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Molecular characterization of the *Aedes aegypti* odorant receptor gene family

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Abstract

The olfactory-driven blood-feeding behaviour of female *Aedes aegypti* mosquitoes is the primary transmission mechanism by which the arboviruses causing dengue and yellow fevers affect over 40 million individuals worldwide. Bioinformatics analysis has been used to identify 131 putative odourant receptors from the *A. aegypti* genome that are likely to function in chemosensory perception in this mosquito. Comparison with the *Anopheles gambiae* olfactory subgenome demonstrates significant divergence of the odourant receptors that reflects a high degree of evolutionary activity potentially resulting from their critical roles during the mosquito life cycle. Expression analyses in the larval and adult olfactory chemosensory organs reveal that the ratio of odourant receptors to antennal glomeruli is not necessarily one to one in mosquitoes.

Keywords

odourant receptor; olfaction; genome; *Aedes aegypti*; *Anopheles gambiae*

Introduction

Olfaction is the chemosensory modality dedicated to the detection of small airborne chemicals called odourants, which is of fundamental importance in the life cycle and life histories of insects. *Aedes aegypti* is responsible for the transmission of arboviral pathogens associated with dengue haemorrhagic fever, yellow fever and Rift Valley encephalitis. Olfactory signal transduction plays a critical role in female behaviours such as the search for oviposition sites or host selection and thus directly impacts their overall ability (vectorial capacity) to transmit pathogenic agents (Zwiebel & Takken, 2004).

Insect olfactory systems have been the subject of intense study in models such as *Drosophila melanogaster* as well as a range of both medically and economically important systems. At the molecular level, olfactory signal transduction is mediated by a complex and still enigmatic suite of proteins that are hypothesized to act through G-protein coupled receptor (GPCR) mediated pathways (van Der Goes van Naters & Carlson, 2006). These include a varied set of small molecular weight odourant binding proteins, olfactory arrestins,

G-proteins, biotransformation enzymes as well as large and highly diverse families of putative odourant receptors (Ors) (reviewed in Rutzler & Zwiebel, 2005). In *Drosophila*, the two main insect olfactory appendages: the antennae and maxillary palps are covered by various morphological and functional classes of olfactory sensilla housing up to four primary olfactory receptor neurones (ORNs) (Shanbhag *et al.*, 1999; Stocker, 1994). First order ORNs project to the antennal lobe (AL) where they synapse locally to interneurons and projection neurons in morphological structures called glomeruli (Shanbhag *et al.*, 1999). With one notable exception (Goldman *et al.*, 2005), the olfactory organization in the adult and the larva is linear: individual ORNs express one type of OR while their axons stereotypically converge to a unique glomerulus, thus giving rise to the 'one neurone—one receptor—one receptor—one glomerulus' paradigm (Couto *et al.*, 2005; Fishilevich & Vosshall, 2005).

The identification of the first vertebrate *Ors* (Buck & Axel, 1991) led to a major effort by several groups interested in identifying insect *Or* genes. Nearly a decade later, the release of the *D. melanogaster* genome facilitated the identification of a family of *Ors* from *D. melanogaster* (Clyne *et al.*, 1999b; Gao & Chess, 1999; Vosshall *et al.*, 1999). The completion of the genome of the malaria mosquito, *An. gambiae* (Holt *et al.*, 2002) led to the description of a second complete insect *Or* gene family and allowed the first comparative genomic analysis between two related insects (Fox *et al.*, 2001; Hill *et al.*, 2002). Multiple *Ors* have now been identified in species from at least four other insect orders, including Diptera, Lepidoptera, Hymenoptera and Coleoptera (Krieger *et al.*, 2003; Robertson & Wanner, 2006; Wanner *et al.*, 2007; Hallem *et al.*, 2006) where a significant body of work has described insect *Or* genes, their patterns of expression, functions and implications for odour coding (Robertson *et al.*, 2003; Jacquin-Joly & Merlin, 2004; Hallem *et al.*, 2004a,b; Rutzler & Zwiebel, 2005; Ache & Young, 2005; Dahanukar *et al.*, 2005; Jefferis, 2005). Additional studies have characterized one *Or* that is highly conserved across insect orders, widely coexpressed with other more diverse *Ors* and which is required for their function (Krieger *et al.*, 2003; Pitts *et al.*, 2004; Larsson *et al.*, 2004; Jones *et al.*, 2005; Xia & Zwiebel, 2006; Benton *et al.*, 2006). It is apparent that with several additional insect genome projects nearly complete or about to be initiated, the future of comparative genomics is certain to yield new and exciting information.

Here we report the manual annotation and characterization of the *Or* gene family in the genome of *Ae. aegypti* (Liverpool strain). As a result of their role in olfactory signal transduction cascades, *Ae. aegypti* odourant receptors (*AaOrs*) exert significant effects throughout the mosquito life cycle and, accordingly, are expected to have a dramatic impact on the potential for the mosquito to transmit diseases.

Results

The Ae. aegypti odourant receptor family comprises 131 members

We have identified a total of 131 candidate *AaOr* genes in the genome of *Ae. aegypti*. Of these, 100 genes encode putative, complete functional proteins, 10 are incompletely annotated genes, and 21 are apparent pseudogenes (Table S1). While we believe these represent the complete *Or* repertoire in the *Ae. aegypti* genome, we acknowledge this annotation is dependent on the maturity of the current genome assembly, the inevitable refinement of which may ultimately affect changes in the complexity of the *AaOr* gene family.

The C-terminus is the most conserved region among *AaOrs* and, in most cases, includes an SYT or SYS motif near the extreme C-terminus of the conceptual translation, a feature that is conserved in both *An. gambiae* and *D. melanogaster* (Hill *et al.*, 2002; Robertson *et al.*,

2003; Severson *et al.*, 2004). The complete list of *AaOrs*, including their predicted amino acid sequences, is available as supporting online material (Table S1). As is the hallmark of all GPCRs, all of the *AaOR* peptides contain multiple *trans*-membrane (TM) regions based on TMPred predictions (Hofmann & Stoffel, 1993). Chemosensory receptors, comprising the *AaOrs* and a large family of 88 gustatory receptors encoded by 79 *Gr* genes (Kent L.B. and Robertson H.M., unpublished data), belong to the most numerous of the GPCR classes in the *Ae. aegypti* genome (Hill, personal communication).

It was not possible to annotate the full-length sequence of all *AaOrs*, especially in 5'- and 3'-regions, and a number of incompletely annotated genes were identified. Presumably, this was due in some cases to lack of sequence conservation among insect *Ors*. In addition, the presence of large introns and/or incomplete assembly of genomic sequences may have complicated exon identification. *AaOrs126*, *127*, *130* and *131* are very short incompletely annotated genes, each encoding fewer than 100 amino acids with one or two recognizable exons (Table S1). These genes may represent *Ors* with extremely large introns or exons that are difficult to identify for the reasons mentioned previously. Alternatively, they may be pseudogenes or may not be *Ors* at all, although in a couple of instances they are located near other *AaOrs* on the same supercontig with which they share high similarity. In any case, these short incomplete protein sequences were not used in phylogenetic analyses. Because no complete physical/cytological map is yet available in *Ae. aegypti*, *AaOr* names were assigned, when possible, based on phylogenetic relationships (Table S1, Figs 1–3).

Pseudogenes are rare occurrences in both the *D. melanogaster* and *An. gambiae* genomes (Krzywinski *et al.*, 2001). We identified 21 potential *Or* pseudogenes (Table S1), based on the occurrence of premature stop codons, mutated start codons, or small nucleotide insertion/deletion (indels) in their open-reading frames (Table S1). Thirteen of these genes, *AaOrs* 22, 29, 38, 51, 54, 57, 64, 73, 77, 82, 83, 112 and 116, were detected as reverse transcription–polymerase chain reaction (RT–PCR) products in antennal tissue (Fig. 4). Sequencing the *AaOr38* RT–PCR product revealed a CAG codon in the Costa Rica strain where an in-frame TAG stop-codon exists in the Liverpool strain, thus demonstrating that *AaOr38* is likely to be an intact gene in the Costa Rica strain (data not shown). A similar result was observed with *AaOr29* for which the Costa Rica strain contains TGG instead of a TGA stop codon at position 31 in the Liverpool strain. Further investigation is required to document whether the other potential *AaOr* pseudogenes show similar polymorphisms. We have also observed regular occurrences of single nucleotide polymorphisms (SNPs) in other *AaOr* RT–PCR amplicons in this study (data not shown). The existence of SNPs between and within strains of species has been recognized in *DmOrs* (Berger *et al.*, 2001), *AgOrs* (Hill *et al.*, 2002), and *C. elegans* chemoreceptors (Stewart *et al.*, 2005), so their presence here is not without precedent.

