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Odorant receptors and antennal lobe morphology offer a new approach to understanding olfaction in the Asian longhorned beetle

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Abstract

The Asian longhorned beetle *Anoplophora glabripennis* (Motschulsky) is an exotic forest pest that has repeatedly invaded North America and Europe from Asia, and has the potential to kill millions of trees and cause billions of dollars in damage. Traps baited with an attractive mixture of volatile organic compounds (VOCs) from hosts have been of limited success in monitoring invasion sites. We propose that lures might be improved through studying the olfactory system of adult beetles, especially the gene family of odorant receptors (ORs) and the structure of the antennal lobes of the brain. Here, we report identification of 132 ORs in the genome of *A. glabripennis* (inclusive of one Orco gene and 11 pseudogenes), some of which are orthologous to known pheromone receptors of other cerambycid beetles. We also identified three ORs that are strongly biased toward expression in the female transcriptome, and a single OR strongly biased toward males. Three-dimensional reconstruction of the antennal lobes of adults suggested a male-specific macroglomerulus and several enlarged glomeruli in females. We predict that functional characterization of ORs and glomeruli will lead to identification of key odorants in the life history of *A. glabripennis* that may aid in monitoring and controlling future invasions.

Keywords

Anoplophora glabripennis; Cerambycidae; olfactory receptor; antennal lobe morphology; pheromone

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Introduction

The Asian longhorned beetle, *Anoplophora glabripennis* (Motchulsky), is a destructive insect native to Asia that has repeatedly become established in urban areas of North America and Europe (Haack et al. 2010). *A. glabripennis* is considered to be among the most serious forestry pests on account of its ability to infest and kill healthy trees of numerous genera, including widely planted species of *Acer*, *Salix*, and *Populus* (Hu et al. 2009). To date, invasions have been demarcated and monitored using traps baited with an attractive mixture of volatile organic compounds (VOCs) produced by the host and two components of the male-produced aggregation-sex pheromone, 4-(*n*-heptyloxy)butan-1-ol and 4-(*n*-heptyloxy)butanal (e.g., Nehme et al. 2010, 2014). This mixture, however, shows low efficacy of attraction (<100 specimens over multiple seasons; Meng et al. 2014; Nehme et al. 2014) compared to that of host VOC/pheromone blends that target related genera such as *Monochamus* (e.g., >2000 specimens, Allison et al. 2012), which suggests that a more optimal mixture or attractant likely remains to be discovered. Female-associated VOCs (Wickham et al. 2012), a trail pheromone (Hoover et al. 2014), and a putative third component of the male-produced aggregation pheromone (Crook et al. 2014) have been recently identified, although their effectiveness in monitoring invasion sites has yet to be tested.

We propose that the discovery of additional attractive VOCs might be hastened through studies of the olfactory system of *A. glabripennis*. Neopteran insects detect VOCs by means of sensory receptor neurons expressing olfactory receptors (ORs), ionotropic receptors (IRs), and a few gustatory receptors (Leal 2013; Missbach et al. 2014). The ORs appear to be primarily responsible for detection of VOCs (Leal 2013), while the IRs appear to be sensitive to VOCs as well as abiotic factors such as humidity and temperature (Benton et al. 2009; Min et al. 2013; Enjin et al. 2016). In insects, ORs are usually expressed in the antennae, where a single OR gene and an *Orco* co-receptor are expressed on the dendrites of an individual odorant-sensitive receptor neuron (OSN) (Vosshall and Stocker 2007). In some situations, multiple ORs can be expressed on the same OSN (Couto et al. 2005; Fishilevich and Vosshall 2005; Goldman et al. 2005). Each OSN projects its axon to the antennal lobe (AL) of the brain, and the axons of each OSN type converge in one of the discrete, condensed neuropil structures in the AL the olfactory glomeruli (Martin et al. 2011). Thus, each glomerulus represents the combined inputs of OSNs expressing the same OR gene, or same sets of OR genes (c.f., Koutroumpa et al. 2014). The size of the glomerulus is thus correlated to the number of associated OSN axons, which in turn may reflect the importance of the odorant information it receives with respect to the life history of the insect (Dekker et al. 2006), especially when the glomerulus is involved in detecting pheromones (e.g., Christensen et al. 1995). By functionally characterizing the ORs and/or glomeruli, we may identify novel volatiles that can influence the behavior of an insect species.

Here, we present the OR family of *A. glabripennis*, annotated from the recent Asian longhorned beetle genome project (McKenna et al. 2016). We compare these OR genes in the transcriptomes of male and female *A. glabripennis* to identify potential ORs that may be biased toward expression in one sex. Finally, we present an initial map of the AL of male

and female *A. glabripennis* in order to identify enlarged glomeruli, which may be sites of primary neural processing of sensory information about key odorants in the life history of this invasive pest.

Methods and Materials

Annotation of ORs

Genomic data were made available through the Asian longhorned beetle genome project (McKenna et al. 2016). Using TBLASTN (Altschul et al. 1997), we searched the first draft of the *A. glabripennis* genome (NCBI: BioProject PRJNA167479) against a database of available coleopteran OR sequences: *Tribolium castaneum* (Herbst) (Engsontia et al. 2008); *Megacyllene caryae* (Gahan) (Mitchell et al. 2012); and *Ips typographus* (L.) and *Dendroctonus ponderosae* (Hopkins) (Andersson et al. 2013). All of these beetles belong to a single species-rich clade (series Cucujiformia) containing ~190,000 described extant species (McKenna et al. 2015). Models of ORs were constructed from a combination of predictions by MAKER 2.0 (Canterel et al. 2008), transcriptome support (McKenna et al. 2016), and manual annotation in Geneious R6.1 (Biomatters, Ltd., Auckland, NZ). We discarded most partial models that were <100 amino acids in length or consisted of only a single apparent exon, to avoid designating separated exons of one receptor with two names. Pseudogenes were designated when regions of the genome presented strong sequence similarity to a receptor gene, but with nonsense mutations and/or missing splice site sequences.

