Insect Molecular Biology (2009) **18**(2), 233–242

Transcriptional diversity and allelic variation in nicotinic acetylcholine receptor subunits of the red flour beetle, *Tribolium castaneum*

F. D. Rinkevich and J. G. Scott

Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY, USA

Abstract

Sequence analysis of 168 cDNA clones encoding 12 nicotinic acetylcholine receptor subunits, *Tcasα1* – *Tcasα11* and *Tcasβ1*, from the red flour beetle, *Tribolium castaneum*, revealed extensive post-transcriptional modification and multiple alleles. The greatest diversity was found for *Tcasa6*, where 18 unique transcripts, as a result of alternative and optional exon usage, were seen. A novel alternative exon 8d was found in one *Tcasα6* transcript. *Tcasα5* transcripts did not contain previously reported exons 8–10. Six subunits had transcripts that contained unspliced introns, which introduced premature stop codons. Intron 3′ splice site variants were seen at six intron boundaries across five subunits. A-to-I RNA editing was seen only in *Tcasα6*. Alleles were found for all subunit genes, except *Tcasα1* and *Tcasα10*. Transcriptional and allelic diversity are discussed with respect to receptor function and potential interactions with insecticides.

Keywords: RNA editing, nicotinic acetylcholine receptor alleles, red flour beetle, alternative exon usage.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are cationic selective members of the cys-loop ligand-gated ion channel superfamily. They facilitate rapid cholinergic transmission in the insect central nervous system by binding acetylcholine (ACh). Receptor complexes in insects can be heteropentamers consisting of two α and three β subunits (Thany *et al*., 2006) or homopentamers of only α subunits (Fayyazuddin *et al*., 2006). The α subunits are defined by a characteristic YxCC motif in the ACh-binding extracellular loop C, whereas β subunits lack this feature (Karlin, 2002). Receptor subunits consist of four transmembrane segments (TM1–4) and an extracellular ligand binding domain (loops A–F) at the N-terminus (Karlin, 2002). The second transmembrane domain lines the pore of the receptor complex (Imoto *et al*., 1988). Hydrophobic amino acids in the intracellular linker between the third and fourth transmembrane domain are important for cell surface expression of certain subunits (Ren *et al*., 2005).

The nAChRs are a diverse family of ion channels with many subunits found in every multicellular animal species. In insects, there are 12 subunit genes in *Bombyx mori* (Shao *et al*., 2007) and *Tribolium castaneum* (Jones *et al*., 2007), 11 in *Apis mellifera* (Jones *et al*., 2006) and 10 in *Anopheles gambiae* (Jones *et al*., 2005) and *Drosophila melanogaster* (Sattelle *et al*., 2005). Transcripts of these genes, especially α6 subunit orthologues, may be modified by alternative exon usage and/or A-to-I RNA editing (Sattelle *et al*., 2005; Jin *et al*., 2007). In *D. melanogaster*, more than 30 000 transcriptional variants of *Dα6* may be produced from combinations of alternative exon usage and A-to-I RNA editing (Grauso *et al*., 2002). Transcriptional diversity of orthologous *α6* subunits is conserved across widely divergent insects (Jin *et al*., 2007). Alternative exon use and A-to-I RNA editing may affect subunit coassembly (Lansdell & Millar, 2000) and agonist-induced currents (Saragoza *et al*., 2003). A deficit in RNA editing is the underlying cause of amyotrophic lateral sclerosis (Kawahara *et al*., 2004).

nAChR transcripts containing one or more introns have been reported from a number of α subunits from insects (Grauso *et al*., 2002; Jones *et al*., 2005, 2006; Gao *et al*., 2007b). In each of these cases, this leads to a stop codon being introduced, which eliminates crucial motifs such as transmembrane segments and ligand-binding domains that are required for proper receptor assembly and function. Despite the loss of these critical motifs caused by unspliced introns, the fact that they have been observed in multiple α subunits from highly divergent insects suggests a possible

Correspondence: Dr Jeffrey G. Scott, Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853-0901, USA. Tel.: +1 607 2557340; fax: +1 607 255-0939; e-mail: jgs5@cornell.edu

functional role for these truncated transcripts. It is unknown if these transcripts are subject to nonsense-mediated decay. Although no physiological studies have identified a role for these truncated transcripts in insects, it has been suggested they code for proteins that may moderate synaptic events in a similar manner to ACh-binding protein in *Lymnaea stagnalis* (Sattelle *et al*., 2005) or α7 in mice (Saragoza *et al*., 2003).

Spinosad and imidacloprid are two commercially important insecticides [a macrocyclic lactone and neonicotinoid, respectively (Nauen & Denholm, 2005; Tomizawa & Casida, 2005)]. Both insecticides act primarily on the nAChR, but on different types of nAChRs (Salgado & Saar, 2004). Studies on a lab-selected, imidacloprid resistant strain of the brown plant hopper (*Nilaparvata lugens*) indicated that the highly conserved tyrosine in extracellular loop B of Nlα1 and Nlα3 was important for imidacloprid binding and toxicity as well as inheritance of resistance (Liu *et al*., 2005). ethyl methanesulfonate (EMS) mutagenized *Drosophila* with various mutations in *Dα1* or *Dβ2* all resulted in strains with resistance to imidacloprid and other neonicotinoids (Perry *et al*., 2008). A *pogoR11* element-mediated knock-out mutation in *Dα6* where the protein is truncated after second transmembrane segment (TM2), resulted in 1180-fold resistance to spinosad (Perry *et al*., 2007). Spinosad resistant house-flies derived from field-collected resistant populations, however, showed no differences in the coding sequences, post-transcriptional modifications or levels of *Mdα6* transcripts compared to a genetically related susceptible strain (Gao *et al*., 2007b). As such, the mechanism(s) of spinosad resistance in house-flies and other pest insects remains unknown (Scott, 2008).

The genome of *T. castaneum* has recently been published (*Tribolium* Genome Sequencing Consortium, 2008) and 12 nAChR subunit genes were identified (Jones *et al*., 2007). *T. castaneum* is an ideal study organism because it is easy to rear, its genetics and genome are well understood and it is easy to manipulate with molecular techniques such as RNA interference (RNAi), gene-knockouts and germ-line transformation (*Tribolium* Genome Sequencing Consortium, 2008). Besides being an ideal model organism, *T. castaneum* is also a serious pest of stored products causing more than $US 1 billion in damage annually (Throne *et al*., 2003). Although sequences of nAChR subunit gene transcripts have been reported (Jones & Sattelle, 2007), the presence of additional transcript variations that have been reported in other subunits from other animals (Grauso *et al*., 2002; Jones *et al*., 2005; Gao *et al*., 2007b; Jin *et al*., 2007) have not been investigated. Applying similar approaches as Jones & Sattelle (2007), but using a more genetically diverse strain may yield novel transcripts and alleles. Spinosad and imidacloprid are promising control agents against *T. castaneum* (Toews *et al*., 2003). As both compounds act on nAChRs, it would be prudent to make use of genomic information as a guide to help provide the ground work for understanding the role of nAChR subunits in the toxicity of spinosad and imidacloprid. Herein, we report the full length sequences for the open reading frames of all 12 nAChR subunit cDNAs from *T. castaneum*, compare our findings to previous results (Jones & Sattelle, 2007) and identify other novel transcripts to establish a solid framework for future studies on nAChRs in *T. castaneum*.

