome assembly

	Hymenoptera	**Insecta**
Complete BUSCOs (C)	3002	1358
Complete and single-copy BUSCOs (S)	2388	1068
Complete and duplicated BUSCOs (D)	614	290
Fragmented BUSCOs (F)	450	77
Missing BUSCOs (M)	963	223
Total BUSCO groups searched (N)	4415	1658
Result Summary	C:68.0% [S:54.1%, D:13.9%], F:10.2%, M:21.8%, N:4415	C:81.9% [S:64.4%, D:17.5%], F:4.6%, M:13.5%, n:1658

1027 Table S3. Detailed information about the hymenopteran OR phylogenetic tree

OR Subfamily	Bootstrap	CfusOr	CsolOr	NvitOr	MdemOr	AmelOr	DnovOr	HsalOr	Total ORs
Orco_Orco	100	1	1	1	1	1	1	1	7
XXX_Zb	67	0	0	24	9	0	0	0	33
XII_F	71	4	3	27	20	1	1	1	57
XXII_D	100	2	2	12	0	0	0	4	20
XV_E	77	8	9	37	35	6	9	26	130
XXXI_-	100	2	1	2	0	0	0	0	5
XXXII_-	100	1	0	3	0	0	0	0	4
XXVIII_Y	34	0	0	0	0	0	0	4	4
XXIII_N	17	1	1	1	1	0	0	0	4
XXIV_O	40	1	5	3	7	0	0	0	16
III_V	87	8	3	11	0	6	8	54	90
VI_T	78	8	8	23	9	2	3	9	62
IV_U	88	2	1	7	3	1	5	36	55
XXV_R	29	0	0	0	9	0	0	2	11
XXVI_S	100	1	2	4	13	0	0	1	21
XXIX_Za	46	0	0	1	2	0	0	1	4
IX_K	100	0	0	1	1	2	2	2	8
X_L	89	2	2	8	13	58	15	56	154
II_I	100	1	0	2	1	1	1	1	7
XIX_W	100	0	0	0	1	1	1	1	4
XXXIII_-	98	0	0	2	0	0	0	0	2
I_A	67	0	0	0	0	3	2	17	22
XVII_G	77	0	0	0	5	3	0	0	8
XXVII_X	88	1	3	15	0	0	0	0	19
XXI_J	100	1	0	3	3	23	12	2	44
Xl_9-exon	29	6	7	90	31	43	21	130	328
XIV_C	4	0	0	0	1	1	1	1	4
XIII_B	67	0	0	0	27	1	1	1	30
XVI_Z	98	5	7	19	0	1	0	0	32
XX_orphan	100	0	0	2	0	1	1	0	4
VII_M	97	0	0	0	0	1	1	4	6
VIII_P	100	0	0	0	0	5	1	10	16
V_Q	63	0	0	1	0	1	1	3	6
XVIII_H	58	1	1	1	11	14	3	9	40
HsalOr215		0	0	0	0	0	0	1	1
DnovOr143like_1		0	0	0	0	0	1	0	1
DnovOr181PC		0	0	0	0	0	1	0	1
NvitOr43		0	0	1	0	0	0	0	1
									1261
Xa	97	0	0	0	0	14	0	21	35
Xb	100	0	0	0	0	22	5	17	44
Xrest		2	2	8	13	22	10	18	75
									154

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1029

1030 Table S4. Number of annotated ORs in specialist and generalist Hymenoptera

S. NO.	Name of the Species	Number of ORs	Specialist/ Generalist
	BEES		
1	*Apis cerana cerana*	119	Generalist
2	***Apis mellifera***	177	Generalist
3	*Apis florea*	180	Generalist
4	***Dufourea novaeangliae***	112	Oligolege of pickerel weed
5	*Habropoda laboriosa*	151	oligolectic
6	*Megachile rotundata*	254	Generalists
7	*Bombus impatiens*	159	Generalists
8	*Bombus terrestris*	228	Generalists
	ANTS		
9	*Atta cephalotes*	376	Generalists
10	*Acromyrmex echiniator*	385	Generalists
11	*Pogonomyrmex barbatus*	344	Generalists