Peptide sequences of the *A. glabripennis* ORs were aligned using MUSCLE (10 iterations; gap score 3; Edgar 2004) and subsequent manual adjustment. ORs of *A. glabripennis* were separately aligned with the three functionally characterized ORs from *M. caryae*, the OR genes available from the genome of *T. castaneum*, and published ORs from transcriptomes of three other major beetle families: Chrysomelidae (*Ambrostoma quadriimpressum* Motchulsky; Wang et al. 2016), Scarabaeidae (*Anomala corpulenta* Motchulsky; Li et al. 2015), and Curculionidae (*D. ponderosae*). Only genes longer than 100 amino acids were included in this analysis. Phylogenetic trees were constructed from the alignments using FastTree 2.1 at its default settings (Price et al. 2010) with nodal support values based on a Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999). Trees were visualized and edited in FigTree v1.4.2 (Rambaut 2014).

OR genes from *A. glabripennis* were designated with the prefix AglaOR and numbered consecutively down the tree so that in most cases, genes similar in number also share a predicted phylogenetic placement. The AglaOR1 designation was reserved for the conserved OR co-receptor (Orco) gene, following Engsontia et al. (2008). Gene names were provided suffix codes of three letters to indicate missing regions of the model (NTE, INT, CTE for N-terminal, internal, or C-terminal gaps), or single letters in the case of multiple missing exons (e.g., NC = NTE + CTE). We assigned OR genes to subfamilies based on their phylogenetic placement and following the naming scheme initiated by Engsontia et al. (2008). This scheme divides ORs among seven major subfamilies (dubbed “Group 1”, “Group 2”, etc.), six of which were annotated from the genome of *T. castaneum* (Engsontia et al. 2008). The

final subfamily in the scheme, Group 7, was subsequently identified from several other beetle families (Mitchell et al. 2012; Andersson et al. 2013; Li et al. 2015) but apparently is absent in *T. castaneum*.

Expression of ORs

We approximated the expression of each OR by searching the unassembled reads of whole-body transcriptomes of an adult male and female *A. glabripennis* (McKenna et al. 2016). We recorded 100% matches to peptide sequences of each OR and four housekeeping genes (cytoplasmic actin, EF1-alpha, and ribosomal proteins L32 and S3). Hits were recorded only in the event of a perfect match. We took the number of reads as a rough estimate of transcriptional activity. This number was scaled to the read count of each housekeeping gene to generate an average ratio of reads between sexes (female/male), which was expressed as $\log_{10}$ to indicate female vs. male expression (positive for female, negative for male). Sex-biased transcriptional activity was estimated by observing for genes that were present in a single sex, or, for genes present in both sexes, log ratios that exceeded two standard deviations from the mean activity (exceeding 95% of the expected distribution).

Source of insects for AL morphology

A. glabripennis adults were reared separately at the USDA-APHIS CPHST Lab (“Otis Lab”; Otis ANGB, MA, USA) and Pennsylvania State University (“PSU”; University Park, PA). Beetles at the Otis Lab were reared on a diet similar to that of Dubois et al. (2002) for ~3 mo, chilled at 10 °C for 11 wk, and allowed to complete development on the same diet. Adults were fed twigs of striped maple (*Acer pensylvanicum* L.) until dissection. Beetles were reared at PSU on a pourable modification of a diet designed for *Enaphalodes rufulus* (Haldeman) for 90 days (Keena 2005), chilled at 10 °C for 90 days, and allowed to develop until pupation on the same diet. Upon pupation they were transferred to 50 ml Falcon centrifuge tubes and incubated at 27.5 °C until adult eclosion. Adults were fed red maple (*Acer rubrum* L.) twigs until dissection.

Preparation of ALs

We initially attempted to stain glomeruli with nc82 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), an antibody with an affinity for synaptic neuropils in insects (Wagh et al. 2006). However, the antibody failed to detect a target antigen in the ALs of *A. glabripennis*. We instead visualized the glomeruli by fixing with glutaraldehyde, which produces fluorescence throughout the AL, but such that the more condensed neuropil of the glomeruli stand out against other tissues (e.g., Sombke et al. 2012).

Live, adult beetles of both sexes were decapitated with a razor blade, and the brain was accessed either directly through the exposed occipital foramen, or by cutting along the gena and frons. Brains were carefully dissected from the head capsule under 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1M Sorensen’s phosphate buffer (pH 7.4; Electron Microscopy Sciences) and, using forceps, cleaned of trachea, fat deposits, and other accessory tissues that adhered to the surface. Dissections at PSU were conducted in Tucson

Ringer (Waldrop et al. 1987), but subsequently incubated in glutaraldehyde solution. Paired ALs were located prominently on the ventral side of the brain relative to the neuraxis, which lies against the dorsal-anterior surface of the head capsule. We discarded samples if these structures were visibly damaged. Successful dissections were fixed in fresh glutaraldehyde solution at 1–3 °C for at least 48 hours, dehydrated by a series of 10-min treatments in solutions of increasing ethanol concentration, and cleared in methyl salicylate (Sigma-Aldrich, St. Louis, MO).

Imaging and analysis of ALs

Prepared brains of four male and four female beetles were imaged on a Zeiss Meta 510 LSM confocal microscope (Carl Zeiss AG, Jena, Germany) or an Olympus FluoView FV1000 confocal microscope (Olympus Corporation, Center Valley, PA). Brains were oriented so that the ventral surface of the brain, and the ALs, were facing the microscope objective and imaged on a transverse plane. Consistent positioning of the brain at this stage was critical for comparing later reconstructions of the AL. This preparation was illuminated by a HeNe laser at 633 nm (Otis) or a combination of 543 nm and 405 nm (PSU). Excitation of the glutaraldehyde background alone was sufficient to visualize glomeruli, which appeared as bright, discrete clusters in the ALs.