Results

All 12 nAChR subunit full length cDNAs, including transcripts with numerous post-transcriptional modifications were successfully cloned and sequenced using reverse transcription followed by PCR based on published sequences. All subunits except *Tcasα1* contained novel alleles or transcriptional modifications distinct from those previously reported (Jones & Sattelle, 2007). The cloning results are summarized in Table 1. GenBank accession

Table 1. Variation in *Tribolium castaneum* (*Tcas*) nicotinic acetylcholine receptor (nAChR) cDNAs. Underlined modifications indicate previously reported post-transcriptional modifications

Subunit	*n*†	Modifications
Tcasα1	12	None
Tcasα2	10	Six alleles, intron 3 splice variant
Tcasα3	11	Two alleles, unspliced introns 9 and 10, intron 4 splice variant
Tcasα4	15	Two alleles, alternative exons, unspliced intron 7
Tcasα5	11	Three alleles, Δexons$^{8-10}$*
Tcasα6	39	Two alleles, alternative exons‡, RNA-editing, Δexons^{3,4,5,6,7,8}, splice variants of introns 5 and 7
Tcasα7	10	Two alleles
Tcasα8	10	Five alleles, intron 1 splice variant
Tcasα9	11	Five alleles, unspliced introns 5 + 6
Tcasα10	12	Unspliced introns 2 + 3, intron 5 splice variant
Tcasα11	10	Two alleles, unspliced intron 6
Tcasβ1	18	Two alleles, unspliced intron 6

*seen in all clones.
†total number of clones sequenced.
‡see Fig. 2.
Δ = deleted/missing.

numbers for all transcripts and alleles are listed in the Supporting Information (Table S1).

Alternative exon usage

Alternative exon use was only found in *Tcasα4* and *Tcasα6*. The nucleotide sequence of alternative exons 4 and 4′ of *Tcasα4* were identical to what was previously reported (Jones & Sattelle, 2007). Exon 4 of *Tcasα4* codes for loops E and B as well as the cys-loop, which are important for ligand binding (Shimomura *et al.*, 2005). Alternative exons were observed for exons 3 and 8 of *Tcasα6* as previously reported (Jones & Sattelle, 2007), except exon 8c was not detected in any transcripts ($n = 39$). We found a novel exon 8d in one of the 39 clones. Whereas exons 8a, 8b and 8c shared 55–63% nucleotide and 65–79% amino acid similarity, exon 8d shared only 21–25% nucleotide and 10–14% amino acid similarity with exons 8a, 8b and 8c. The nucleotide sequence of exon 8d is 31 bp longer than exons 8a, 8b and 8c, which are all 88 base pairs (bp) in length. Based on the published *T. castaneum* genome sequence (*Tribolium* Genome Sequencing Consortium, 2008), exon 8d is separated from exon 8c by 982 bp, whereas 8a and 8b are separated by 337 bp and 8b and 8c are separated by 544 bp. The sequence of exon 8d indicates its first codon would be a stop codon, thus truncating the protein at this point (Fig. 1).

For *Tcasα6*, we detected previously identified isoforms I to IV, but isoform V (Grauso *et al.*, 2002) was not found. Unique exon combinations and 13 novel isoforms were identified for *Tcasα6* (Fig. 2). Five of 39 clones did not contain exon 3, 10 clones did not contain exon 5 and five clones did not contain exon 8. Eight of 10 clones missing exon 5 also had exon 3b. The two other clones missing exon 5 had exon 3a or 3x (no exon 3). A full description of each *Tcasα6* transcript (including schematic diagrams) showing the frequency of observed exon combinations and frequency of alternative exon usage is given in Fig. 2 and Table 2, respectively.

Previously reported exons 8, 9 and 10 for *Tcasα5* (Jones & Sattelle, 2007) were not found in any of the 11 clones that were sequenced. The absence of these exons did not affect the open reading frame of *Tcasα5* and increased the similarity to *α5* orthologues in *D. melanogaster* [+8.2% (Grauso *et al.*, 2002)] and *An. gambiae* [+2.5% (Jones *et al.*, 2005)], but decreased similarity to *Ap. mellifera* [–0.9% (Jones *et al.*, 2006)]. To verify that these previously reported exons were not present, primers were designed based on the sequence of previously reported exons 8, 9 and 10 and used in combination with primers used for full length amplification and sequencing of *Tcasα5* (Fig. 3). Primers sets specific for exons 3 + 12 (primers Tcasa5IF and Tcasa5FullR), or 8 + 10 (Tcasa5Exon8F and Tcasa5Exon10R) successfully amplified their intended targets (Fig. 3, lanes 2 and 5). However, primer sets specific for exons 3 + 10 (Tcasa5IF and Tcasa5Exon10R), or 8 + 12 (Tcasa5Exon8F and Tcasa5FullR) failed to generate a PCR product (Fig. 3, lanes 1 and 6). The fragment generated by primers Tcasa5Exon78F (spanning exons 7 and 8) and Tcasa5Exon10R [exon 10 (Fig. 3, lane 3)] was cloned and sequenced. The sequence showed that primer Tcasa5Exon78F actually annealed between bases 86 to 106 of exon 9. Tcasa5Exon8F also annealed to exon 9 at positions 94 to 114 although it was specifically designed for the beginning of exon 8. The *T. castaneum* genomic sequence shows that exons 8 and 9 share an identical sequence of 141 bp. Exon 9 has an additional 93 bp on the 5′ end of the alignment overlap between these two exons whereas exon 8 extends 43 bp at the 3′ end. The 93 bp 5′ extension of exon 9 overlaps with 100% identity to the 3′ end of intron 7 and the 43 bp 3′ extension of exon 8 shares the exact same sequence as the 5′ end of intron 9. This explains why PCR products of the observed sizes were produced and demonstrates

Table 2. Exon usage frequency in *Tribolium castaneum* nicotinic acetylcholine receptor subunit *α6* (*Tcasα6*) transcripts ($n = 39$)

Exon	Frequency
3a	0.23
3b	0.61
3ab	0.03
3x	0.13
4	0.95
4x	0.05
5	0.74
5x	0.26
6	0.90
6x	0.10
7	0.90
7x	0.10
8a	0.38
8b	0.46
8d	0.03
8x	0.13

x, exon not used in transcript.
Exon 8c was not observed in any clones.