12	***Harpegnathos saltator***	426	Generalists
13	*Linepithema humile*	367	Generalists
14	*Solenopsis invicta*	400	Generalists
15	*Micropilitis demolitor*	203	Generalists
16	*Cerapachys biroi*	369	Generalists
17	*Cardiocondyla obscurior*	309	Generalists
18	*Monomorium pharaonis*	240	Generalists
19	*Camponotus floridanus*	352	Generalists
	WASPS		
20	*Aenasius bambawale*	226	Solitary parasitoid / Generalist
21	***Microplitis demolitor***	214	Parasitoid wasp
22	*Cotesia vestalis*	158	Endoparasitic wasp
23	***Nasonia vitripennis***	225	Parasitoid wasp
24	***Ceratosolen solmsi***	56	Specialist
25	***Ceratosolen fusciceps***	63	Specialist

1031

1032 Table S5. Proportional abundance (%) of VOC's from receptive (B-phase) of *Ficus racemosa*
1033 trees (Means ± SD, n=19 for SI, n=17 for NE; RI=Retention Index) from North-east
1034 (Meghalaya) and South India (Bangalore)

S.NO	Compounds	R I	South India (SI)	Northeast India (NE)
I	**Fatty acid derivatives**			
1	(Z)-3-hexen-1-ol	856	2.53±1.41	0.05±0.20
2	Hexanol	868	0.19±0.20	-
3	1-octen-3-ol	978	-	0.33±0.90
4	3-octanone	979	-	0.22±0.92
5	octan-3-ol	997	-	0.02±0.10
6	hexyl acetate	1007	0.59±0.97	0.23±0.85
7	(Z)-3-hexenyl acetate	1009	54.41±22.54	-
8	nonanal	1105	6.43±8.56	1.79±3.55
9	decanal	1204	0.28±0.54	0.19±0.76
10	(Z)-hex-2-en-1-yl benzoate	1573	0.11±0.21	-
	Total		**64.53±34.44**	**2.83±7.27**
II	**Aromatics (Shikimic acid pathway)**			
11	Benzaldehyde	953	0.57±1.66	1.96±2.35
12	benzyl alcohol	1034	2.47±4.33	3.35±4.74
13	2-phenylethanol	1111	0.08±0.24	-
14	benzyl acetate	1163	1.58±3.22	-
15	methyl salicylate	1195	0.20±0.87	-

16	Indole	1289	0.16±0.47	1.28±2.60
	Total		**5.07±10.79**	**6.59±9.69**
III	**Monoterpenes**			
17	a-thujene	920	-	0.27±0.77
18	α-pinene	932	0.21±0.41	0.39±0.95
19	sabinene	971	-	0.50±0.78
20	β -pinene	974	-	0.08±0.20
21	6-methyl-5-hepten-2-one	988	1.22±1.78	2.09±6.26
22	myrcene	988	-	0.58±1.12
23	6-methyl-5-hepten-2-ol	993	0.81±1.24	0.38±0.63
24	Limonene	1029	1.15±2.62	0.65±1.23
25	1,8-cineole	1031	0.03±0.12	2.97±3.90
26	(Z)-β-ocimene	1040	0.50±0.61	0.80±1.05
27	(E)-β-ocimene	1049	20.52±20.98	14.98±20.51
28	NI 1	1056	-	0.31±1.27
29	Linalool	1099	1.09±2.25	0.40±1.35
30	α-terpineol	1187	0.04±0.17	-
	Total		**25.57±30.19**	**24.63±40.85**
III	**Sesquiterpenes**			
31	(E)-4,8-dimethyl-1,3,7-nonatriene	1117	1.81±4.11	15.16±21.45
32	Geraniol	1256	-	0.22±0.83
33	α-ylangene	1375	0.02±0.05	-