AaOrs are highly divergent

Phylogenetic analysis reveals several species-specific *Or* expansions in *Ae. aegypti* and *An. gambiae* (Fig. 1). The *AaOr* family is significantly expanded relative to the *Or* families of *An. gambiae* (79 *AgORs*) (Hill *et al.*, 2002) and *D. melanogaster* (62 *DmORs*) (Robertson, 2001) and is dominated by a large set of 40 *AaOrs* (86–125) with no closely related *An. gambiae* *Ors* (Fig. 1). The expansion and diversification of mosquito *Ors* reflects the apparent rapid evolution of the insect chemosensory receptors (Robertson *et al.*, 2003). *Or* diversity between these two mosquitoes indicates that many species-specific differences in sensory perception have arisen in the 140–200 million years (Myr) since the Anophelinae/Culicinae divergence (Krzywinski *et al.*, 2001). Furthermore, short branch lengths observed between many *Ors* within one species (Fig. 1) suggest numerous recent gene duplication events. This raises the possibility that any behaviours associated with these novel receptors have undergone recent or rapid shifts both between *An. gambiae* and *Ae. aegypti*, and

probably also within these mosquito subfamilies. The diversification of the *Or* family is more pronounced in *Ae. aegypti*, suggestive of the increased potential of this mosquito to exploit a wider array of ecological niches. While it is tempting to speculate that the life history of *Ae. aegypti* includes a need for greater peripheral chemosensory capacity, further analyses that include *AaOr* deorphanization will be necessary to understand the significance of this large increase in *Or* complexity.

Ae. aegypti* and *An. gambiae* share a subset of conserved *Ors

As is the case for other insect *Ors*, *AaOrs* display a high degree of divergence (Robertson *et al.*, 2003; Clyne *et al.*, 1999a). Indeed, the majority of *AaOrs* share less than 20% identity at the amino acid level with each other as well as with *Or* peptides from *An. gambiae* and *D. melanogaster* (data not shown). Based on phylogenetic analysis, we identified 18 *Aedes/Anopheles* orthologous subgroups including the highly conserved *Or7* gene (Figs 1 and 2). Along with the nonconventional *Drosophila DmOr83b* gene (Gao & Chess, 1999; Larsson *et al.*, 2004; Pitts *et al.*, 2004), these genes are clear orthologs based on conserved sequence homology (Melo *et al.*, 2004), microsynteny (Fig. 2) and, in the case of *Drosophila*, an essential requirement for olfactory signal transduction (Jones *et al.*, 2005; Benton *et al.*, 2006). The evolutionary relationships to other apparent orthologs are more complex due to lower sequence conservation and sometimes to the presence of neighbouring paralogs (Fig. 2). Another closely related group is the OR2/OR10 paralogous clade; they include *AaOR10/AgOR10*, *AaOR9/AgOR10* and *AaOR2/AgOR2*, which share 71.5%, 65% and 70.5% identity, respectively. The closest *Drosophila* relative of *AaOR2/AgOR2* is *DmOR43a* (< 40% identity; Hill *et al.*, 2002); however, these receptors do not appear to share odourant ligand specificity (Wetzel *et al.*, 2001; Hallem *et al.*, 2004b). The remaining 20 *Ae. aegypti-An. gambiae Or* orthologs have no counterparts in *D. melanogaster*, raising the interesting possibility that these genes may be associated with conserved functions between *Ae. aegypti* and *An. gambiae*. As such, they may offer important insight into comparative studies of mosquito olfaction. Finally, it should be noted that there is potentially a second copy of *AaOr7* in the *Ae. aegypti* genome located on supercontig 1.2048 (data not shown), which would be unique among insects to date. However, inasmuch as this very small (only 15 kb in length) supercontig only contains a partial length *AaOr7* sequence and may therefore represent an anomalous sequence assembly.

***AaOrs* are clustered in the genome**

Although the *AaOrs* have not been mapped cytologically to the *Ae. aegypti* chromosomes, *AaOr* localization on super-contigs revealed that 12 *AaOrs* are paired in tandem arrays, seven are found as triplets and many others as larger clusters of up to 11 genes (Table S1). This is similar to the organization of the *AgOrs* (Hill *et al.*, 2002) but in contrast to that of the *DmOrs*, which are typically dispersed as single genes throughout the genome and at most are found in triplets (Clyne *et al.*, 1999a; Robertson *et al.*, 2003). Overall, 18 conserved *AaOrs* share microsynteny with their *AgOr* counterparts and neighbouring genes further supporting the evolutionary relationships of these receptors (Fig. 2). Taken together, genomic clustering, conserved gene orientation, and sequence similarity provide strong evidence that these groups have arisen via gene duplication.

Ae. aegypti introns are on average larger than those of *An. gambiae* (Nene *et al.*, 2007). and, not surprisingly, the gene architecture of *AaOrs* differs markedly from that of *AgOrs*. In general, *AaOrs* contain introns that are significantly larger, where the average is 544 bp, than those observed in *An. gambiae* where the average is 119 bp. The largest *AaOr* intron ($\approx$ 30 kb) is found in *AaOr7* and is nearly 10 times larger than the largest intron in *AgOr7*. We identified 18 conserved intron positions between *Ae. aegypti* and *An. gambiae* (Fig. 3). Of these 18, introns o, p, q and s are present at the base of the *Or* lineage and are also shared

with the *Drosophila Ors* (Fig. 3). Intron loss is a dominant feature of the recent evolution of eukaryotes (Roy & Penny, 2006), and this phenomenon is reflected in various *Or* lineages where numerous instances of independent intron losses have occurred (Fig. 1). Furthermore, there are five and nine idiosyncratic intron locations specific to *Ae. aegypti* and *An. gambiae Ors*, respectively.

AaOrs are expressed during the aquatic and terrestrial stages

To validate our gene models and to explore the spatial localization and distribution of the *AaOrs*, we used RT-PCR to determine the expression profiles of 123 *AaOrs* across the olfactory apparatus of *Ae. aegypti*. Like most insects, the antennae of *Ae. aegypti* and *An. gambiae* constitute the principal olfactory appendages, as reflected by the sheer number of olfactory sensilla and wide olfactory response profiles (Pappenberger *et al.*, 1996; Meijerink *et al.*, 2000; Hallem *et al.*, 2004b) contained within. In addition, insects manifest important olfactory responses in their maxillary palps, which display fewer olfactory sensilla and reduced olfactory response profiles (Kellogg, 1970; Grant *et al.*, 1995). Interestingly, another population of ORNs has been identified on the proboscis (labellum) of *An. gambiae* (Pitts *et al.*, 2004) that have recently been shown to harbour olfactory responses that are potentially novel to mosquitoes (Kwon *et al.*, 2006).

Eighty-three of the 123 surveyed *AaOrs* (70%, excluding the previously characterized *AaOr7*) are expressed in the antennae (Fig. 4, Table 1). Of these, nine (*AaOr22*, 36, 62, 96, 105, 109, 116, 121 and 123) appeared to be expressed only in females, as they were undetectable in male antennae and other olfactory appendages. The expression of two antennal *AaOrs* (*AaOr84* and *AaOr113*) was further mapped to discrete sets of *Ae. aegypti* ORNs using *in-situ* hybridization (Fig. S4). In these studies, *AaOr84* and *AaOr113* were expressed in a relatively small number of ORN cell bodies spanning multiple antennal segments (data not shown), where they are consistently colocalized with the *AaOr7*, which serves as a general ORN marker protein in *Ae. aegypti* (Melo *et al.*, 2004).