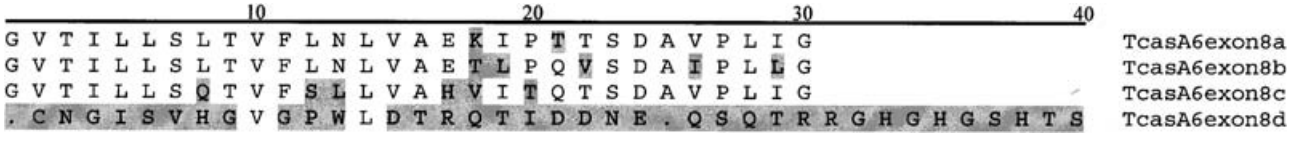

Figure 1. Alignment of deduced amino acids from exons 8a, b, c and d of *Tribolium castaneum* nicotinic acetylcholine receptor subunit *α6* (*Tcasα6*). Amino acids divergent from the consensus are shaded. Exon 8d shows < 14% amino acid similarity to the other exons and introduces multiple stop codons that are indicated by full stops.

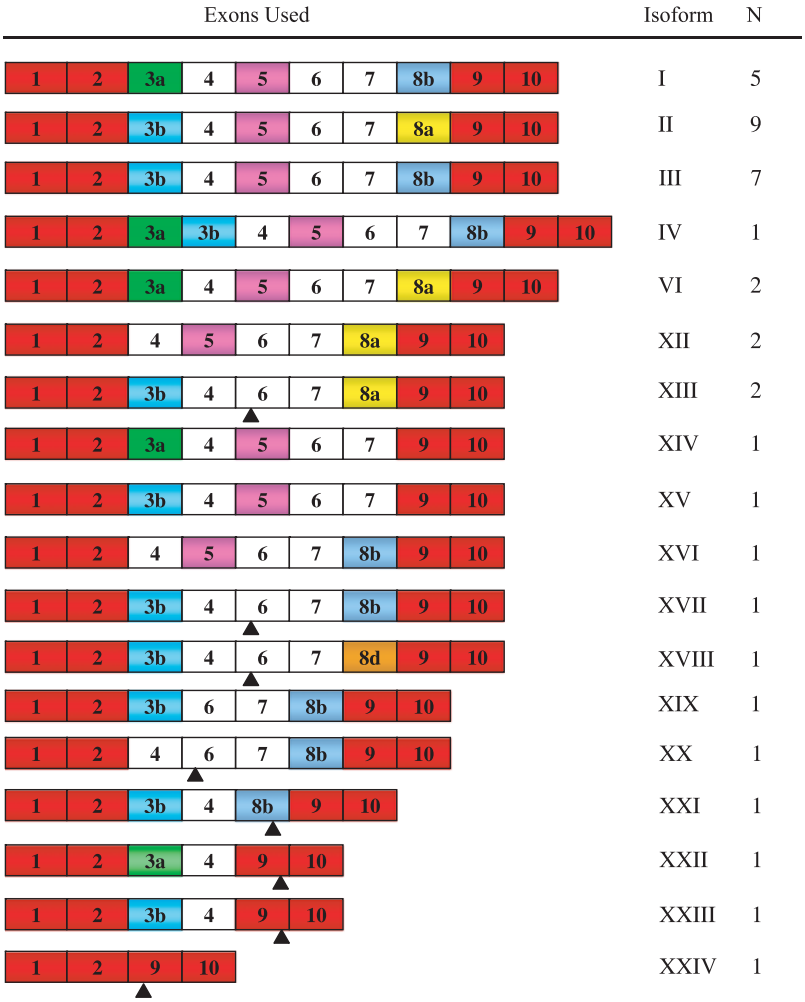

Figure 2. Schematic diagrams and frequency of variable exon isoforms of *Tribolium castaneum* nicotinic acetylcholine receptor subunit *α6* (*Tcasα6*). Numbered boxes indicate exons found in that particular isoform. Conserved exons found in every isoform are coloured red. Alternative exons 3a, 3b, 8a, 8b and 8d are coloured green, light blue, yellow, dark blue and orange, respectively. Optional exon 5 is coloured pink. Isoform numbering follows the convention put forth by Grauso *et al.* (2002; isoforms I–V) and Gao *et al.* (2007b; isoforms VI–XI). Isoforms V and VII–XI are not listed because they were not detected. Isoforms XII to XXIV are arbitrarily numbered. The sizes of the exon boxes are not proportional to their nucleotide length. Triangles below exons indicate the approximate location of the introduction of a stop codon because of a frame shift resulting from the loss of one or more exons.

that previously reported exons 8 to 10 are likely not to be included in transcripts containing other exons in this strain. The results in Fig. 3 show that previously reported exons 8, 9 and 10 are not found in transcripts containing other *Tcasα5* exons, but that there is a transcript containing only exons 8–10. The previously reported intron between exons 9 and 10 is not spliced out but remains in this transcript. It does not appear this is an alternatively spliced intron because of the presence of a single band on the gel. The function of this transcript is unknown.

Intron 3′ splice site variation

Splice variants at the 3′ splice sites of introns were observed in *Tcasα2*, *α3*, *α6*, *α8* and *α10*. Most splice variants resulted in the introduction of a premature stop codon. Intron 4 of *Tcasα3* was spliced out 10 bp upstream in two clones (out of 11) and led to the introduction of a premature stop codon. Introns 5 and 7 in *Tcasα6* were spliced out 179 and 91 bp upstream, respectively, in two separate clones. Both splice variants for introns 5 and 7 introduced a premature stop codon. Intron 1 of *Tcasα8* was spliced out 21 bp upstream [relative to previously reported sequences (Jones & Sattelle, 2007)] in all 10 clones we sequenced, but remained in-frame. The resulting seven amino acid deletion resulted in a protein that was ~1% less similar to *α8* orthologues in *An. gambiae*, *Ap. mellifera* and *D. melanogaster* (actually *Dβ2*). *Tcasα10* intron 5 has a splice variant that is 13 bp downstream of the normal boundary and introduces a premature stop codon. Splice variants from all subunits are preceded by the typical 3′–AG intron boundary as deduced from genomic DNA sequences. The only exception to the boundary rule is seen in *Tcasα2* where intron 3 is spliced out 5 bp downstream of the typical 3′–AG intron boundary. The splice variant is preceded by a –TG boundary when compared to other cDNA and genomic sequences.

