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The red flour beetle's large nose: An expanded odorant receptor gene family in *Tribolium castaneum*

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Abstract

The *Tribolium castaneum* genome sequence reveals a large number of odorant receptor (Or) genes compared to those found in other insects whose olfactory genomes have been studied—341 Or genes and pseudogenes, encoding 259 intact odorant receptor proteins. An RT-PCR study of larvae and adults revealed that only 145 (64%) of 233 genes with successful genomic DNA amplifications were expressed. No expression of the other 87 genes was detected at any age, suggesting either that these genes are not expressed in this particular strain, or that they are induced only in certain environmental or developmental conditions. *TcOR1*, the ortholog of the *Drosophila Or83b* (*DmOr83b*) gene, which is required for the function of olfactory receptor proteins in *Drosophila*, was expressed in extracts from adult and larval heads and in extracts from adult bodies. Expression of 41 TcOr genes was detected in extracts from larval head tissue and 111 in extracts from adult head tissue (both figures exclude *TcOr1*). Twenty-eight TcOrs were detected only in adult bodies. Beetle pupae were injected with *TcOr1* dsRNA; unlike sham-injected and control beetles, these knock-down beetles showed no significant response to the *Tribolium* aggregation pheromone, supporting the hypothesis that *TcOr1* plays a similar decisive role in olfaction to *DmOr83b*. The substantial number of Ors poses the question of why *Tribolium* has such a large olfactory receptor repertoire, and underlines the need for more studies of the natural history of this species.

Keywords: *Tribolium*; Insect; Odorant receptor; Olfaction; Behaviour; Olfactory genome; Smell; Aggregation pheromone

1. Introduction

Olfaction represents one of the key interfaces between animals and the environment, as the organism uses this sense to detect stimuli that provide it with information about food, predators and potential mates. Studying the genes that code for olfactory receptor proteins in different species provides an insight not only into the evolutionary relationships between those organisms but also into how organism–environment interactions have evolved. As each of the major insect genomes have been completed, particular attention has focused on the olfactory genome. *Drosophila melanogaster* has 62 odorant receptor (Or) genes (Robertson et al., 2003), while there are around 80 in the mosquito *Anopheles gambiae* (Hill et al., 2002), 131 in *Aedes aegypti* (Bohbot et al., 2007) and substantially more—around 170—in the honey bee, *Apis mellifera* (Robertson and Wanner, 2006). An incomplete analysis of the genome of the silkworm, *Bombyx mori*, revealed 48 Or genes, suggesting that the final number will be higher (Wanner et al., 2007).

The relation between Or sequence and function is the focus of intense research (e.g. Keller et al., 2007), and no functional orthologs of Ors have yet been established across species, even for 12 closely related species of *Drosophila* (Nozawa and Nei, 2007). One set of striking exceptions are the orthologs of the *Drosophila Or83b* gene. The OR83B protein does not appear to encode an olfactory receptor *per se*, but in *D. melanogaster* acts as a co-factor

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enabling the OR protein to integrate correctly into the cell membrane (Benton et al., 2006). *Or83b* orthologs have been shown to be highly conserved in all insect olfactory genomes thus far studied (Krieger et al., 2003), and can retain their function across 250 million years of evolution when expressed in *D. melanogaster* (Jones et al., 2005) although there is as yet no direct proof of their function *in vivo* in any other species apart from *D. melanogaster*.

Tribolium castaneum offers the possibility of genetic manipulation through RNAi (Bucher et al., 2002) and transposon-mediated transformation (Lorenzen et al., 2003; Pavlopoulos et al., 2004). Coupled with its position as a representative of the largest order of eukaryotic organisms—the beetles—these techniques make *T. castaneum* an excellent model for testing the current hypotheses regarding the genomics and neurobiology that underlie olfactory behaviour. As a first step towards the study of the neurobiology and evolutionary biology of *Tribolium* olfaction, we describe the *T. castaneum* olfactory genome, provide a preliminary analysis of the range of tissues in which Or gene expression can be detected using RT-PCR and make an initial investigation of the genetic bases of olfactory function in this species.

2. Materials and methods

2.1. Bioinformatics and phylogenetics

These were essentially the same as for the *D. melanogaster* (Robertson et al., 2003), *An. gambiae* (Hill et al., 2002), *B. mori* (Wanner et al., 2007), *A. mellifera* (Robertson and Wanner, 2006) and *Ae. aegypti* (Bohbot et al., 2007) Or gene families, except that gene fragments were given names in the TcOr numerical series. Briefly, gene models were built in the text editor of PAUP*v4.0b10 (Swofford, 2001) using these other insect Ors as guides plus the Splice Site Prediction by Neural Network server at the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html) to help identify intron splice sites. The manually curated gene models were compared with those generated by automated pipelines for the main genome paper (Tribolium Genome Consortium, submitted) and updated versions will be submitted to GenBank at NCBI therewith. Phylogenetic sequence similarity analysis used a reduced representative set of DmOR, AgOR, and AmOR proteins to provide context and multiple alignment in CLUSTALX (Jeanmougin et al., 1998) using default settings. Corrected distances were obtained using the maximum likelihood models in TreePuzzle v5.2 (Schmidt et al., 2002) with the BLOSUM62 amino acid exchange matrix and otherwise default settings. A minimum evolution tree was generated using the heuristic search with tree-bisection-and-reconnection branch swapping in PAUP*v4. Branch support was determined from 1000 replications of uncorrected distances. The DmOR83b/TcOR1 phylogenetic tree presented in Fig. 2 was manually generated using ClustalW (http://www.ebi.ac.uk/clustalw/) and Tree Editor (http://evolve.zoo.ox.ac.uk/software/TreeEdit/main.html). DmOR83 homologues were BLAST identified using http://flybase.bio.indiana.edu/blast/.

2.2. T. castaneum husbandry

Stock cultures of *T. castaneum* were a kind gift from the Natural Resources Institute, The University of Greenwich at Medway, Kent, UK. The stocks were originally from Ghana and have been cultured in the UK since 1996. Beetles were maintained in a growth chamber at 30 °C under a 12:12 light:dark phase and were reared in transparent plastic boxes on 95% organic whole wheat flour, seeded with 5% by weight brewer's yeast, without overcrowding.

