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Sequence similarity and functional comparisons of pheromone receptor orthologs in two closely related *Helicoverpa* species

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ABSTRACT

The olfactory system of moth species in subfamily Heliothinae is an attractive model to study the evolution of the pheromone reception because they show distinct differentiation in sex pheromone components or ratios that activate pheromone receptors (PRs). However, functional assessment of PRs in closely related species remains largely untried. Here we present a special cloning strategy to isolate full-length cDNAs encoding candidate odorant receptors (ORs) from *Helicoverpa armigera* (Harm) and *Helicoverpa assulta* (Hass) on the basis of *Heliothis virescens* ORs, and investigate the functional properties of PRs to determine how the evolution of moth PRs contribute to intraspecific mating choice and speciation extension. We cloned 11 OR orthologs from *H. armigera* and 10 from *H. assulta*. We functionally characterized the responses of PRs of both species to seven pheromone compounds using the heterologous expression system of *Xenopus* ooctyes. HassOR13 was found to be highly tuned to the sex pheromone component Z11-16:Ald, and unexpectedly, both HarmOR14b and HassOR16 were specific for Z9-14:Ald. However, HarmOR6 and HassOR6 showed much higher specificity to Z9-16:OH than to Z9-16:Ald or Z9-14:Ald. HarmOR11, HarmOR14a, HassOR11 and HassOR14b failed to respond to the tested chemicals. Based on our results and previous research, we can show that some PR orthologs from *H. armigera*, *H. assulta* and *H. virescens* such as OR13s have similar ligand selectivity, but others have different ligand specificity. The combined PR function and sex pheromone component analysis suggests that the evolution of PRs can meet species-specific demands.

1. Introduction

Insects have evolved an acute olfactory system for survival and reproduction, including food source identification, oviposition site selection, mate choice, kin recognition and predator avoidance. The entire olfactory system relies to a great extent on the types of receptors expressed on peripheral olfactory sensory neurons (OSNs) (Leal, 2013). The odorant receptor (OR) expressed in an OSN determines the sensitivity and specificity of the ORN, which in turn governs natural and learned olfactory behaviors, such as attraction to food and pheromones and avoidance of repellents (Hallem and Carlson, 2006; Semmelhack and Wang, 2009).

Insect ORs form an enormous and highly divergent gene family, with no close orthologs (aside from Orco) or apparent subfamily structure conserved across insect orders (Hasson and Stensmyr, 2011). Therefore, genome sequencing, expressed sequence tag (EST) sequencing and transcriptome sequencing have been largely used to identify the complete OR repertoires in some insect species such as moths, honey bees, mosquitoes, ants, and beetles. In Lepidoptera, the OR genes that have been isolated by these methods include 66 in *Bombyx mori*, 21 in *Heliothis virescens*, 43 in *Cydia pomonella*, 66 in *Manduca sexta*, 41 in *Spodoptera littoralis*, and 47 in *Helicoverpa armigera* (Tanaka et al., 2009; Krieger et al., 2002, 2004; Bengtsson et al., 2012; Grosse-Wilde et al., 2011; Howlett et al., 2012; Legeai et al., 2011; Jacquin-Joly et al., 2012; Liu et al., 2012). Although these species belong to the same order, most ORs are highly divergent and share low similarity with each other except for closely related species such as *H. virescens* and *H. armigera*. However, their pheromone receptors (PRs) are a little more conserved and show relatively high sequence identity. Traditional homology-based cloning strategy using degenerate primers has

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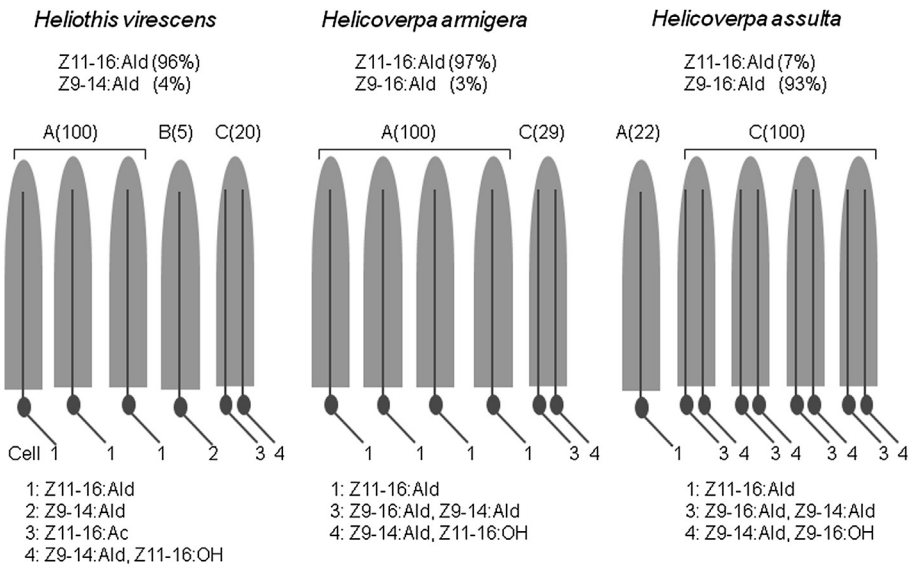


Fig. 1. Schematic comparision of the sensillar arrangements of the types A, B, and C trichoid sensilla and their compartmental ORNs in male antennae of *Heliothis virescens*, *Helicoverpa armigera*, and *Helicoverpa assulta*. Just beneath the name of three species are the principal sex pheromone components and their ratios (Vetter and Baker, 1983; Wang et al., 2005). The number in parentheses of A, B and C is the proportion of each type of sensilla based on Baker et al. (2004) and Wu et al. (2013). The indicated compounds to which various OSNs respond are from Baker et al. (2004), Berg et al. (2005) and our unpublished data.

successfully identified some PR genes in *Plutella xylostella*, *Mythimna separata*, *Diaphania indica*, *H. armigera*, *Helicoverpa assulta*, *Spodoptera exigua* and *Ostrinia* moths (Mitsuno et al., 2008; Miura et al., 2009, 2010; Zhang et al., 2010; Liu et al., 2013a,b). Most PR genes show male-biased expression patterns and are expressed at higher levels in the antennae of male moths. Since Sakurai T et al. discovered the first sex pheromone receptor BmOR1 from *B. mori* in 2004 (Sakurai et al., 2004), the PRs in a few moth species have been identified and characterised using heterologous expression. For example, experiments with HvOR13, HvOR14 and HvOR16 expressed in a modified HEK293 cell line (not co-expressing Orco) and *Xenopus* oocytes (co-expressing Orco) indicate that these ORs are tuned to Z11-16:Ald, Z11-16:OAc and Z11-16:OH, respectively, which are the pheromone components in *H. virescens* (Grosse-Wilde et al., 2007; Wang et al., 2010). Other functionally characterized PRs of moths include BmOR3 responding to bombykal in *B. mori* (Nakagawa et al., 2005), PxOR1 responding to Z11-16:Ald in *P. xylostella* (Mitsuno et al., 2008), MsOR1 responding to Z11-16:OAc in *M. separate* (Mitsuno et al., 2008), DiOR1 responding to E11-16:Ald in *Diaphania indica* (Mitsuno et al., 2008), OlatOR1