Unspliced introns

Tcasα3, *α4*, *α9*, *α10*, *α11* and *β1* have transcripts which contain an intron. These transcripts are not a result of genomic DNA contamination as they do not possess any other introns. Introns that were unspliced all had the typical GT–AG intron boundaries, a premature stop codon and were of short

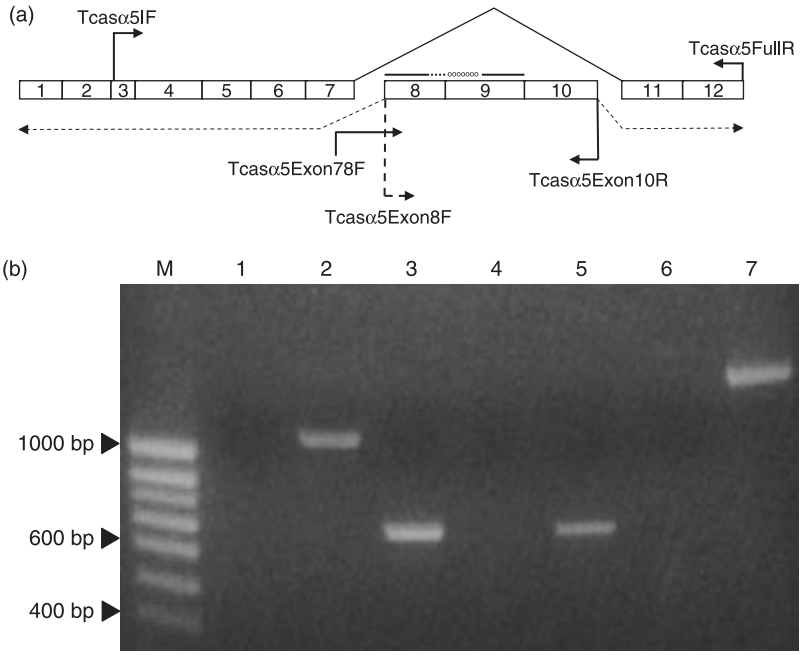

Figure 3. Schematic diagram (A) and gel photograph of PCR products (B) used to validate exons 8–10 of *Tribolium castaneum* nicotinic acetylcholine receptor subunit $\alpha 5$ (*Tcasα5*) were missing. (A) Tcasα5Exon78F spans the ends of exons 7 and 8. Solid lines over exons 8 and 9 represent the sequence of 141 identical bases shared between both exons. The dashed line over exon 8 represents the sequence of 43 identical bases shared between exon 8 and the 5′ end of intron 9. The circles over exon 9 represent the sequence of 93 identical bases shared between exon 9 and the 3′ end of intron 7. (B) The banding patterns show that previously reported exons 8, 9 and 10 are not included in transcripts containing other exons used in other full-length transcripts. (Lane M) 5 μl HyperLadder IV (Bioline, Taunton, MA, USA): (1) Tcasα5IF/Tcasα5Exon10R; (2) Tcasα5IF/Tcasα5FullR; (3) Tcasα5Exon78F/Tcasα5Exon10R (4) Tcasα5Exon78F/Tcasα5FullR; (5) Tcasα5Exon8F/Tcasα5Exon10R; (6) Tcasα5Exon8F/Tcasα5FullR; and (7) Tcasβ1FullF/Tcasβ1FullR as a positive control.

Table 3. Length and intron number of unspliced introns from nicotinic acetylcholine receptor (nAChR) subunits in *Tribolium castaneum* (*Tcas*). All unspliced introns introduce premature stop codons

Subunit	Unspliced intron length (bp)	Intron #	Detection rate
Tcasα3	44 + 45	9 + 10	1/11
Tcasα4	45	7	1/15
Tcasα9	49 + 44	5 + 6	1/11
Tcasα10	50 + 44	2 + 3	1/12
Tcasα11	70	6	2/10
Tcasβ1	45	6	2/18

length (44–70 bp). A summary of these unspliced introns is shown in Table 3. The unspliced introns do not have any polymorphisms associated with the intron or the adjacent exon sequences that could be associated with the inefficient splicing. Out of the subunits that exhibit unspliced introns, *Tcasα4* was the only subunit that had alternative exon usage. Therefore, it is unlikely that alternative exon usage is an important factor for retaining introns. Intron location also does not appear to be important for retaining introns, as they can appear early or late in the transcript. The only consistent variable in retaining introns in *T. castaneum* is short intron length (Table 3). Intron 9 was unspliced in both *Tcasα3* and *Amelα3* (Jones *et al.*, 2006). Although intron 9 in *Tcasα3* and *Amelα3* varies in length and 3′ splice site, the fact that the same intron is unspliced in two different taxa suggests a possible biological role for these truncated proteins. Although a premature stop codon is introduced, unspliced intron 9 in both *Amelα3* and *Tcasα3* would still produce a transcript coding for a protein with signal peptides, extracellular binding domains and three transmembrane domains. Retention of introns 5 and 6 of *Tcasα9* disrupts the reading frame immediately before TM2 and introduces a premature stop codon. A *Tcasα10* transcript containing introns 2 + 3 codes for a protein containing only the signal peptide and loop D. The transcript of *Tcasα11*, which contains intron 6, is immediately truncated just after TM3. Retention of intron 6 in *Tcasβ1* truncates the protein immediately before TM2. Intron 6 is immediately downstream and adjacent to the codons for the conserved GEK motif, which is integral for selective ion permeability (Jensen *et al.*, 2005). Retained introns did not encode motifs such as N-glycosylation or phosphorylation sites or novel transmembrane domains.

RNA editing

A-to-I RNA editing was found only for *Tcasα6*, and was similar to a previous report (Jones & Sattelle, 2007). All editing sites for *Tcasα6* are on exon 5. Editing sites 4, 5 and 6 [numbering based on all previously reported A-to-I RNA editing sites in insects (Jin *et al.*, 2007)] were edited at frequencies of 86, 51 and 55%, respectively, based on *Tcasα6* transcripts containing exon 5 ($n = 29$). Transcripts containing edits at sites 4, 5, 6, 4 + 5, 4 + 6, 5 + 6 and 4 + 5 + 6 were edited at frequencies of 14, 0, 7, 21, 17, 0 and 31%, respectively. Editing at site 5 was always in conjunction with editing at site 4. Previously reported (Jones & Sattelle, 2007) editing of *Tcasβ1* at nucleotide 155 (resulting in a K to R substitution) was not seen in any of the 18 clones that we sequenced. Thus, the editing frequency of *Tcasβ1* is < 6% in the GA-1 strain of *T. castaneum*.

Table 4. Summary of *Tribolium castaneum* nicotinic acetylcholine receptor (nAChR) subunit alleles. Only nonsynonymous substitutions are listed. A full list of alleles is in Supporting Information Table S1

Subunit	Base substitution*	Amino acid substitution	Detection rate
α5	G268A	G90R	3/11
α6	T128C	V43A	2/39
α8	A314G	E105G	2/10
	T775C	C259R	2/10
α9	A1069G^{v5}	T357A	1/11
	A1069T^{v3}	T357S	1/11
α11	G36A	M12I	5/10

*bases are numbered relative to the translation start site of *T. castaneum* subunits.
Substitutions are for allele v2 for each subunit gene unless otherwise noted.