2.3. Extraction of total RNA and genomic DNA

For RNA extraction males and females of larval stages 4 and 5 were combined together (approximately 15–20 days old) and 1-week-old adults were used. Heads and bodies were removed and placed immediately in liquid nitrogen and total RNA extracted for RT-PCR analysis. Briefly, 100 mg of adult and larval heads and bodies, without heads, were flash frozen by placing them into liquid nitrogen, after which total RNA was extracted in TRI reagent according to the manufacturers protocol (Sigma). Total RNA was treated with Turbo DNase (TURBO DNA-free, Ambion) for 30 min at 37 °C to eliminate genomic DNA. RNA was quantified using a Nanodrop ND-1000 (Nanodrop) and 4 μg of RNA was used in a 20 μl cDNA synthesis reaction. Total RNA was reverse transcribed using oligo $(dT)_{20}$ primers and cDNA synthesised using ThermoScript RT, according to manufacturers instructions (Invitrogen). For genomic DNA extraction, 25 pupae were used for the extraction using a modified *D. melanogaster* DNA extraction protocol (Bender et al., 1983).

2.4. PCR amplification of odorant receptors

Two microlitres of each cDNA or genomic DNA sample were used in 25 μl PCR reactions consisting of 0.2 mM of each gene specific primer, 0.2 mM dNTPs and standard polymerase buffer and Taq (Bioline). PCR conditions varied according to primer pairs but generally the programme used was: 2 min at 94 °C, 35 cycles of 94 °C for 30 s, 55–60 °C for 40 s and 72 °C for 30 s, followed by an incubation for 1 min at 72 °C. Amplicons were run on 2.0% agarose and ethidium bromide gels and sized according to the hyperladder IV (Bioline) DNA marker and visualised using a Gene Flash gel documentation system (Syngene Bioimaging). For the purpose of the detection of amplicons, imaging was standardised so that no bias in band presence was created across gene subfamilies. The primer sequences used can be found in Supplementary data. Predicted sizes of cDNA or genomic DNA amplicons can

be found in Supplementary data. Predicted odorant receptor gene regions were analysed to establish exon/intron borders. From this, primer sets were designed to amplify bands of differing sizes for genomic and cDNA-derived amplicons, establishing whether there was genomic DNA contamination in our total RNA preparations. The smallest intron that could easily be detected on an agarose gel was selected and primers designed around it so that the amplicon was approximately 400 bp. The expression pattern of each gene was studied 3–5 times. For a gene to be classified as detected in a particular tissue extract, bands had to be visualised in at least two replicate PCRs. Full details of the number of times each band was detected in a given tissue are given in Supplementary information: Table S1.

2.5. RNAi procedures

The procedure adopted followed that outlined in Bucher et al. (2002). Briefly, *TcOr1* was sub-cloned into the Stratagene pSC-A strataclone vector. The resultant pSC-A TcOr1 plasmid was linearised and *in vitro* transcribed using T3 and T7 RNA polymerases. Single-stranded sense and anti-sense strands of RNA from each *in vitro* reaction were combined, denatured at 100 °C and allowed to cool to room temperature slowly over 4 h, allowing annealing. ds-RNA was quantified by a Nanodrop spectrophotometer to be approximately 1 μg/μl. Final phase pupae of both sexes were taken from breeding boxes, cleaned of excess flour using a small paintbrush and placed in a petri dish. The pupae were then fixed to a glass slide by adhesive, and approximately 0.8 μl of injection buffer containing phenol red, or *TcOr1* ds-RNA plus injection buffer with phenol red was injected into the abdomen using a glass needle and a micro-manipulator. Testing took place when adult beetles were 3–5 days old, or at least 1 week after injection.

2.6. Behavioural measures

A simple plastic olfactometer, 15 cm long and 0.7 cm in diameter, was used to measure olfactory responses. The bottom of the olfactometer was covered with filter paper, which was changed after each trial. A single *T. castaneum* adult aged at least 5 days old was introduced into the middle of the tube, and an average of 24.25 (±1.23) mg of the *T. castaneum* volatile aggregation pheromone, 4,8-dimethyldecanal (DMD) (Pest Patrol, from Insects Limited, Indiana, USA) was placed at one end, separated from the main olfactometer area by a thin sheet of gauze. Although DMD is not produced by mature females (Arnaud et al., 2002), both sexes were used, as both respond to DMD (Obeng-Ofori, 1991). The beetle was allowed to migrate in the olfactometer for 5 min and its position was noted every 30 s. The amount of time each beetle spent on the odour side and the non-odour side was calculated. Ten wild-type beetles were tested, 16 beetles that had been injected with RNAi buffer ("sham") and 16 RNAi *TcOr1* knock-down beetles. All data were tested against the null hypothesis (equal time on each side), using one-sample *t*-tests.

3. Results

3.1. Genomic analysis

Three hundred and forty-one genes were annotated in the Or gene family, including 79 pseudogenes (of which 37 are considered to be gene fragments because they encode less than half of the normal length of an Or), yielding 259 intact full-length Ors. Sequence similarity trees are presented in Figs. 1a–c. Most TcOrs form six lineage-specific subfamily expansions (labelled 1–6 on Figs. 1a–c) relative to the other insect Ors, ranging in size from the tiny set of *TcOr167* and three related pseudogenes (labelled 3a) to an expansion of 150 genes in Fig. 1c (labelled 4–6). The vast majority are in tandem gene arrays in the genome. The tree is rooted with the *DmOr83b* lineage which is known to be highly conserved in all insects that have been studied (Krieger et al., 2003; Jones et al., 2005) and apparently serves a role as chaperone/heterodimer with all the other OR proteins (Benton et al., 2006). Following Krieger et al. (2003) the *Tribolium* ortholog is named *TcOr1*; it shares 66% amino acid identity with *DmOr83b*.