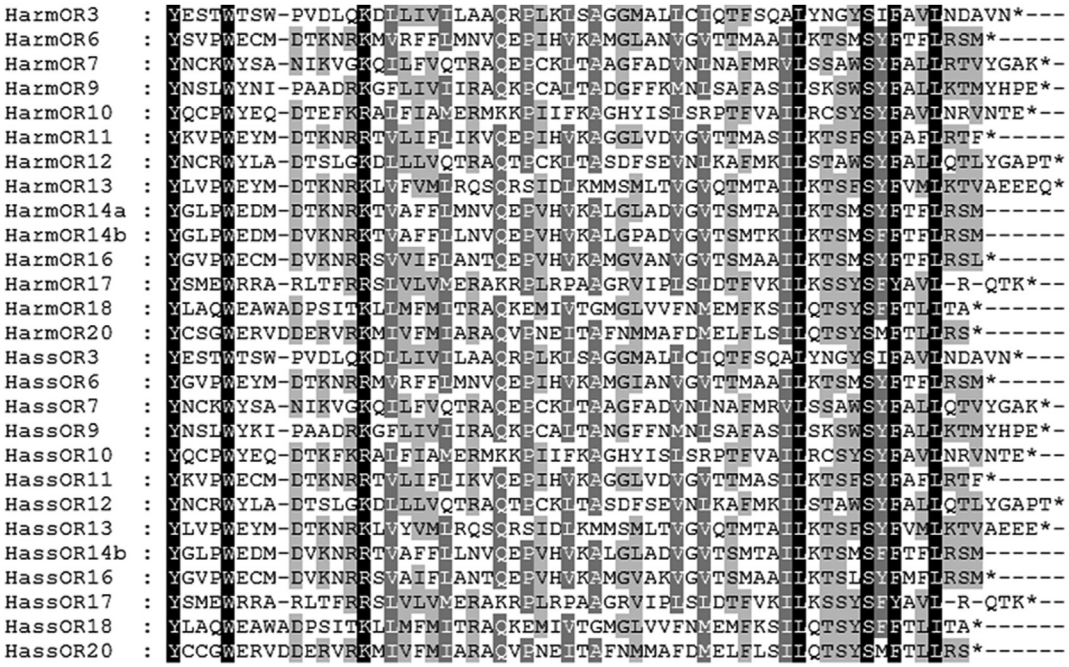


Fig. 2. A conserved amino acids motif within the C-terminal region of HarmORs and HassORs. Amino acids identical in all sequences are shown in white typing on a black background. Residues shared among most of OR sequences are marked with gray.

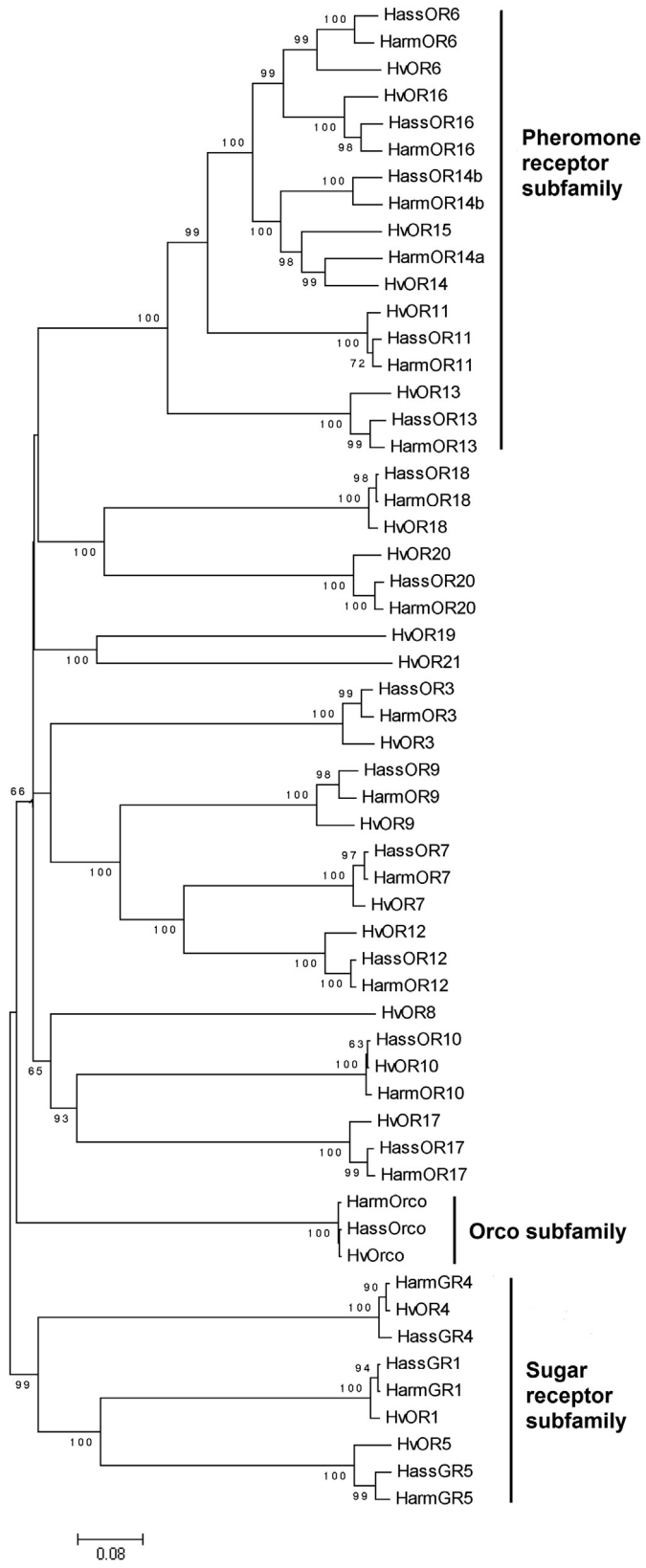


Fig. 3. Neighbor-joining tree of *Helicoverpa armigera*, *Helicoverpa assulta* and *Heliothis virescens* ORs identified to date. The distance tree is calculated by using the MEGA program and is based on a CLUSTAL alignment of 56 chemosensory receptors. The clusters of sugar receptors, Orco and pheromone receptor lineages are indicated with bars on the right.

responding to E11-14:OH in *Ostrinia latipennis* (Miura et al., 2009), OscaOR4 responding to E11-14:OAc in *Ostrinia scapulalis* (Miura et al., 2010), OnOR1 and OnOR6 in *Ostrinia nubilalis* responding to E12-14:OAc and Z11-14:OAc, respectively (Wanner et al., 2010), and SlitOR6 responding to Z9,E12-14:OAc in *S. littoralis* (Montagné et al., 2012).

The two closely related species, *H. armigera* and *H. assulta*, are sympatric pest species in and around China. They use (Z)-11-hexadecenal (Z11-16:Ald) and (Z)-9-hexadecenal (Z9-16:Ald) as their sex pheromone components but in almost reversed ratios (Wang et al., 2005). (Z)-9-tetradecenal (Z9-14:Ald) is reported as a behavioral agonist at 0.25% and behavioral antagonist at 5% in the sex pheromone blend of *H. armigera* (Zhang et al., 2012; Kehat and Dunkelblum, 1990). Peripheral olfactory sensory neurons housed in the trichoid sensilla of male moths are used to detect sex pheromones. In *Heliothis*/*Helicoverpa* species, there are three basic types of pheromone-responsive sensilla with different populations in male antennae, called A, B, and C types (Fig. 1). *H. virescens* has all three, the most abundant A type housing a neuron responding to the principal component, Z11-16:Ald, the B type containing a neuron responding to the secondary component, Z9-14:Ald, and the two neurons in the C type respond to Z9-14:Ald, Z9-16:Ald, and some behavioral antagonists; while *H. armigera* and *H. assulta* have the A and C types of sensilla but their populations are just reverse in the two species (Berg and Mustaparta, 1995; Berg et al., 2005; Wu et al., 2013). In each sensillum of *Heliothis*/*Helicoverpa* species usually there are two colocalized neurons mediating conspecific and interspecific signal information. Ligands stimulating the neuron colocalized with that tuning to Z11-16:Ald have not yet been identified. In *H. armigera* the neuron specific for Z9-16:Ald/Z9-14:Ald is colocalized with the neuron specific for the behavioral antagonists (Z11-16:Ac/Z11-16:OH/Z9-14:Ald) (data not published). Similarly in *Helicoverpa zea* and in *H. assulta* the neuron specific for Z9-16:Ald is colocalized with the neuron specific for the behavioral antagonists (Z9-14:Ald/Z9-16:OH) (Berg and Mustaparta, 1995; Berg et al., 2005). Responses to the pheromones are transmitted from OSNs to a major sub-region of male antennal lobe called the macroglomerular complex (MGC). In each of the two species, the major pheromone component activates the cumulus (main glomerulus of the MGC). In *H. armigera*, the OSNs tuned to the secondary pheromone component, Z9-16:Ald, project to the dorsomedial glomerulus (a smaller subunit of MGC) (Wu et al., 2013). In *H. assulta*, the OSNs responding to the secondary pheromone component, Z11-16:Ald, and the behavioral antagonists, Z9-14:Ald and Z9-16:OH, target the ventral and the dorsomedial glomerulus (Berg et al., 2005; Lee et al., 2006; Wu et al., 2013). However, the molecular basis of sex pheromone reception and signal transduction is still unclear in moths and in particular in the two *Helicoverpa* species. As a first step to understand the molecular mechanisms that enable male moths to respond specifically to the pheromone blend produced by their conspecific females, it is essential to identify and compare functional characterization of the ORs responsive to these pheromone components in *H. armigera* and *H. assulta*.