Image stacks were loaded into the software package Reconstruct (v1.1.0.0; Fiala 2005) and the outline of each glomerulus was manually traced on each section of every stack to create a three-dimensional model of each AL. We compared the resulting three-dimensional models and identified prominent glomeruli of consistent position and size in each brain as landmarks. Some landmark glomeruli appeared consistently greater in size than other glomeruli in the AL, and we noted these as “enlarged” if they exceeded 90% of the range of glomerular size in at least two individuals of either sex, and “macroglomeruli” if they exceeded three times the interquartile range (extremely conservative outliers; Hoaglin et al. 1986). Remaining glomeruli were classified by their spatial relationship to landmark glomeruli. Glomeruli were classified and named following Ghaninia et al. (2007). Landmark glomeruli were designated as Class 1 and the other spatially consistent glomeruli as Class 2, while the remaining structures that varied in location or presence across individuals were designated as Class 3. We named glomeruli by their spatial relationship to the neuraxis, with three capital letters indicating the position relative to each axis: anterior/posterior (A, P), ventral/dorsal (V, D), lateral/medial (L, M), or central (C) (Ghaninia et al. 2007). All subsequent description of the brain is presented relative to the neuraxis unless otherwise specified.

Results

Annotation of ORs

We annotated 132 genes with sequences similar to the family of insect ORs (Fig. 1, Online Resource 1A), comprising the single expected ortholog to the *Orco* gene (Vosshall and Hansson 2011; AglaOR1\Orco), eleven apparent pseudogenes (AglaOR22, 23, 34, 67, 75, 83, 88, 104, 111, 119, and 122; designated by suffix PSE), and 120 ORs. Eighty-one of these

receptors were annotated with full-length ORFs in the genome. Sixteen additional ORs were nearly complete and lacked only a short initial or terminal exon (Online Resource 1A). The remaining 24 receptors are likely to be complete genes, but with one or more exons missing in unassembled regions of the genome (Online Resource 1A). We were conservative in assigning names to receptor fragments, because fragments with very high sequence similarity to full-length AglaORs may be assembly errors involving different alleles of the same gene. OR models and sequences may be viewed as part of the *A. glabripennis* genome project (McKenna et al. 2016; NCBI: BioProject PRJNA167479), and FASTA files of peptide and nucleotide sequences are included here as supplementary materials (Online Resources 1B, C).

The *Anoplophora* ORs could be placed almost entirely within the seven established groups of coleopteran ORs (Figs. 1, 2.; Engsontia et al. 2008; Mitchell et al. 2012; McKenna et al. 2016). Groups 1 (AglaOR54-68), 3 (AglaOR73-100), and 7 (AglaOR37-53) were clearly defined with high support values. In contrast, Groups 4, 5, and 6 placed within a single, well-supported lineage of AglaORs (AglaOR104-132), defined by the outgroup of AglaOR101-103. Group 2 was only weakly supported, but consisted of two major subgroups that included AglaOR2-28 and AglaOR29-36. AglaOR69-72 was recovered in a position separate from the seven established groups. When receptors from other beetle lineages were included (Fig. 2), AglaOR71-2 resolved as members of the 3A subgroup (Engsontia et al. 2008). AglaOR69-70 apparently were not part of any existing clade of ORs, but grouped with some OR genes in the beetle families Curculionidae (weevils) and Chrysomelidae (leaf beetles), both near relatives of the Cerambycidae.

Seven AglaOR genes were similar to three functionally characterized ORs from the cerambycid beetle *Megacyllene caryae*, included in Fig. 2. AglaOR29 was orthologous to McarOR3, a receptor sensitive to the compound 2-methylbutan-1-ol, while AglaOR35-36 were sister to McarOR5, which is sensitive to 2-phenylethanol (Mitchell et al. 2012). Finally, AglaOR65-68 formed a small clade that also included McarOR20, a receptor sensitive to stereoisomers of 2,3-hexanediol and 3-hydroxyhexan-2-one (Mitchell et al. 2012). The above are all volatile compounds produced by male *M. caryae* as part of this species' aggregation-sex pheromone (Lacey et al. 2008; Mitchell et al. 2012).

Expression of ORs

Of the 120 putative ORs, 89 were present as at least one unique read in the transcriptomes (Fig. 3, Online Resource 2). Most ORs were present minimally, with a median of only four reads. Nine ORs were recovered only from the male transcriptome and 36 ORs were recovered only from the female, but only two of these sex-specific ORs (AglaOR99 and AglaOR43; female) were recovered as more than ten reads (Fig. 2). Thus, the sex bias of the other receptors is questionable, because it is possible they may have been present but not sequenced from the transcriptome of the other sex. Forty-four receptors were transcribed in both sexes and overall were slightly biased toward the female transcriptome, with an average log ratio of 0.29. Log ratios of 1.62 and -1.02 were calculated as cutoffs for bias in females and males, respectively. AglaOR18, 25, and 28 exceeded the threshold for a bias in females (log ratios 1.85, 1.99, 1.81), and AglaOR16 exceeded the threshold in males (-1.56).

AglaOR102, 38, and 39 were not biased toward either sex, but presented a notably high read count (>100) relative to other ORs in the transcriptomes of both sexes. The actual expression of AglaOR38-39 is unclear because many reads corresponded to a region of shared sequence, but unique reads were assigned to both genes. Thirty-two genomic ORs were not recovered by the transcriptomes, and are probably associated with other developmental stages that were not targeted in this study (e.g., larva; Engsontia et al. 2008).