Representative Ors from other insect species are included on Figs. 1a–c, but none of their lineage-specific expansions. A central clade of TcOrs (labelled 3 on Fig. 1b) nestles within Or genes from *Aedes, Heliothis, Apis* and *Drosophila*. The position of this clade suggests that these may be the oldest of the TcOrs, while the substantial expansion of clades 4–6 is relatively distant from the Ors of any other insects that have been thus far studied. As with the honey bee, there are a few highly divergent Or singletons or pairs, e.g. *TcOr74PSE*, *TcOr275FIX*, *TcOr46/64*, *TcOr65/66*, and *TcOr71PSE/72* which show no close relationships to other Ors.

Compared to 23 other endopterygotan *DmOr83b* orthologs, TcOr1 is the closest to the outgroup we used for our analysis, the *DmOr83b* ortholog from the human louse, *Pediculus humanus* (Exopterygota = Hemimetabola) (Fig. 2). However, most of the species for which sequences are available are dipterans, with only two hymenopterans and six lepidopterans, providing an unrepresentative sample of the Endopterygota.

3.2. Expression patterns of TcORs using RT-PCR

Our bioinformatic analysis predicted 259 intact and presumably functional odorant receptor genes. We made a preliminary investigation of the expression patterns of all 259 genes using RT-PCR on RNA extracted from both adult and larval tissue, with head and bodies separated for each stage. Fig. 3 gives examples of the variety of expression patterns we detected.

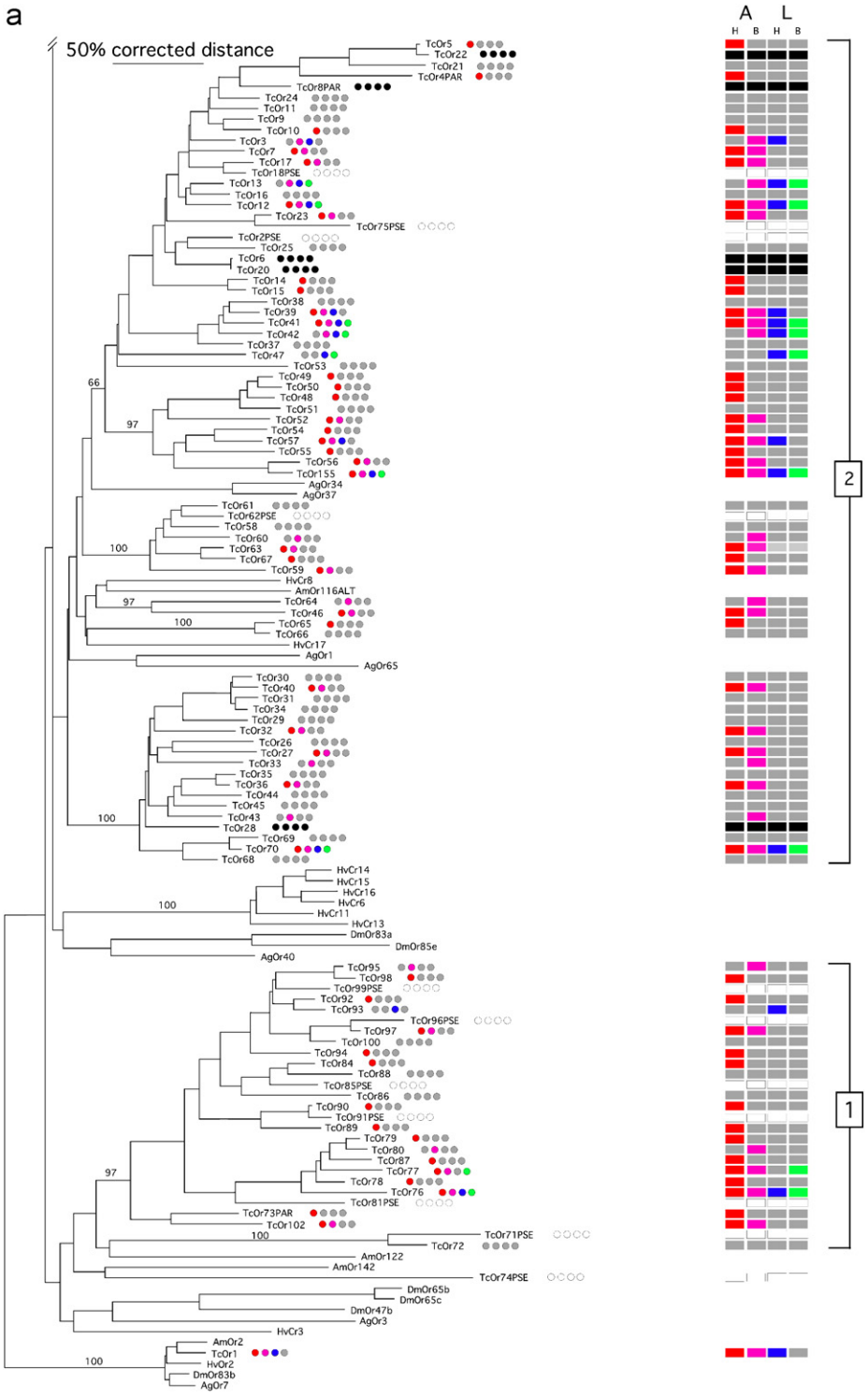

Fig. 1. (a) Sequence similarity tree of 341 TcOR proteins (excluding fragments) together with *Drosophila*, moth and *Apis* OR proteins. The expression pattern of each gene, as determined by RT-PCR, is given in the grid on the right-hand side, and in blobs after each gene name. The corrected distance tree was rooted by declaring the DMOR83b protein and its orthologs as the outgroup. Scale bar shows 50% of corrected distance (corrected for amino acid substitutions) obtained using the maximum likelihood models in TreePuzzle v5.2 (Schmidt et al., 2002). Representatives of major insect Or clades are also presented, but for the sake of clarity, not all proteins from other species are presented. Numbers give bootstrap support for TcOR lineages. PSEU to the right of a gene name indicates it is a pseudogene with one or more in-frame stop codons, frame-shifting indels, or unacceptable intron splice sites. Expression patterns for each gene, as determined by RT-PCR on RNA extracted from adult (A) and larval (L) head (H) and body (B) tissues are indicated as follows: grey = no expression detected; colour = expression detected in that tissue; black = no amplification possible; white = pseudogene). For ease of study, expression in each tissue is indicated by a different colour; for example, expression in adult head is indicated by red, in adult body by magenta. For indicative analysis, TcORs are grouped into *ad hoc* clades, as indicated by right-hand bar and numbers.