Alleles

Alleles were observed for *Tcasα2*, *α3*, *α4*, *α5*, *α6*, *α7*, *α8*, *α9*, *α11* and *β1*. Alleles were designated for substitutions that were not previously reported as A-to-I RNA editing sites and were shared between two or more independent clones. The existing sequence in GenBank was named allele 1. Only seven out of 51 polymorphisms observed in the alleles resulted in an amino acid substitution (Table 4). *Tcasα6*, *α8*, *α9* and *α11* contained alleles with nonsynonymous substitutions. Although transitions were more frequent than transversions, there was no statistical bias toward any specific nucleotide substitution across all alleles (one-way ANOVA, $P > 0.05$). Of the 51 polymorphic sites, 80% occurred at the third codon position, of which, only A1069G/T in *Tcasα9* resulted in an amino acid substitution.

Discussion

The sequences of nAChR subunit cDNAs that we report exhibit an unprecedented amount of variation in these genes from a single organism. Although our work confirms a previous report (Jones & Sattelle, 2007), there are many novel differences in our data set. We observed a greater amount of variation in alternative exon usage in *Tcasα6* and identified 13 new isoforms. Although more than 256 splice variants are mathematically possible from this arrangement of alternative exons, 18 isoforms are likely to be an accurate measure of isoform diversity given the large number of clones we sequenced.

Although duplicated exons are commonly spliced in a mutually exclusive manner (Letunic *et al.*, 2002), we identified a transcript that contained both exons 3a and 3b (3ab). This pattern of alternative splicing of exon 3 in the α6 subunit was seen in *D. melanogaster* (Grauso *et al.*, 2002), *Musca domestica* (Gao *et al.*, 2007b) and *B. mori* (Jin *et al.*, 2007; Shao *et al.*, 2007), but not previously described in *T. castaneum* (Jones & Sattelle 2007). Splicing together exons 3a and 3b is developmentally regulated in *B. mori* (Jin *et al.*, 2007). Having both exons 3a and 3b would duplicate the acetylcholine binding loop D. It is unknown if subunits with repeated ligand binding loops would alter ACh binding or not. Much like in *B. mori*, we found that exon 3 can be excluded from mature transcripts altogether (Shao *et al.*, 2007). Exclusion of exon 3 does not cause a frame shift in the transcript and otherwise retains nucleotide and amino acid identity to other transcripts. These transcripts would produce proteins that lack ligand binding loop D. Thus, the ligand binding capacity and affinity of these subunits would most likely be altered.

We observed alternative exons 8a and 8b in *Tcasα6.* The frequency of exon usage (Table 2) shows that 8b is found in transcripts more frequently than 8a. Exon 8c was not found in any clone that was sequenced, but a novel exon 8d was detected. The frequency of exon 8 usage we found in *Tcasα6* is 8b > 8a >> 8d, thus ensuring most *Tcasα6* transcripts would produce proteins with TM2 that contains amino acids crucial to normal receptor function. This is in agreement with previous reports from *T. castaneum*, *Ap. mellifera*, *B. mori* and *D. melanogaster* (Jin *et al.*, 2007), with the exception that exon 8c was found in the other insects instead of 8d. Although we did not find exon 8c, it has been shown that exon 8c is present infrequently in other insects (Gao *et al.*, 2007b; Jin *et al.*, 2007). There were three clones that did not contain exon 8. Exon 8 was also excluded from isoform IX in *M. domestica* (Gao *et al.*, 2007b), but transcripts missing exon 8 may be nonfunctional as they lack TM2 and the well-conserved glutamic acid residue (encoded by transcripts containing exons 8a and 8b) that is involved in ion conductance (Imoto *et al.*, 1988). Exclusion of exon 8 does not cause a frame shift in the sequence. These two physical alterations in the resulting protein may form complexes with altered pharmacological properties. Neither *Bmα5* from *B. mori* (Shao *et al.*, 2007) nor *Agα1* from *Aphis gossypii* (Li & Han, 2005) possess transcripts that code for TM4 in any mature transcripts; therefore, it may be possible to generate smaller subunits lacking TMs that may retain function.

The previously undescribed exon 8d from *Tcasα6* appears to be an anomaly rather than a true alternative exon. The low nucleotide and amino acid similarity to 8a, 8b and 8c, longer exon length, longer intron length between exons, immediate introduction of a stop codon and low detection rate (one out of 39 clones) indicate that exon 8d is either a splicing error or an optional exon rather than a true alternative exon that arose through duplication. This is similar to the observation of exon 9b in the *α7* subunit from mice (Saragoza *et al.*, 2003). However, these two exons are not analogous as *Tcasα6* 8d truncates the protein at the beginning of TM2, whereas mouse *α7* terminates just after TM3.

The observation of optional exons 5 and 6 + 7 for *Tcasα6* is a rare event relative to other insect nAChRs. The only

other examples of optional exon use are exon 2 of *Dα4* (Lansdell & Millar, 2000), exons 5–7 of *Mdα6* (Gao *et al*., 2007b) and exons 2 and 3 of *Agβ1* (Li & Han, 2005). Although *Tcasα6*, *Dα4* and *Mdα6* are alternatively spliced, *Agβ1* is not. Thus, alternative exon use is not a requirement for optional exon use.

Exon 5 of *Tcasα6* is an optional exon missing from 25% of the clones. Deletion of exon 5 introduces a premature stop codon. Exon 5 codes for extracellular loops E and B that are important for ligand binding. Given that exon 5 codes for amino acids crucial for receptor function and diversity through ligand binding and RNA-editing, it is unclear why it would be excluded from nine of the isoforms. However, mutual exclusion of exon 4 and exon 5, as in the case of isoform XIX, does not disrupt the open reading frame of the transcript. However, exclusion of exon 3 and exon 5 does introduce a premature stop codon. Exon 5 is always missing from transcripts that are also missing both exons 6 and 7. Exon 6 codes for extracellular loop F and exon 7 codes for loop C, which contains the α-subunit specific YxCC motif.

Anopheles gambiae (Jones *et al*., 2005), *Ap. mellifera* (Jones *et al*., 2006), *D. melanogaster* (Grauso *et al*., 2002) and *B. mori* (*S*hao *et al*., 2007) *α6* orthologues have identical genomic architectures. Exons 10, 11 and 12 of these *α6* orthologues in other insects are fused into a single exon 10 in *T. castaneum*. This is an interesting observation because *T. castaneum* is placed phylogenetically above *Ap. mellifera* and below the other insects. Exon boundaries for other orthologous nAChR subunits do not share the same conserved genomic architecture as seen in α6 orthologues.