Imaging and analysis of ALs

Glomeruli were clearly visible in the ventral sections of the ALs of both sexes (Fig. 4A), but the signal strength decreased as imaging proceeded dorsally (Fig. 4B), leaving some uncertainty to the form and structure of the glomeruli deeper in the AL. The visible glomeruli presented as a hollow, spherical cluster, consistent with observations of isomorphic glomeruli in other insect species (Rospars and Hildebrand 2000; Dreyer 2010). We identified an average of 60 (range ± 2) glomeruli in females, with the median size of glomeruli in an individual beetle ranging between 55,124–79,568 μm^3 . Males presented 55 ± 3 glomeruli, with a median size range of 64,022–90,570 μm^3 (Fig. 5).

Many glomeruli were inconsistent in location, size, and even presence among individuals in the study, hindering our comparison of the structures between sexes. We ultimately named 18 glomeruli that appeared to be consistent in their large size and/or general location between the sexes, and two additional glomeruli that were apparently unique to females. These glomeruli are described briefly below and illustrated in Figs. 4C and 4D. Three-dimensional reconstructions, confocal image stacks, and additional descriptions are available in the supplementary materials (Online Resources 3–5).

In both sexes, the AL was clearly bounded by a crest of four class 1 glomeruli along its posterior surface (PCC1-4; Fig. 4C,D). The large glomerulus PVL1 was situated directly anterior to this crest and acted as a landmark glomerulus to define the class 2 cluster of PVL2-5. Another large class 1 glomerulus, PVM1, was situated medial to PVL1 and, in females, was tightly appressed to two class 2 glomeruli, PVM2 and 3. The class 1 glomerulus CVC1 was positioned at the ventral apex of the AL, and CVC2-3 were immediately posterior and anterior to CVC1. The final class 1 glomerulus, ACD1, was situated at the anterior margin of the AL, near the antennal nerve. Four class 2 glomeruli, AMD1-4, were defined by their proximity to CVC3 and ACD1.

Male and female beetles also differed in their apparent macroglomeruli (Fig. 5A,B). PVL1 exceeded the threshold for a macroglomerulus in one brain of a female and was greatly enlarged in the remaining three (Fig. 5A). This glomerulus, though larger than average in males, was never more than twice the median size. In contrast, we classified ACD1 as a macroglomerulus in males because it greatly exceeded our threshold in all four specimens (Fig. 5B), but was mostly indistinct in females (with one exception; Fig. 5A, Sample 4). PVM1 exceeded this threshold in a single male, and was enlarged in two other males and a female, suggesting it as a second potential macroglomerulus. Additionally, CVC1 and CVC3 were enlarged in multiple males, while PVL2, PVL3, PVM2, and PCC2 were enlarged in females, and PVL4 was enlarged in one individual of each sex.

Discussion

Our genomic and neuroanatomical survey describes a potentially diverse olfactory repertoire for *A. glabripennis* and highlights several receptors that may play a key role in the ecology of this invasive pest. Notably, the single receptor AglaOR16 was strongly biased towards males, and a single glomerulus in the ALs of males (ACD1) was vastly expanded relative to other glomeruli. Highly expressed, male-biased receptors may be associated with pheromone production by females (Wanner et al. 2007). Furthermore, a male-specific macroglomerular complex (MGC) in many lepidopteran species receives and processes signals from OR neurons that are specialized to detect the key components of a female-produced pheromone (Christensen et al. 1995; Namiki et al. 2014). Brains of *A. glabripennis* males did not contain a distinct MGC, in that we did not observe the strong sexual dimorphism in both shape and size that defines this region in Lepidoptera (Rospars and Hildebrand 2000). Instead, each macroglomerulus observed here appears to correspond to a similar but diminished counterpart in the opposite sex, perhaps suggesting a conserved olfactory function, but with differing sensitivity or downstream circuitry (e.g., Datta et al. 2008). Nevertheless, the male-specific macroglomerulus, coupled with the single male-biased OR, hints at a female-produced sex pheromone: perhaps one or more of the oxidized aldehydes that are associated with virgin females and attractive to males (Wickham et al. 2012). Similarly, three receptors were more common in the female transcriptome, and female brains contained a potential macroglomerulus (PVL1). This may be sensitive to the male-produced pheromone components, or to other odorants important to females such as oviposition cues.

The size of a glomerulus can correlate to its importance in the life history of an insect (Ibba et al. 2010). We identified several enlarged glomeruli that may thus be responsive to volatiles that strongly influence the behavior of *A. glabripennis*. Many of the largest glomeruli were located near the surface of the AL, potentially making them more accessible to future characterization via calcium imaging (Galizia et al. 1999) or neural recording and dye injection (Reisenman et al. 2005). A single, unnamed glomerulus deeper in the AL was also enlarged (Fig. 5B, Sample 3), but because of limitations in imaging, some dorsal glomeruli were less distinct and we were unable to consistently locate them or assign names. Future surveys of the AL with more powerful imaging equipment (e.g., multiphoton microscopy) or effective antibody stains will be necessary for better characterization of this region.

Nevertheless, the total number of glomeruli identified in our analysis was relatively consistent across specimens. The number of glomeruli in the AL can approximate the number of ORs expressed in the antennae (Vosshall and Stocker 2007), and indeed we identified approximately 55 glomeruli in the brains of males, and reads corresponding to 52 ORs. However, although the most complex female AL contained 62 glomeruli, we identified 79 ORs from this transcriptome. One explanation for this is that some glomeruli were not successfully mapped or visualized. In particular, the dorsolateral portion of the AL (Fig. 4B, bottom right) transitioned into a region of indiscrete structures, which were occasionally difficult to distinguish from “true” glomeruli. This region probably marks the boundary of the antennal mechanosensory and motor center (Homberg et al. 1989), but may include

additional structures of the olfactory neuropil. Some ORs may also be co-expressed in the same neurons (Couto et al. 2005; Fishilevich and Vosshall 2005; Goldman et al. 2005; Koutroumpa et al. 2014), which is another possible reason for the lack of a strict 1:1 ratio of ORs to glomeruli. Additionally, some OR genes may be expressed elsewhere in the body (e.g, Engsontia et al. 2008), and thus not linked to the deutocerebrum, and such site specificity would have been overlooked by the whole-body transcriptomes conducted here.