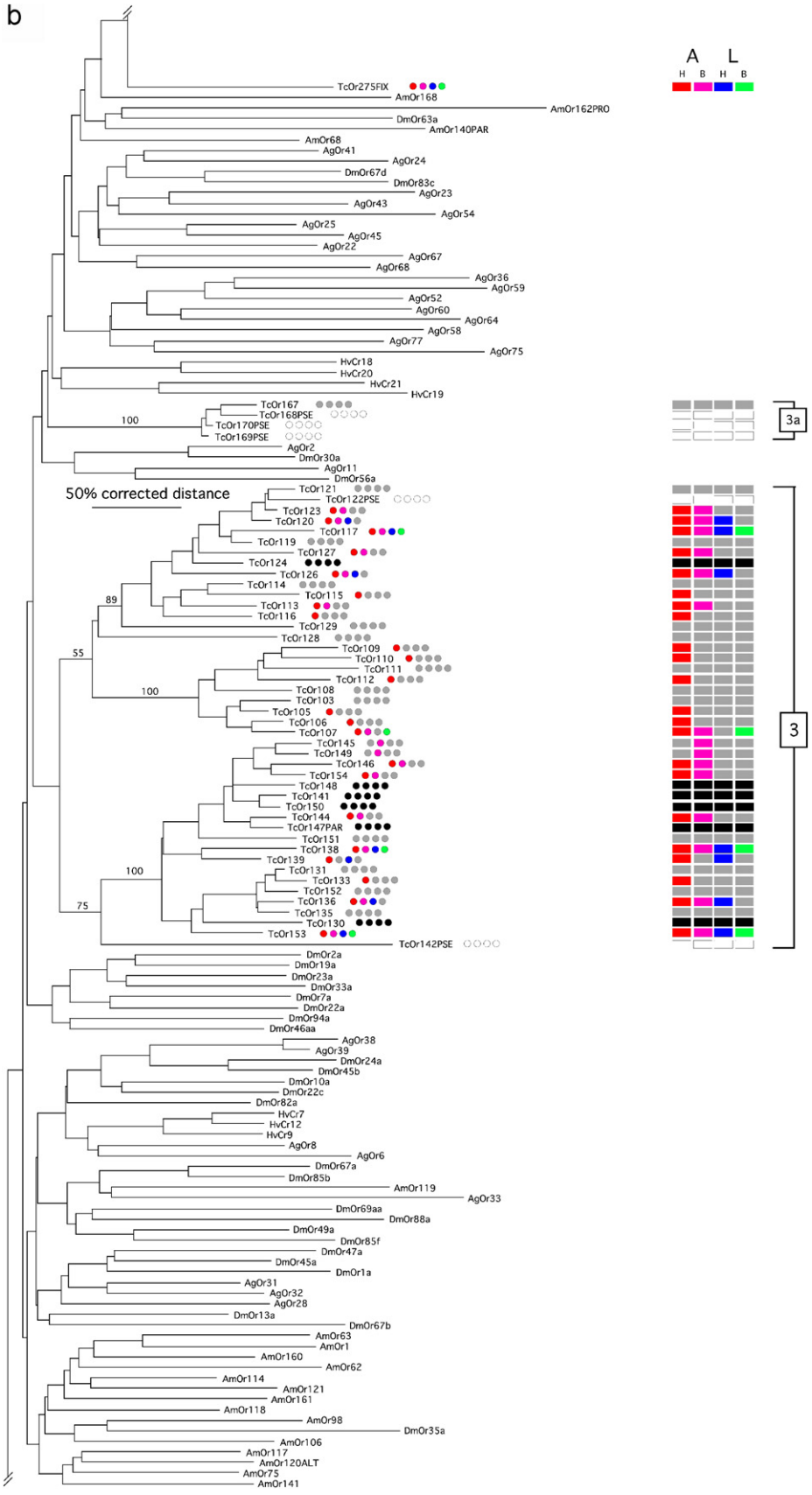

Fig. 1. (*Continued*)