In this work, we have undertaken a special cloning strategy to isolate full-length cDNAs encoding candidate chemoreceptors from *H. armigera* and *H. assulta*. Among the present and previous cloned genes, we identified pheromone receptors (PRs) by using *Xenopus* oocytes and two-electrode voltage-clamp recording, and studied their expression by using qRT-PCR and *in situ* hybridization. Furthermore, to determine if sequence similarity among presumptive orthologs, proteins showing significant conservation in sequence and structure, can predict the function of PRs in closely related species, we conducted a functional

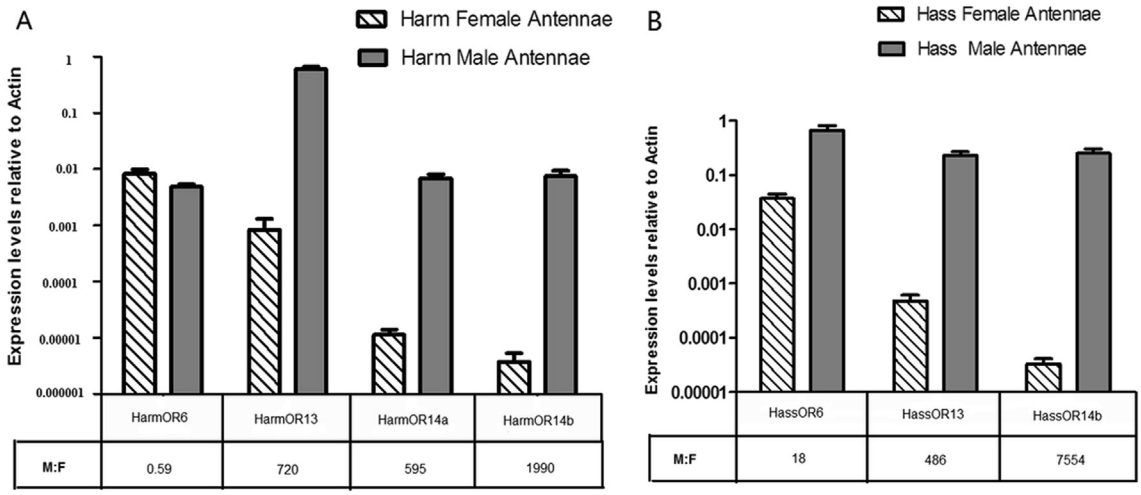


Fig. 4. *Helicoverpa armigera* and *Helicoverpa assulta* OR gene expression levels in male and female moth antennae. Gene expression, determined by real-time quantitative PCR with SYBR green, is reported relative to the reference gene Actin on a logarithmic scale. Expression values are presented as averages (with standard error bars) of three biological replicates and three technical replicates. Ratios of male to female expression (M:F) or *H. armigera* to *H. assulta* are presented below each bar. (A) Comparison of OR expression between male and female antennae of *H. armigera*. (B) Comparison of OR expression between male and female antennae of *H. assulta*.

comparison of PR orthologs from *H. armigera*, *H. assulta* and *H. virescens*.

2. Materials and methods

2.1. Insects

H. armigera and *H. assulta* were reared at the Institute of Zoology, Chinese Academy of Sciences, Beijing. The larvae were fed with an artificial diet, mainly constituted by wheat germ, yeast and tomato paste for *H. armigera*, wheat germ, yeast and chilli for *H. assulta*. Rearing took place at a temperature of 27 ± 1 °C with a photoperiod of 16 h:8 h, L:D. Pupae were sexed and males and females were put into separate cages for eclosion. 10% honey solution was used as diet for adults. One-to-three-day-old virgin adults were used in all the experiments.

2.2. Cloning of candidate odorant receptors of H. armigera and H. assulta

Based on the nucleotide sequences of ORs in *H. virescens*, specific primers were designed to amplify orthologous cDNAs in *H. armigera* and *H. assulta*. Full-length sequences were obtained through 5′ and 3′ RACE PCR. Nested PCR was used to increase the specificity and sensitivity of RACE products. All primers were designed with Primer Premier 5.0 software, UPM and NUP were used as universal primer and nested universal primer, respectively. The primer sequences are reported in Supplementary Table 1.

Total RNA was isolated from antennae collected from 30 female and 30 male moths for each species, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacter's instructions. RACE first-strand cDNA was synthesized with SMARTScribeTM reverse transcriptase (Clontech, Mountain View, CA, USA). The 5′-RACE cDNA reaction vessel contained 1–2 μg of total RNA, 1.2 μM of the 5′ oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′), 1.2 μM of oligo-dT18 primer and 1 μl of reverse transcriptase in a final volume of 10 μL. The 3′-RACE cDNA was synthesized using 1.2 μM of oligodT-adaptor primer (5′-AAGCAGTGGTATCAACGCAGAGTAC $(T)_{30}$VN). The following cycling parameters were used: 42 °C for 60 min to produce cDNA and 70 °C for 15 min to terminate the reaction. 5′-RACE PCR reactions were performed in 2 × GC Buffer and LATaq polymerase (Takara shuzo, Shiga, Japan). The downstream regions to the 3′-end of most genes were amplified by LATaq polymerase and 10× LA PCR Buffer (Takara shuzo). The PCR programme included initial denaturation at 94 °C for 2 min, followed by 5 cycles of 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C; and a final extension step of 8 min at 72 °C.

2.3. Phylogenetic analysis

Phylogenetic analysis of ORs and GRs was performed based on the amino acid sequences. The data contained 17 full-length receptor sequences from *H. assulta,* 18 from *H. armigera*, and 21 from *H. virescens*. GenBank accession numbers of all the receptor sequences are reported in Supplementary Table 1. Protein sequences were aligned using ClustalX. The phylogeny tree was constructed by the neighbor-joining method using the software MEGA 5. Node support was assessed using a bootstrap procedure based on 1000 replicates.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from 30 pairs of adult male antennae and 30 pairs of adult female antennae was isolated and purified using RNAprep Pure Tissue Kit (Tiangen, Beijing, China). RNA quality and quantity were determined by absorbance at 260 nm, 280 nm, and 230 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized from the total RNA with PrimeScript® RT reagent Kit (TaKaRa shuzo) where genomic DNA was removed by gDNA Eraser. qRT-PCR was performed with LightCycler480 and 96 well plates (Roche, Branford, CT, USA). All reactions were performed in triplicate in a total volume of 20 μL containing 10 μL SYBR Premix Ex TaqII (TaKaRa shuzo) and 0.4 μM of each primer under the following conditions: 95 °C for 30 s followed by 45 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 15 s. The melting curve analysis was performed to ensure the specificity of primers. The threshold cycle (C_T) was plotted against the log of the template dilution and primers with slopes ranging from 3.1 to 3.5 were used (a slope of 3.3 represents 100% efficiency). Expression levels of HassOR6, HassOR13 and HassOR14b were calculated relative to the control gene, actin,