The maxillary palps of *Ae. aegypti* mediate carbon dioxide and other chemical responses (Kellogg, 1970; Grant *et al.*, 1995), are sexually dimorphic, and harbour a small subset of olfactory sensilla basiconica on the fourth segment (McIver, 1971). In addition to the widely expressed nonconventional *AaOr7* gene, only the two non-antennal *AaOr9* and *AaOr49* (Fig. 4, asterisks) were found in the palpi of both sexes (Fig. 4, Table 1). Of these, only *AaOr8*, which has a potential ortholog in *An. gambiae*, is palp specific. *AaOr49* is also expressed in the proboscis (Fig. 4, Table 1). *In-situ* hybridization showed that *AaOr49* was found to be expressed throughout the fourth maxillary palpomere of females, which has been reported to be the sole olfactory sensilla-bearing segment (McIver, 1982). The proboscises of *Ae. aegypti* and *An. gambiae* are homologous structures and the expression of multiple *AgOrs* and robust olfactory responses, perhaps involved in mediating the penultimate stages of blood feeding behaviours, have been described on the labellum of *An. gambiae* (Kwon *et al.*, 2006). Similarly, we have identified a subset of 12 *AaOrs* (excluding *AaOr7*) expressed in the proboscis of *Ae. aegypti*. It is interesting to note that, of these, only *AaOr6* has a potential *AgOr* ortholog that displays labellum expression (Kwon *et al.*, 2006). This conservation of mosquito proboscis *Or* expression and presumptive olfactory function is, to date, unique among dipterans and further underscores important differences in the olfactory processes between fruit flies and mosquitoes. Indeed, such differences may be associated with the anautogenous requirement for blood meals that underlies the vectorial capacity of these mosquitoes. Not including *AaOr7*, we found 23 *AaOrs* in the larval antenna. Fifteen of these (1, 9, 14, 34, 37, 40, 48, 58, 60, 61, 73P, 74, 75, 89 and 90) are larval specific (Fig. 4). Most larval *Or* genes form monophyletic clusters and/or are linked on the genome (Figs 1 and 2). Such is the case for the *AaOr1/34/37*, *AaOr2/9*, *AaOr7/40*, *AaOr3/14/15/48*, *AaOr60/61*, *AaOr89/90*, *AaOr1/34/37* and *AaOr58/73P/74/75/78*.

Discussion

The *Aedes Or* gene repertoire is 60% larger than its *Anopheles* counterpart. Phylogenetic comparison demonstrates that the vast majority of ORs in each subgenome is species-specific, indicating a higher degree of evolutionary activity in *Ors* that is likely to reflect their critical roles during the mosquito life cycle. At the other end of the spectrum, the observed conservation of a subset of ORs in these two distant species may also reflect common biological constraints. One caveat in this, or indeed any, comparison of the two mosquito genomes is that future refinements in the overall assemblies may necessitate the re-evaluation of the *Or* annotations used in this study. Errors of assembly such as false duplications or simple gaps in sequence data may obscure the actual number of *Ors* in either mosquito genome. This may be especially relevant for the *Ae. aegypti* WGS assembly, which lacks a corresponding physical map, is much larger in size and considerably more difficult to assemble because of the large amounts of repetitive DNA in the genome (Nene *et al.*, 2007).

Expression of 20 *AaOrs* ($\approx 16\%$) is undetectable in adult and larva mosquitoes. This may in part reflect low levels of transcripts distributed across a limited number of ORNs, thereby confounding amplification by means of RT-PCR. In other instances, it is likely that a subset of *AaOrs* are specifically expressed in wings or legs as observed in the legs of *An. gambiae* (Pitts *et al.*, 2004). We cannot exclude the possibility that these genes have been rendered nonfunctional (pseudogenes) through mutations outside of their open-reading frame or through mis-sense mutations. Taken together, the differential *AaOr* expression patterns within the peripheral olfactory apparatus provide a molecular basis for the response profiles of these tissues, as has been demonstrated for *D. melanogaster* (Krzywinski *et al.*, 2001; Hallem *et al.*, 2004b) in which the odour space of each olfactory tissue is determined by the spectrum of *Ors* expressed. Moreover, if one accepts that the peripheral segregation of *AaOrs* into the antennal and non-antennal structures observed here is nonrandom, and may support broad biological functions for each appendage (e.g. host, mate or oviposition site recognition), then it reflects a biologically constrained topography for *AaOr* expression in the periphery.

It is reasonable to suggest that the observed female-specific expression, detected by RT-PCR, may indicate the involvement of these *AaOrs* in female-specific, olfactory-mediated behaviours. Moreover, these data provide an additional departure from the accepted 'one receptor-one neurone' and 'one receptor-one glomerulus' paradigms of olfactory organization in that one might also expect this sexual dimorphism to be manifest in AL anatomy. However, the only apparent AL sexual dimorphism is restricted to a single additional glomerulus in male *Ae. aegypti* (Ignell *et al.*, 2005). Alternatively, the observed difference in antennal PCR signals between female and male may just be a reflection of the lower number of olfactory sensilla and ORNs in males (McIver, 1982). The absence of male-specific *Ors* suggests that both male and female mosquito olfactory-mediated behaviours are able to utilize a common *Or* array as shown in *Drosophila* (Ejima *et al.*, 2007). While *Ors* expression has been reported in the larva of *Drosophila* (Fishilevich & Vosshall, 2005; Kreher *et al.*, 2005), this is the first time that *Ors* have been shown to express in aquatic insects. The larval antenna of Diptera harbours two chemosensory organs. The peg organ is assumed to be a contact chemosensory sensillum based on the presence of a single terminal pore while the sensory cone is multiporous consistent with olfaction (Nicastro *et al.*, 1998). The cone of *Ae. aegypti* is innervated by approximately 13 receptor neurones (Zacharuk *et al.*, 1971). This amount does not account for the number of expressed *AaOrs* in the larval antenna. However, mosquito *Ors* are also expressed in the adult proboscis, which has been considered a gustatory organ (Pitts *et al.*, 2004; Kwon *et al.*, 2006). It is therefore possible that *AaOrs* may be found in the larval peg organ as well or

that more than one *AaOr* per neurone is expressed in the cone. We found a direct correlation between larval *Or* expression, sequence conservation and genomic proximity suggesting that larval *Or* genes may be under the control of larval-specific regulatory elements. A recent study demonstrated that the expression of *Ors* in adult *Drosophila* is under a hierarchical code of regulatory elements and transcription factors dictating organ and neurone specificity (Ray *et al.*, 2007).

In *Drosophila*, transgenic reporter lines have facilitated systematic analyses of the anatomical relationships between discrete ORNs and their synaptic targets within AL glomeruli. The results of these studies have largely adhered to the one receptor–one neurone and one glomerulus–one receptor principles that are characteristic of mammalian olfactory systems (Couto *et al.*, 2005; Fishilevich & Vosshall, 2005; Kreher *et al.*, 2005). This paradigm is also likely to be valid in the honeybee *Apis mellifera*, where between 156 and 166 AL glomeruli (Galizia *et al.*, 1999) and 163 intact *Or* genes have recently been identified (Robertson & Wanner, 2006). In contrast, mosquito olfactory networks seem to be paradoxical in this regard as the *Or* repertoires of both *An. gambiae* and *Ae. aegypti* are significantly greater than the approximately 50 AL glomeruli present in both vector species (Ignell *et al.*, 2005). This raises the intriguing possibility that there may be significant differences across insect taxa in how chemosensory information is encoded.

Defining and spatially characterizing the complete repertoire of the *AaOr* transcriptome is a critical step toward the functional characterization of these receptors. Further study of the highly divergent *Or* gene families will reveal ways in which these receptors influence chemosensory-driven behaviours, some of which are likely to be unique to certain mosquito species and may have a bearing on their ability to transmit disease. Accordingly, the characterization of this gene family in *Ae. aegypti* has potentially far-reaching implications for basic research, vector control and global health.

Experimental procedures

Gene identification and annotation

The *Ae. aegypti* (Liverpool strain) whole genome sequencing database at the Broad Institute (www.broad.mit.edu/annotation/disease_vector/aedes_aegypti/) was queried using previously described OR sequences from *An. gambiae* (Hill *et al.*, 2002), *D. melanogaster* (Clyne *et al.*, 1999b; Vosshall *et al.*, 1999; Zwiebel & Takken, 2004) and *Heliothis virescens* (Krieger *et al.*, 2003) by tBLASTn (Altschul *et al.*, 1990). Recently, trace amine-associated receptors (TAARs) have been implicated in odour recognition in mice (Liberles & Buck, 2006) and we used these sequences from vertebrates (Lindemann & Hoener, 2005; Gloriam *et al.*, 2005) to query the *Ae. aegypti* genome. Our searches resulted in the identification of several class-A biogenic amine receptors (data not shown; Nene *et al.*, 2007). Putative *Or* sequences were manually annotated using Sequencher v4.5 (Gene Codes, Inc., Ann Arbor, MI) and refined using the Softberry Splice Site Prediction program (www.softberry.com/berry.phtml?topic=fsplce&group=programs&subgroup=gfind) and TMPred (Hofmann & Stoffel, 1993). All *Ae. aegypti* ORs identified in this manner were used in successive tBLASTn searches to identify other candidate sequences, which were annotated as above. Final AaOR annotations were compared with the AaegL1.1 Genebuild at Vectorbase (<http://www.vectorbase.org>). The ClustalW algorithm (Thompson *et al.*, 1994) was used to align protein sequences and predict the exon/intron structures (see ‘Intron/exon analysis’ below) to further support and refine the final annotation predictions. In some cases, it was possible to confirm whole or partial *Or* gene predictions using tentative consensus sequences identified from the TIGR *Ae. aegypti* Gene Index (AeGI Release 5.0, 29 June 2005; http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=a_aegyptii).

