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Conservation of Indole Responsive Odorant Receptors in Mosquitoes Reveals an Ancient Olfactory Trait

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Abstract

Aedes aegypti and *Anopheles gambiae* are among the best-characterized mosquito species within the Culicinae and Anophelinae mosquito clades which diverged ~150 million years ago. Despite this evolutionary distance, the olfactory systems of these mosquitoes exhibit similar morphological and physiological adaptations. Paradoxically, mosquito odorant receptors, which lie at the heart of chemosensory signal transduction pathways, belong to a large and highly divergent gene family. We have used 2 heterologous expression systems to investigate the functional characteristics of a highly conserved subset of *Ors* between *Ae. aegypti* and *An. gambiae* to investigate whether protein homology correlates with odorant-induced activation. We find that these receptors share similar odorant response profiles and that indole, a common and ecologically relevant olfactory cue, elicits strong responses from these homologous receptors. The identification of other highly conserved members of this *Or* clade from mosquito species of varying phylogenetic relatedness supports a model in which high sensitivity to indole represents an ancient ecological adaptation that has been preserved as a result of its life cycle importance. These results provide an understanding of how similarities and disparities among homologous OR proteins relate to olfactory function, which can lead to greater insights into the design of successful strategies for the control of mosquito-borne diseases.

Key words: *Aedes aegypti*, *Anopheles gambiae*, indole, mosquito, odorant-receptor, olfaction, oviposition

Introduction

In many parts of the world, a diverse spectrum of blood-feeding mosquitoes present a serious challenge to the economic and physical well being of human populations; each year, hundreds of millions of people contract mosquito-borne diseases including malaria, dengue, lymphatic filiariasis, rift valley, West Nile, chikungunya, and other maladies (Snow et al. 2005; Weissenbock et al. 2009). The vectors for the majority of these disease-causing agents belong to the Anophelinae and the Culicinae subfamilies, which include *Aedes aegypti*, *Culex pipiens quinquefasciatus*, and the malaria vector mosquito, *Anopheles gambiae* sensu stricto.

In spite of distinct evolutionary histories, these species share a number of general properties insofar as life cycle (Clements 1999). The need to efficiently meet these complex ecological demands is, in part, the task of the mosquitoes' sensory systems that acquire and process a wide array of environmental information pertaining to mating, resource acquisition, and other aspects of the ecological niches occupied by these mosquitoes. Olfaction, in particular, is a central component of this system as it facilitates nectar feeding (Davis 1977; Foster and Hancock 1994) and mating (Cabrera and Jaffe 2007) as well as female-specific behaviors including host seeking, blood feeding (Takken 1991), and oviposition (Bentley and Day 1989).

The general ultrastructure of the olfactory apparatus of mosquitoes is largely conserved as is the qualitative and

quantitative characteristics of olfactory sensilla (McIver 1982; Pitts and Zwiebel 2006). At a physiological level, mosquitoes and other blood-feeding arthropods exhibit overlapping receptive fields for many aromatic compounds such as indole (Blackwell and Johnson 2000; Jeanbourquin and Guerin 2007; Harraca et al. 2009), 3-methylindole (sometimes referred to as skatole; Mboera et al. 2000; Jeanbourquin and Guerin 2007; Harraca et al. 2009), and 4-methyl-phenol (4-MP; sometimes referred to as *p*-cresol; Bentley et al. 1979; Blackwell and Johnson 2000; Jeanbourquin and Guerin 2007; Harraca et al. 2009). A variety of sensory modalities mediate the oviposition behavior of mosquitoes (O'Gower 1963). Olfaction plays a central role in facilitating both attraction to specific aqueous sites as well as stimulation of egg-laying behavior itself (Lindh et al. 2008; Ponnusamy et al. 2008). Indole and 4-MP are both derived from bacterial degradation (Isenberg and Sundheim 1958; Lindh et al. 2008; Mackie et al. 1998) of tryptophan (Elgaali et al. 2002) and tyrosine (Curtius et al. 1976), respectively. Indole is also a by-product of a wide variety of plants (Frey et al. 2000; Schmelz et al. 2003). An odor blend consisting of phenol, 4-MP, 4-ethylphenol, indole, and 3-methylindole from grass infusion attracts female *C. quinquefasciatus* (Millar et al. 1992; Du and Millar 1999). Of these, 3-methylindole alone mediates long-range attraction in several culicine species. Other aromatics such as 4-MP, a compound found in hay infusion (Bentley et al. 1979; Millar et al. 1992) and in human sweat (Cork and Park 1996) also play an important role as an oviposition attractant for various mosquito genera including *Aedes*, *Culex*, and *Anopheles* (Bentley et al. 1979; Blackwell and Johnson 2000; Poonam et al. 2002).

In order to examine evolutionary aspects of odor sensitivity in vector mosquitoes, we have focused on a subset of their olfactory repertoire that defines precise ecological niches. Aromatics and heterocyclics occupy a large portion of the characterized odor space of *An. gambiae* (Carey et al. 2010; Wang et al. 2010) and play a role in attracting mosquitoes from various lineages in the context of host detection (Cork and Park 1996; Takken et al. 2001), larval behavior (Xia et al. 2008), and oviposition (Du and Millar 1999; Lindh et al. 2008). Indole, in particular, is an aromatic heterocyclic organic compound that elicits strong responses in adult antennal trichoid sensilla of *An. gambiae* (Blackwell and Johnson 2000; Meijerink et al. 2000; Qiu et al. 2006) and activates olfactory receptor neurons (ORNs) in *Ae. aegypti* (Siju et al. 2010), *C. quinquefasciatus* (Hill et al. 2009; Syed and Leal 2009), and *C. tarsalis* (Du and Millar 1999).