34	α-copaene	1378	0.09±0.38	0.63±0.71
35	Daucene	1385	0.11±0.21	1.29±1.76
36	NI 2	1383	-	0.91±1.44
37	α-duprezianene	1387	0.09±0.17	0.87±1.12
38	7-epi-a-cedrene	1407	0.02±0.05	1.08±0.89
39	cis-α-bergamotene	1408	0.03±0.09	0.31±0.40
40	β-funebrene	1418	0.16±0.32	2.61±1.89
41	β-ylangene	1420	0.34±0.69	9.32±6.13
42	β-duprezianene	1421	-	0.57±1.37
43	β-cedrene	1425	0.01±0.05	-
44	β-copaene	1427	0.05±0.11	1.97±1.43
45	β-gurjunene	1436	0.02±0.08	1.36±2.22
46	NI 3	1438	0.07±0.17	-
47	α-guaiene	1440	-	0.33±0.51
48	(Z)-β-farnesene	1450	0.12±0.49	-
49	epi-prezizaene	1454	0.08±0.17	2.25±2.11
50	epi-zizaene	1456	0.21±0.50	8.15±7.06
51	(E)-β-farnesene	1458	0.14±0.49	-
52	α-acoradiene	1460	0.09±0.26	0.79±2.42
53	zizaene (= khusimene)	1466	0.93±2.05	13.95±12.63
54	NI 4	1468	-	0.95±1.81
55	β-acoradiene	1467	0.03±0.13	-

56	10-epi-β-acoradiene	1480	0.07±0.16	0.52±1.05
57	γ-curcumene	1483	0.01±0.04	0.14±0.26
58	ar-curcumene	1486	0.00±0.02	0.08±0.13
59	(E,E)-α-farnesene	1496	0.03±0.14	-
60	(Z)-γ-bisabolene	1510	0.24±0.71	-
61	α-alaskene	1511	-	1.05±2.64
62	β-curcumene	1516	0.03±0.13	0.29±0.38
63	β-sesquiphellandrene	1529	0.03±0.10	0.73±0.88
64	(E)-nerolidol	1569	-	0.04±0.10
65	zizanone	1679	-	0.59±1.67
	Total		**4.83±11.87**	**65.95±74.45**

1035

1036 Table. S6. Primer sequences for OR genes

S. No	Name	Sequence	Start Position	Strand	Length	Primer Tm	Amplicon size
1	Orco_L1	GGCTGCGTACTCCTGCCATT	1239	forward	20	59.92	150
	Orco_R1	GCTCCGAGAACCGAGGCAAA	1369	reverse	20	59.85	
2	Or3C_L1	TGGAAGACCAGCCAGAGCTT	170	forward	20	58.1	144
	Or3C_R1	ACCAGCAGGAATCGCACAATG	293	reverse	21	58.46	
3	Or7C_L1	ACTTTCATGTGAGCACATTTAACAA	284	forward	25	54.58	182
	Or7C_R1	AACACTTGAACATAGTACAAGCATAC	440	reverse	26	54.28	