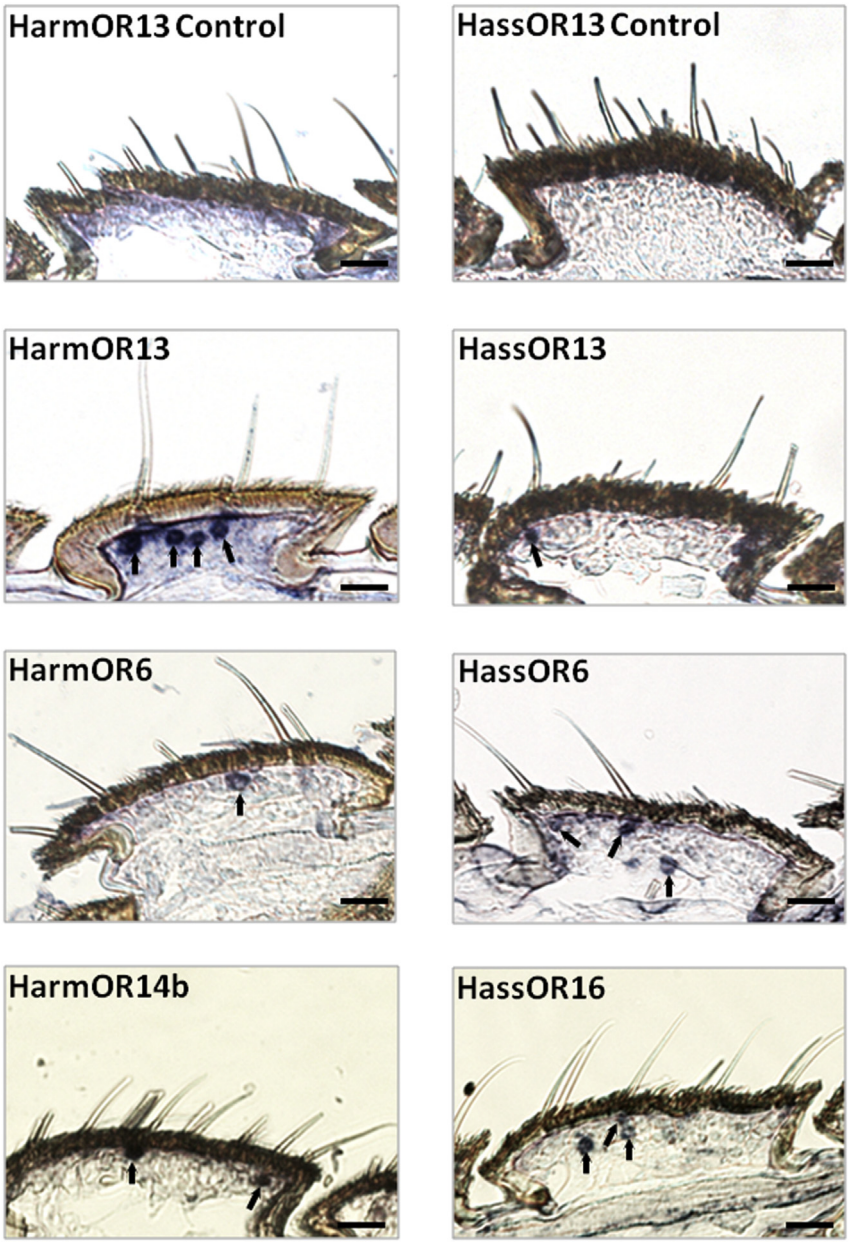


Fig. 5. Topographical expression of pheromone receptors in the antennae of male *H. armigera* and *H. assulta*. *In situ* hybridization was performed on the longitudinal tissue sections using DIG-labeled antisense probes. Top panel: no cells were labeled using sense OR13 probes. Middle two panels: localization of OR13 and OR6 in male antennae of *H. armigera* and *H. assulta* using anti-sense probes. Lower panel: labeling pattern of OR14b in male antennae of *H. armigera* and OR16 in male antennae of *H. assulta*. Bold arrows point to the cell bodies stained by anti-sense riboprobes. Scale bar represents 20 μm.

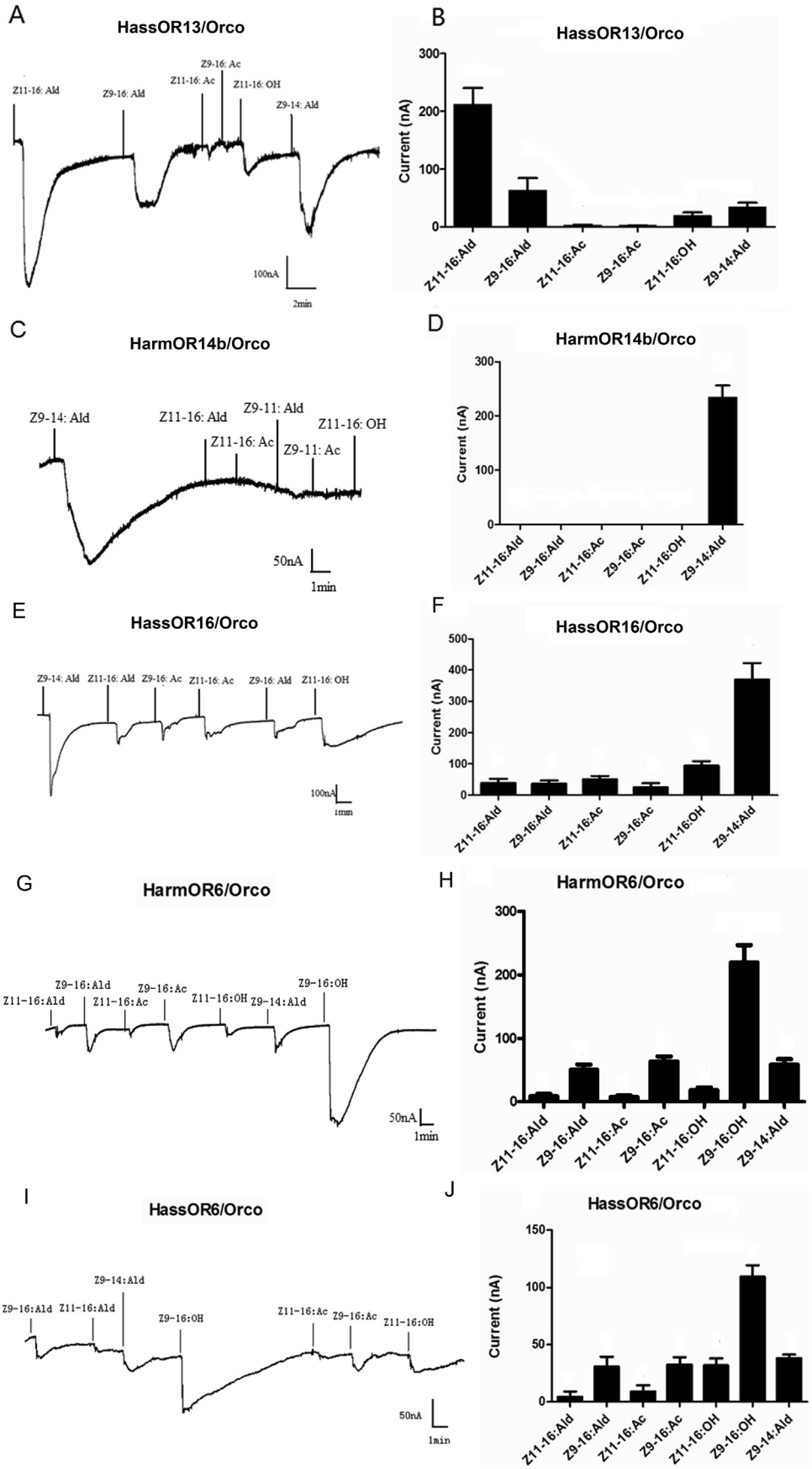
using the $2^{-\Delta CT}$ method. The experiments were repeated three times using three independent RNA samples, and three technical replicates were run.

2.5. Pheromone components and related chemicals

(Z)-11-hexadecenal (Z11-16:Ald), (Z)-9-hexadecenal (Z9-16:Ald), (Z)-9-tetradecenal (Z9-14:Ald), (Z)-11-hexadecenol (Z11-16:OH), (Z)-9-hexadecenal (Z9-16:OH), (Z)-11-hexadecenyl acetate (Z11-16:Ac), and (Z)-9-hexadecenyl acetate (Z9-16:Ac) were purchased from Shin-Etsu Chemical (Tokyo, Japan). Stock solutions (200 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Before the experiments, stock solutions were diluted in ND96 Buffer (96 mM NaCl/2 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/5 mM HEPES pH 7.5)0.1 × ND96 Buffer containing 0.1% DMSO was used as a negative control.