Exons 8 to 10 of *Tcasα5* were not seen in any of the 11 clones we sequenced. We conclude that reported exons 8 to 10 are probably very rare exons for four reasons. First, exons showing such a high similarity at the nucleotide level would probably indicate alternative exons as alternative exons 4 and 4′ of *Tcasα4* share 70% nucleotide similarity, whereas exons 3a/b and 8a/b/c of *Tcasα6* share 66 and 60–65% similarity, respectively. The discrepancy in the length of exons 8 and 9, however, is not indicative of alternative exons because all other alternative exons in *T. castaneum* are exactly the same length. Second, the similarity of the adjacent areas of both exons exactly matches nearby introns suggesting this stretch of DNA may have undergone recent duplication. This is substantiated by the fact that sequence comparison of both exons 8 and 9 with the adjacent introns (ie intron 7/exon 8/intron 8 vs. intron 8/exon 9/intron 9) share 85% similarity. Third, the primers designed specifically for exon 8 annealed to exon 9 without generating a double banding pattern on the gel, suggesting that exon 8 is not transcribed. Fourth, transcripts containing exons 9 and 10 do not contain other exons that are found in mature transcripts which may indicate these exons are processed differently than transcripts that do not contain them.

Splice variants in the 3′ splice site of introns were found in four subunits. For *Tcasα3*, *Tcasα6* and *Tcasα10*, the splice variants disrupted the open reading frame and introduced a premature stop codon. All clones that we sequenced for *Tcasα8* had 'splice variants' compared to previously reported sequences. It is unlikely that this is a true splice variant, but rather that it is actually the normal splicing boundary as it is seen in all clones. The length of this 'splice variant' coded for an additional seven amino acids in the highly variable N-terminal signal leader peptide and did not disrupt the open reading frame. Therefore, the differences between the deduced amino acid sequence previously reported (Jones & Sattelle, 2007) and ours would not be expected to have a dramatic effect on the normal function of the receptor complex, but might alter its cellular trafficking. It is difficult to reconcile the differences seen in *Tcasα8* with the previous report because the beetle strains used in our study and by Jones & Sattelle (2007) are highly related (ie GA-2 is derived from GA-1) and the techniques used in both studies are very similar. Although the 'splice variants' we reported for *Tcasα8* had decreased similarity to other orthologous subunits, it does not create significant changes in overall similarity (1.0 to 1.3%).

Unspliced introns were found in six nAChR subunit transcripts in *T. castaneum* and they all introduced premature stop codons. This represents the largest collection of truncated nAChR transcripts as a result of unspliced introns in any insect and may be a reflection of the large number of clones we sequenced. Unspliced introns were also reported from *Dα7* (Grauso *et al*., 2002), *Agamα7* (Jones *et al*., 2005), *Amelα3* and *α7* (Jones *et al*., 2006) and *Mdα6* isoform VIII (Gao *et al*., 2007b). All unspliced introns introduce a premature stop codon shortly after the introduction of the intron into the open reading frame except in *Agamα7*, which remains in frame for an additional 86 amino acids. Intron 9 in *Mdα6 is*oform VIII was only 59 bp in length. Although we did not observe any retained introns in *Tcasα7*, intron 5 was unspliced in *Agamα7*, *Amelα7* and *Dα7* with lengths of 7292, 3666 and 62 bp, respectively. Thus, it appears intron length may be a species-specific determinant to leave introns unspliced from mature transcripts. Unspliced intron 9 in *Tcasα3* and *Amelα3* and intron 6 of *Tcasα11* produce transcripts that are truncated just after TM3, whereas unspliced intron 7 of *Tcasα4* is truncated halfway through TM3. Similarly truncated transcripts of *Dα1* abolished ACh-mediated inward currents (Schulz *et al*., 2000). EMS mutagenesis that produced truncated *Dβ2* between TM3 and TM4 in *D. melanogaster* created a strain resistant to imidacloprid and other neonicotinoids (Perry *et al*., 2008). Thus, investigating the role of orthologous subunits of *T. castaneum* in insecticide toxicity would be of great value.

Alleles were found for 10 nAChR subunit genes and some of the nonsynonymous substitutions could affect receptor function. In *Tcasα5*, the G90R substitution occurs

at a highly conserved glycine residue between loops D and A. Glycine at position 90 (based on *Tcasα5* numbering) is highly conserved in *T. castaneum* (11/12 nAChRs), and alignments show that it is also conserved in *Dα1-7*, *Dβ1* and *2, Hvα7-1* and *2* (*Heliothis virescens*), *Ggα7* (*Gallus gallus*) *Tcα1* [*Torpedo californica* (Grauso *et al.*, 2002)], *Agamα1-8* and *β1* (Jones *et al.*, 2005), *Amelα1-9* and *Amelβ1* (Jones *et al.*, 2006), *Bmα1-*, *Bmβ1-3* (Shao *et al.*, 2007), *Cfα1-5,7,8* and *β1* [*Ctenocephalides felis* (Bass *et al.*, 2006)] *Mdα6* (Gao *et al.*, 2007b), *Mdα5* and *β3* (Gao *et al.*, 2007a) and *Mpα1-5* [*Myzus persicae* (Huang *et al.*, 1999)]. The striking conservation within and across insect and vertebrate taxa suggests that G90 has an important role in subunit structure and function. The effect of the G90R substitution is not known. The addition of a charge at such a conserved site, however, is likely to cause some structural change. The V43A substitution in *Tcasα6* falls between the signal peptide and loop D. Substitution E105G in *Tcasα8* occurs two residues downstream of the well-conserved glycine residue mentioned in *Tcasα5*. This residue can be acidic or basic, so replacing glutamic acid with glycine at this position may affect proper folding of the extracellular loops. A fairly conserved cysteine (nine out of 12 subunits in *T. castaneum*) at position 259 found in TM1 is changed to arginine in *Tcasα8*. Position 1069 in *Tcasα9* is the only allele that has a double variant (A1069G/T). Both of these substitutions result in T357A/S at the amino acid level. This is located adjacent to a potential phosphorylation site (SFH motif). The T357A/S substitutions occur at the intracellular linker between TM3 and TM4, which is important for cell surface expression (Ren *et al.*, 2005). The M12I substitution resulting from the G36A allelic variant of *Tcasα11* occurs in the highly variable signal peptide. The codon location of the alleles is biased at the third position. As only one out of 35 substitutions at the third codon position create an amino acid substitution, they are likely to be under little selective pressure and thus able to accumulate at this position. Nearly 80% of *T. castaneum* nAChR alleles are substitutions at the third codon position.

nAChR subunits in the GA-1 strain of *T. castaneum* undergo extensive post-transcriptional modification and some contain multiple alleles. Despite a large amount of transcript variation, a large proportion of these transcripts code for proteins with unknown functions. Comparison of results between the GA-1 (presented here) and GA-2 (Jones & Sattelle, 2007) strains shows that although strains may be highly related, there may be dramatic differences between RNA processing and allelic content. Future research on nAChRs is needed to shed light on the functional role of transcripts with alternative exons, retained introns and splice variants. The easy manipulation of *T. castaneum* using RNAi, chemical mutagenesis and binary transposon systems (*Tribolium* Genome Sequencing Consortium, 2008) presents an excellent opportunity to study these interactions.