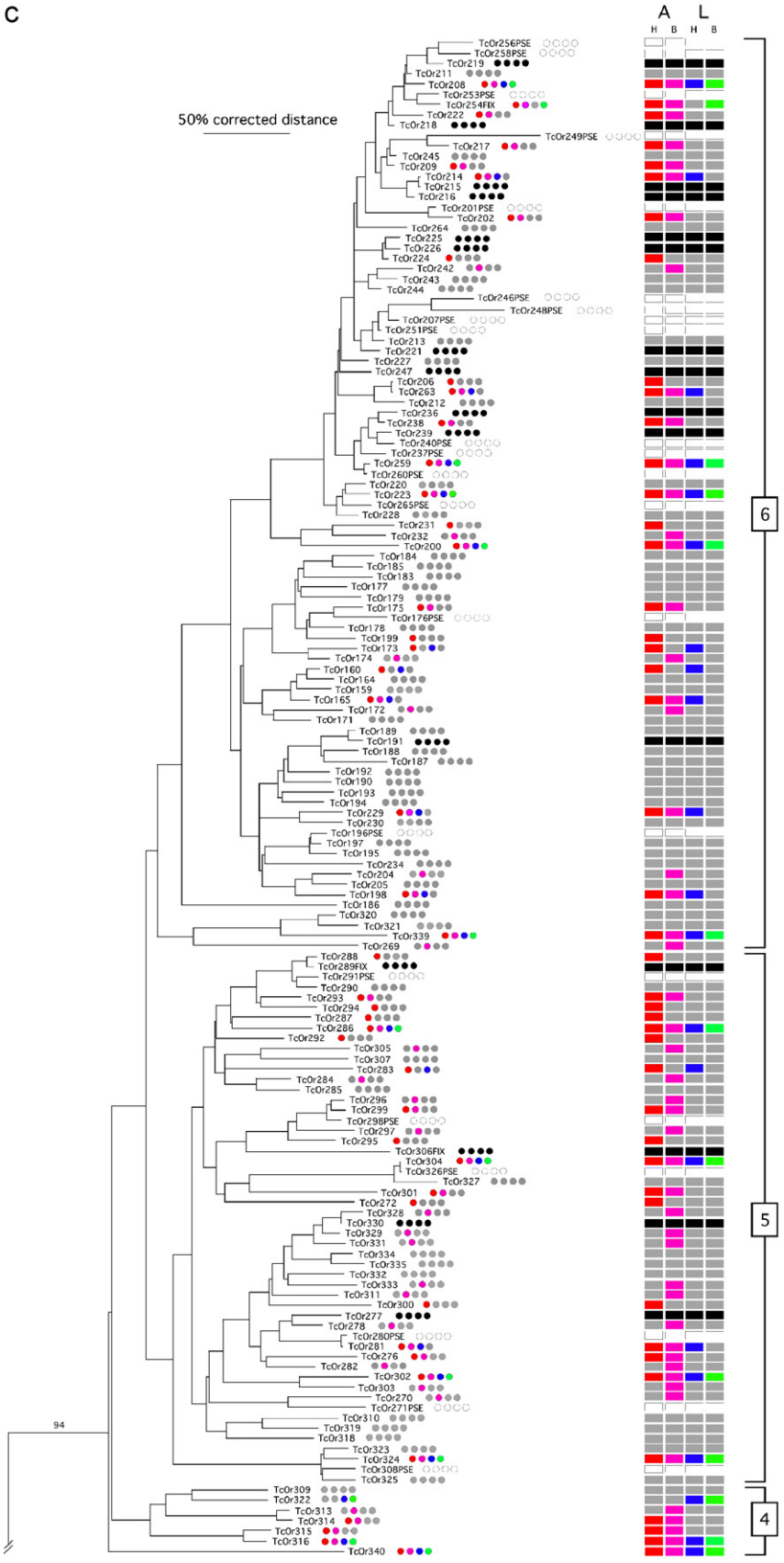

Fig. 1. (*Continued*)

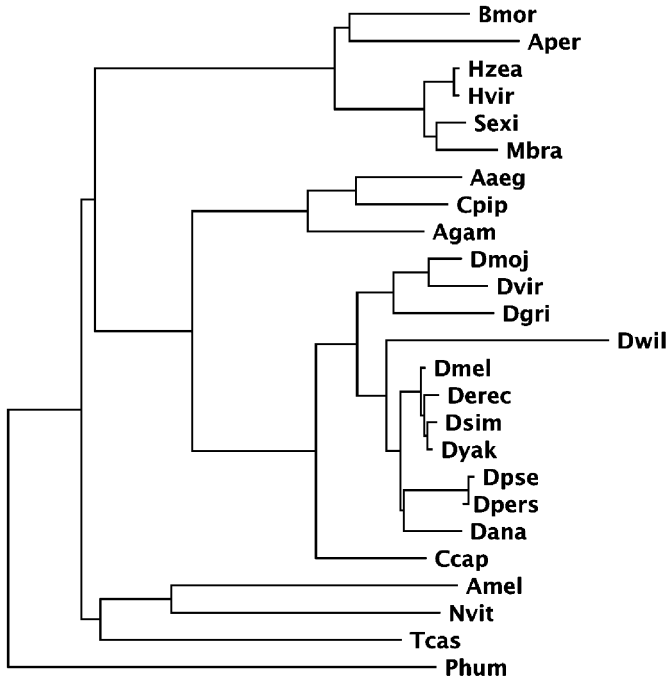

Fig. 2. TcOR1/DmOR83b ortholog phylogenetic tree. Showing the relationship between protein sequences of the family members plotted using Tree Editor v1.0a10 and ClustalW. The TcOR1/DmOR83b ortholog of the human body louse *Pediculus humanus* (Phum) (Exopterygota = Hemimetabola), has been placed at the root of the tree. See Supplementary data: Fig. 1 for ClustalW alignment of all sequences represented here.