At a molecular level, mosquito olfactory signal transduction begins on the surface of ORN dendrites that lie within antennal, labellar, and maxillary palp sensilla. Although the precise mechanisms underlying this process are still emerging (Sato et al. 2008; Wicher et al. 2008), it is clear that odorant receptors (ORs) play a significant, if not central role. As is the case for all insect systems, mosquito ORs form heteromeric complexes of unknown stoichiometry, consisting of at least one conventional and one nonconventional OR (Benton et al. 2006). Conventional ORs are thought to be the ligand-binding components of the complex, whereas the nonconventional OR is necessary for the proper function of this assembly (Rutzler and Zwiebel 2005; Benton et al. 2006). Mosquito and other insect ORs are encoded by large and highly divergent gene families that are unrelated to vertebrate ORs (Mombaerts 1999; Benton et al. 2006).

The characteristic divergence of insect ORs is likely to reflect rapid changes in ecological and other life cycle considerations that help to drive speciation (Clark et al. 2007; Guo and Kim 2007; McBride 2007; Gardiner et al. 2008; de Bruyne et al. 2010). Indeed, a phylogenetic comparison between conventional *Ae. aegypti* and *An. gambiae Or* genes demonstrate that with the exception of a subset of 12 *Aedes*/*Anopheles* presumably orthologous *Or* pairs most of the predicted proteins encoded by these genes share less than 20% amino acid identity (Bohbot et al. 2007). This high level of divergence among conventional OR proteins may reflect both the evolutionary distance (Krzywinski et al. 2001a) and the diversity of chemical signals encountered by each species. As would be expected, genes encoding mosquito *Or7* proteins, the ortholog of the nonconventional *Drosophila melanogaster Or83b* (*DOr83b*) which is the requisite functional partner of most conventional ORs (Larsson et al. 2004) are extremely conserved at both the amino acid sequence (Melo et al. 2004; Xia and Zwiebel 2006; Bohbot et al. 2007) and functional levels (Jones et al. 2005).

Beyond the *Or83b*/*Or7* orthologous group, the most closely related group of ORs between *Ae. aegypti* and *An. gambiae* is represented by the OR2/OR10 clade which shares an average of 69% or greater amino acid identity (Bohbot et al. 2007). Recently, conserved members of the OR2/OR10 clade have been identified in the southern house mosquito *C. pipiens quinquefasciatus* (Pelletier et al. 2010). When viewed within the overall context of *Or* gene divergence, it is evident that strong selective pressure has maintained the high level of sequence conservation within the OR2/OR10 clade. This could arise from shared ecological constraints that require a set of common olfactory responses that predate the Anophelinae/Culicinae split ~150 million years ago (Krzywinski et al. 2001a). A prediction of this hypothesis would be that OR2/OR10 would share similar activation profiles between *Ae. aegypti* and *An. gambiae* and moreover, that additional members of this gene subfamily are present in the olfactory repertoire of other mosquito species.

To examine this question, we have used heterologous expression in 2 distinct systems to functionally characterize the odorant response profiles of OR2/OR10 members from *Ae. aegypti* and *An. gambiae*. These studies establish broad and commonly held functional relationships between the OR2/OR10 clade's amino acid sequence and its odorant response profiles. We have also identified OR2/OR10 homologs from additional mosquito species across variable evolutionary distances. From a biological perspective, the

functional conservation of the OR2/OR10 clade in both zoophilic and anthropophilic mosquitoes suggests that while the role of this group of ORs is not strictly associated with host selection, it is nonetheless crucial within the entire family of Culicidae. These studies provide an example of how comparative studies can inform our understanding of the role of ORs in the evolution of chemosensory pathways as well as reveal structure–function relationships of OR proteins in mosquito vectors.

Materials and methods

Mosquito rearing

Aedes aegypti (Costa Rica strain), *An. gambiae* sensu stricto (Suakoko strain), *An. quadriannulatus*, and *An. stephensi* were reared as described in Fox et al. 2001. *Anopheles gambiae* (SUA2La; MRA765), *An. quadriannulatus* (SUAQUA; MRA-761), and *An. stephensi* (IV; MRA-314) were provided by The Malaria Research and Reference Reagent Resource Center (MR4). For stock propagation, 4- to 5-day-old female mosquitoes were blood-fed for 30–45 min on anesthetized mice, following the guidelines set by Vanderbilt Institutional Animal Care and Use Committee.

Molecular cloning

AqOr2 and AsOr2 cloning

Polymerase chain reaction (PCR) templates were prepared from 908 hand-dissected female antennae of *An. quadriannulatus* and 561 hand-dissected female antennae of *An. stephensi* mosquitoes. Collected tissues were used to generated total RNA using the RNeasy (Qiagen) protocol followed by cDNA synthesis using the BD Smart RACE cDNA Amplification Kit (BD Biosciences Clontech) generating 5′ and 3′ cDNA pools. The same 2 degenerate primers and amplification protocols described above were used in subsequent PCR amplifications. Full length *AqOr2* cDNA were obtained using Rapid Amplification of cDNA Ends (RACE) in a GeneAmp PCR system 9700 (Applied Biosystems) under conditions as described in the BD Smart Race cDNA Amplification Kit and with Adaptor primer Universal Primer Mix (UPM) and *AqOr2*-specific RACE primers—3′ RACE primer 1: TTCACCAGCTTCTACGCGACCTG and 5′ RACE primer 2: CAGCAGTGCGCACAGCATCATC. A second nested PCR RACE amplification was carried out using *AqOr2*-specific RACE primers—3′ RACE primer 3: TCGTCCAGATAGCGGCCCTAAAGC and 5′ RACE primer 4: CAGCAGTGCGCACAGCATCATC both with UPM. All experimental-specific PCR products were gel-purified using QIAquick gel extraction reagents (Qiagen), cloned into the pCRII-TOPO cloning vector (Invitrogen), and subsequently sequenced in the DNA Core Facility at Vanderbilt University. The same procedure was applied for *AsOr2* using the following RACE primers—3′ RACE primer 1: GTTCACCAGCTTCTACGCGACCTG and 5′ RACE primer 2: CACAGCATCATCCCGAACGACAAG. A second nested PCR RACE amplification was carried out using *AsOr2*-specific RACE primers—3′ RACE primer 3: ACTCTGTTCGCCGAGCTGAAGGAG and 5′ RACE primer 4: TCGAGCAAACACAGATGGGTGACG both with UPM. The complete nucleotide sequences have been deposited to GenBank (accession numbers: FJ008067, FJ008068, FJ008071, and FJ008072).