The *Anoplophora* ORs are only the second complete set of receptors annotated from a coleopteran genome, and thus offer the first proper comparison to the unusually large olfactory suite of *Tribolium castaneum* (Engsontia et al. 2008). Our analysis confirms speculation by Engsontia et al. (2008) that the prominent expansions of *Tribolium* are lineage-specific, and in fact, suggests that the major OR Groups 4, 5, and 6 might be better unified as a single group of receptors, along with the recently-proposed Group 9 (Antony et al. 2016). This unified group is defined by a small outgroup of AglaOR101-103, which also includes conserved representatives from other beetles including TcasOR275 (Engsontia et al. 2008) and McarOR44 (Mitchell et al. 2012). Unfortunately, few members of Groups 4–6 were present in the published transcriptomes of chrysomelid, scarabaeid, and curculionid beetles, so additional beetle genomes must be annotated to further elucidate the extent of this family.

The remaining Groups 1, 2, 3, and 7 were generally well-maintained. Group 3 included the largest expansion of AglaORs, including a tandem array of AglaOR89-98 that shared a high degree of sequence similarity and may be recent duplications. This cluster may thus be under active selection in *A. glabripennis*, although no members were prominent in the transcriptomes. Group 2 is recovered with strong statistical support in other studies (Engsontia et al. 2008; Andersson et al. 2013; Li et al. 2015; RFM unpub. data), so its poor support in the present study is questionable, but it may reflect a division that will become evident as additional beetle genomes are sequenced. The inclusion of TcasOR71-72 in Group 7 is also probably in error, because their placement is inconsistent in studies to date (Engsontia et al. 2008; Wang et al. 2016; RFM unpub. data). Some authors have even gone so far as to entirely remove them from analyses (Andersson et al. 2013; Li et al. 2015). It is possible TcasOR71-72 are remnants of a separate, diminished group of receptors in *Tribolium* that are lost in *Anoplophora* and the other beetle families included in our analyses.

In fact, our phylogeny suggests two such groups of coleopteran ORs that are separate from the seven primary radiations. The enigmatic Group 3A, identified in the original *Tribolium* genome project by TcasOR167, is joined by AglaOR71-72, and its relationship with Group 3 is poorly supported here. Group 3A also includes a large expansion of scarab (staphyliniform) genes, suggesting it may be a lineage that is diminished in cucujiform beetles. Similarly, we identified a small, orphaned family of receptors including AglaOR69-70, AquaOR11-12, and DponOR15. Again, genomic data from additional families and suborders of beetles will be necessary to properly define these potentially novel receptor groups. We therefore refrain from naming them here.

The ligands of coleopteran ORs remain largely unknown. To date only the three receptors McarOR3, 5, and 20 have been characterized, and they are sensitive to pheromone components produced by the cerambycid beetle *M. caryae* (Mitchell et al. 2012). McarOR3 and McarOR5 were highly similar to AglaOR29 and AglaOR35-36, suggesting that these genes may be related to pheromone biology. However, the pheromone components in question (2-methylbutan-1-ol and 2-phenylethanol) are also widespread floral compounds (El Sayed 2016) with little structural similarity to the known pheromone components of *A. glabripennis*. Neither of these receptors appeared to be highly expressed in *A. glabripennis*, in contrast to what might be expected for a receptor sensitive to pheromone components (Wanner et al. 2007; Mitchell et al. 2012), but quantitative PCR of antennal RNA will be necessary to clearly establish the relative expression of these and other OR genes identified in this study.

McarOR20 is sensitive to the conserved pheromone components 2,3-hexanediol and 3-hydroxyhexan-2-one produced by many species in the cerambycid subfamilies Cerambycinae and Prioninae (Millar and Hanks 2016), and it is interesting that McarOR20 shares similarity with AglaOR65-68. These are not known to be produced as pheromone components by species in the cerambycid subfamily Lamiinae (including *A. glabripennis*); nonetheless, AglaOR65-68 may be tuned to detect similar pheromone components produced by the many sympatric cerambycine and prionine species in Asia (Wickham et al. 2014, 2016), perhaps to enforce mating isolation or aid in identifying host plant material.