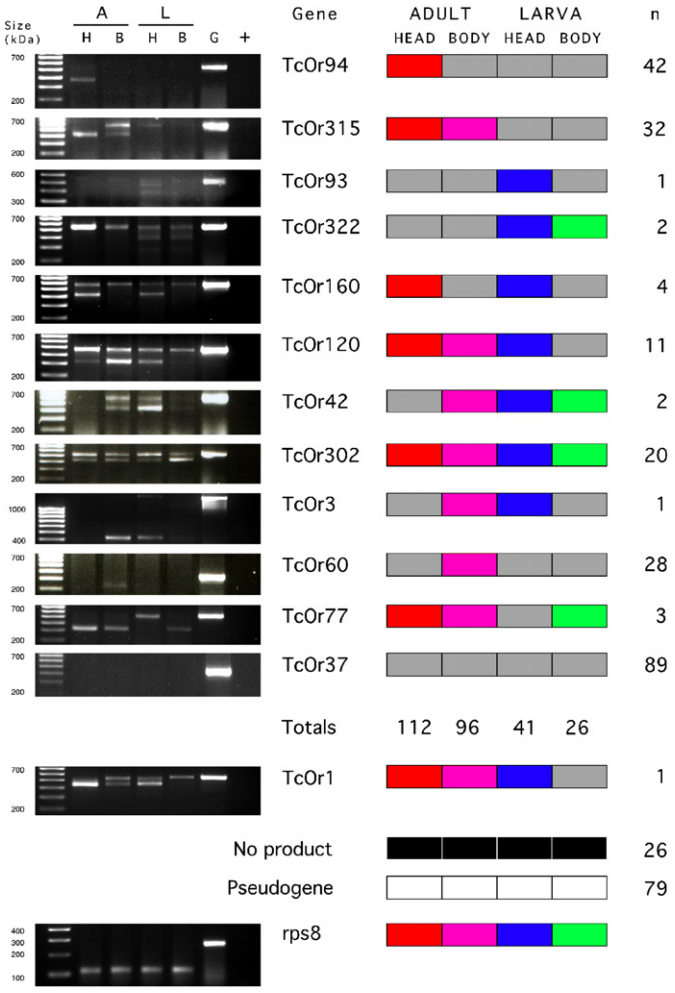

Fig. 3. Representative results of *Tribolium castaneum* Or gene expression patterns detected by RT-PCR on RNA extracted from larval and adult head and body tissues, as shown in Figs. 1a–c. Left lane = size ladder (in kD); A = adult, L = larva, H = head, B = body; G = genomic DNA, + = water control. One gel is shown for each of the 12 classes of expression patterns shown in Figs. 1a–c. Coloured bars show expression patterns; grey = not expressed; colour = expressed in that tissue; black-no primers could be designed or no product was detected in genomic DNA (see text); white = pseudogenes. *n* = the number of genes showing a given expression pattern. Rps8 = ribosomal protein s8, used as a control. The *TcOr93* gel shows expression in the adult body but this gene is not classified as expressing in this tissue; this was the only time out of four replicates that this band was visualised in extracts from the adult body. For a gene to be classified as expressing, it was required to be detected at least twice (see Section 2).

The expression data were plotted onto the sequence similarity tree (Figs. 1a–c), and are also presented in order of gene number (see Supplementary data: Table S1); a summary of the expression patterns and the number of genes showing each profile is given in Fig. 3. Twelve genes (TcOr20, TcOr191, TcOr215, TcOr216, TcOr218, TcOr225, TcOr226, TcOr236, TcOr239, TcOr241, TcOr289 and TcOr330) showed very high levels of sequence similarity, presumably reflecting recent duplication events, and we were unable to design primer sets that could discriminate between them. No expression data are available for these genes. In a further 14 genes (TcOr6, TcOr8, TcOr28, TcOr124, TcOr130, TcOr141, TcOr147, TcOr148, TcOr150, TcOr219, TcOr221, TcOr247, TcOr277, TcOr306) we were unable to obtain any amplicons for cDNA or genomic DNA. The primers may have been inappropriate in some unknown way, or these genes may show intraspecific variability in the primer binding sites we targeted.

Two hundred and thirty-three genes (including TcOr1) were amplified from genomic DNA extracts, but only 146 (plus TcOr1) could be amplified from cDNA. Of the 87 genes, which we report as having no expression, 14 were observed to produce a band in at least one extract in one of the 3–5 replications (see Supplementary information). Because this fell below our expression threshold of visualising a band in at least two replicates (see Section 2), these genes were still classified as showing no expression. To control for developmental effects in expression pattern, we made single-strand cDNA from first instar larvae and attempted to amplify the 87 genes for which we found no expression, together with *TcOr1* and a handful of genes that showed larval-specific expression. We found no differences with the data shown in Figs. 1a–c (data not shown). We conclude that the 87 genes shown with four grey boxes in Figs. 1a–c are not expressed under our laboratory conditions.

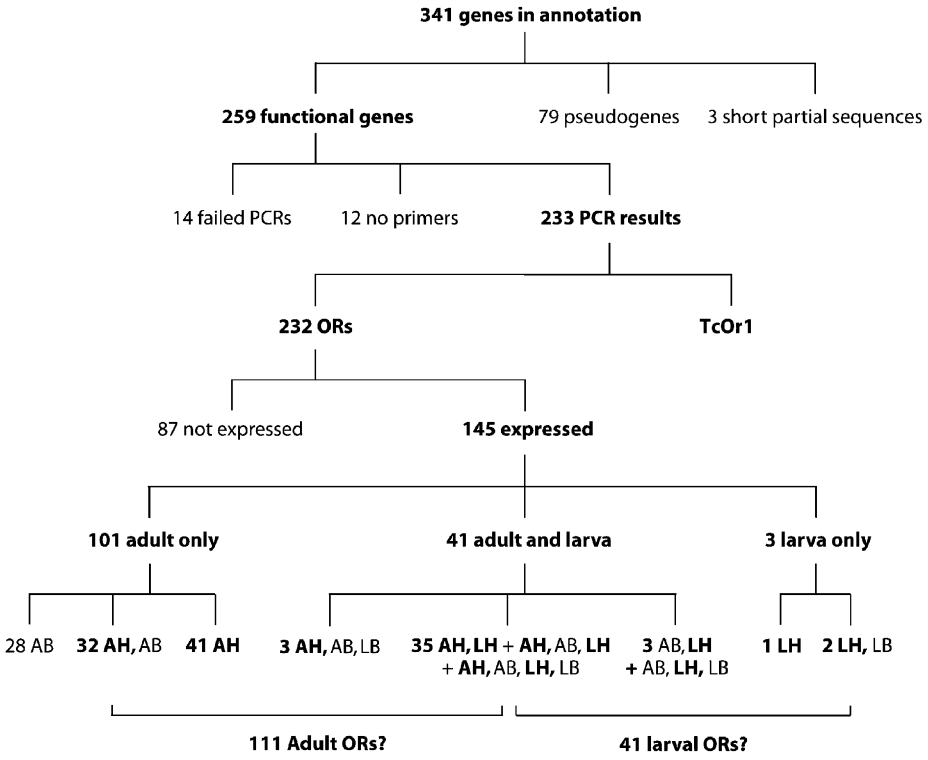

Fig. 4. Flow diagram of gene numbers represented in each class between TcOr gene family. *TcOr1* is assumed not to directly detect odours, so is treated separately. Putative functional olfactory receptors are represented in bold. AH = adult head; AB = adult body; LH = larval head; LB = larval body.

TcOr1 was found to express in adult and larval head tissue, and in adult body tissue (Fig. 3). We observed 28 genes that were detected only in RNA extracted from adult body tissue; 42 genes were expressed only in RNA extracted from head tissue from adults (41) or larvae (1). The remaining 75 Or genes were expressed in both head and body extracts. Twenty genes were detected in all extracts. Assuming that *TcOr1*, like *Drosophila Or83b*, plays a structural role in forming the olfactory receptor (Benton et al., 2006), rather than directly detecting odours, the maximum number of functional ORs in this species would be 145. If olfactory sensory neurons are restricted to head tissues, this figure would be 117; 111 in the adult, 41 in the larva, with 35 ORs shared between the two stages. Fig. 4 summarises this analysis. There is no obvious patterning to the distribution of these expression patterns on the sequence comparison tree (Figs. 1a–c).