AqOr10 and *AsOr10* cloning

AqOr10 and *AsOr10* were amplified from the same cDNA pools as described above using the following 2 degenerate primers: forward primer 5′-CCTGTACCGGGCCTGGGGNAAVAT-3′ and reverse primer 5′-GAGGCGTTCAGCAGGGACTGRAACATYTC-3′. The PCR products were gel-purified using QIAquick gel extraction reagents, cloned into the pCRII-TOPO cloning vector, and subsequently sequenced in the DNA Core Facility at Vanderbilt University. 5′ and 3′ missing fragments for *AqOr10* and *AsOr10* were amplified from the cDNA pools using the Advantage 2 Polymerase Mixes and PCR Kit (Clontech) combined with touchdown PCR following the manufacturer procedure. RACE primers for *AqOr10* included—3′ RACE primer 1: AACGAGGTGCGGGAGGAAAGC and 5′ RACE primer 2: TTGATCTGCACCAGCCCGAACAG. A second nested PCR RACE amplification was carried out using *AqOr10*-specific RACE primers—3′ RACE primer 3: ACCGTGGCTGAATGTGGATGAAAC and 5′ RACE primer 4: GCCAGGTTGGAGATGGACAGGAAG both with UPM. RACE primers for *AsOr10* included—3′ RACE primer 1: ACGAGGTGCGTGAGGAAAGCATGG and 5′ RACE primer 2: CGAACAGCGTGCTCGAGGTGAA. A second nested PCR RACE amplification was carried out using AsOr10-specific RACE primers—3′ RACE primer 3: ACAGTGGACCGTGGCTCAATGTGG and 5′ RACE primer 4: GAAGTGGGCCCGTTTGGTGTACG both with UPM. In all cases, cDNA and genomic DNA sequences were amplified, cloned, and sequenced. Full-length cDNA and genomic clones were obtained using gene-specific primers, and nucleotide sequences have been deposited to GenBank (accession numbers: FJ008069, FJ008070, FJ008073, and FJ008074).

Receptor expression in *Xenopus laevis* oocytes and 2-electrode voltage-clamp electrophysiological recording

Full-length coding sequences of *AaOr2*, *AaOr9*, *AaOr10*, *AgOr2*, and *AgOr10* were PCR amplified from antennal cDNA. PCR were first cloned into pENTR/D-TOPO (Invitrogen) and then subcloned into pSP64DV by means of the Gateway LR reaction (Lu et al. 2007). Complementary RNA (cRNA) was synthesized from linearized vectors using the mMESSAGE mMACHINE SP6 kit (Ambion). Mature healthy oocytes (stage V–VII) were treated with 2 mg/mL collagenase S-1 in washing buffer (96 mm NaCl, 2 mm KCl, 5 mm $MgCl_2$ and 5 mm

N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.6]) for 1–2 h at room temperature. Oocytes were later microinjected with ~28 nL cRNA. After injection, oocytes were incubated for 3–5 days at 18 °C in 1× Ringer's solution (96 mm NaCl, 2 mm KCl, 5 mm $MgCl_2$, 0.8 mm $CaCl_2$, and 5 mm HEPES [pH 7.6]) supplemented with 5% dialyzed horse serum, 50 mg/mL tetracycline, 100 mg/mL streptomycin, and 550 mg/mL sodium pyruvate. Whole-cell currents were recorded from the *Xenopus* oocytes injected with corresponding cRNAs by using a 2-electrode voltage clamp as described in Lu et al. (2007). The data were first analyzed using Clampfit. A Tukey multiple comparison test ($P < 0.001$) was used to compare the mean EC_{50} values of each OR-odorant couple.

Cell culture and Ca^{2+} fluorometry

To create a cell culture expression vector capable of coexpressing AgOR7 in conjunction with a conventional ORx, pcDNA5/FRT/TO (Invitrogen) was modified to create 2 individual expression cassettes each under the control of separate CMV/TetO2 promoters and bovine growth hormone (BGH) polyadenylation signals. Flp-In T-REx 293 cell lines (Invitrogen) were transfected with the modified pcDNA5 plasmid along with POG44 (a plasmid encoding FLP recombinase) to facilitate site-specific recombination. Stable cell lines were selected using hygromycin B (Invitrogen). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% Tetracycline-free FBS (HyClone).

For the fluorometric measurements of Ca^{2+} mobilization, stable lines expressing OR7/ORX were seeded at 20 000 cells per well in black wall, poly-lysine coated 384-well cell culture plates (Greiner) and treated with 0.3 μg/μL tetracycline (Sigma) overnight to induce OR expression. Cells were dye-loaded with 1.8 μM Fluo-4 AM (Molecular Probes) for 45 min at 37 °C prior to each assay, and Ca^{2+} mobilization was assayed in an FDSS6000 plate reader (Hammamatsu). Baseline readings were taken for 20 s before automated addition of 2×10^{-3} M compound previously diluted in dimethyl sulfoxide and assay buffer (20 mM HEPES, 1× Hanks' balanced salt solution). Ratios were described as maximum/minimum response, and each response was normalized to the maximum responder. Each odor was assayed in triplicate per plate and 3 plates were run per cell line. Concentration response curves (CRCs) were run similarly.

Chemicals

All odorants were >99% pure or of the highest grade commercially available. Please see Supplementary Table 1 for a complete list of odorants used in this study including their corresponding CAS numbers.