Intron/exon analysis

The predicted structure of each gene was reconstructed as described above. We first compared intron locations for each monophyletic clade within a species and then representatives of individual clades. Species comparisons were carried out in a similar fashion between *Ae. aegypti* and *An. gambiae*, and only the most parsimonious intron locations were considered to be conserved. All other were considered idiosyncratic.

Phylogenetic analysis

Deduced amino acid sequences of *AaOrs* were aligned with AgORs using ClustalW (Thompson *et al.*, 1994) and phylogenetic analysis was performed using distance methods in PAUP* v4.0b10 (Swofford, 1998). For the details of corrected distance phylogenetic methods, see Fig. S1 legend and (Hill *et al.*, 2002). Bootstrap analysis was used to assess statistical support for relationships via uncorrected neighbour-joining analysis of 1000 pseudo-replicated data sets.

Expression analysis using RT-PCR

Twenty-five antennae, proboscises, and maxillary palps each from 5-, 6-, 7- and 8-day-old *Ae. aegypti* were hand dissected. Three separate collections were done per sex. We also hand dissected three separate collections of larval antennae (larval stages 3 and 4). Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from RNA samples using SuperScript III RT according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). To control for genomic DNA contamination, each cDNA synthesis was carried out in duplicate: one with RT (experimental) and one without (no RT control). We used the *Ae. aegypti* housekeeping gene for Lysosomal Aspartic Protease (*AaLAP*: (13)) to control the quantity of each cDNA sample. PCR cycling conditions were as follows: 94 °C (2 min) followed by 35 cycles (40 cycles for proboscis, palp and larval antenna cDNAs) at 94 °C (25 s), 55 °C (20 s), and 72 °C (1 min). PCR products were then visualized with ethidium bromide on 2% agarose gels.

Confirmation of Or sequence

Aedes aegypti Or PCR amplicons were sequenced to confirm predicted coding regions for a subset of genes. Twenty-seven *AaOr* PCR products corresponding to predicted cDNA amplicons were gel-extracted (QiaexII, Qiagen) and directly sequenced at Vanderbilt University.

In situ hybridization and immunolabelling—cDNA fragments were amplified utilizing the oligonucleotides 5'-CTC AGG GAT TTT TGT AGC AGG AGC A-3' and CAC TCC GTT GAT GGT GTT GTG C-3' (*AaOr113*), 5'-GTG GAA AGG ATG ACG CAA AAG CA-3', and 5'-ACG GAA CCA AAA ACC CTG TTG AG-3' (*AaOr84*), 5'-GC GAG TTG ATC TAC TTT ACC G-3' and 5'-GGT TCC AAG TAC CAG GGA TAG C-3' (*AaOr49*). For synthesis of digoxigenin-labelled sense and anti-sense RNA probes, amplification products were cloned into the pCRII-TOPO vector (Invitrogen). *In situ* hybridization and probe preparation were carried out as described previously (Kwon *et al.*, 2006). *AaOr* signals were visualized by peroxidase-coupled antidigoxigenin antibodies (Roche, Indianapolis, IN, USA) at 1 : 200 dilution. Peroxidase signals were detected using tyramide signal amplification plus cyanine 3 system (Perkin-Elmer, Wellesley, MA, USA) according to the manufacturer's instructions. *AaOr7* and *nervana*/HRP immuno-labelling, respectively, was carried out using anti-AgOR7 antibodies and anti-HRP antibodies, respectively, as previously described (Melo *et al.*, 2004; Rutzler *et al.*, 2006). We used anti-HRP instead of anti-AgOR7 as it has been previously reported to weakly labels the cell body of the capitite pegs in the maxillary palp of *Ae. aegypti* (Melo *et al.*, 2004). All presented

images were captured within the dynamic signal range and have not been adjusted in contrast. Optical sections of 1.2 μm are shown.

Mosquito rearing

Aedes aegypti (Costa Rica strain), originally established in 2001 (Perich *et al.*, 2003) was kindly provided by Mark Q. Benedict (Centers for Disease Control and Prevention, Atlanta, GA, USA). Embryos were hatched in flat plastic pans with distilled water. Costa Rica strain larvae were reared on a 2 : 1 mixture of ground fish food (Aquaricare Koi floating blend, Victor, NY, USA) and bakers yeast, and Liverpool larvae were reared on ground guinea pig food (Nutriphase products, Phoenix, AZ, USA). Pupae were transferred to plastic cups within 4-litre plastic containers, from which newly emerged adults were collected the next morning. Adult mosquitoes were maintained in 4 litre plastic containers at relative humidity under a 12 h/12 h photoperiod and provided with a 10% dextrose solution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ache BW, Young JM. Olfaction: diverse species, conserved principles. *Neuron*. 2005; 48:417–430. [PubMed: 16269360]
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990; 215:403–410. [PubMed: 2231712]
- Benton R, Sachse S, Michnick SW, Vosshall LB. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. *PLoS Biol*. 2006; 4:e20. [PubMed: 16402857]
- Berger J, Suzuki T, Senti KA, Stubbs J, Schaffner G, Dickson BJ. Genetic mapping with snp markers in *Drosophila*. *Nat Genet*. 2001; 29:475–481. [PubMed: 11726933]
- Buck L, Axel R. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell*. 1991; 65:175–187. [PubMed: 1840504]
- Clyne PJ, Certel SJ, De Bruyne M, Zaslavsky L, Johnson WA, Carlson JR. The odor specificities of a subset of olfactory receptor neurons are governed by acj6, a pou-domain transcription factor. *Neuron*. 1999a; 22:339–347. [PubMed: 10069339]
- Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron*. 1999b; 22:327–338. [PubMed: 10069338]
- Couto A, Alenius M, Dickson BJ. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr Biol*. 2005; 15:1535–1547. [PubMed: 16139208]
- Dahanukar A, Hallem EA, Carlson JR. Insect chemoreception. *Curr Opin Neurobiol*. 2005; 15:423–430. [PubMed: 16006118]
- Ejima A, Smith BP, Lucas C, Van Der Goes Van Naters W, Miller CJ, Carlson JR, et al. Generalization of courtship learning in *Drosophila* is mediated by cis-vaccenyl acetate. *Curr Biol*. 2007; 17:599–605. [PubMed: 17363250]
- Fishilevich E, Vosshall LB. Genetic and functional subdivision of the *Drosophila* antennal lobe. *Curr Biol*. 2005; 15:1548–1553. [PubMed: 16139209]