3.3. RNAi of TcOr1 to determine function

To test the assumption that the high degree of conservation of *TcOr1* reflects conserved function (Fig. 2 and Supplementary data: Fig. S1), and that this gene acts as a homologue of *DmOr83b*, we attempted to block the expression of *TcOr1* using RNAi, following the technique of Bucher et al. (2002). Fig. 5 shows that both wildtype and sham-operated (saline-injected) beetles showed a significant preference for the aggregation pheromone ($t_9 = 2.41$, $p = .039$, $t_{15} = 3.11$, $p = .007$, respectively), while the behaviour of *TcOr1* knock-down beetles was not significantly different from a random distribution ($t_{15} = .896$, $p =$ n.s.). This strongly suggests that *TcOr1* knock-down

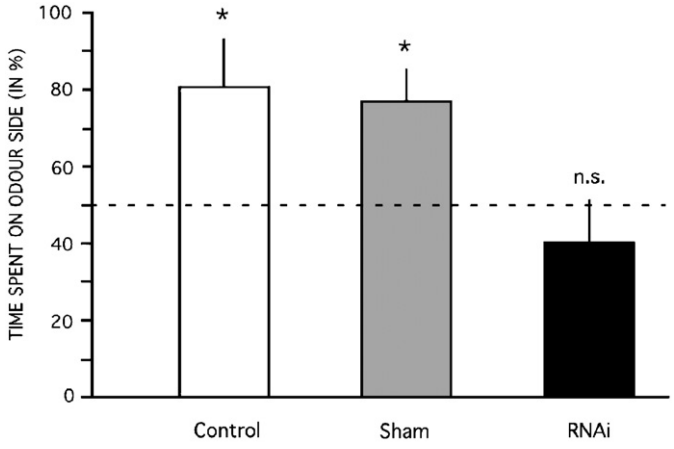

Fig. 5. Aggregation pheromone response to wild-type and *TcOr1* RNAi adult animals. Mean responses to the *Tribolium castaneum* aggregation pheromone, DMD. Single beetles were allowed to migrate in a linear olfactometer for 5 min. The amount of time spent on either side of the apparatus was noted. Dotted line = 50% (null hypothesis). Error bars = s.e. Control = no injection; Sham—injected only with buffer. RNAi—injected with buffer + *TcOr1* ds-RNA. Adult beetles were tested at least 1 week after injection into the pupa. For full details, see Materials and methods. * = different from null hypothesis at $p < .05$; n.s. = not significantly different from the null hypothesis. For full details of procedures, see Section 2.

beetles were unable to detect the aggregation pheromone because of their inability to smell, due to the absence of TcOr1 protein.

4. Discussion

The olfactory genomic data presented here are surprising on three inter-related counts. Firstly, the unexpectedly large number of apparently functional TcOr genes identified in our bioinformatic analysis—259—is substantially greater than that found in any other insect thus far studied (*D. melanogaster* has around 60 Ors, the mosquito *An. gambiae* has about 80, the mosquito *Ae. aegypti* has 131, while the honey-bee *A. mellifera* has around 160—Robertson et al., 2003; Hill et al., 2002; Bohbot et al., 2007; Robertson and Wanner, 2006, respectively). Second, under our laboratory conditions not all these genes appear to code for functioning olfactory receptor proteins: the results of our RT-PCR expression study suggest that around 37% (87/232) of the TcOr genes are not expressed in any tissue. This proportion is over double that seen in *Ae. aegypti* (Bohbot et al., 2007); no unexpressed Ors are found in *D. melanogaster* (Robertson et al., 2003). Finally, 28 genes were detected only in extractions from the adult body, and a further 69 were detected in the adult body as well as in the adult or larval head.

The genes that we failed to detect may be present in such low levels—for example, in a single OSN on each individual—that they fell below the threshold sensitivity of our system. Another possibility is that these genes are non-functional because of undetected mutations in regulatory regions or outside their open reading-frame. Finally, some or all of these genes may be conditionally expressed only under certain developmental or environmental conditions. However, none of the "non-expressed" genes was detected in RNA extracted from first instar larvae (data not shown), suggesting that expression patterns in at least two larval stages are constant. The distribution of "non-expressed" TcOrs appears to be random across the major clades shown in Figs. 1a–c. There is no significant difference in the frequency of non-expressed genes in what may be the most ancestral clade of Ors (labelled 3 on Fig. 1b) (12/36) and that seen in what appears to be one of the most recent expansions (labelled 4–6 on Fig. 1c) (47/111) ($\chi^2 = 1.06$, $p =$ n.s.). We suggest that these levels of non-expression may indicate either that these genes represent a relatively recent adaptation and are induced in environmental conditions that did not apply in our laboratory, or that they are expressed at a particular age which we did not study.

Twenty-one of the 28 genes showing expression only in the adult body were found in clades 4–6. This frequency (21/112) was not significantly different from that seen in clade 3 (2/37) ($\chi^2 = 3.79$, $p = 0.051$), suggesting that these genes are randomly distributed. The frequency of TcOr detection in apparently non-olfactory tissue (i.e. the adult body) may indicate either that chemosensory structures can be found on or in the adult body (including in sperm—e.g. Spehr et al., 2003), as well as on the adult and larval head, or that these genes have a non-chemosensory function in the body. Finally, 23% (79/341) of the annotated *T. castaneum* Or genes were pseudogenes. In comparison, there are no pseudogenes in the *D. melanogaster* olfactory genome (Robertson et al., 2003) while 16% (12/131) of the candidate Ors in the *Ae. aegypti* genome appear to be pseudogenes (Bohbot et al., 2007).