Gene identification and sequence analyses

The primary amino acid sequence of *D. melanogaster* Or43a protein was retrieved from GenBank (NP_523647). The *CqOr10b*, *CqOr2*, and *CqOr9* genes were identified using the AaOR10 protein to tBLASTn query the *C. quinquefasciatus* (Johannesburg strain) database located at the BROAD Institute (http://www.broad.mit.edu/annotation/genome/culex_pipiens/Home.html). Matches were manually annotated using ClustalW and refined using the Softberry Splice Site Prediction program (http://linux1.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind). ClustalW was used to predict the exon/intron structures of each individual *Or* genes. Deduced amino acid sequences of mosquito *Ors* were aligned using ClustalW, and the resulting data matrix were submitted to the MEGA4 software (Tamura et al. 2007). A neighbor-joining tree was constructed using a pairwise distance method and gaps handled by pairwise deletion. Inferred relationships were tested by bootstrapping based on 10 000 pseudoreplicates.

Results

OR2/OR10 proteins are highly conserved in mosquitoes

Approximately 95% of *Ae. aegypti* ORs share less than 20% amino acid sequence identity with the *An. gambiae* OR repertoire (Supplementary Figure S1) with *Aedes*- or *Anopheles*-specific *Or* gene expansions accounting for most of this diversity (Bohbot et al. 2007). Notwithstanding the extraordinary conservation of *Ag/AaOr7* genes, a subset of 5 anopheline ORs stand out due to their unusually high sequence identity (above 50%) as compared with their homologs in *Ae. aegypti*. These OR homologs belong to the OR2/OR10, OR8, and OR11 protein groups, respectively. Both AaOR10/AgOR10 and AaOR2/AgOR2 share 71% amino acid identity, whereas all OR2s, AaOR9, and OR10s share between 51% and 67% amino acid sequence identity. Of these, only OR2s and OR10s display 70–79% amino acid identity representing 0.02% of all 10 349 possible comparisons of the 131 AaORs with the 79 AgORs (Supplementary Figure S1). It is noteworthy that AgOR9 (named numerically in order of discovery) is not part of this clade and the genome of *An. gambiae* appears to lack a homolog of AaOR9.

A survey of the *Or2/Or10* genes was carried out in 3 additional species of the Anophelinae and the Culicinae lineages. Using bioinformatics and molecular approaches, *Or2*, *Or9*, and *Or10* genes were identified from *C. pipiens quinquefasciatus*, *An. quadriannulatus*, and *An. stephensi* (Figure 1A). *Drosophila melanogaster* OR43a (DOR43a) was also included in this phylogenetic analysis as it represents the closest *Drosophila* homolog to the mosquito OR2/OR10 group. An alignment of the amino acid sequences was carried out (data not shown) to build a sequence-based phylogenetic tree (Figure 1A). All 5 mosquito species studied herein contain one member of the *Or2* gene lineage and at least one member of the *Or10* lineage, which has apparently expanded in the Culicinae subfamily resulting in an additional *Or10* homolog (*CxOr10b*) in *C. quinquefasciatus*. However, *Or9* homologs were only