- Fox AN, Pitts RJ, Robertson HM, Carlson JR, Zwiebel LJ. Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proc Natl Acad Sci USA*. 2001; 98:14693–14697. [PubMed: 11724964]
- Galizia CG, McIlwrath SL, Menzel R. A digital three-dimensional atlas of the honeybee antennal lobe based on optical sections acquired by confocal microscopy. *Cell Tissue Res*. 1999; 295:383–394. [PubMed: 10022959]
- Gao Q, Chess A. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. *Genomics*. 1999; 60:31–39. [PubMed: 10458908]
- Gloriam DE, Bjarnadottir TK, Yan YL, Postlethwait JH, Schioth HB, Fredriksson R. The repertoire of trace amine G-protein-coupled receptors: Large expansion in zebrafish. *Mol Phylogenet Evol*. 2005; 35:470–482. [PubMed: 15804416]
- Goldman AL, Van Der Goes Van Naters W, Lessing D, Warr CG, Carlson JR. Coexpression of two functional odor receptors in one neuron. *Neuron*. 2005; 45:661–666. [PubMed: 15748842]
- Grant AJ, Wigton BE, Aghajanian JG, O'Connell RJ. Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. *J Comp Physiol A*. 1995; 177:389–396. [PubMed: 7674195]
- Hallem E, Fox AN, Zwiebel LJ, Carlson JR. A mosquito odorant receptor tuned to a component of human sweat. *Nature*. 2004a; 427:212–213. [PubMed: 14724626]
- Hallem E, Ho MG, Carlson JR. The molecular basis of odor coding in the *Drosophila* antenna. *Cell*. 2004b; 117:965–979. [PubMed: 15210116]
- Hallem EA, Dahanukar A, Carlson JR. Insect odor and taste receptors. *Annu Rev Entomol*. 2006; 51:113–135. [PubMed: 16332206]
- Hill CA, Fox AN, Pitts RJ, Kent LB, Tan PL, Chrystal MA, et al. G protein-coupled receptors in *Anopheles gambiae*. *Science*. 2002; 298:176–178. [PubMed: 12364795]
- Hofmann K, Stoffel W. Tmbase – a database of membrane spanning proteins segments. *Biol Chem Hoppe-Seyler*. 1993; 374:166.
- Holt RA, Subramanian GM, Halpern A, Sutton GG, Charlab R, Nusskern DR, et al. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science*. 2002; 298:129–149. [PubMed: 12364791]
- Ignell R, Dekker T, Ghaninia M, Hansson BS. Neuronal architecture of the mosquito deutocerebrum. *J Comp Neurol*. 2005; 493:207–240. [PubMed: 16255032]
- Jacquín-Joly E, Merlin C. Insect olfactory receptors: Contributions of molecular biology to chemical ecology. *J Chem Ecol*. 2004; 30:2359–2397. [PubMed: 15724962]
- Jefferis GSXE. Insect olfaction: a map of smell in the brain. *Curr Biol*. 2005; 15:R668–R670. [PubMed: 16139199]
- Jones WD, Nguyen TA, Kloss B, Lee KJ, Vosshall LB. Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr Biol*. 2005; 15:R119–R121. [PubMed: 15723778]
- Kellogg FE. Water vapour and carbon dioxide receptors in *Aedes aegyptii*. *J Insect Physiol*. 1970; 16:99–108. [PubMed: 5417711]
- Kreher SA, Kwon JY, Carlson JR. The molecular basis of odor coding in the *Drosophila* larva. *Neuron*. 2005; 46:445–456. [PubMed: 15882644]
- Krieger J, Klink O, Mohl C, Raming K, Breer H. A candidate olfactory receptor subtype highly conserved across different insect orders. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*. 2003; 189:519–526. [PubMed: 12827420]
- Krzywinski J, Wilkerson RC, Besansky NJ. Toward understanding anophelinae (diptera, culicidae) phylogeny: insights from nuclear single-copy genes and the weight of evidence. *Syst Biol*. 2001; 50:540–556. [PubMed: 12116652]
- Kwon HW, Lu T, Rutzler M, Zwiebel LJ. Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA*. 2006; 103:13526–13531. [PubMed: 16938890]
- Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron*. 2004; 43:703–714. [PubMed: 15339651]

- Liberles SD, Buck LB. A second class of chemosensory receptors in the olfactory epithelium. *Nature*. 2006; 442:645–650. [PubMed: 16878137]
- Lindemann L, Hoener MC. A renaissance in trace amines inspired by a novel GPCR family. *Trends Pharmacol Sci*. 2005; 26:274–281. [PubMed: 15860375]
- McIver S. Comparative studies on the sense organs on the antennae and maxillary palps of selected male culicine mosquitoes. *Can J Zool*. 1971; 49:235–239. [PubMed: 4395988]
- McIver SB. Sensilla of mosquitoes (diptera: Culicidae). *J Med Entomol*. 1982; 19:489–535. [PubMed: 6128422]
- Meijerink J, Braks MA, Braak AA, Adam W, Dekker T, Posthumus MA, Beek TA, Van Loon JJA. Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. *J Chem Ecol*. 2000; 26:1367–1382.
- Melo AC, Rutzler M, Pitts RJ, Zwiebel LJ. Identification of a chemosensory receptor from the yellow fever mosquito, *Aedes aegypti*, that is highly conserved and expressed in olfactory and gustatory organs. *Chem Senses*. 2004; 29:403–410. [PubMed: 15201207]
- Nicastro D, Melzer RR, Hruschka H, Smola U. Evolution of small sense organs: sensilla on the larval antennae traced back to origin of the diptera. *Naturwissenschaften*. 1998; 85:501–505.
- Pappenberger B, Geier M, Boeckh J. Responses of antennal olfactory receptors in the yellow fever mosquito *Aedes aegypti* to human body odours. *Ciba Found Symp*. 1996; 200:254–63. Discussion 263–6, 281–284. [PubMed: 8894302]
- Perich MJ, R.N.O. Castro AL, Alfaro AW, Platt KB, Solano T, Rowley WA. Evaluation of the efficacy of lambda-cyhalothrin applied by three spray application methods for emergency control of *Aedes aegypti* in Costa Rica. *J Am Mosq Control Assoc*. 2003; 19:58–62. [PubMed: 12674536]
- Pitts RJ, Fox AN, Zwiebel LJ. A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector, *Anopheles gambiae*. *Proc Natl Acad Sci USA*. 2004; 101:5058–5063. [PubMed: 15037749]
- Ray A, Van Der Goes Van Naters W, Shiraiwa T, Carlson JR. Mechanisms of odor receptor gene choice in *Drosophila*. *Neuron*. 2007; 53:353–369. [PubMed: 17270733]
- Robertson HM. Taste: independent origins of chemoreception coding systems? *Curr Biol*. 2001; 11:R560–R562. [PubMed: 11509254]
- Robertson HM, Wanner KW. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: Expansion of the odorant, but not gustatory, receptor family. *Genome Res*. 2006; 16:1395–1403. [PubMed: 17065611]
- Robertson HM, Warr CG, Carlson JR. Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc Natl Acad Sci USA*. 2003; 100(Suppl. 2):14537–14542. [PubMed: 14608037]
- Roy SW, Penny D. Large-scale intron conservation and order-of-magnitude variation in intron loss/gain rates in apicomplexan evolution. *Genome Res*. 2006; 16:1270–1275. [PubMed: 16963708]
- Rutzler M, Zwiebel L. Molecular biology of insect olfaction: Recent progress and conceptual models. *J Comp Physiol A Neuroethol Sens Neural Beh Physiol*. 2005:1–14.
- Rutzler M, Lu T, Zwiebel LJ. Gal4 encoding gene family of the malaria vector mosquito *Anopheles gambiae*. Expression analysis and immunolocalization of agal4 and agal4a in female antennae. *J Comp Neurol*. 2006; 499:533–545. [PubMed: 17029251]
- Severson DW, DeBruyn B, Lovin DD, Brown SE, Knudson DL, Morlais I. Comparative genome analysis of the yellow fever mosquito *Aedes aegypti* with *Drosophila melanogaster* and the malaria vector mosquito *Anopheles gambiae*. *J Hered*. 2004; 95:103–113. [PubMed: 15073225]
- Shanbhag SR, Müller B, Steinbrech RA. Atlas of olfactory organs of *Drosophila melanogaster*. 1. Types and external organization, innervation and distribution of olfactory sensilla. *Int J Insect Morphol Embryol*. 1999; 28:377–397.
- Stewart MK, Clark NL, Merrihew G, Galloway EM, Thomas JH. High genetic diversity in the chemoreceptor superfamily of *Caenorhabditis elegans*. *Genetics*. 2005; 169:1985–96. [PubMed: 15520260]
- Stocker RF. The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell Tissue Res*. 1994; 275:3–26. [PubMed: 8118845]

- Swofford, DL. Paup*: Phylogenetic Analysis Using Parsimony and Other Methods. 4th edn.. Sinauer Press; New York: 1998.
- Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; 22:4673–4680. [PubMed: 7984417]
- van Der Goes van Naters W, Carlson JR. Insects as chemosensors of humans and crops. *Nature.* 2006; 444:302–307. [PubMed: 17108954]
- Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R. A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell.* 1999; 96:725–736. [PubMed: 10089887]
- Wanner KW, Anderson AR, Trowell SC, Theilmann DA, Robertson HM, Newcomb RD. Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. *Insect Mol Biol.* 2007; 16:107–119. [PubMed: 17257213]
- Wetzel CH, Behrendt H, Gisselmann G, Storkuhl KF, Hovemann B, Hatt H. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. *Proc Natl Acad Sci USA.* 2001; 98:9377–9380. [PubMed: 11481494]
- Xia Y, Zwiebel LJ. Identification and characterization of an odorant receptor from the West Nile virus mosquito, *Culex quinquefasciatus*. *Insect Biochem Mol Biol.* 2006; 36:169–176. [PubMed: 16503478]
- Zacharuk RY, Yin LR-S, Blue SG. Fine structure of the antenna and its sensory cone in the larvae of *Aedes aegypti* (1.). *J Morphol.* 1971; 135:273–298. [PubMed: 5126255]
- Zwiebel LJ, Takken W. Olfactory regulation of mosquito–host interactions. *Insect Biochem Mol Biol.* 2004; 34:645–652. [PubMed: 15242705]

Figure 1.