2.6. Protein expression in Xenopus laevis oocytes and electrophysiological recordings

Full-length coding sequences of HarmOR and HassOR cDNAs amplified from antenna by RT-PCR were first cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and then subcloned into pCS2+ vector. cRNAs were synthesized from linearized modified pCS2+ vectors with mMESSAGE mMACHINE SP6 (Ambion, Austin, TX, USA). Mature healthy oocytes were treated with 2 mg/mL of collagenase typeI(Sigma–Aldrich) in Ca^{2+}-free saline solution (82.5 mM NaCl/2 mM KCl/1 mM $MgCl_2$/5 mM


A
HassOR13/Orco
Z11-16: Ald
Z9-16: Ald
Z9-16: Ac
Z11-16: Ac
Z11-16: OH
Z9-14: Ald
100nA
2min
B
HassOR13/Orco
Current (nA)
300
200
100
0
Z11-16:Ald
Z9-16:Ald
Z11-16:Ac
Z9-16:Ac
Z11-16:OH
Z9-14:Ald
C
HarmOR14b/Orco
Z9-14: Ald
Z11-16: Ald
Z9-11: Ald
Z11-16: Ac
Z9-11: Ac
Z11-16: OH
50nA
1min
D
HarmOR14b/Orco
Current (nA)
300
200
100
0
Z11-16:Ald
Z9-16:Ald
Z11-16:Ac
Z9-16:Ac
Z11-16:OH
Z9-14:Ald
E
HassOR16/Orco
Z9-14: Ald
Z11-16: Ald
Z9-16: Ac
Z11-16: Ac
Z9-16: Ald
Z11-16: OH
100nA
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HassOR16/Orco
Current (nA)
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200
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Z11-16:Ald
Z9-16:Ald
Z11-16:Ac
Z9-16:Ac
Z11-16:OH
Z9-14:Ald
G
HarmOR6/Orco
Z11-16:Ald
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Z11-16:OH
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HassOR6/Orco
Current (nA)
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Z11-16:Ald
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Z11-16:Ac
Z9-16:Ac
Z11-16:OH
Z9-16:OH
Z9-14:Ald

HEPES, pH 7.5) for 1–2 h at room temperature. Oocytes were later microinjected with 25 ng OR cRNA and 25 ng Orco cRNA. Injected oocytes were incubated for 3–7 days at 18 °C in bath solution (96 mM NaCl/2 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/5 mM HEPES, pH 7.5) supplemented with 100 μg/mL gentamycin and 550 μg/mL sodium pyruvate. Whole-cell currents were recorded with a two-electrode voltage clamp. Intracellular glass electrodes were filled with 3 M KCl and had resistances of 0.2–2.0 MΩ. Signals were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT, USA) at a holding potential of −80 mV, low-pass filtered at 50 Hz and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1322A and pCLAMP software (Axon Instruments Inc., Foster City, CA, USA). Dose-response data were analyzed using GraphPad Prism 6.

2.7. In situ hybridization

The experimental procedures were adopted from Krieger et al., 2002 with minor modifications. Briefly, the sequence for probe synthesis spanned variable length (HarmOR13 and HassOR13: 1220 nucleotides; HarmOR6 and HassOR6: 678 nucleotides; HarmOR14b: 836 nucleotides; HassOR16: 668 nucleotides) at 5′-end of the pheromone receptors. Digoxigenin (Dig)-labeled probes of were synthesized with DIG RNA labeling Kit version 12 (SP6/T7) (Roche, Mannheim, Germany). The fresh antennae, which were dissected from virgin male adults, were imbedded in JUNG tissue freezing medium (Leica Microsystems, Germany). Sections of 12 μm in the 30–60 annuli of antennal flagella from the proximal were prepared with Leica CM 1950 at −22 °C, and then, mounted onto the Superfrost Plus Glass Slides (Electron Microscope Science, Hatfield, USA). Afterward, slides were dried in the air fixed by 4% paraformaldehyde treated with phosphate buffer saline (PBS) and 0.2 M HCl and at last, washed with PBS twice for 30 s. After the rinse in 50% formamide deionized (MP Biomedicals, Solon, OH, USA)/5 × SSC for 15 min, each slide was given 100 μl hybridization buffer (Boster, Wuhan, China) containing DIG-labeled probes. The slides were incubated at 55 °C. Subsequently, the slides were washed twice against 0.1 × SSC for 30 min at 60 °C, rinsed shortly in TBS buffer, and incubated in 1% blocking reagent (Roche, Mannheim, Germany) in TBS plus 0.03% Triton-X100 (Merck, Darmstadt, Germany) for 30 min at room temperature. Then, each slide was incubated with 100 μl anti-dioxigenin alkaline phosphatase conjugated antibody (Roche, Mannheim, Germany) diluted 1:500 in1% blocking reagent in TBS plus 0.03 Triton-X100 at 37 °C for 1 h. After three washes for 5 min against TBS plus 0.05% Tween-20 and shortly rinsed in DAP buffer, the signals were visualized by nitro-blue tetrazolium (NBT)/5-brom-4-chlor-3-indolyl phosphate (BCIP) (Promega, Madison, WI, USA). Finally, pictures were taken by Leica DM 2500 microscope and adjusted contrast and brightness only by Adobe Photoshop CS3.