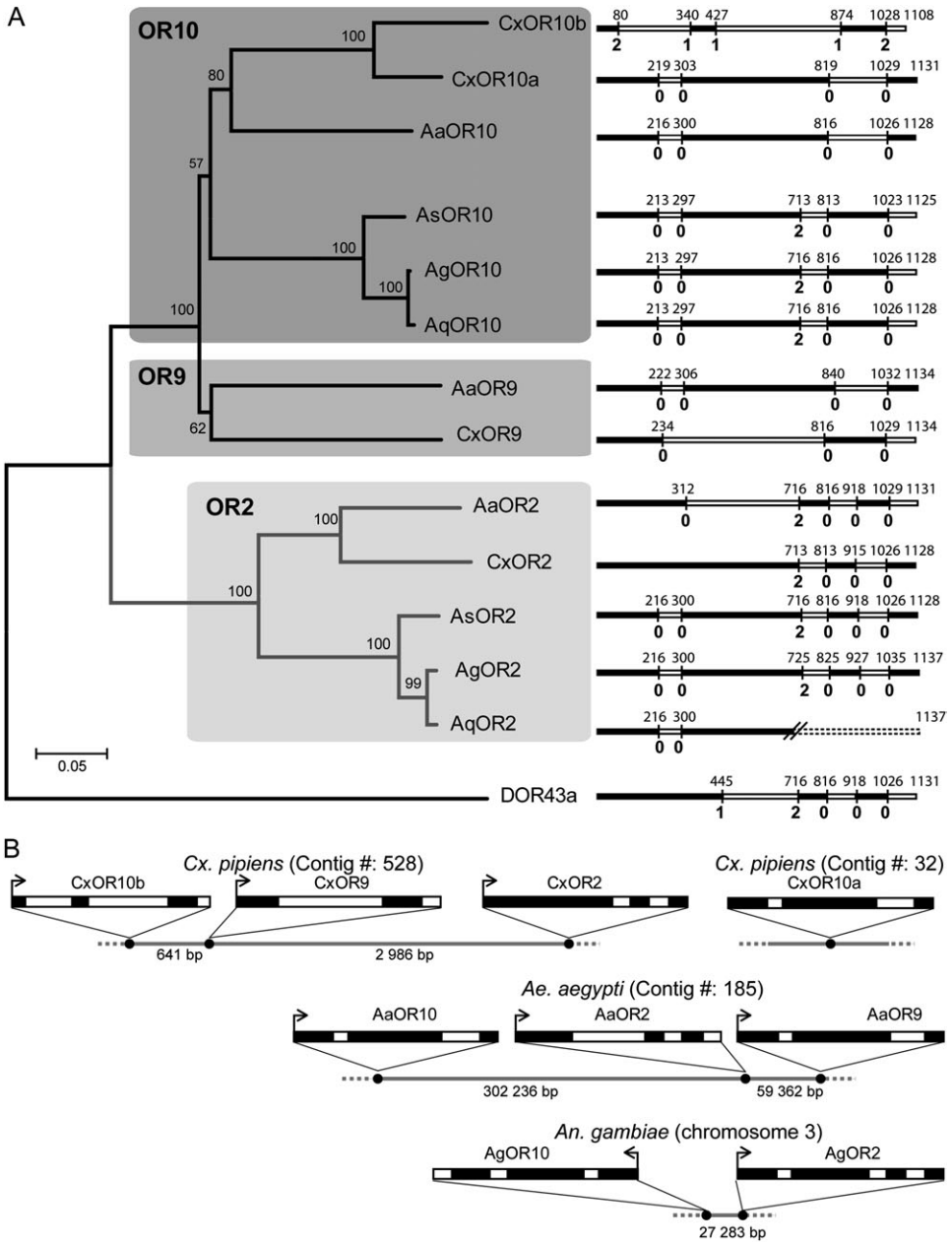

Figure 1 The OR2/9/10 clade predates the Anophelinae/Culicinae split. **(A)** Phylogenetic relationships of the mosquito OR2 (light gray shaded area), OR9 (medium gray shaded area), and OR2 (dark gray shaded area) clade of mosquitoes. Aa: *Aedes aegypti*; Cx: *Culex quinquefasciatus*; Ag: *An. gambiae*; Aq: *An. quadriannulatus*; As: *An. stephensi*. The P-distance tree was generated using MEGA 3.1 using a Neighbor-joining model. Branch lengths are proportional to the scale of sequence distance indicated by the bar below the tree. Bootstrap values (%) are based on 10 000 replicates. Gene structures are indicated by black and whites boxes. Intron positions and protein lengths are indicated above the gene structure. Intron phases are indicated in bold below the gene structure. Complete cDNA sequence was characterized for *AqOr2*, whereas an incomplete genomic DNA sequence was obtained thus providing only the position of the first 2 introns. **(B)** Microsynteny and gene structure of *Ae. aegypti Or2/10*, *C. quinquefasciatus Or2/9/10*, and *An. gambiae Or2/10* genes. Each filled black and white squares represent an exon. Distance between genes is indicated below individual contig.

identified in aedine mosquito species including *Ae. aegypti* and *C. quinquefasciatus*. Sequence identity (Supplementary Figure S1), commonalities in gene structure (Figure 1A), and conserved syntenic relationships (Figure 1B) are consistent with the phylogenetic analysis. Overall, insofar as primary protein sequence is concerned, OR9 is more similar to OR10 than to any of the OR2 predicted proteins (Figure 1A). For example, *AaOr9* encodes a protein that is more similar to AaOR10 (69% amino acid identity) than to AaOR2 (51% amino acid identity).

Based on the unusual sequence similarity (Supplementary Figure S1) and the phylogenetic relationships between OR2s,

OR10s, and AaOR9 (Bohbot et al. 2007), we hypothesized that the OR2/OR10 paralogs would manifest distinct odorant response profiles while the potential Aa/AgOR2 and Aa/AgOR10 pairs would share common odorant sensitivity profiles. To further examine these questions, we carried out a detailed functional characterization within the OR2/OR10 clade to assess odorant sensitivities using 2 heterologous expression systems.

OR2/10 clade exhibits overlapping odor-response profiles

In order to establish the receptive range for divergent members of the OR2/OR10 clade, stable lines of HEK cells functionally expressing AaOR2, AgOR2, AaOR9, AaOR10, or AgOR10 along with AgOR7 were established and challenged with a 30 odor panel (Figure 2). As expected, the tuning curves of orthologous pairs (Ag/AaOR10 and Ag/AaOR2) were strongly concordant in their ability to detect a subset of compounds in this panel (Figure 2). There were no instances in which an odor activated one ortholog and failed to activate its heterospecifc counterpart, although some differences in absolute response levels were observed.

When paralogs were compared, the OR2 and OR10 clades showed considerable overlap in their ability to detect the panel, which is consistent with their overall relatedness. The tuning curve of AaOR9 closely resembled that of the OR10 clade's (Figure 2). There were a few odorants (Figure 2, arrows) for which one set of paralogs responded significantly more strongly than the other pair, and in these instances, AaOR9 possessed an intermediate coding capacity in that it responded to virtually all activators of the 2/10 clade (Figure 2).

To further demonstrate that the specificity of the receptive capacity of these ORs, AgOr8, which has previously been shown to be an octenol receptor (Lu et al. 2007) with less than 20% amino acid identity with OR2/10, was included in this survey. In HEK cells stably expressing AgOR7/AgOR8 proteins, odorant-induced responses were largely indifferent to the principal activators of the OR2/9/10 clade (Figure 2) and instead, closely mirrored the response profiles of AgOR8-expressing oocytes (Wang et al. 2010) and fly ORNs (Carey et al. 2010). All 3 expression systems manifested a significant proportion of the odorant responses directly recorded in vivo from AgOr8-expressing ORNs on the maxillary palp pegs of *An. gambiae* (Lu et al. 2007), which validates the use of cell-based OR expression. In all cases, control HEK-293 cells were indifferent to any odor tested (data not shown).

Indole is an important activating compound of the *Ae. aegypti* and *An. gambiae* OR2/OR10 clade

Our hypothesis was that sequence homology and divergence of the mosquito OR2/OR10 proteins would be reflected in their respective odorant sensitivities. Indeed, using the *Xenopus* oocyte expression system, we observed significant sensitivity differences in response to indole between OR2/9/10 paralogs when expressed together with AgOR7 (Figure 3A). Several odorants including indole, 4-MP, and benzaldehyde have been identified as strong ligands for AgOR2 and AgOR10 in oocytes (Xia et al. 2008; Wang et al. 2010) as well as in the *Drosophila* empty neuron expression system (Carey et al. 2010). In the current study, AgOR2 and AaOR2 exhibited highest sensitivity to indole (Figure 3A and 3C; Supplementary Table 2), whereas AaOR9 and AaOR10 exhibited nearly identical responsiveness that were approximately 10-fold less sensitive than those observed for Aa/AgOR2 (Figure 3A and 3C). Of the paralogous ORs tested, AgOR10 was the least sensitive to indole (Figure 3A; Supplementary Table 2) while we observed uniform albeit relatively low sensitivity in response to benzaldehyde and 4-MP, each of which elicited reduced responses at high concentrations from all ORs tested. CRCs were also generated in HEK cells in order to examine whether sensitivity differences existed between AgOR10 and AaOR10 as well as to validate our oocyte-based assays. In these studies, the OR2/10 clade was once again most sensitive to indole, followed by 4-MP and lastly benzaldehyde. Overall, the OR2/10 clade agonist rankings were comparable with those obtained using *Xenopus* oocytes (Figure 3B and 3D) further demonstrating the consistency of these analyses across multiple functional outputs.