We employed a promising new approach for elucidating the olfactory biology of a devastating forest pest, and it has yielded numerous candidate receptors that may be associated with the detection of pheromones or other behaviorally-active volatiles. Our data suggest the presence of a key female-produced volatile that is detected by males and has perhaps not been chemically or behaviorally characterized. None of the recently characterized components of the trail sex pheromone produced by females (Hoover et al. 2014) seem to be implicated here as the missing female volatile, because males appear to use mostly their maxillary and labial palps and not their antennae for detecting and responding to this pheromone (Graves et al. 2016). Functional characterization of receptors such as AglaOR16 should be given top priority because they may reveal *A. glabripennis* pheromone components or other attractants and improve efforts to monitor for invasive populations of the Asian longhorned beetle worldwide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Allison JD, McKenney JL, Millar JG, McElfresh JS, Mitchell RF, Hanks LM. Response of the woodborers *Monochamus carolinensis* and *Monochamus titillator* (Coleoptera: Cerambycidae) to known cerambycid pheromones in the presence and absence of the host plant volatile α -pinene. *Environ Entomol.* 2012; 41:1587–1596. [PubMed: 23321107]
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997; 25:3389–3402. [PubMed: 9254694]
- Andersson MA, Grosse-Wilde E, Keeling CI, Bengtsson JM, Yuen MMS, Li M, Hillbur Y, Bohlmann J, Hansson B, Schlyter F. Antennal transcriptome analysis of the chemosensory gene families in the tree killing bark beetles, *Ips typographus* and *Dendroctonus ponderosae* (Coleoptera: Curculionidae: Scolytinae). *BMC Genomics.* 2013; 14:198. [PubMed: 23517120]
- Antony B, Soffan A, Jakše J, Abdelazim MM, Aldosari SA, Aldawood AS, Pain A. Identification of the genes involved in odorant reception and detection in the palm weevil *Rhynchophorus ferrugineus*, an important quarantine pest, by antennal transcriptome analysis. *BMC Genomics.* 2016; 17:69. [PubMed: 26800671]
- Benton R, Vannice KS, Gomez-Diaz C, Vossall LB. Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell.* 2009; 136:149–162. [PubMed: 19135896]
- Cantarel BL, Korf I, Robb SM, Parra G, Ross E, Moore B, Holt C, Sanchez Alvarado A, Yandell M. MAKER: an easy-to-use annotation pipeline designed for emerging model organism genomes. *Genome Res.* 2008; 18:188–96. [PubMed: 18025269]
- Christensen TA, Harrow ID, Cuzzocrea C, Randolph PW, Hildebrand JG. Distinct projections of two populations of olfactory receptor axons in the antennal lobe of the sphinx moth *Manduca sexta*. *Chem Senses.* 1995; 20:313–323. [PubMed: 7552040]
- Crook DJ, Lance DR, Mastro VC. Identification of a potential third component of the male-produced pheromone of *Anoplophora glabripennis* and its effect on behavior. *J Chem Ecol.* 2014; 40:1241–1250. [PubMed: 25432666]
- Couto A, Alenius M, Dickson BJ. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr Biol.* 2005; 15:1535–47. [PubMed: 16139208]
- Datta RD, Vasconcelos ML, Ruta V, Luo S, Wong A, Demir E, Flores J, Balonze K, Dickson BJ, Axel R. The *Drosophila* pheromone cVA activates a sexually dimorphic neural circuit. *Nature.* 2008; 452:473–477. [PubMed: 18305480]
- Dekker T, Ibba I, Siju KP, Stensmyr MC, Hansson BS. Olfactory shifts parallel superspecialism for toxic fruit in *Drosophila melanogaster* sibling, *D. sechellia*. *Curr Biol.* 2006; 16:101–109. [PubMed: 16401429]
- Dreyer D, Vitt H, Dippel S, Goetz B, el Jundi B, Kollmann M, Huetteroth W, Schachtner J. 3D standard brain of the red flour beetle *Tribolium castaneum*: a tool to study metamorphic development and adult plasticity. *Front Syst Neurosci.* 2010; 4:3. [PubMed: 20339482]
- Dubois T, Hajek AE, Smith S. Methods for rearing the Asian longhorned beetle (Coleoptera: Cerambycidae) on artificial diet. *Ann Entomol Soc Am.* 2002; 95:223–230.
- Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004; 32:1792–97. [PubMed: 15034147]
- El-Sayed, AM. [Accessed 18 June 2016] The Pherobase: Database of Pheromones and Semiochemicals. 2016. <http://www.pherobase.com>
- Engsontia P, Sanderson A, Cobb M, Walden KKO, Robertson HM, Brown S. The red flour beetle's large nose: an expanded odorant receptor gene family in *Tribolium castaneum*. *Insect Biochem Mol Biol.* 2008; 38:387–397. [PubMed: 18342245]
- Enjin A, Zaharieva EE, Frank DD, Mansourian S, Suh GSB, Gallio M, Stensmyr MC. Humidity sensing in *Drosophila*. *Curr Biol.* 2016; 26:1352–1358. [PubMed: 27161501]