3. Results and discussion

3.1. Candidate odorant receptor isolation

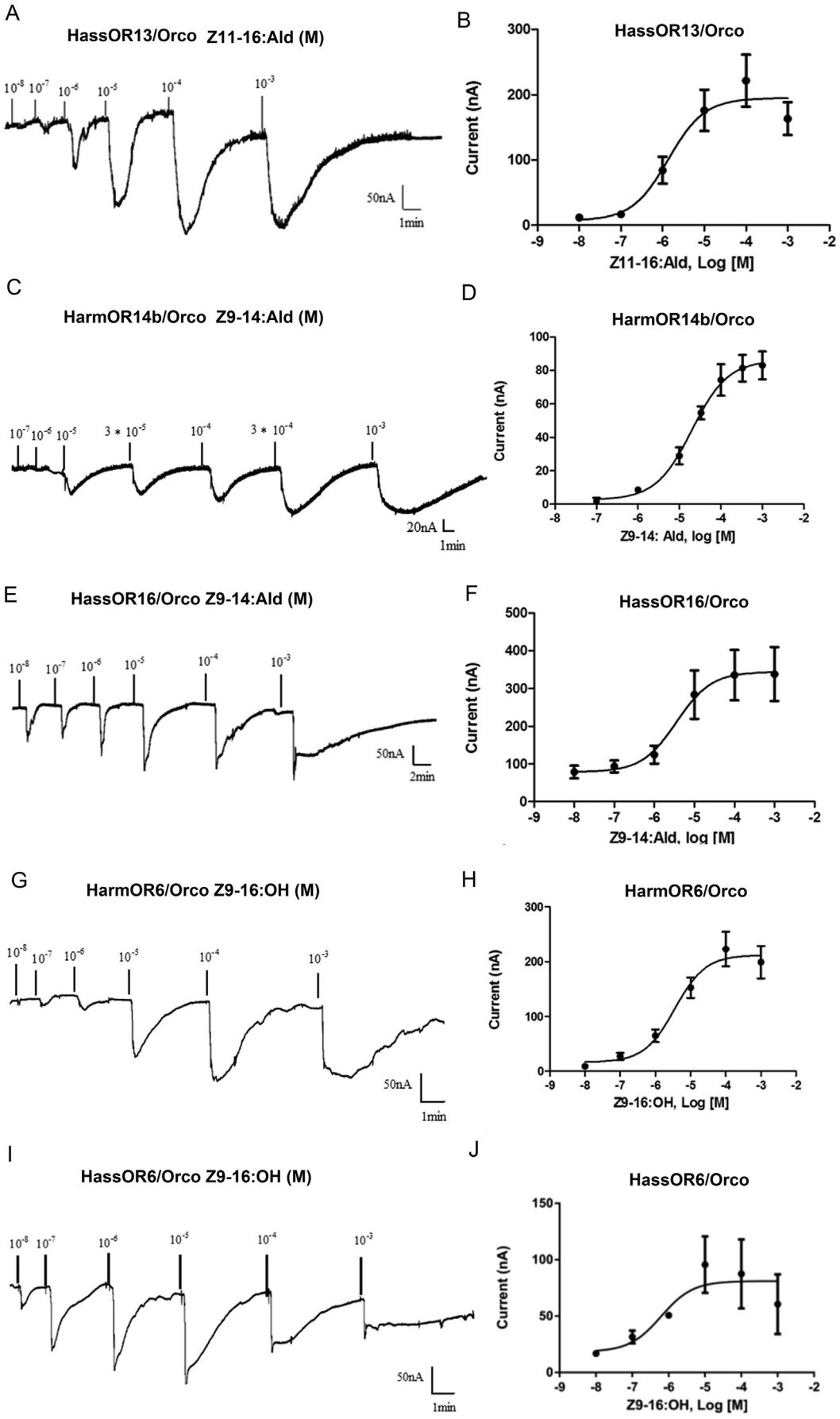
A special approach was used to identify the greatest possible number of candidate chemosensory receptors in the absence of whole genome sequencing, EST sequencing and transcriptome sequencing. Sequence comparisons of the published orthologous odorant receptors from *H. armigera, H. assulta* and *H. virescens* revealed that not only the alignment of amino acid sequences but also nucleotide sequences had very high similarity. Based on the assumption that OR orthologs from closely related species might share high nucleotide sequence identity with each other, full-length sequences of 11 and 10 putative olfactory receptors in the moths *H. armigera* and *H. assulta* were identified by RACE PCR using the specific primers designed on the corresponding ORs of *H. virescens*, where there were 21 chemosensory receptors including 17 ORs, 3 GRs and ORco (Krieger et al., 2004; Bengtsson et al., 2012). We named these genes as HarmOR3, HarmOR6, HarmOR7, HarmOR9, HarmOR10, HarmOR12, HarmOR14a, HarmOR14b, HarmOR17, HarmOR18, HarmOR20 in *H. armigera* (GenBank Accession numbers JX982524–JX982534) and HassOR3, HassOR6, HassOR7, HassOR9, HassOR10, HassOR12, HassOR14b, HassOR17, HassOR18, HassOR20 in *H. assulta* (GenBank Accession numbers JX982538–JX982547), using the same number as in their *H. virescens* orthologs. OR13, OR11 and OR16 of both Harm and Hass (GenBank Accession numbers ACS45304-ACS45309) were previously reported as OR1, OR2, and OR3, respectively (Zhang et al., 2010).

3′RACE PCR only succeed for OR8 and OR21. However, full-length cDNAs of OR15 and OR19 have been recently obtained in *H. armigera* by assembling the adult male and female antennal transcriptomes (Liu et al., 2013a,b). Two presumptive homologs of HvOR14 were identified in *H. armigera* and named as HarmOR14a and HarmOR14b. HarmOR14a recently described in Liu et al. was named HarmOR14 (Liu et al., 2012, 2013). HarmOR14b was not previously identified in *H. armigera* transcriptome and could be from a new gene. In *H. assulta* only HassOR14b was cloned. HarmOR14b and HassOR14b were named as OR14b because of their relatively low similarity to HvOR14. 47 ORs were identified in *H. armigera* by transcriptomic sequencing (Liu et al., 2012). Most of other 26 OR orthologs except 21 reported genes should be easily cloned not only in *H. assulta* but also in the closely related genus *Heliothis* by this method.

The deduced amino acid sequences of HarmORs and HassORs showed high degrees of identity (67–98%) to their orthologs of *H. virescens*. Higher degrees of identity (90–99%) were shared between the orthologs from the two sibling species *H. armigera* and *H. assulta*. Within the same species, OR sequences can be highly divergent with amino acid identities between 4% and 72%, and can be segregated into two groups. The first group contains OR7 and OR12, sharing 49% of their amino acids, and the second includes six receptors (OR6, OR11, OR13, OR14a, OR14b and OR16), exhibiting at least 40% of amino acids identity between pairs. The second group was considered as the pheromone receptor subfamily. Alignment of amino acid sequences showed that these ORs share a conserved amino acids motif (Y-X3-W-X8-K/R-X31-L-X4-S-X-F-X2-L/I) within the C-terminal region (Fig. 2).

All identified chemosensory receptors of *H. armigera* and *H. assulta* were used to construct a phylogenic tree with the known ORs of *H. virescens* (Fig. 3). HvOrco, HarmOrco and HassOrco grouped together in a single lineage named Orco subfamily. OR6 , OR11 , OR13 , OR14 , OR15 and OR16 from the three species were clustered in the pheromone receptor subfamily, which included the functionally identified PRs in *H. virescens*. These ORs in the sibling species were considered as the putative pheromone receptors for further analysis. In addition, several GRs of the three species were members of the insect sugar receptor subfamily based on functional characterization of *B. mori* sugar receptors (Sato et al., 2011; Wanner and Robertson, 2008).

Fig. 6. Responses of *Xenopus* oocytes with co-expressed HassOR13/Orco (A, B), HarmOR14b/Orco (C, D), HassOR16/Orco (E, F), HarmOR6/Orco (G, H), and HassOR6/Orco (I, J) to stimulation with pheromone compounds and analogs. (A, C, E, G, I) Inward current responses of *Xenopus* oocytes expressing the PR/Orco in response to 10^{-4} M solution of six or seven chemicals. (B, D, F, H, J) Response profile of *Xenopus* oocytes expressing the PR/Orco. Error bars indicates SEM (HassOR13/Orco, $n = 9$; HarmOR14b/Orco, $n = 5$; HassOR16/Orco, $n = 7$; HarmOR6/Orco, $n = 10$; HassOR6/Orco, $n = 4$).


A
HassOR13/Orco Z11-16:Ald (M)
10-8 10-7 10-6 10-5 10-4 10-3
50nA
1min
B
HassOR13/Orco
Current (nA)
Z11-16:Ald, Log [M]
C
HarmOR14b/Orco Z9-14:Ald (M)
10-7 10-6 10-5 3 * 10-5 10-4 3 * 10-4 10-3
20nA
1min
D
HarmOR14b/Orco
Current (nA)
Z9-14: Ald, log [M]
E
HassOR16/Orco Z9-14:Ald (M)
10-8 10-7 10-6 10-5 10-4 10-3
50nA
2min
F
HassOR16/Orco
Current (nA)
Z9-14:Ald, log [M]
G
HarmOR6/Orco Z9-16:OH (M)
10-8 10-7 10-6 10-5 10-4 10-3
50nA
1min
H
HarmOR6/Orco
Current (nA)
Z9-16:OH, Log [M]
I
HassOR6/Orco Z9-16:OH (M)
10-8 10-7 10-6 10-5 10-4 10-3
50nA
1min
J
HassOR6/Orco
Current (nA)
Z9-16:OH, Log [M]

3.2. Differential expression of candidate pheromone receptor genes

Quantitative real-time PCR (qRT-PCR) was used to measure expression levels of genes encoding the odorant receptors HarmOR6, HarmOR14a, HarmOR14b, HassOR6, and HassOR14b. OR gene expression in male and female adult antennae was normalized to the internal control gene actin using the equation $2^{-\Delta CT}$ (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). As a control we measured expression levels of HarmOR13 and HassOR13 which exhibited a male-biased expression patterns in adult antennae (Zhang et al., 2010; Liu et al., 2013a,b). As predicted by the sexual dimorphism of male moth olfactory systems, we found that OR13 and OR14 were expressed at much higher levels in male antennae than in female antennae in both species (Fig. 4A, B). However, OR6 was strongly expressed at comparable levels in male and female antennae, and HarmOR6 was expressed at a little higher level in *H. armigera* female antennae than in male antenna (Fig. 4A). HarmOR6 reported in Liu et al. was weakly expressed in male antennae because the primers for qRT-PCR expression analysis were near the 5′end of ORF (Liu et al., 2013a,b). In the present study, we used the primers for qRT-PCR expression analysis of HarmOR6 and HassOR6 near the 3′end of ORF. It suggested that mRNAs of HarmOR6 and HassOR6 were not stable. All candidate pheromone receptor genes showed significant expression in adult male antenna of both species (Fig. 4A, B).