Discussion

The increasing availability of whole insect genomes has provided novel opportunities for examining the evolutionary concepts of orthology and paralogy (Zdobnov and Bork 2007). Current paradigms put forward the view that orthologous proteins retain the same function, whereas paralogs tend to develop new ones (Koonin 2005). In terms of insect olfaction, this concept is best illustrated by the highly conserved nonconventional OR subclade that was first described in terms of its original member *Or83b* from *Drosophila* (Vosshall 2000). Subsequent homologs were identified as *Or7* in mosquitoes (Hill et al. 2002; Melo et al. 2004; Xia and Zwiebel 2006) or *Or2* in moths (Krieger et al. 2004; Nakagawa et al. 2005) and hymenoptera (Robertson and Wanner 2006; Robertson et al. 2010). Members of the *OR83b* family are expressed in a majority of insect ORNs (Vosshall 2000; Pitts et al. 2004), functionally conserved (Jones et al. 2005) and are required for general olfactory signaling (Larsson et al. 2004). Even so, these orthologous ORs are likely not to be directly involved in odorant recognition but rather are required for the proper translocation of the OR complex to the ORN dendrite membrane (Larsson et al. 2004) as well as functioning as a cation channel component of OR complexes (Sato et al. 2008; Wicher et al. 2008).

Despite an abundance of apparent OR ortho/paralogs in *Drosophila* (Clark et al. 2007; McBride 2007; Guo and Kim 2007; Nozawa and Nei 2007; Gardiner et al. 2008)

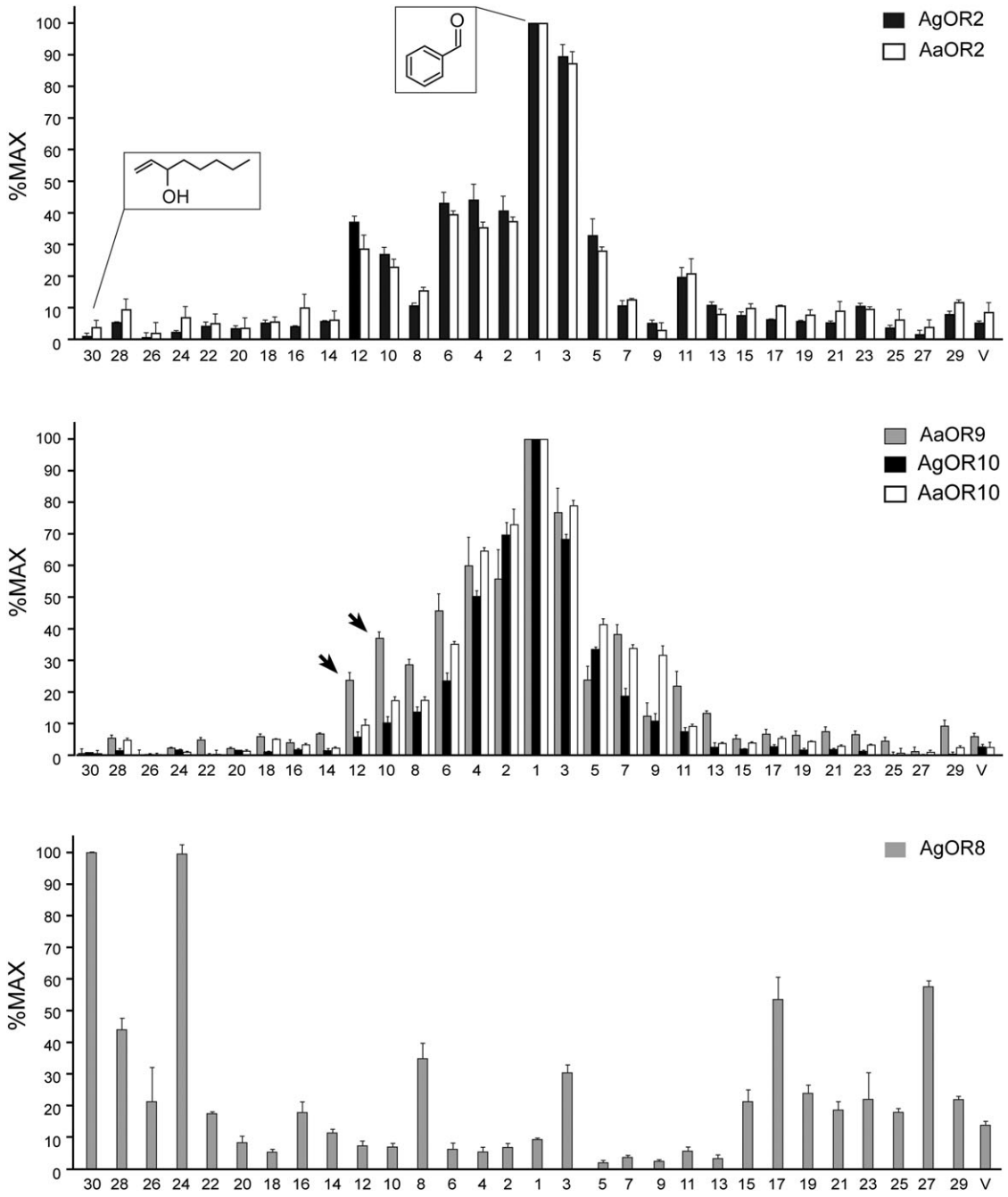

Figure 2 Members of the OR2/10 clade exhibit overlapping sensitivities. Tuning curves of AaOR2, AgOR2, AaOR9, AaOR10, AgOR10, and AgOR8. The 30 odorants are ordered along the *x* axis, with those eliciting the strongest responses for the OR2/10 clade near the center. Benzaldehyde and 1-octen-3-ol (chemical structures shown) elicited the highest response for the OR2/10 clade and OR8, respectively. Arrows indicate odorants for which AaOR9 responses are more similar to OR2s than to OR10s.