Phylogenetic relationships of the *Ae. aegypti* and *An. gambiae* odourant receptor (*Or*) families. Corrected distance tree generated using *PAUP** v4 using distances corrected in TreePuzzle v5 using a maximum-likelihood model and the BLOSUM62 amino acid matrix [(Hill *et al.*, 2002; Robertson *et al.*, 2003; Robertson & Wanner, 2006) for methods]. The tree is rooted using the *AaGPRor7* and *AgGPRor7* pair based on the basal position of their highly conserved ortholog *DmOr83b* in the *Or* family in a phylogenetic tree of the entire superfamily (Robertson *et al.*, 2003). Bootstrap support from 1000 replications of neighbour-joining with uncorrected distances is shown on the relevant branch points. *Ae. aegypti* (bold branches) and *An. gambiae* (thin branches) gene expansions are indicated by vertical black bars. Brackets indicate apparent orthology between *Ae. aegypti* and *An. gambiae* OR proteins. Protein names are abbreviated to AaOr and AgOr, with suffixes: (P) – pseudogene; (C) (N) – lacking sequence at C- or N-terminus, respectively. Introns are named according to Fig. S3, and their presence within a lineage is indicated by bold letters. Inferred intron losses are indicated by italicized letters on the relevant branches.

Figure 2.

Analysis of microsynteny between orthologous and paralogous *Ae. aegypti* and *An. gambiae* Or genes. Or genes (black arrows) are abbreviated to AaOr and AgOr. Interspecific neighbouring homologs that share at least 50% amino acid identity are shaded in grey. The *Anopheles* neighbouring genes are labelled by the last five digits of their ENSANG number preceded by an underscore character (e.g. ENSANG00000008672 abbreviated to _08672). Gene orientation is indicated by arrows. *Ae. aegypti* supercontig number, *An. gambiae* scaffold number, and chromosome number are on the right side. Centromeres are indicated by a dot.

Figure 3. Intron analysis of *Ae. aegypti* and *An. gambiae* *Or* genes. (A) The intron positions and phases of *Ae. aegypti* and *An. gambiae* (*AaOrs* and *AgOrs*) are shown relative to a scale of the average receptor size in amino acids. The ancestral intron locations o, p, q and s (arrowheads) are shared by the two mosquito subfamilies, while idiosyncratic introns (small letters with asterisk and prime symbols) are species specific. The total number of *Ors* harbouring each conserved intron is indicated in (B).

Figure 4.

Peripheral topography of the *Or* transcriptome in adult and larval olfactory organs of *Ae. aegypti*. Schematic representation of a larval and chimeric adult *Ae. aegypti* heads. Left and right sides represent male and female olfactory appendages, respectively. Non-antennal expression is indicated with an asterisk. *AaOr8* was detected only in the maxillary palp. Female-specific and larval-specific *Ors* are indicated by an arrowhead and 'L', respectively. Olfactory sensilla bearing segments are in shaded in grey.

AaOr expression survey in olfactory organs by RT-PCR. Positive expression is highlighted in grey. The number of (+) symbols represents the number of positive signals for each individual biological replicate performed; (–) negative results in any of the three biological replicates; nd, not determined. Additional information regarding these analyses may be found in the supporting text

Table 1

Gene	Adult antenna		Adult proboscis		Adult palps		Larval antenna	Forward primer	Reverse primer
	male	female	male	female	male	female			
<i>AaGPRor1</i>	-	-	-	-	-	-	+++	ACGGGAGATCGAGAGAACAG	TGGTAGGAAGATCTGCGTC
<i>AaGPRor2</i>	+++	+++	-	-	-	-	+++	TGCTTGCTCGATTTTGAC	AACCTCGCGAGCAATG
<i>AaGPRor3</i>	+++	+++	+++	+++	-	-	+++	ACGCTGTTTGTGTTGGAAC	TTCATAACGACGTAAGCCG
<i>AaGPRorH</i>	+++	+++	+++	+++	-	-	-	TCATTCTCTGCTACTACGGTG	ACGTCCGAATGGTTATGG
<i>AaGPRor5</i>	+++	+++	+++	+++	-	-	-	TCATTCTCTGCTACTACGGTG	AAGTAGGACCATGATGTTTGC
<i>AaGPRor6N</i>	+++	+++	+++	+++	-	-	-	ACCAGTCCGGACAAATTATC	TGAAATACGACCCACGATGC
<i>AaGPRor7</i>	+++	+++	+++	+++	+++	+++	+++	AGAAAGCGATGACCATATCC	TTCAACTGCACCAACACC
<i>AaGPRor8</i>	-	-	-	-	+++	+++	+++	ACGGAAACAAATTGATTGAAGC	TGGCCAGCTCGAGTCATC
<i>AaGPRor9</i>	-	-	-	-	-	-	+++	TAGTGGACCATGGATTGAAC	TCATAGGATAGACGTTACCCAC
<i>AaGPRor10</i>	+++	+++	-	-	-	-	-	TCCTGCTGATAATTCTACGGG	ATAAACCCGACGCGAGC
<i>AaGPRor11</i>	+++	+++	-	-	-	-	-	ACGACTGGTATAACTATCCCG	AGCTTGTTCAGGATCGTG
<i>AaGPRor12C</i>	nd	nd	nd	nd	nd	nd	nd		
<i>AaGPRor13</i>	+++	+++	-	-	-	-	-	TGTTTGAACGGAACTGCTCTC	TCGTGTAGCCCGTTGAATC
<i>AaGPRor14</i>	-	-	-	-	-	-	++	TGGATTGCGTACTCATATGG	TGTGATACCTTCTTTCGGTTG
<i>AaGPRor15</i>	+++	+++	+++	+++	-	-	+++	TCGTGGCACTTACCCGG	ACTTTGCTGCTGTGATACCTTC
<i>AaGPRor16</i>	+++	+++	-	-	-	-	+++	TGGATCTACCTGTTCAACGG	TATTCTCGCGCTGTTACAAG
<i>AaGPRor17</i>	+++	+++	-	-	-	-	-	AGCCTCATCCCAAAATTTC	TCCTTTAACGTGCTGGGAC
<i>AaGPRor18C</i>	nd	nd	nd	nd	nd	nd	nd		
<i>AaGPRor19</i>	+++	+++	-	-	-	-	-	AGGAACGCTCTTATTGATAAAGG	TCCTCATGTTCATAAATCCG
<i>AaGPRor20</i>	+++	+++	-	-	-	-	-	TCCTTGCTACTTCTCAGTCTCCG	AGCAGCATAATCCGGAGTAAAC
<i>AaGPRor21</i>	+++	+++	-	-	-	-	-	TTTACTTCTGAACGGTGCCG	AGCATGCAAAATCGAAATACAGAG
<i>AaGPRor22P</i>	-	+++	-	-	-	-	-	AGCAAAGGACATTGATGAGG	ACGCAGATGTGAGCTATGG
<i>AaGPRor23</i>	+++	+++	-	-	-	-	-	ACTGGCACTCAATCTCAACTC	CTAGTTGAAACTCTGGAGCATTG
<i>AaGPRor24</i>	+++	+++	-	-	-	-	-	AGGAGATTGGAGGTGGATG	TCCCGTTGAAAGTTGTTTCG
<i>AaGPRor25</i>	-	-	-	-	-	-	-	ACCGGATAAGATCTATGCTCTCG	TGGAGTAGTACGAAAATGCCG
<i>AaGPRor26</i>	+++	+++	-	-	-	-	-	AGTTTTCACCTCAACCG	TGACCTTGGTCTCGATCTTC