- Fiala JC. Reconstruct: a free editor for serial section microscopy. *J Microsc.* 2005; 218:52–61. [PubMed: 15817063]
- Fishilevich E, Vosshall LB. Genetic and functional subdivision of the *Drosophila* antennal lobe. *Curr Biol.* 2005; 15:1548–53. [PubMed: 16139209]
- Galizia CG, Sachse S, Rappert A, Menzel R. The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*. *Nature Neurosci.* 1999; 2:473–478. [PubMed: 10321253]
- Ghaninia M, Hansson BS, Ignell R. The antennal lobe of the African malaria mosquito, *Anopheles gambiae* – innervation and three-dimensional reconstruction. *Arthropod Struct Dev.* 2007; 36:23–39. [PubMed: 18089085]
- 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; 46:661–666. [PubMed: 15944133]
- Graves F, Baker TC, Zhang A, Keena M, Hoover K. Sensory aspects of trail-following behaviors in the Asian longhorned beetle, *Anoplophora glabripennis*. *J Insect Behav.* 2016; 29:615–628.
- Haack RA, Hérard F, Sun J, Turgeon JJ. Managing invasive populations of Asian longhorned beetle and citrus longhorned beetle: a worldwide perspective. *Annu Rev Entomol.* 2010; 55:521–546. [PubMed: 19743916]
- Hoaglin DC, Iglewicz B, Tukey JW. Performance of some resistant rules for outlier labeling. *J Am Stat Assoc.* 1986; 81:991–999.
- Hoover K, Keena M, Nehme M, Wang S, Meng P, Zhang A. Sex-specific trail pheromone mediates complex mate finding behavior in *Anoplophora glabripennis*. *J Chem Ecol.* 2014; 40:169–180. [PubMed: 24510414]
- Homborg U, Christensen TA, Hildebrand JG. Structure and function of the deutocerebrum in insects. *Annu Rev Entomol.* 1989; 34:477–501. [PubMed: 2648971]
- Hu J, Angeli S, Schuetz S, Luo Y, Hajek AE. Ecology and management of exotic and endemic Asian longhorned beetle *Anoplophora glabripennis*. *Ag For Entomol.* 2009; 11:359–375.
- Ibba I, Angioy AM, Hansson BS, Dekker T. Macroglomeruli for fruit odors change blend preference in *Drosophila*. *Naturwissenschaften.* 2010; 97:1059–1066. [PubMed: 20972770]
- Keena MA. Pourable artificial diet for rearing *Anoplophora glabripennis* (Coleoptera: Cerambycidae) and methods to optimize larval survival and synchronize development. *Ann Entomol Soc Am.* 2005; 98:536–547.
- Koutroumpa FA, Kárpáti Z, Monsempes C, Hill SR, Hansson BS, Jacquín-Joly E, Krieger J, Dekker T. Shifts in sensory neuron identity parallel differences in pheromone preference in the European corn borer. *Front Ecol Evol.* 2014; 2:65.
- Lacey ES, Moreira JA, Millar JG, Hanks LM. A male-produced aggregation pheromone blend consisting of alkanediols, terpenoids, and an aromatic alcohol from the cerambycid beetle *Megacyllene caryae*. *J Chem Ecol.* 2008; 34:408–417. [PubMed: 18253798]
- Leal WS. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annu Rev Entomol.* 2013; 58:373–391. [PubMed: 23020622]
- Li X, Ju Q, Jie W, Li F, Jiang X, Hu J, Qu M. Chemosensory gene families in adult antennae of *Anomala corpulenta* Motschulsky (Coleoptera: Scarabaeidae: Rutelinae). *PLoS ONE.* 2015; 10:e0121504. [PubMed: 25856077]
- Martin JP, Beyerlein A, Dacks AM, Reisenman CE, Riffell JA, Lei H, Hildebrand JG. The neurobiology of insect olfaction: sensory processing in a comparative context. *Prog Neurobiol.* 2011; 95:427–447. [PubMed: 21963552]
- McKenna DD, Scully ED, Pauchet Y, Hoover K, Kirsch R, Geib SM, Mitchell RF, Waterhouse RM, et al. Genome of the Asian longhorned beetle (*Anoplophora glabripennis*), a globally significant invasive species, reveals key functional and evolutionary innovations at the beetle-plant interface. *Genome Biol.* 2016; 17:227. [PubMed: 27832824]
- McKenna DD, Wild AL, Kanda K, Bellamy CL, Beutel RG, Caterino MS, Farnum CW, Hawks DC, Ivie MA, Jameson ML, Leschen RAB, Marvaldi AE, McHugh JV, Newton AF, Robertson JA, Thayer MK, Whiting MF, Lawrence JF, lipi ski A, Maddison DR, Farrell BD. The beetle tree of life reveals Coleoptera survived end Permian mass extinction to diversify during the Cretaceous terrestrial revolution. *Syst Entomol.* 2015; 40:1–46.

- Meng PS, Trotter RT, Keena MA, Baker TC, Yan S, Schwartzberg EG, Hoover K. Effects of pheromone and plant volatile release rates and ratios on trapping *Anoplophora glabripennis* (Coleoptera: Cerambycidae) in China. *Environ Entomol.* 2014; 43:1379–1388. [PubMed: 25259696]
- Millar, JG., Hanks, LM. Chemical ecology of cerambycid beetles. In: Wang, Q., editor. *Cerambycidae of the world: biology and management*. CRC Press/Taylor & Francis; Boca Raton: 2016. (in press)
- Min S, Minrong A, Shin SA, Sug GSB. Dedicated olfactory neurons mediating attraction behavior to ammonia and armines in *Drosophila*. *Proc Natl Acad Sci USA.* 2013; 110:E1321–E1329. [PubMed: 23509267]
- Missbach C, Dweck HKM, Vogel H, Vilcinskas A, Stensmyr MC, Hansson BS, Grosse-Wilde E. Evolution of insect olfactory receptors. *eLife.* 2014; 3:e02115. [PubMed: 24670956]
- Mitchell RF, Hughes DT, Luetje CW, Millar JG, Soriano-Agatón F, Hanks LM, Robertson HM. Sequencing and characterizing odorant receptors of the cerambycid beetle *Megacyllene caryae*. *Insect Biochem Mol Biol.* 2012; 42:499–505. [PubMed: 22504490]
- Namiki S, Daimon T, Iwatsuki C, Shimada T, Kanzaki R. Antennal lobe organization and pheromone usage in bombycid moths. *Biol Lett.* 2014; 10:20140096. [PubMed: 24759369]
- Nehme ME, Keena MA, Zhang A, Baker TC, Xu Z, Hoover K. Evaluating the use of male-produced pheromone components and plant volatiles in two trap designs to monitor *Anoplophora glabripennis*. *Environ Entomol.* 2010; 39:169–176. [PubMed: 20146854]
- Nehme ME, Trotter RT, Keena MA, McFarland C, Coop J, Hull-Sanders HM, Meng P, De Moraes CM, Mescher MC, Hoover K. Development and evaluation of a trapping system for *Anoplophora glabripennis* (Coleoptera: Cerambycidae) in the United States. *Environ Entomol.* 2014; 43:1034–1044. [PubMed: 24960252]
- Price MN, Delah PS, Arkin AP. FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLoS ONE.* 2010; 5:e9490. [PubMed: 20224823]
- Rambaut, A. FigTree v1.4.0, a graphical viewer of phylogenetic trees. Edinburgh: University of Edinburgh; 2014. Available at: <http://tree.bio.ed.ac.uk/software/figtree/> [Accessed 17 June 2016]
- Reisenman CE, Christensen TA, Hildebrand JG. Chemosensory selectivity of output neurons innervating an identified, sexually isomorphic olfactory glomerulus. *J Neurosci.* 2005; 25:8017–8026. [PubMed: 16135759]
- Rospars JP, Hildebrand JG. Sexually dimorphic and isomorphic glomeruli in the antennal lobes of the sphinx moth *Manduca sexta*. *Chem Senses.* 2000; 25:119–29. [PubMed: 10781018]
- Shimodaira H, Hasegawa M. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol Biol Evol.* 1999; 16:1114–1116.
- Sombke A, Lipke E, Kenning M, Müller CHG, Hansson BS, Harzsch S. Comparative analysis of deutocerebral neuropils in Chilopoda (Myriapoda): implications for the evolution of the arthropod olfactory system and support for the Mandibulata concept. *BMC Neurosci.* 2012; 13:1. [PubMed: 22214384]
- Vosshall LB, Hansson BS. A unified nomenclature system for the insect olfactory coreceptor. *Chem Senses.* 2011; 36:497–498. [PubMed: 21441366]
- Vosshall LB, Stocker RF. Molecular architecture of smell and taste in *Drosophila*. *Annu Rev Neurosci.* 2007; 30:505–533. [PubMed: 17506643]
- Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle M-C, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron.* 2006; 49:833–44. [PubMed: 16543132]
- Waldrop B, Christensen TA, Hildebrand JG. GABA-mediated synaptic inhibition of projection neurons in the antennal lobes of the sphinx moth, *Manduca sexta*. *J Comp Physiol A.* 1987; 161:23–32. [PubMed: 3039128]
- Wanner KW, Nichols AS, Walden KKO, Brockmann A, Luetje CW, Robertson HM. A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. *Proc Natl Acad Sci USA.* 2007; 104:14383–14388. [PubMed: 17761794]