To verify the expression of pheromone receptors in the antennae, *in situ* hybridization was performed to check the topological expression pattern of variable PRs in the male antennae of *H. armigera* and *H. assulta* (Fig. 5). The cell bodies labeled by (DIG)-labeled antisense probes were detected at the base of sensilla trichodea. There was no labeling obtained when sense probes of OR13 were applied (Fig. 5), and the same with sense probes of other PRs (data not shown). Four cell bodies were labeled by OR13 antisense probes in a segment, while only one cell body was labeled in *H. assulta*. The cells expressing HarmOR13 were the most abundant among all of the tested PRs. Conversely, the cells reactive to the OR6 anti-sense probes in *H. armigera* were much less than those in *H. assulta*. The anti-sense probes of HarmOR14b and HassOR16 both labeled 2 or 3 cells per antennal segment. These results were well compatible with the qRT-PCR expression results.

3.3. Functional assay of HarmORs and HassORs

Recently, it has been reported that HarmOR13 and HarmOR16 in *H. armigera* are respectively tuned to Z11-16:Ald and Z11-16:OH (Liu et al., 2013a,b). According to the neighbor-joining tree of ORs and the result of real-time PCR, we hypothesized that HarmOR6, HarmOR11, HarmOR13, HarmOR14a, HarmOR14b, HarmOR16 in *H. armigera*, and HassOR6, HassOR11, HassOR13, HassOR14b, HassOR16 in *H. assulta* could be pheromone receptors. To check if the other ORs were the pheromone receptors, we examined electrophysiological responses of *Xenopus* oocytes co-expressing ORs and Orco to the following seven sex-pheromone constituents in the two species: Z11-16:Ald and Z9-16:Ald, the behavioral antagonists: Z11-16:Ac and Z11-16:OH for *H. armigera*, Z9-16:OH and Z9-14:Ald for *H. assulta*, Z9-16:Ac. We confirmed that adult male antennae of both species respond to these 7 compounds using electroantennography and single-sensillum recordings (data not published).

HassOR13/Orco cells responded robustly to Z11-16:Ald and to a much lesser extent to Z9-16:Ald, Z9-14:Ald and Z11-16:OH at a concentration of 10^{-4} M (Fig. 6A, B). Z11-16:Ald is the second component of the *H. assulta* sex pheromone. The Z11-16:Ald-induced current increased from the lowest threshold concentration of 10^{-8} M to 10^{-4} M in a dose-dependent manner with an EC_{50} value of 1.36×10^{-6} M (Fig. 7A, B). The observed EC_{50} value was close to that measured for Z11-16:Ald and its receptor HarmOR13 in *H. armigera* (1.82×10^{-6} M) (32).

Both HarmOR14b and HassOR16 were tuned to Z9-14:Ald. HarmOR14b cells showed a specific response to 10^{-4} M Z9-14:Ald and no response to the other chemicals (Fig. 6C, D). In dose–response studies, the lowest concentration of Z9-14:Ald, 10^{-6} M, elicited measurable responses from oocytes with co-expressed HarmOR14b/Orco, with an EC_{50} value of 2.00×10^{-5} M (Fig. 7C, D). The cells expressing HassOR16/Orco responded best to 10^{-4} M Z9-14:Ald, but also responded to the other six components (Fig. 6E, F). The threshold for Z9-14:Ald was 10^{-8} M and the EC50 value was 3.59×10^{-6} M (Fig. 7E, F).

HarmOR6/Orco and HassOR6/Orco cells strongly responded to Z9-16:OH at a concentration of 10^{-4} M while displaying much smaller responses to Z9-16:Ald, Z9-14:Ald, Z9-16:Ac (Fig. 6G–J). In dose–response analyses, we measured thresholds as low as 10^{-8} M, with EC_{50} values of 3.56×10^{-6} M and 6.17×10^{-7} M for HarmOR6/Orco and HassOR6/Orco responses to Z9-16:OH, respectively (Fig. 7G–J). The other four candidate pheromone receptors, HarmOR11, HarmOR14a, HassOR11 and HassOR14b, failed to respond to the tested chemicals (Suppl. Fig. 1). Perhaps the ligands of these PRs are other still untested odorants. Alternatively, we cannot exclude the hypothesis that these PRs, especially HarmOR14a and HassOR14b, have lost their functions during evolution.

3.4. Sequence similarity and the functional properties of PR orthologs

To determine if sequence similarity among orthologs can predict the function of PRs in closely related species, we compared the functional properties of orthologous PRs from *H. armigera*, *H. assulta* and *H. virescens*. According to our data and previous literature, there are 7 PR orthologs that we have functionally characterized using heterologous expression in *Xenopus* oocytes: four of them are present in all three species, two only in *H. armigera* and *H. virescens* and one only in *H. armigera* and *H. assulta*. Amino acid identities between PRs orthologs and paralogs from the three species are reported in Table 1. Sequence similarity of PRs in *H. armigera*, *H. assulta* and *H. virescens* ORs and their response profiles to each of chemicals at the concentration of 10^{-4} M are listed in Fig. 8.

OR13 orthologs present high amino acid identities (up to 90%) between the three species and are all specifically tuned to Z11-16:Ald, the principal sex pheromone component in the three species. HarmOR6 and HassOR6 which share 92% of amino acid identity are both rather specific to Z9-16:OH (the behavioral antagonist in *H. assulta*), but are also weakly activated by other pheromone components, such as Z9-16:Ald and Z9-14:Ald. HvOR6 is narrowly tuned to Z9-14:Ald (the sex pheromone component in *H. virescens* and *H. armigera*) although it is 88% identical with HarmOR6. HarmOR16 and HassOR16 share 93% of their residues, but are less similar to HvOR16 (88 and 87%, respectively). HarmOR16 and

Fig. 7. Dose responses of *Xenopus* oocytes with co-expressed HassOR13/Orco (A, B), HarmOR14b/Orco (C, D), HassOR16/Orco (E, F), HarmOR6/Orco (G, H), and HassOR6/Orco (I, J) to stimulation with the most active pheromone compounds and analogs. (A, C, E, G, I) *Xenopus* oocytes expressing the PR/Orco stimulated with a range of concentrations. (B, D, F, H, J) Dose-response curve of *Xenopus* oocytes expressing the PR/Orco to the related compounds. HassOR13/Orco to Z11-16:Ald, $EC_{50} = 1.36 \times 10^{-6}$ M, $n = 7$; HarmOR14b/Orco to Z9-14:Ald, $EC_{50} = 2.01 \times 10^{-5}$ M, $n = 4$; HassOR16/Orco to Z9-14:Ald, $EC_{50} = 3.59 \times 10^{-6}$ M, $n = 6$; HarmOR6/Orco to Z9-16:OH, $EC_{50} = 3.56 \times 10^{-6}$ M, $n = 7$; HassOR6/Orco to Z9-16:OH, $EC_{50} = 6.17 \times 10^{-7}$ M, $n = 4$. Error bars indicate SEM.

Table 1
Amino acid identity matrix for *H. armigera*, *H. assulta* and *H. virescens* orthologs and paralogs of PRs.

