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The nicotinic acetylcholine receptor subunits *Mdα5* and *Mdβ3* on autosome 1 of *Musca domestica* are not involved in spinosad resistance

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Abstract

Spinosad is a relatively new insecticide that exerts its toxic action via nicotinic acetylcholine receptors (nAChRs). Spinosad resistance in house flies appears to be due to an altered target site. To evaluate the molecular basis of spinosad resistance, two nAChR subunit genes, *Mdα5* and *Mdβ3* were cloned and characterized from an insecticide-susceptible (aabys) and spinosad resistant (rspin) strain of the house fly, *Musca domestica*. The *Mdα5* and *Mdβ3* cDNAs encode proteins of 781 and 432 amino acid residues, respectively. Phylogenetic analysis with insect nAChR subunits suggested that Mdα5 and Mdβ3 are most closely related to Dα5 and Dβ3 of *Drosophila melanogaster*, respectively. *Mdβ3* is intronless, which is unique among all previously described nAChR genes. A-to-I RNA editing was found at 13 sites in *Mdα5*, eleven of which resulted in amino acid substitutions. No evidence for A-to-I RNA editing was found in *Mdβ3*. *Mdα5* expression, quantified by real time PCR, was 340- and 23-fold higher in the head and thorax than in the abdomen. *Mdβ3* expression was more uniform, being only 2.4-fold higher in the head and 1.4-fold lower in the thorax, compared to the abdomen. There was no difference in the expression of *Mdα5* and *Mdβ3* between the aabys and rspin strains. Although *Mdα5* and *Mdβ3* both map to the same chromosome as spinosad resistance, there were no unique features of either gene in rspin, relative to the aabys strain. This suggests neither *Mdα5* nor *Mdβ3* is responsible for spinosad resistance in house flies.

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Introduction

Nicotinic acetylcholine receptors (nAChR) play a number of essential physiological roles in both vertebrates and insects, including the well characterized mediation of fast excitatory neurotrasmission at cholinergic synapses (Karlin, 2002). nAChRs are ligand-gated ion channels of the Cys-loop superfamily that include γ-aminobutyric acid (GABA)-gated Cl^- channels, glycine receptors, glutamate-gated Cl^- channels and 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors (Lester *et al.*, 2004). Vertebrate nAChRs exist as pentameric proteins consisting of homologous subunits, typically 2 α and 3 non-α subunits (receptors consisting of only α subunits are also known) and insect nAChRs are thought to have a similar configuration (Couturier *et al.*, 1990; Marshall *et al.*, 1990). The α subunits are characterized by the presence of two adjacent cysteine residues in loop C, while the non-α subunits lack this cysteine doublet. Each subunit possesses a large N-terminal extracellular domain that includes components forming the acetylcholine (ACh) binding site, and four hydrophobic transmembrane domains (TM1-4) with TM2 contributing most of the amino acids that line the ion channel (Karlin, 2002). The ACh binding site is located at the interface of two subunits and is possibly contributed by three loops (loops A–C) of an α subunit and by three loops (loops D–F) of another (α or non-α) subunit (Grutter & Changeux, 2001). In addition to multiple subunit genes, at least some nAChRs are diversified through post-transcriptional modifications, such as RNA editing and alternative splicing (Grauso *et al.*, 2002; Sattelle *et al.*, 2005; Gao *et al.*, 2007a,b).

Spinosad is a relatively new insecticide derived from the bacterium *Saccharopolyspora spinosa* and is used to control a wide range of insects. The primary target of spinosad appears to be nAChRs (Salgado & Sparks, 2005). Resistance to spinosad has been reported from US field populations of the diamondback moth, *Plutella xylostella*, (Zhao *et al.*, 2002, 2006) and beet armyworm, *Spodoptera*

exigua, from the US and Thailand (Moulton *et al*., 2000). Spinosad was registered for house fly (*Musca domestica*) control in the US in 2005. The rapid development of resistance to spinosad in field and laboratory populations of several insect species (Moulton *et al*., 2000; Zhao *et al*., 2002; Shono & Scott, 2003; Hsu & Feng, 2006) highlights the need to develop monitoring programs. However, monitoring spinosad resistance with insecticide bioassays is problematic, given the highly recessive nature of this trait (Deacutis *et al*., 2007). Thus, development of an allele specific molecular monitoring tool is highly desirable.