Experimental procedures

Beetle rearing

The wild-type, insecticide susceptible GA-1 strain of red flour beetles (*T. castaneum*) was obtained from Kathy Leonard at Kansas State Univ. The GA-1 strain has been reared in the laboratory for more than 28 years without exposure to insecticides. Beetles were reared in 475 ml mason jars (Kerr, Jackson, TN, USA) with approximately 75 g of organic golden buffalo flour (Heartland Mill, Merienthal, KS, USA) with 5% (wt/wt) of dried baker's yeast (MP Biomedicals, Solon, OH, USA). The top of the jar was covered with a fine mesh cloth and sealed with a canning band. Jars were held at 30 °C with 65% ± 5% humidity in continuous darkness. Adult beetles were transferred every four weeks to new rearing jars using the paper transfer method with a strip of paper towel (USDA, 2008 http://bru.gmprc.ksu.edu/proj/tribolium/wrangle.asp). Three colony jars were used for each transfer.

RNA isolation

Total RNA was isolated from five, unsexed, 1–5-day-old adults using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. Total RNA was resuspended in 100 μl of diethylpyrocarbonate-treated H_2O. RNA concentration was measured on a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA) by dividing the absorbance at 260 nm by the specific absorption coefficient of RNA (Gallagher & Desjardins, 2006).

Reverse transcription, PCR, cloning and sequencing

Reverse transcription (RT) was performed using 5 μg of total RNA using SuperScript III according to the manufacturer's directions (Invitrogen, Carlsbad, CA) with the RT time extended to 3 h. Two μl of cDNA were used in PCR to amplify specific subunits (see Supporting Information Table S2 for primer sets) using 100 μl volumes and GoTaq Green Master Mix (Promega, Madison, WI, USA). PCR products were visualized on 1% agarose gels stained with 50 μg/ml ethidium bromide under UV light. PCR products were directly cloned into pGEM-T Easy cloning vector (Promega) according to the manufacturer's directions for chemically competent cells, except that the ligation step was performed at 4 °C > 12 h. Individual colonies were screened for the presence of an insert using the colony as a template in PCR. Colony PCR was conducted in 15 μl volumes using GoTaq and T7 and SP6 primers. PCR products were visualized on 1% agarose gels and positive colonies were incubated at 37 °C overnight in 3 ml of Luria broth medium containing 100 μg/ml ampicillin. Plasmid DNA was isolated using the Wizard Plus SV Minipreps (Promega) and eluted in 100 μl H_2O. Positive clones were sequenced from both ends using T7 and SP6 primers as well as an internal gene-specific primer (Table S2) at Cornell's Biotechnology Resource Center. Sequences for each subunit were aligned using MegAlign (DNA Star, Madison, WI, USA) in order to determine the full-length sequence of each subunit. The electropherograms were manually examined for clones if sequences differed from each other or those previously reported. A minimum of 10 clones were sequenced for each subunit. If only one clone had a substitution (compared to the other clones), this nucleotide difference was assumed to be a Taq error which happens with a frequency of about 10^{-4} (Eckert & Kunkel, 1990; Chen *et al.*, 1991). If two or more clones shared a nucleotide difference, they were considered to be a polymorphism. A-to-I RNA-editing was evaluated at sites previously

shown to be edited in *T. castaneum* (Jones & Sattelle, 2007), *Ap. mellifera* (Jones *et al*., 2006), *B. mori (*Shao *et al*., 2007), *D. melanogaster* (Grauso *et al*., 2002; Hoopengardner *et al*., 2003) and *M. domestica* (Gao *et al*., 2007a,b). Polymorphisms not previously associated with RNA editing in other species were considered alleles.

Acknowledgements

We thank Kathy Leonard for providing the GA-1 strain of *T. castaneum*. This work was supported by a Sarkaria Fellowship (FDR).

References

Bass, C., Lansdell, S.J., Millar, N.S., Schroeder, I., Turberg, A., Field, L.M. *et al*. (2006) Molecular characterisation of the nicotinic acetylcholine receptor subunits from the cat flea, *Ctenophalides felis* (Siphanaptera: Pulicidae). *Insect Biochem Molec Biol* **36**: 86–96.

Chen, J., Sahota, A., Sambrook, P.J. and Tischfield, J.A. (1991) Polymerase chain reaction amplification and sequence analysis of human mutant adenine phosphoribosyltransferase genes: The nature and frequency of errors caused by Taq DNA polymerase. *Mutat Res* **249**: 169–176.

Eckert, K.A. and Kunkel, T.A. (1990) High fidelity DNA synthesis by *Thermus aquaticus* DNA polymerase. *Nucleic Acids Res* **18**: 3739–3744.

Fayyazuddin, A., Zaheer, M.A., Hiesinger, P.R. and Bellen, H.J. (2006) The nicotinic acetylcholine receptor Dalpha7 is required for an escape behavior in *Drosophila*. *PLoS Biol* **4**: e63.

Gallagher, S.R. and Desjardins, P.R. (2006) Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. In *Current Protocols in Molecular Biology* (Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., eds), pp. 1–21. John Wiley and Sons, Inc., Hoboken NJ.

Gao, J.-R., Deacutis, J.M. and Scott, J.G. (2007a) Characterization of the nicotinic acetylcholine receptor subunits *Mdalpha5* and *Mdbeta3* on autosome 1 of *Musca domestica* indicate they are not involved in spinosad resistance. *Insect Molec Biol* **16**: 691–701.

Gao, J.-R., Deacutis, J.M. and Scott, J.G. (2007b) The nicotinic acetylcholine receptor subunit *Mdα6* from *Musca domestica* is diversified via post transcriptional modification. *Insect Molec Biol* **16**: 325–334.

Grauso, M., Reenan, R.A., Culetto, E. and Sattelle, D.B. (2002) Novel putative nicotinic acetylcholine receptor subunit genes, Dα5, Dα6 and Dα7, in *Drosophila melanogaster* identify a new and highly conserved target of adenosine deaminase acting on RNA-mediated A-to-I pre-mRNA editing. *Genetics* **160**: 1519–1533.

Hoopengardner, B., Bhalla, T., Staber, D. and Reenan, R. (2003) Nervous system targets of RNA editing identified by comparative genomics. *Science* **301**: 832–836.