	HassOR6	HvOR6	HarmOR11	HassOR11	HvOR11	HarmOR13	HassOR13	HvOR13	HarmOR14a	HvOR14	HarmOR14b	HassOR14b	HarmOR16	HassOR16	HvOR16
HarmOR6	92	80	49	49	49	38	39	37	57	57	54	54	72	71	72
HassOR6		77	48	48	48	37	38	36	57	57	53	53	70	70	72
HvOR6			46	46	46	37	39	37	54	53	52	53	66	65	67
HarmOR11				98	95	39	39	39	44	46	41	42	48	46	47
HassOR11					94	38	39	39	44	46	40	42	48	46	47
HvOR11						38	39	39	45	46	41	42	47	46	47
HarmOR13							95	90	38	38	39	37	40	39	39
HassOR13								90	37	38	38	37	40	39	40
HvOR13									37	37	36	35	39	38	39
HarmOR14a										83	67	68	57	56	58
HvOR14											68	67	56	54	57
HarmOR14b												90	54	55	56
HassOR14b													55	55	56
HarmOR16														93	88
HassOR16															87

The identities of amino acid sequences were analyzed by GeneDoc software.

HvOR16 are tuned to Z11-16:OH (the behavioral antagonist in *H. armigera* and *H. virescens*), but HassOR16 robustly responds to Z9-14:Ald (the behavioral antagonist in *H. assulta*). HarmOR14a and HvOR14 share 88% identity. HvOR14 strongly responds to Z11-16:Ac (the behavioral antagonist in *H. virescens*), but HarmOR14a does not show any response to the tested chemicals, including Z11-16:Ac. A similar situation was observed with HarmOR14b which is 90% identical with HassOR14b. HarmOR14b is a highly specific receptor tuned to Z9-14:Ald, but HassOR14b did not respond to any of the odorants tested in this study. In addition, HarmOR14b, HassOR16 and HvOR6 that show strong response to Z9-14:Ald have relatively low amino acid identities between each other (53–65%). From our analysis, some PR orthologs are tuned to the same ligand, but others respond to different ligands, suggesting that many PRs show dynamic functional changes during evolution. Therefore, sequence similarities between PR orthologs in closely related moth species cannot be used to predict their functional characteristics.

Sequence similarities of PR combined with functional analysis also show that some PRs with low amino acid identities are tuned to the same ligand. BmOR1 of *B. mori* and DmelOR7a of *D. melanogaster*, sharing only 17% of their amino acids, respond to the same ligand, bombykol (Sakurai et al., 2004; Syed et al., 2010). PxOR1 of *P. xylostella* is a receptor for Z11-16:Ald, as are HarmOR13, HassOR13 and HvOR13, which exhibit identities with the first protein of about 44% (Mitsuno et al., 2008; Grosse-Wilde et al., 2007; Wang et al., 2010; Liu et al., 2013a,b; Sun et al., 2013). Notably, in *Ostrinia* species a single mutation to OR3 produces a major shift for specificity to the sex pheromone components of closely related species (Leary et al., 2012). In *Ostrinia nubilalis* the amino acid residue corresponding to position 148 in transmembrane domain 3 of OR3 is alanine, while in *Ostrinia furnacalis* OR3 has a threonine (T) in the same position. Mutation of this residue from A to T alters the pheromone recognition pattern of OR3, from response strongly to E11-14: Ac and generally to Z11-14:Ac, E12-14:Ac, and Z12-14:Ac shifting to E12-14:Ac and Z12-14:Ac, and selectively reducing the E11-14: Ac response about 14-fold. These facts support the idea that the ligand specificity of the sex-pheromone receptors is determined by local amino acid sequences rather than global ones (Mitsuno et al., 2008; Miura et al., 2010).

3.5. Functional comparisons of PRs from three Heliothinae moths explain the evolution of sex pheromone systems

We functionally characterized five PRs, HarmOR6, HarmOR14b, HassOR6, HassOR13 and HassOR16, by expressing them in *Xenopus* oocytes with Orco but in the absence of PBPs. Up to now, the receptor proteins for all pheromone components except for Z9-16:Ald have been identified in the closely related species, *H. armigera*, *H. assulta* and *H. virescens*. OR13s, which are the receptors of Z11-16:Ald, the most abundant pheromone component of *H. armigera* and *H. virescens*, were the only homologous ORs that shared the common ligand in three species. HvOR13 mapped to the sex chromosome in *H. virescens* (Gould et al., 2010). Phylogenetic analysis shows that although OR6, OR11, OR13, OR14, OR16 cluster together in the pheromone receptor subfamily, OR13s fall into a subgroup separate from other PRs (Figs. 3 and 8). These findings suggest that the acquirement of OR13 genes occurred before speciation within the *Heliothinae*. OR16 orthologs were particularly interesting as they showed dynamic functional changes in three species. Both HarmOR16 and HvOR16 shared Z11-16:OH as their ligand, an inhibitor in *H. armigera* and *H. virescens* when added to attraction blends (Kehat and Dunkelblum, 1990; Wu et al., 1997; Vetter and Baker, 1983). However, HassOR16 were obviously tuned to a different ligand, Z9-14:Ald, which acts as behavioral

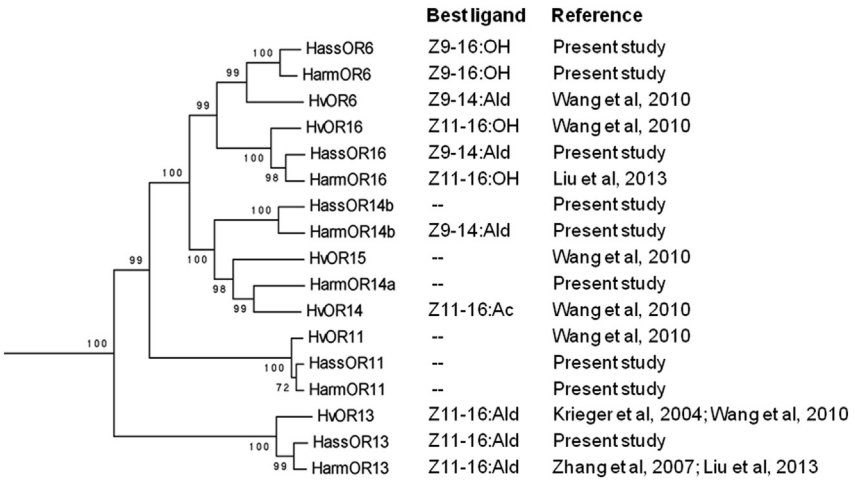


Fig. 8. Sequence similarity of PRs in *Helicoverpa armigera*, *Helicoverpa assulta* and *Heliothis virescens* ORs and their best ligands identified to date. – no responses with tested chemicals.

antagonist in *H. assulta* (Cork et al., 1992; Boo et al., 1995). There was no OSN tuned to Z11-16:OH on the antennae of male *H. assulta* (Berg and Mustaparta, 1995; Berg et al., 2005). Thus HassOR16 evolved a new function to adapt to sex pheromone communication of *H. assulta* although it has 93% amino acid identity with HarmOR16. HvOR14 was tuned to Z11-16:Ac, the behavioral antagonist in *H. virescens* (Vickers and Baker, 1997), but HarmOR14a that is 83% identical with HvOR14 did not respond to any of the nine chemicals tested, including Z11-16:Ac (Liu et al., 2013a,b). Likely, HarmOR14b was specially tuned to Z9-14:Ald, but HassOR14b had no response to Z9-14:Ald, whose receptor was HassOR16 in *H. assulta*. Surprisingly, Z9-14:Ald was the ligand of *H. virescens* HvOR6 (Wang et al., 2010), whose orthologs are tuned to Z9-16:OH in *H. armigera* and *H. assulta*. It seems that in the evolution of *Heliothis*/*Helicoverpa* species, functional similarity of OR superfamily is related to lineage-by-descent evolution in the large context, but in many cases OR genes that are similar in sequence may not be similar in function. Some PR orthologs have changed their functions during evolution to satisfy the need of specific-species pheromone reception. It is possible that the interplay of speciation events leads to the functional divergence of PR orthologs.

The insect olfactory receptor repertoire is an excellent model to study evolution, because ORs have been subjected to rapid evolution over relatively short time spans, probably caused by changes of the olfactory system to adapt to the environment. At present we use functional comparisons of PR orthologs from three *Heliothinae* moths to explain how well their sex pheromone systems evolve. We also recognize the limitations of our extrapolation from only three closely related species. In the near future, it will be important to build on these results by analyzing olfactory differentiation in more *Heliothinae* species and by exploring functional divergence in more ORs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2014.02.010

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