A strain of house fly that is > 150-fold resistant to spinosad was selected from field populations in 1998. The resistance in house flies is monofactorial, recessive and cannot be overcome with insecticide synergists, suggesting resistance is due to an altered target site (Shono & Scott, 2003). This is similar to resistance in diamondback moth, tobacco budworm, *Heliothis virescence* (Salgado & Sparks, 2005) and oriental fruit fly, *Bactrocera dorsalis* (Hsu & Feng, 2006) and suggests a common mechanism (target insensitivity) in these species. In house fly, spinosad resistance is linked to autosome 1 (Shono & Scott, 2003). Based on a *D. melanogaster*/*M. domestica* homology map (Foster *et al*., 1981), autosome 1 should have three nAChR subunit genes, α6, α5 and β3. Strains of *D. melanogaster* that lack *Dα6* are highly resistant to spinosad (Perry *et al*., 2007). However, *Mdα6* does not appear to be responsible for spinosad resistance in house flies (Gao *et al*., 2007b). These results led us to analyze the other two nAChR subunit genes (α5 and β3) predicted (by *D. melanogaster*/*M. domestica* homology maps) to be on autosome 1. Here we report the cloning and characterization of *Mdα5* and *Mdβ3* nAChR subunit genes from house flies; including RNA editing, expression and linkage analysis in insecticide-susceptible and spinosad resistant strains of house flies.

Table 1. Sequences of primers used in this study

Name	Sequence
MaF2	5′-ATGAARTTYGGNWSNTGGACNTAYGA-3′
MaR1	5′-GCNACCATRAACATDATRCARTTRAA-3′
3′M5Race1	5′-TCAACGGCGAGTGGGAACTACTGG-3′
5′M5Race3	5′-CAACGTTCGTCGTCGTATGATGATGGCA-3′
5′M5Race4	5′-TCGATATACGGTTCCGGACAGCAGTTG-3′
5′M5Race6	5′-GTCCTTTGGTTGTCAGTAACGGTAGTAG-3′
5′M5Race7	5′-GTTGGTGAGGACGATAGCGGCGATGAC-3′
5′M5Race10	5′-GTGCGGTTTGTGACAATGCGGTTC-3′
5′M5Race11	5′-GCATACAGTGTTGCAATTGCGGTCA-3′
5′M5Race15	5′-CCACTAAGATGGTGCTCCACATTGCT-3′
M5F1	5′-GACAGGGAGAGAGAGAGAGTATAAAGCT-3′
M5R4	5′-GAGAGGTTAGGACAGCAGTGAAACATC-3′
M5R1	5′-GATAAAACCAATAACAATAACCACCTCTCTC-3′
qMa5F2	5′-GGTATTTCTATGTTGGTTACCATGGA-3′
qMa5R2	5′-GAACATGGCGTCGATGTGAA-3′
gM5IF1	5′-GAACCGCATTGTCACAAACCGCAC-3′
5′M5Race8	5′-GTGGTGGTGGTGGCAGCGATGGAG-3′
gM5I7F2	5′-CATTGCCGGCCAATGAAATTTTGAA-3′
gM5VIIIR3	5′-CATTCGGACATTTCGTGTGTATCAGCA-3′
gM5F1	5′-ACGTGTGGTATTTCTATGTTGGTTAC-3′
gM5I11R	5′-TTGAACTACAAACATCTTGAACTTACCTG-3′
gM5XIF	5′-TGGACCTAATGACACACGAATTGACATA-3′
MBF1	5′TGGATGCCNCCNGCNGTNTAYAC-3′
MBR2	5′GGNGTRTADATNACNGCNGTRTACAT-3′
5′MBRace1	5′GTACATCGAAGAGTAGCGTTGAAGTTGGA-3′
3′MBRace1	5′TCAAGGAAAGCATCGATTACAAGGA-3′
MB3F1	5′TTTCGTGGATATTGTGCGGCGT-3′
MB3F2	5′-TGCCGCAAACAATTGTGGATTTAAT-3′
MB3R1	5′GACATATTTTTGGTTTTCGAATGGTAGA-3′
MB3R1a	5′-TATTTACATATTAAATTTGCAATACAAAAATG-3′
qMBF1	5′-AGCTCGCACCCCATGGT-3′
qMBR1	5′-TCGATGTAGTTGACGGCTATGC-3′