4	Or8_L1	TGGCTTCGCTATTTGTGTGAGCA	804	forward	23	59.83	144
	Or8_R1	TGTAAGGTCACTTCGTTGCCGAAA	924	reverse	24	59.49	
5	Or12_L1	CGTTTGTCTTTGTAGCGTTGG	45	forward	21	60.7	155
	Or12_L1	CATCAATGTCGCCCCAATA	205	reverse	19	60.7	
6	Or17F_L1	TTGGCAGTGTTACTATTCTGAACAAGA	24	forward	27	57.04	112
	Or17F_R1	AGCAGTAATAGTACCTTGTGCTAAACC	109	reverse	27	57.21	
7	Or32_L1	ACGCACTCACTTTCGGCTTGT	230	forward	21	60	138
	Or32_R1	ACTTGGCTGCGATACTGCGT	348	reverse	20	59.57	
8	Or40F_L1	TCGTGCACGCATTCATTTGCT	110	forward	21	59.01	138
	Or40F_R1	TGAGCTCAGACATTCGCTCCA	227	reverse	21	58.37	
9	Or41_L1	TCGGTCGCACTGAACCTCAC	409	forward	20	59.51	130
	Or41_R1	TGGACCAATGGACGGCTCCT	519	reverse	20	60.35	
10	Or46_L1	ATGCGAGCCCATGCCAAGAT	361	forward	20	59.63	128
	Or46_R1	GGTCCCAATTGACGCTCGCT	469	reverse	20	60.2	
11	Or52_L1	TCGACTTTGCTAGCAGGATTGATAGT	400	forward	26	58.31	121
	Or52_R1	ACGTTGACCAGTACGTTGCATGT	498	reverse	23	59.77	
12	Or57_L1	TGTGTGCTTCGCTGGATTTCA	816	forward	21	57.71	159
	Or57_R1	ACGTGGCTTGCAATGTCTTCA	954	reverse	21	57.98	
13	Or60C_L1	TCGTCTATATATTCGGTGGAATGGTT	460	forward	26	56.02	180
	Or60C_R1	ACCTATAAGTATCATTGCCAAAGACCA	613	reverse	27	56.45	
14	Or62_L1	ATCGGCATCGGTTAGAAGAA	699	forward	20	59.67	127

	Or62_R1	TTCTGGTTGGTGATTGATGC	825	reverse	20	59.5	
15	Or64C_L1	TTACGCAAGTGAAGGGGTGT	510	forward	20	60	187
	Or64C_R1	TTCTGGTTGGTGATTGATGC	696	reverse	20	59	
16	Or66_L1	ATGGTAGGCAATCACCGTTC	464	forward	20	59	142
	Or66_R2	CGTAATGCGTTCACCTTTGA	605	reverse	20	59	
17	ActinB_L1	TCCAAGCAGGAGTACGACGAGT	231	forward	22	59.81	123
	ActinB_R2	GCTGCGTCCGTCTGGTCTTT	334	reverse	20	60.13	

1037

1038 Table S7. Statistical analysis of qPCR analysis of Cfus ORs

S.No.	**Tissue.OR**	**t.value**	**df**	**p.value**	**Ci**
1	Antennae Or8	2.936263782	7.536281753	0.020094997	0.515800628
2	Antennae Or17F	-0.262141839	7.979369325	0.799852482	-1.548517024
3	Antennae Or62	-2.463655031	5.516060008	0.052403039	-9.545712287
4	Abdomen Or32	4.256625878	4.862871734	0.008552016	1.988323515
5	Abdomen Or52	-5.334500758	6.061498041	0.001714609	-5.410499235
6	Abdomen	1.048156743	5.495509708	0.338459597	-

	Or64C				1.573352984
7	Head Or7C	6.198296123	5.63145322	0.001032104	1.921208019
8	Head Or66	0.851910286	7.693376814	0.419990046	-2.854401539
9	Legs Or32	3.889037215	7.496063338	0.005236058	0.583257426
10	Legs Or40F	-0.006271035	4.427533078	0.995269025	-5.983315411
11	Thorax Or32	3.962965567	5.125021903	0.010195157	1.192154172
12	Thorax Or52	-2.673543157	7.853484152	0.028673697	-4.122393316
13	Wings Or17F	2.492512289	5.372349683	0.051643369	-0.015914636
14	Wings Or46	-3.522524439	6.074505048	0.012229063	-3.913261564
15	Wings Or60C	3.030817998	7.93497619	0.016445407	0.713231669
16	Ovipositor Or3c	8.935890391	7.821167467	2.25E-05	3.789005443
17	Ovipositor Or40F	10.17491556	5.90223613	5.82E-05	4.021812919

18	Ovipositor Or64c	- 8.611515876	5.997559156	0.000135208	- 3.335637244

1039

1040 **Additional File 2**

1041 Annotation of *C.fusciceps* olfactory receptors

Additional file 2.xlsx

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1043 **Additional File 3**

Additional file 3.xls

1044

1045 Expression matrix containing transcripts per million (TPM) values for *C.fusciceps* olfactory

1046 receptors

1047