Gene	Adult antenna		Adult proboscis		Adult palps		Larval antenna	Forward primer	Reverse primer
	male	female	male	female	male	female			
<i>AaGPRor27</i>	+++	+++	-	-	-	-	-	TCGTGCATATTCCAGACCTG	TCCA CTGACCACTCGTACC
<i>AaGPRor28</i>	-	-	-	-	-	-	-	TTCCAAAGTGTGTGCTCAGG	TTGAGCCCGTTGTCCAC
<i>AaGPRor29P</i>	+++	+++	-	-	-	-	+++	ACCGAATGCCCTCAACGTGATG	ATGGTTGTAGATGGCATCGCTAAC
<i>AaGPRor30</i>	-	-	-	-	-	-	-	TTCGTCTACTCCATGCTCG	TAGGATGATTTCGTGTTCGC
<i>AaGPRor31</i>	+++	+++	-	-	-	-	-	TGCGGGCTCAAAGTTC	AGCAACGTACGAGGCCAG
<i>AaGPRor32P</i>	-	-	-	-	-	-	-	TGGTCTACTCTATGTTGAGCACC	TTGCCAGTAGTAAGCTTCGC
<i>AaGPRor33</i>	+++	+++	-	-	-	-	-	TGGTGCTATTATGGAACACAGAG	ACTTGAATGGCTTCTGCG
<i>AaGPRor34</i>	-	-	-	-	-	-	+++	AACGACCGAAAGATCTG	ACCGAATAGGCTGTGTTTAGAG
<i>AaGPRor35C</i>	nd	nd	nd	nd	nd	nd	nd		
<i>AaGPRor36</i>	-	+++	-	-	-	-	-	TCGGTTGTGCGTTATTCTAC	AGTCGCGCACAAATGTATCG
<i>AaGPRor37</i>	-	-	-	-	-	-	+++	TCCAAGATATCGAACGAGTCTAC	ACAACAGGTACATCATATCCC
<i>AaGPRor38P</i>	+++	+++	-	-	-	-	-	ACGGCTTGTTTCATCGGAGTTTGTC	ATGCCACGCGAGTACCAACAC
<i>AaGPRor39N</i>	+++	+++	-	-	-	-	-	GGTGACGGTTTGTTTCATCGGAGTTT	CGGTAGCGATACCAAGGAAAAATCGT
<i>AaGPRor40</i>	-	-	-	-	-	-	+++	ACCGCTAAAGCTAAACCGC	TTAACTAGTATGGCGACTACCG
<i>AaGPRor41</i>	+++	+++	-	-	-	-	-	TTGAAAGCCAAACAGCGAC	ATTAAGCGTCATCATTCCTCC
<i>AaGPRor42</i>	-	-	-	-	-	-	-	AATGACGCTGTTGTGCTAC	ATATCGTACACCTCTCTGGATG
<i>AaGPRor43</i>	-	-	-	-	-	-	-	TGCTAGAAATGTGTTTGCTCG	ATCAAATACCAAGTCCATGCC
<i>AaGPRor44</i>	+++	+++	-	-	-	-	-	TGGAGTCTGTTTGCTTGGGACAG	TGTTCAATGGTGCAGTCCACC
<i>AaGPRor45</i>	+++	+++	-	-	-	-	-	TCTAACTGTGGGAGGCTTTG	TCATCATAGCGAAGAATTGG
<i>AaGPRor46</i>	-	-	+++	+++	-	-	-	AGACCTTCTCATCAAAATGGG	AACATTTTCGCTGCTCGG
<i>AaGPRor47</i>	+++	+++	+++	+++	-	-	+++	TTGCTTGACCATGATGACC	AAGATGTTACGCTGCTCTGG
<i>AaGPRor48</i>	-	-	-	-	-	-	+++	TGGCAGATGAGATCTACAGC	AATGAGTAAAGTGTTTGGACG
<i>AaGPRor49</i>	-	-	++	-	+++	+++	-	TCAATGGAGATGAGCTAAGCG	TAATACCGATCTTCCGGCTGC
<i>AaGPRor50</i>	+++	+++	-	-	-	-	-	TGGGAACGGAAGTATAAGTG	TCATCCTCTGTGATCTTCCC
<i>AaGPRor51P</i>	+++	+++	-	-	-	-	-	TGGTGGCAAAATGCGGTCTATGAGG	TGAAGTAAAGACATCGTGTGCGGAC
<i>AaGPRor52</i>	+++	+++	-	-	-	-	-	TGCTGGAGTCTTACTGGTTCTG	ACAA TCAGCAGCGTCTCC
<i>AaGPRor53</i>	+++	+++	nd	nd	nd	nd	-	TTTCGATGCTGGGAGCTGAATC	GACGGTCGGCTCTCATTTTCATG
<i>AaGPRor54P</i>	+++	+++	-	-	-	-	-	ACATGAGGTTTGCTTCCGATCTGC	GGCAGCCATTCGGTTGTACCAATT
<i>AaGPRor55</i>	+++	+++	+++	+++	+++	+++	-	TGCCGAAAGACGTTTGC	TCCGTAGAACTGCTGCATTATC
<i>AaGPRor56</i>	+++	+++	+++	+++	+++	+++	-	ATGTTGCTTCTGCAATATACCG	AACAACAGCAATCGGCTCTCGGTAG

Gene	Adult antenna		Adult proboscis		Adult palps		Larval antenna	Forward primer	Reverse primer
	male	female	male	female	male	female			
<i>AaGPRor57P</i>	+++	+++	-	-	-	-	-	ATGGTCGTAATCGCATCG	ATCCGATCTCAGCTTCATG
<i>AaGPRor58</i>	-	-	-	-	-	-	+++	TCGAAGCCAAACCCAGGAC	ACAGCCGATGGTTGATGGATGAC
<i>AaGPRor59</i>	+++	+++	-	-	-	-	-	ACTTTAAGACAAAGCCCTTCGC	AGCACAGAGCATGGATCCC
<i>AaGPRor60</i>	-	-	-	-	-	-	+++	TTGTAAACA TGGTGTCTGCTGTG	ATTCCATCAGCTCGATTAGTATGC
<i>AaGPRor61</i>	-	-	-	-	-	-	+++	TTC ACTCCAAAAATTCGACAGC	TGCTGTATCTCAAAATCGACTCC
<i>AaGPRor62</i>	-	+++	-	-	-	-	-	TGAACATCGCAGCTGGAG	AGGCCGAAAAGACCGGATATATTG
<i>AaGPRor63</i>	+++	+++	-	-	-	-	-	TCTAGGAACAGCTTTGACGC	TGTTTCAAGAGAGCCACGC
<i>AaGPRor64P</i>	+++	+++	-	-	-	-	-	ATCCGTGTCATGCTCAGTATG	TGCAGTACAAAATTTCAACCG
<i>AaGPRor65</i>	-	-	-	-	-	-	-	ATTTATGCTATCAACTGGCACC	TTGTGATGAGCATCGCC
<i>AaGPRor66</i>	+++	+++	-	-	-	-	-	TGACTGGTACGCAACATATG	TCGGTAATAAAATGCCATGG
<i>AaGPRor67</i>	-	-	-	-	-	-	-	TGGTCGACATTTATGCTATCAAC	ATTGTGATGAGCATCGCC
<i>AaGPRor68P</i>	-	-	-	-	-	-	-	TGTTGTGAATTTCCGTGG	ACATGATTGGTGTCTCGATG
<i>AaGPRor69</i>	+++	+++	-	-	-	-	-	TGATGCGATGCTTTTGG	AAATTTAACGGAGCAAAACCC
<i>AaGPRor70</i>	+++	+++	-	-	-	-	-	TCGTCTTCACCTGGCGTATTC	TTTGAGCAAAATGCCACG
<i>AaGPRor71</i>	+++	+++	-	-	-	-	-	GTACTGGGGACGGAGTTGAGT	GCCGATCCAGAAACATCTTTGAGCA
<i>AaGPRor72</i>	-	-	-	-	-	-	-	TTGTGTTCA TCGGTTGTGC	TGCTTCCGTGAGTTTGG
<i>AaGPRor73P</i>	-	-	-	-	-	-	+++	CTGCTGTACGGATTCTTGTGCGA	
<i>AaGPRor74</i>	-	-	-	-	-	-	+++	CAAGCGGTGCGTTGATCGTC	CCGTGTAAGGGAACCAACACC
<i>AaGPRor75</i>	-	-	-	-	-	-	+++	GGCATTTGGTGTTCGGGACAA	TGCCCTCATCATCA CAATCAGCA
<i>AaGPRor76</i>	-	-	-	-	-	-	-	GCGTTTCGACGGCACCCAG	CAGCAGCGTGAATGCCGAGT
<i>AaGPRor77P</i>	+++	+++	-	-	-	-	-	GCGTCATCAGAGGTTGCTGGAA	GATGCCGCCACAAGTGAATCC
<i>AaGPRor78</i>	+++	+++	-	-	-	-	+++	GCAGGCTTGTCATCTGTTCC	TTCCGCTCGTCGCAATTCACA
<i>AaGPRor79</i>	+++	+++	-	-	-	-	-	CCTGCTGGTGGTTCAGCTTCG	TCGCCACGTCCCTGATCG
<i>AaGPRor80</i>	+++	+++	-	-	-	-	-	GCATTCTGTTTCATCTGTTGGAGCA	CATTCCACCAACTCTGAACCTTGATTCC
<i>AaGPRor81</i>	-	-	-	-	-	-	-	GACGGATTGCTGGAA GTACGAGGA	TCCGAAAATATACTGGATCGCCATAA
<i>AaGPRor82P</i>	+++	+++	+++	+++	-	-	-	CGCATCCGAAACCTACCTGTGTT	TGTCATGTCGTACCGCACAAAAGC
<i>AaGPRor83P</i>	+++	+++	+++	+++	-	-	-	CATCCGAAACCTACCTGTGTTGC	ACCGATTTCATCATCTGTTGGCAAC
<i>AaGPRor84</i>	+++	+++	-	-	-	-	-	TCGGGTTGTTGCTA TCGTTGATGA	ACGGAACCAAAAAACCTGTTGAG
<i>AaGPRor85</i>	+++	+++	-	-	-	-	-	GACGCTTTGGGACTGTTGGTT	TCCAAATCCCTGCTGAGCTTGT
<i>AaGPRor86N</i>	+++	+++	-	-	-	-	-	TGCGACAGAGCTTACAATAGCAT	TTCTCTGTGGGGAAATAATCTGTGA