Results and discussion

Cloning and characterization of Mdα5

The full length (4157-bp) cDNA of *Mdα5* (accession no. EF203213) from aabys flies consists of a 2343-bp ORF (Fig. 1), a 1647-bp 5′UTR and a 167-bp 3′UTR. The 5′UTR contains 14 in-frame stop codons (TAA, TAG and TGA) upstream of the position nt –990, indicating the ORF is complete. It also contains an in-frame start codon (ATG) at nt –912 to –910, which is unlikely to be the correct start codon because the resulting deduced amino acid sequence would lack a signal peptide. The 3′UTR contains two polyadenylation siginals (AATAAA) at nt 2429–2434 and 2443–2448, respectively and ends with a 29-adenine tail. Numerous single nucleotide polymorphisms (SNPs) in the ORF were identified (Table 1), and at least 13 (in aabys) and 12 (in rspin) of them resulted from A-to-I RNA editing (discussed below). A 15-nt deletion was found at a frequency of 14% (7/51 transcripts) in aabys flies and 21% (3/14 transcripts) in rspin flies at nt 2041 to 2055, resulting in a loss of YLENL residues (681–685). Comparison with genomic DNA sequences revealed that this deletion was generated by an alternative use of donor (5′) splice sites (gtattta or gtaatac) of intron 10. The use of the first donor splice site seems unique for *Mdα5* when compared to its orthologs in *D. melanogaster*, *Anopheles gambiae* and *Aedes aegypti* (Grauso *et al*., 2002; Jones *et al*., 2005). In addition, nucleotide triplet deletions, although at a very low frequency, were also found at nt –352 to –354, –172 to –174, 498–500, 565–567 and 688–690 in different aabys transcripts (one each of these deletions was found in a single transcript). These triplet deletions occurred within $(AAC)_4$, $(CAA)_5$, $(ACA)_5$, $(CAG)_5$ and $(CCA)_5$ triplet repeat regions, respectively. The latter three of them are in the ORF and resulted in loss of single amino acids. A single triplet deletion (nt 688–690) was found in 14% (2/14) of the transcripts from the rspin flies. Comparisons of the different sequences in aabys and rspin demonstrate there is transcript variability within and between strains. However, given that rspin and aabys express many of the same transcripts, suggests that changes in the nt sequence of *Mdα5* are not responsible for spinosad resistance.