and mammalian systems (Branscomb et al. 2000; Churcher and Taylor 2009; Dong et al. 2009; Ohara et al. 2009), functional characterization of ortho/paralogs ORs are scarce in insects (Jones et al. 2005; Lu et al. 2007; Bohbot et al. 2007; Pelletier et al. 2010) as well as in mammals (Krautwurst et al. 1998; Schmiedeberg et al. 2007). In one example of such an analysis the I7 receptors of mouse and rat manifest differential odorant response profiles despite sharing 94% identity at the amino acid level; the rat I7 receptor is more sensitive to octanal as compared with heptanal, whereas mouse I7 displays the opposite sensitivities (Krautwurst et al. 1998). In addition, while the mouse Olfr43 and human

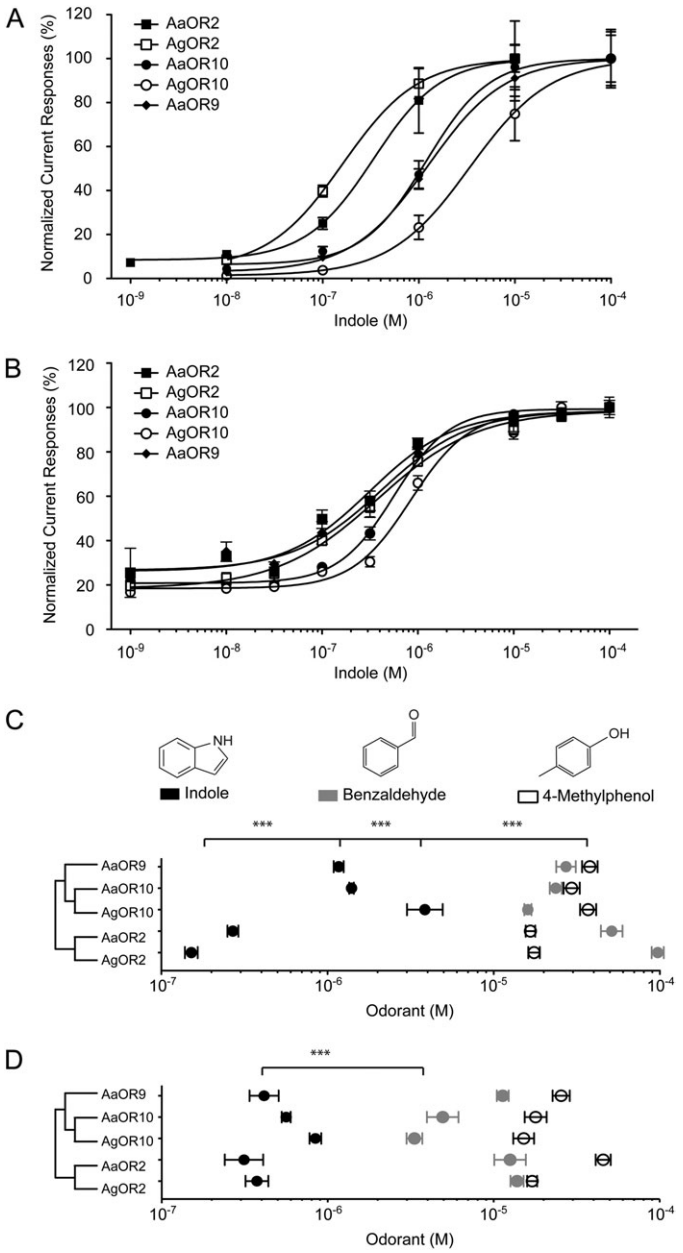

Figure 3 Indole activation correlate with the primary structure of the OR2/9/10 protein. Normalized CRCs of OR2s, OR9, and OR10s expressed in *Xenopus* oocytes **(A)** and in HEK cells **(B)** in response to indole (n = 4–7). Odorant concentrations were plotted on a logarithmic scale. **(C)** Scatter chart displaying 3 functional OR groups: the OR2, AaOR9/AgOR10, and AaOR10 groups (n = 4–8) expressed in *Xenopus* oocytes. **(D)** Scatter chart displaying the sensitivity of the OR2/10 clade expressed in HEK cells in response to indole, 4-MP, and benzaldehyde (n = 3–8). Three asterisks, $P <$ 0.001 (analysis of variance test with Tukey post test). The mean EC_{50} values and standard error of the mean of their scatter were determined using Prism5.

OR1A1 orthologous pair selectively detect (*S*)-(–)-citronellol, a single amino acid change between human OR1A1 and OR1A2 paralogs is responsible for their differential response toward this compound (Schmiedeberg et al. 2007).

These few examples illustrate the caveats involved in predicting function solely from sequence alignments and phylogenetic analyses. In *Drosophila*, a recent study has examined the functional aspects of conserved classes of ORNs and associated *Or* genes across a range of species spanning ~40 million years of evolution (de Bruyne et al. 2010). With notable differences, in vivo ORN responses were found to be largely conserved over this evolutionary time span and attributed to *Or* gene loss or duplication and were generally correlated with primary sequence conservation of homologous ORN/*Or* pairs. Furthermore, by comparing the limited amount of primary sequence divergence between orthologous *Ors* with similar functional characteristics as well as paralogous *Ors* with differential odorant response profiles, the authors identified the amino acid residues associated with general *Or* functionality as well as those linked to determining odorant specificity (de Bruyne et al. 2010). In mosquitoes, OR2 (Pelletier, Guidolin, et al. 2010) and OR8 (Lu et al. 2007; Bohbot and Dickens 2009) are the only instances of demonstrated functional orthology. Overall, these examples suggest that functional conservation is characteristic of orthologous ORs while functional divergence is associated with but not limited to paralogous ORs.