- Wang Y, Chen Q, Zhao H, Ren B. Identification and comparison of candidate olfactory genes in the olfactory and non-olfactory organs of elm pest *Ambrostoma quadrimpressum* (Coleoptera: Chrysomelidae) based on transcriptome analysis. PLoS ONE. 2016; 11:e0147144. [PubMed: 26800515]
- Wickham JD, Harrison RD, Lu W, Guo Z, Millar JG, Hanks LM, Chen Y. Generic lures attract cerambycid beetles in a tropical montane rain forest in southern China. J Econ Entomol. 2014; 107:259–267. [PubMed: 24665709]
- Wickham JD, Millar JG, Hanks LM, Zou Y, Wong JCH, Harrison RD, Chen Y. (2*R*,3*S*)-2,3-octanediol, a female-produced sex pheromone of *Megopis costipennis* (Coleoptera: Cerambycidae: Prioninae). Environ Entomol. 2016; 45:223–228. [PubMed: 26590160]
- Wickham JD, Xu Z, Teale SA. Evidence for a female-produced, long range pheromone of *Anoplophora glabripennis* (Coleoptera: Cerambycidae). Insect Sci. 2012; 19:355–371.

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Fig. 1.

Unrooted phylogram illustrating the OR gene family of *Anoplophora glabripennis*. Pseudogenes are indicated by the suffix PSE, and other suffixes indicate missing exons (see text). Different colors indicate the major groups of coleopteran receptors, with Groups 4–6 depicted as a single radiation. Receptors in black did not correspond to a previously established group. Numbers on nodes indicate Shimodaira-Hasegawa support values >0.90.

Fig. 2.

Unrooted cladogram illustrating the relationships among known ORs from five beetle families: Cerambycidae (*Anoplophora glabripennis*, “Agl”, red); Chrysomelidae (*Ambrostoma quadriimpressum*, “Aqua”, teal), Scarabaeidae (*Anomala corpulenta*, “Acor”, pink), Curculionidae (*Dendroctonus ponderosae*, “Dpon”, yellow), and Tenebrionidae (*Tribolium castaneum*, “Tcas”, blue). Receptor sequences with <100 amino acids were excluded from the analysis. Three additional receptors, coded in black, have been functionally characterized from the longhorned beetle *Megacyllene caryae* (Cerambycidae). Colors and numbers around the perimeter indicate the seven major groups of coleopteran

receptors, but with Groups 4–6 labeled only in *Tribolium*. Large expansions of receptors have been collapsed to improve clarity and are color-coded by beetle family. Numbers alongside major nodes indicate Shimodaira-Hasegawa support values.

Fig. 3.

Abundance and sex bias of ORs in male and female *Anoplophora glabripennis*, as measured by the presence of unique reads of each receptor in the transcriptome. Reads present in males and females are reported according to their normalized log ratio. The solid line above the x-axis is the mean of the distribution of ratios, and dashed lines are two standard deviations above and below the mean. Solid circles are receptors exceeding two standard deviations. Small boxes at the edge of the y-axis include reads that were present only in females (above) or males (below). Prominent receptors are indicated by the number corresponding to their gene name (e.g., 16 = AglaOR16).

Fig. 4. Glomeruli in the left AL of adult *Anoplophora glabripennis*, with orientation given relative to the neuraxis (*A*, anterior; *P*, posterior; *D*, dorsal; *V*, ventral; *M*, medial; *L*, lateral; *AN*: antennal nerve). Ventral sections of the lobe (**A**) were more distinct than the deeper, dorsal sections (**B**). Eighteen named glomeruli (colored) are mapped onto three-dimensional reconstructions of the ALs of a male (**C**) and a female (**D**). Reconstructions are dorsoventrally doubled in length to better visualize the glomeruli.

Fig. 5.

Volumes of glomeruli in four female (**A**) and four male (**B**) *Anoplophora glabripennis*. The grey box encompasses the first and third quartiles and the median is indicated by a solid horizontal line. Vertical lines extending from the box indicate 90th and 10th percentiles. Dashed horizontal lines indicate three times the interquartile range. Glomeruli exceeding the 90th percentile in size are indicated as black circles.