Gene	Adult antenna		Adult proboscis		Adult palps		Larval antenna	Forward primer	Reverse primer
	male	female	male	female	male	female			
<i>AdGPRor87</i>	++	++	-	-	-	-	-	AATCATGCGTCAACATTGAGTTGCTT	TGATAAAACCCCGCAGCTAAATTTG
<i>AdGPRor88</i>	++	++	-	-	-	-	-	TGGGGATAACGGTGTCTTTCATCG	TGGGTGTCGAATCTTCAATTATCATCA
<i>AdGPRor89</i>	-	-	-	-	-	-	+++	ATCTGGATGGACGAGCGATATTCAA	GGGTAGGATTTCTGGAGGATCTGC
<i>AdGPRor90</i>	-	-	-	-	-	-	+++	TCAACAAGGTATTTCGGACCAACGA	CGAGGAAAGCGAAACAGCGTGTA
<i>AdGPRor91N</i>	++	++	-	-	-	-	-	GATTTTCGCCCAAGTAGCCCTGTA	CATCAGGTCCGTCAAATGTTGCTG
<i>AdGPRor92</i>	-	-	-	-	-	-	-	CGTATGTCGCACCTTGTGGAGACC	CACCACAGCATTCAGCGAAAAA
<i>AdGPRor93</i>	++	++	-	-	-	-	-	TGCTAACGGGATGACTCGCTTTG	GCTGCTGATAGTCGGAATGTTCTC
<i>AdGPRor94</i>	++	++	-	-	-	-	-	AAGGATTTTCGTTCCGGCTCGGTA	GCAGCATTTGACCTGTACGAGCA
<i>AdGPRor95</i>	++	++	-	-	-	-	-	GCTATTGGGTTTCATGGTGAATGC	TCCGAATGATGTTTGGCGGAATGT
<i>AdGPRor96</i>	-	-	-	-	-	-	-	ATGCGTTTCCCGTGACATACTACT	GAGATCAGAAATACTCCAGCACAGC
<i>AdGPRor97</i>	++	++	-	-	-	-	-	GTCTCGAGTGCTATTGGTGGTG	CGATCATCAGCGAAAGTACGCATCT
<i>AdGPRor98</i>	++	++	-	-	-	-	-	CGGAGGAATCGGAGGACTGTTT	ACATTTGATTGGTGGTGGTGGGA
<i>AdGPRor99</i>	++	++	-	-	-	-	-	ATACGAAGCGGGTCTGGGTAATG	TTCCGCTCGATGTTGTTGATCGTA
<i>AdGPRor100</i>	++	++	-	-	-	-	-	TGGCAACTACTCCGATATTCTGGTG	TCGAACAGCCCCAGACATCG
<i>AdGPRor101</i>	-	-	-	-	-	-	-	ATGGGAATCAACGCAACTTCTGGA	GTGTTACGAAACAGCAACGAAAGCA
<i>AdGPRor102</i>	-	-	-	-	-	-	-	AGACGCTTGCTCATCTTTGTGCT	TCTGAAACAATCCAGGCGAGTCCA
<i>AdGPRor103</i>	++	++	-	-	-	-	-	CTCTGGGAATGCGGAATGAAGTTGA	CGAAAAATGATGTTTCCCGATGGAAGCA
<i>AdGPRor104</i>	++	++	-	-	-	-	-	GGAATCGGGATGAAACTCGGAATGA	AACAGCCCCAAAGCAATCCACTGAA
<i>AdGPRor105</i>	-	-	-	-	-	-	-	TGTGCTCGTTGCTTTATTCTGTC	AATCCGTTGAATCCCATGTTTTCG
<i>AdGPRor106</i>	++	++	-	-	-	-	-	TGGGAAATCATCAGTCGGGAAATCA	TGAGCGATGAACAGAAATGCTCCA
<i>AdGPRor107</i>	-	-	-	-	-	-	-	CAGGATGTTATCGCACCCGTTTCACA	TGCGGTGTTAAGCAATTCAACGAA
<i>AdGPRor108P</i>	-	-	-	-	-	-	-	TGTGCCGCTACTTTTGCTGTTAAGGA	AGTTCATCAGCATTCCTCGTCGTG
<i>AdGPRor109</i>	-	++	-	-	-	-	-	TTTCAGCATTTGGAACGAGTGGCTA	CATCAGCATCGTGTAGGCAGTGTG
<i>AdGPRor110</i>	++	++	-	-	-	-	-	GTTCGCCAAGATGTTTGAATAACGC	GTGGGATGTCAGTTCATTTCGGATT
<i>AdGPRor111</i>	++	++	-	-	-	-	-	GCCTTGGAACATGCTTTTACCTATCC	CGCATTTGAGGAAAGCTGACAAACG
<i>AdGPRor112P</i>	++	++	-	-	-	-	-	TCGGGAGGTGAGGACTGTTGTG	CGGCTGTAAAGGCACTGTGTC
<i>AdGPRor113</i>	++	++	-	-	-	-	-	TGCATCCGCATTGGCTACTTTG	CACCTCGTTGATGGTGTGTTGTC
<i>AdGPRor114</i>	++	++	-	-	-	-	-	ATTTTAGCCAAACCGCTTTTCGTTTCG	CGGTTGTGGGGCATAAGAAATAGCA
<i>AdGPRor115</i>	++	++	-	-	-	-	-	AAAGTTTtagccAACCGCTTTTCATTC	CGGTTGTGGGGCATAAGAAATAGCA
<i>AdGPRor116P</i>	-	++	-	-	-	-	-	GCCGCTGTTTCGTTCTTCTATTGG	TGTTGATGTCGAATATGCCCATGC

Gene	Adult antenna		Adult proboscis		Adult palps		Larval antenna	Forward primer	Reverse primer
<i>AaGPRor117</i>	-	-	-	-	-	-	-	TCCAAATTTCCTCCGTCCTCCATCATTTCT	CGATAGAAAAGAACGAAACAGCGGGCT
<i>AaGPRor118P</i>	-	-	-	-	-	-	-	TGTGTTGGAGTTTACCCCTCGTTGGGA	CATCAAAAACGGAGTACGCAACATCG
<i>AaGPRor119</i>	-	-	-	-	-	-	-	TCTTCTGGCGTTGACTGGAAATGTTTC	GGGTTACGAAGGTGAATGTGGTGA
<i>AaGPRor120P</i>	nd	nd	nd	nd	nd	nd	nd		
<i>AaGPRor121</i>	-	+++	-	-	-	-	-	TCCTGTAGCCAAAGTTCTGCTCGT	AACAAAAGCCGACAAAAGCAATCGT
<i>AaGPRor122</i>	+++	+++	-	-	-	-	-	TGGTGCCATCTGGTTGATTCGT	CAGAAAACATCAGCACCGAGTAAGCA
<i>AaGPRor123</i>	-	+++	-	-	-	-	-	AGCATGATATTGGCGGACTGTTGAA	TGTTCCGATTGGCCACAAAGTCAAGA
<i>AaGPRor124</i>	+++	+++	-	-	-	-	-	TGTCGTCGGCTTGGAACCTGGAAT	GCTGTGACGGGCGTTAAATCCA
<i>AaGPRor125N</i>	+++	+++	-	-	-	-	-	CTGGATGCCCGCTGATTTGGT	CATTCCAAAAGTGGACGGATCAA
<i>AaGPRor126C</i>	+++	+++	-	-	-	-	-	ATGAAAGCGAATTGTTAGCTTCTGGAA	GCGACTAATGCCAAAAGGTTTCCAT
<i>AaGPRor127</i>	nd	nd	nd	nd	nd	nd	nd		
<i>AaGPRor128P</i>	nd	nd	nd	nd	nd	nd	nd		
<i>AaGPRor129P</i>	nd	nd	nd	nd	nd	nd	nd		
<i>AaGPRor130C</i>	-	-	-	-	-	-	-	GGACCTGCTGATGATTGCTTTTGG	TTTTGCGGTAACACCTTCCCACAG
<i>AaGPRor131P</i>	nd	nd	nd	nd	nd	nd	nd		
Summary	75/123	84/123	13/123	13/123	3/123	24/123			