Huang, Y., Williamson, M.S., Devonshire, A.L., Windass, J.D., Lansdell, S.J. and Millar, N.S. (1999) Molecular characterization and imidacloprid selectivity of nicotinic acetylcholine receptor subunits from the peach-potato aphid *Myzus persicae*. *J Neurochem* **73**: 380–389.

Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J. *et al*. (1988) Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. *Nature* **335**: 645–648.

Jensen, M.L., Schousboe, A. and Ahring, P.K. (2005) Charge selectivity of the Cys-loop family of ligand-gated ion channels. *J Neurochem* **92**: 217–225.

Jin, Y., Tian, N., Cao, J., Liang, J., Yang, Z. and Lv, J. (2007) RNA editing and alternative splicing of the insect nAChR subunit alpha6 transcript: evolutionary conservation, divergence and regulation. *BMC Evol Biol* **7**: 98.

Jones, A. and Sattelle, D. (2007) The cys-loop ligand-gated ion channel gene superfamily of the red flour beetle, *Tribolium castaneum*. *BMC Genomics* **8**: 327.

Jones, A., Brown, L. and Sattelle, D. (2007) Insect nicotinic acetylcholine receptor gene families: from genetic model organism to vector, pest and beneficial species. *Invert Neurosci* **7**: 67–73.

Jones, A.K., Grauso, M. and Sattelle, D.B. (2005) The nicotinic acetylcholine receptor gene family of the malaria mosquito, *Anopheles gambiae*. *Genomics* **85**: 176–187.

Jones, A.K., Raymond-Delpech, V., Thany, S.H., Gauthier, M. and Sattelle, D.B. (2006) The nicotinic acetylcholine receptor gene family of the honey bee, *Apis mellifera*. *Genome Res* **16**: 1422–30.

Karlin, A. (2002) Emerging structure of the nicotinic acetylcholine receptors. *Nat Rev Neurosci* **3**: 102–114.

Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I. and Kwak, S. (2004) RNA editing and death of motor neurons. *Nature* **427**: 801.

Lansdell, S.J. and Millar, N.S. (2000) Cloning and heterologous expression of Da4, a *Drosophila* neuronal nicotinic acetylcholine receptor subunit: identification of an alternative exon influencing the efficiency of subunit assembly. *Neuropharmacology* **39**: 2604–2614.

Letunic, I., Copley, R.R. and Bork, P. (2002) Common exon duplication in animals and its role in alternative splicing. *Hum Mol Gen* **11**: 1561–1567.

Li, F. and Han, Z.J. (2005) Alternative splicing, multiple transcription initiation sites of nicotinic acetylcholine receptor subunits from the cotton aphid *Aphis gossypii*. *Acta Zool Sin* **51**: 867–878.

Liu, Z., Williamson, M.S., Lansdell, S.J., Denholm, I., Han, Z. and Millar, N.S. (2005) A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper). *Proc Natl Acad Sci USA* **102**: 8420–8425.

Nauen, R. and Denholm, I. (2005) Resistance of insect pests to neonicotinoid insecticides: current status and future prospects. *Arch Insect Biochem Physiol* **58**: 200–215.

Perry, T., McKenzie, J.A. and Batterham, P. (2007) A *Dalpha6* knockout strain of *Drosophila melanogaster* confers a high level of resistance to spinosad. *Insect Biochem Molec Biol* **37**: 184–188.

Perry, T., Heckel, D.G., McKenzie, J.A. and Batterham, P. (2008) Mutations in *Da1* or *Db2* nicotinic acetylcholine receptor subunits confer resistance to neonicotinoids in *Drosophila melanogaster*. *Insect Biochem Mol Biol* **38**: 520–528.

Ren, X.-Q., Chen, S.-B., Treuil, M., Mukherjee, J., Rao, J., Braunewell, K.H., Lindstrom, J.M. and Anand, R. (2005) Structural determinants of a4b2 nicotinic acetylcholine receptor trafficking. *J Neurosci* **25**: 6676–6686.

Salgado, V.L. and Saar, R. (2004) Desensitizing and non-desensitizing subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. *J Insect Physiol* **50**: 867–879.

Saragoza, P.A., Modir, J.G., Goel, N., French, K.L., Li, L., Nowak, M.W. *et al*. (2003) Identification of an alternatively processed nicotinic receptor a7 subunit RNA in mouse brain. *Molec Brain Res* **117**: 15–26.

Sattelle, D.B., Jones, A.K., Sattelle, B.M., Matsuda, K., Reenan, R. and Biggin, P.C. (2005) Edit, cut and paste in the nicotinic acetylcholine receptor gene family of *Drosophila melanogaster*. *BioEssays* **27**: 366–376.

Schulz, R., Bertrand, S., Chamaon, K., Smalla, K.-H., Gundelfinger, E.D. and Bertrand, D. (2000) Neuronal nicotinic acetylcholine receptors from *Drosophila*: Two different types of a subunits coassemble within the same receptor complex. *J Neurochem* **74**: 2537–2546.

Scott, J.G. (2008) Unraveling the mystery of spinosad resistance in insects. *J Pestic Sci* **33**: 221–227.

Shao, Y.-M., Dong, K. and Zhang, C.-X. (2007) The nicotinic acetylcholine receptor gene family of the silkworm, *Bombyx mori*. *BMC Genomics* **8**: 324–333.

Shimomura, M., Satoh, H., Yokota, M., Ihara, M., Matsuda, K. and Sattelle, D.B. (2005) Insect-vertebrate chimeric nicotinic acetylcholine receptors identify a region, loop B to the N-terminus of the *Drosophila* Da2 subunit, which contributes to neonicotinoid sensitivity. *Neurosci Lett* **385**: 168–172.

Thany, S., Lanaers, G., Raymond-Delpech, V., Sattelle, D. and Lapied, B. (2006) Exploring the pharmacological properties of insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci* **28**: 14–22.

Throne, J.E., Hallman, G.J., Johnson, J.A. and Follett, P.A. (2003) Post-harvest entomology research in the United States Department of Agriculture-Agricultural Research Service. *Pest Manag Sci* **59**: 619–628.

Toews, M.D., Subramanyam, B. and Rowan, J.M. (2003) Knockdown and mortality of adults of eight species of stored-product beetles exposed to four surfaces treated with spinosad. *J Econ Entomol* **96**: 1967–1973.

Tomizawa, M. and Casida, J.E. (2005) Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu Rev Pharmacol Toxicol* **45**: 247–268.

Tribolium Genome Sequencing Consortium (2008) The genome of the model beetle and pest *Tribolium castaneum*. *Nature* **452**: 949–955.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. GenBank accession numbers for transcripts and alleles described in the text

Table S2. Sequences of primers used in this study

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