Assuming that "classic" olfactory receptors are expressed in head tissue (i.e. where the antennae are housed), we suggest that *T. castaneum* has 111 adult Ors, 41 larval Ors, with only six Ors being specific to the larval stage, and 76 adult-specific Ors (Fig. 4). 35 Ors are shared by the two stages. The proportion of larval-specific TcOrs (6/145) is much lower than that seen in *D. melanogaster* (10/61—excluding *DmOr83b*; Robertson et al., 2003) and presumably indicates that there are very few ecological differences in the life-styles of the two stages of *T. castaneum*. In the only other insect species for which larval data have been fully explored, *Ae. aegypti*, in which the larval stage is aquatic, 15/100 functional Ors are thought to be larval specific (Bohbot et al., 2007). To accurately determine which TcOrs are expressed in which olfactory tissues, *in situ* hybridisation will be required. Our extraction of RNA from adult and larval heads provides an initial, upper estimate of the number of TcOr genes that may be expressed in olfactory tissues. Finally, these figures may constitute an over-estimation, as male- and female-specific expression patterns may further reduce the number of functional Ors in either sex—such effects have been observed in the silkworm, *B. mori* (Wanner et al., 2007).

The numbers of adult and larval Ors carried on the head that we identify here (111 and 41, respectively) are close to the number of antennal lobe glomeruli that have been reported in *T. castaneum* adults and late larvae (Schachtner et al., personal communication), suggesting that *Tribolium* will correspond to the apparent tendency in animals for each glomerulus to receive projections from olfactory receptor neurons expressing a single kind of Or gene, and therefore for there to be roughly equal numbers of Or genes and glomeruli. However, in the two mosquitoes that have been investigated (*An. gambiae* and *Ae. aegyptiae*), the number of functioning Ors appears to be substantially greater than the number of glomeruli found in the antennal lobes of these mosquitoes (Hill et al., 2002; Ignell et al., 2005; Bohbot et al., 2007; Ghaninia et al., 2007). This suggests that our understanding of the neuronal organisation of olfactory systems is still incomplete.

All of the insects for which extensive Or molecular genetic data exist are members of the Endopterygota (= Holometabola) (*Apis*, *Anopheles*, *Aedes*, *Drosophila*, *Heliothis* and now *Tribolium*). These Endopterygotan insect orders are estimated to have had a common ancestor around 300 Myr ago (Robertson, 2005; Gaunt and Miles, 2002), providing ample time for substantial evolutionary change in sets of genes that are at the interface of the

organism and the environment, and will be susceptible to selection pressure from changing ecological conditions. Because of its position within a large series of sets of Ors from other insect species, we assume that the central clade of TcOrs (labelled 3 on Fig. 1b) is the oldest of the groups of TcOrs we have detected. Using a similar logic, clades 1 and 2, which are interspersed with Ors from the other insect species included on the tree (*Apis, Anopheles, Drosophila* and *Heliothis*), are assumed to be of intermediate age, while the most divergent group of TcOrs, compared both to the remainder of the *Tribolium* Ors and those of other insects, is made up of the large beetle-specific expansion constituted by clades 4–6. We assume that these last three clades make up the most recently evolved set of Ors. Testing these hypotheses will require further molecular investigation of *T. castaneum*—for example by looking for intraspecific variation, which should be lower in more recent clades, and also by comparing TcOrs with those of the closely related species, *T. confusum.*

The phylogeny of DmOR83b and its orthologs (Fig. 2) reflects overall trends in insect evolution—the chosen outgroup (*P. humanus*—Exopterygota) differs substantially from the Endopterygota present in the main clade. However, most of the species we analysed are Dipterans, which would tend to bias the result in favour of such a phylogenetic representation. Inclusion of more exopterygotan insects, as well as of more distantly related endopterygotan orders such as Odonata or Ephemeroptera would clarify whether or not the orthologs of this gene, which is presumed to have a conserved function and therefore to be under high selection pressure, shows a variability that consistently reflects phylogeny.

Our RNAi investigation of the role of *TcOr1, the Tribolium* ortholog of *Drosophila Or83b* (Figs. 2 and 5) supports the assumption that this highly conserved gene plays a similar role in all insects (Jones et al., 2005). Although we do not have any molecular data to demonstrate how the gene functions in *Tribolium*, the fact that *TcOr1* knock-down beetles were unable to respond to the *Tribolium* aggregation pheromone suggests that it plays a similar role to *DmOr83b*, which dimerises with functional OR proteins and permits their integration into the cell membrane (Benton et al., 2006). This also confirms the usefulness of dsRNAi as a technique for investigating the effects of single genes on beetle behaviour.

Although demonstrating the behavioural importance of *TcOr1* is relatively straightforward, it seems unlikely that identifying function in any of the other TcOr genes will be quite so simple. The lack of any close evolutionary relationships between the TcOr genes and those of any other species (excluding *DmOr83b*/*TcOr1*) means that it will be difficult to predict the function of any of these genes on the basis of what is already known about receptor–ligand relations. As an additional problem, most *Drosophila* odorant receptors appear to have a relatively broad spectrum of responses, and knocking down one receptor may not lead to a detectable phenotype (Fishilevich et al., 2005). Probably the best approach for detecting function in this set of *Tribolium* genes will be examining the aggregation pheromone response. Given that both larvae and adults respond to this odour (Obeng-Ofori, 1991), it seems probable that one or more of the 35 TcOr genes that are expressed in both stages will be responsible.

More generally, the relatively large number of odorant receptor genes detected in this species underlines our relative ignorance of its evolution, ecology and natural history. Why would a species that apparently spends its whole life-cycle—indeed, can survive for generations on end—within its food source, need so many olfactory receptors? Some, or many, of these receptors may be involved in chemical communication in this species, including perception of components produced in the stink gland (Blum, 1981; Prendeville and Stevens, 2002), but it also seems likely that, prior to the development of agriculture, the original habitat of *T. castaneum* was more varied and patchy than their current niche in human grain stores. Possible original—and, perhaps, current—food sources might include plant seed-heads and rodent food-stores. The size and distribution of these sources will clearly have been very different from those of modern flour and grain stores. Whether *T. castaneum* still uses all its olfactory receptors (and what for) and whether selection has relaxed on the TcOr genes presented here, are tantalising questions that will be addressed in the future, through studies on *T. castaneum* and related species, such as *T. confusum*. A particularly fruitful approach would be to investigate the behaviour and natural history of this species, both today and in its past, prior to its association with humans.

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There is no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibmb.2007.10.005

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