Insect ORs are characteristically divergent with relatively few examples of interspecific primary sequence homology. In order to examine the evolutionary basis for the limited instances of OR conservation that are present and likely to reflect a biological imperative, we have carried out a molecular and functional survey within OR2/OR10 proteins which is the most conserved of the conventional mosquito *Or* subclades. The species utilized for this analysis belong to the 2 major subfamilies covering over 97% of all mosquitoes (Walter Reed Biosystematics Unit, http://www.mosquitocatalog.org/default.aspx?pgID=2). Among the OR2/OR10 clade, we have identified various degrees of sequence conservation likely reflecting both orthologous and paralogous relationships from which we infer the following evolutionary model: the *Or2* and *Or9*/*Or10* genes form 2 distinct monophyletic lineages, the likely product of a gene duplication event that occurred prior to the Anophelinae and Culicinae divergence. A second gene duplication event followed within the Culicinae lineage, giving rise to the *Or10* and *Or9* subgroups. Furthermore, the presence of an *Or9* lineage in both Aedes (*AaOr9*) and Culex (*CxOr9*) coincident with its absence from the *An. gambiae* genome suggest the second duplication occurred prior to the separation of the Culex and Aedes genera ~38 MYA (Besansky and Fahey 1997; Foley et al. 1998). Alternatively, it is also possible that *Or9* was lost sometime in the anopheline lineage in which case it would be expected to be missing in some but not necessary in all other anophelines besides *An. gambiae*. This hypothesis could be directly assessed as additional genomic resources become available. We further posit that the *Or9* lineage represents a case of Culicinae-specific gene expansion that was likely selected by specific ecological requirements. It is possible that

the larval-specific expression of *AaOr9* (Bohbot et al. 2007) is indicative of such a requirement.

To further examine these questions in vector mosquitoes, we have focused on the OR2/OR10 homologous group using independent heterologous expression systems to further demonstrate that sequence conservation correlates with shared odorant-induced activation patterns. When viewed within the context of the highly divergent mosquito *Or* gene families that are typical at both the intra- and interspecific levels (Bohbot et al. 2007; Bohbot and Dickens 2009) and in light of considering the evolutionary distance between these species, the *Or2*/*Or10* gene lineage represents a striking case of structural and functional homology.

From a functional perspective, the aromatic heterocylic indole elicited the strongest responses from Aa/AgOR2 orthologs and to a lesser extent from OR9/10 paralogs. In contrast, other odorants in our panel such as 4-MP and benzaldehyde were observed to be significantly weaker agonists for the OR2/OR9/OR10 lineage with responses reduced between 5- and 1000-fold. Unlike other aromatic compounds that strongly activate a number of AgORs, sensitivity to indole appears to be narrowly restricted to members of the mosquito OR2/OR10 clade, AgOR11 and AgOR13 (Carey et al. 2010; Wang et al. 2010). However, the high responses of AgOR11 and AgOR13 to indole reported by Wang et al. (2010) do not necessarily imply high sensitivity to this odorant as CRCs and EC_{50} values were not reported for these receptors. Indeed, while benzaldehyde elicits high current responses in the OR2/OR10 clade, sensitivity to this compound is low compared to indole (Figure 3). The observation that other mosquito OR2 receptors exhibited similar response profiles supports the hypothesis that indole sensitivity is tightly conserved between and indeed may be restricted to this narrow range of mosquito OR orthologs. This hypothesis is supported both by data reported here as well as the indole sensitivity recently reported for CxOR2 (Pelletier et al. 2010), which overlaps with that of Aa/AgOR2. Aa/AgOR10 activation profiles while slightly different from Aa/AgOR2 maintained an ability to detect indole albeit with lower affinities. Finally, AaOR9 and AaOR10 paralogs responded similarly to indole suggesting that both ORs have redundant biochemical function, perhaps within different developmental contexts (Bohbot et al. 2007). Alternatively, we cannot rule out that the cognate ligands for these 2 receptors may be structurally similar to indole (Hughes et al. 2010). Lastly, we also acknowledge odorant-binding proteins and other cofactors that are highly expressed in olfactory sensilla but are not present in heterologous assay systems may be important modulators of chemosensory sensitivity in vivo (Biessmann et al. 2010; Pelletier et al. 2010).

The functional conservation of indole sensitivity within the Aa/AgOR2/OR10 clade suggests that this response is an ancient trait that was present prior to the Anophelinae/Culicinae split (Krzywinski et al. 2001b). Moreover, the preservation of indole sensitivity within a narrow group of *Or* genes that make up the most highly conserved Aa/Ag/CxORs indicates this is important in the life cycles of mosquitoes. Indeed, indole is a ubiquitous volatile compound that has been linked to host seeking and oviposition in both aedine (Millar et al. 1992; Du and Millar 1999; Hill et al. 2009; Syed and Leal 2009; Siju et al. 2010) and anopheline mosquitoes (Blackwell and Johnson 2000; Meijerink et al. 2000; Meijerink et al. 2001; Takken et al. 2001; Qiu et al. 2006; Lindh et al. 2008).

The original ecological context of this olfactory trait such as detection of oviposition sites, hosts, nectar sources, or other elements of the mosquito life cycle is unknown. Identification and functional characterization of OR2/OR10 homologs in nonblood feeding mosquitoes such as members of the Toxorhynchitinae subfamily that are attracted to water sites containing both phenols and indoles (Collins and Blackwell 2002) may provide key evidence for the ancestral function of indole reception in adult mosquitoes. Such experiments would support the hypothesis that indole reception facilitates mosquito orientation toward key ecological resources using an ancient olfactory mechanism. More studies will be needed to understand how indole detection intersects with other sensory modalities to inform mosquitoes about different environmental contexts.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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