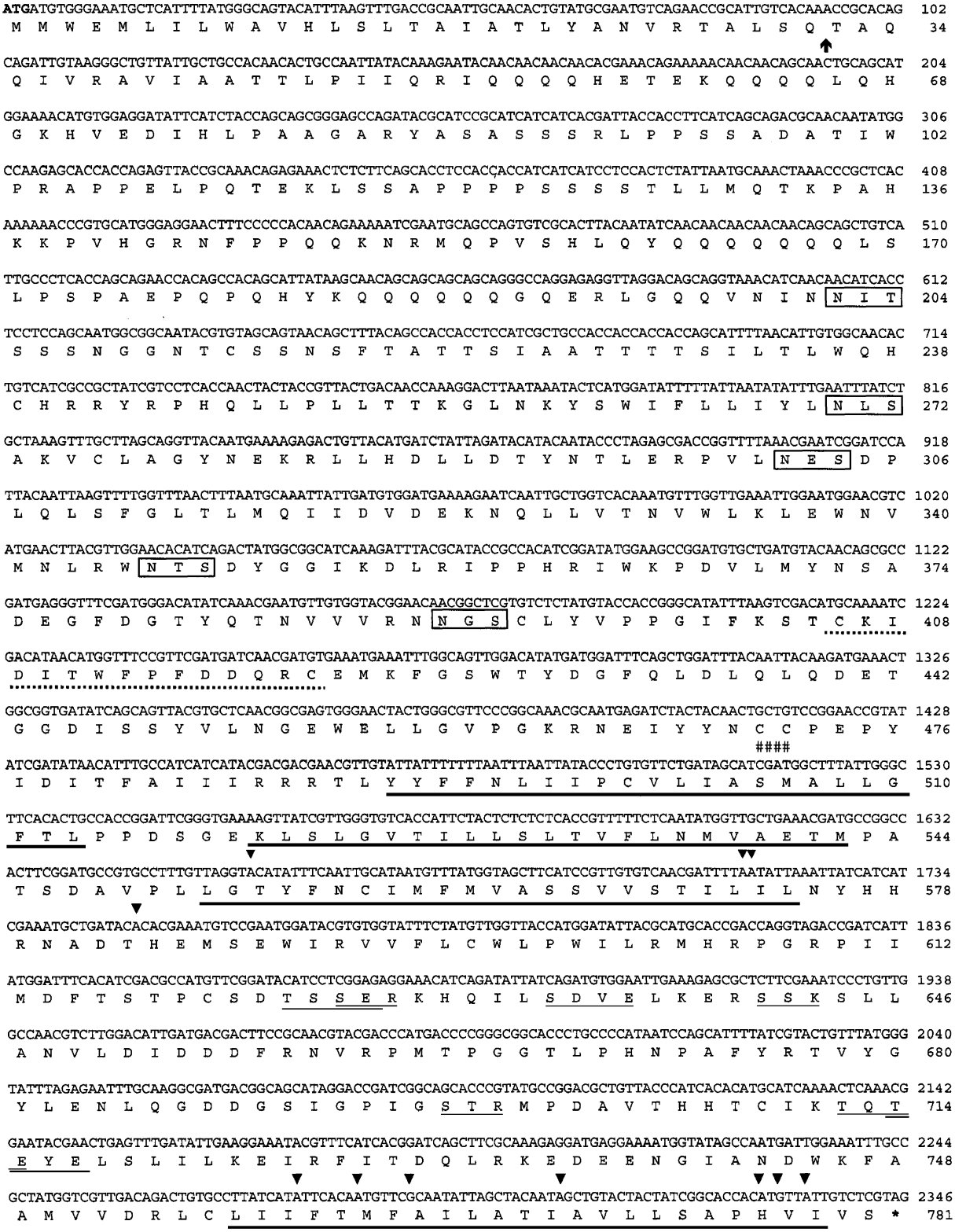

Figure 1. Nucleotide and deduced amino acid sequences of *Mdα5* cDNA from *Musca domestica* (accession no. EF203213). The translation start codon, ATG, is shown in bold. The stop codon, TAG, is indicated by a star. The signal peptide cleavage site is marked with a vertical arrow. Potential N-glycosylation sites are boxed. The potential phosphorylation sites are underlined with a fine line. Transmembrane domains (TM1-4) are underlined with a thick line. The cysteine doublet (characteristics of nAChR α subunits) is marked with as '###'. The Cys loop domain is underlined with a dotted line. A-to-I RNA editing sites are marked with triangles. The 5′ and 3′ UTRs are not shown.

The 2343-bp ORF of *Mdα5* encodes a 781-amino acid proprotein that possesses a 27-aa signal peptide (predicted by SignalP 3.0, www.cbs.dtu.dk/services/SignalP/). Besides the signal peptide, the protein possesses typical characteristics of nAChR α subunits including a long N-terminal extracellular domain having the α subunit signature–YXCC motif, the Cys loop domain and four hydrophobic transmembrane domains (TM1-4). The mature protein has 5 potential N-glycosylation sites in the N-terminal extracellular domain that may be involved in receptor assembly and ligand binding, regulation of receptor desensitization and ion permeability (Chen *et al*., 1998; Nishizaki, 2003; Wanamaker & Green, 2005). The mature protein also has 7 potential phosphorylation sites for protein kinase C and casein kinase II located in the cytoplasmic TM3-TM4 linker. Phosphorylation is important in regulating receptor properties (Swope *et al*., 1992; Fenster *et al*., 1999; Wecker *et al*., 2001; Charpantier *et al*., 2005). The mature protein has a calculated molecular weight of 85.3 kDa and isoelectric point of 6.44. The calculated molecular weight of Mdα5 is greater than Dα5 (56.9 kDa) and Agamα5 (56.7 kDa) (Grauso *et al*., 2002; Jones *et al*., 2005; Wu *et al*., 2005).

A BLASTP search of GenBank with the Mdα5 proprotein sequence revealed that this deduced aa sequence is most similar to *Drosophila* Dα5 (AAM13390), *Anopheles* Agamα5 (AAU12508), Agamα7 (AAU12511), *Drosophila* Dα7 (CAD86936), and *Tribolium* Tcα7 (XP_969669). Mdα5 is most closely related to Dα5 because both have an extended N-terminal extra celular domain, and because of the high percentage of C-terminal sequence identity. Although the N-terminal sequence is variable, when the 503-aa C-terminal sequence was compared (pair wise) with the mature protein of other insect nAChRs, it showed 95%, 86%, 79%, 79% and 78% identity with Dα5, Agamα5, Agamα7, Tcα7 and Dα7, respectively.

Cloning and characterization of Mdβ3

The full length (1744 bp) cDNA of *Mdβ3* (GenBank accession no. EF203216) consists of a 1296-bp ORF, a 304-bp 5′UTR and a 144-bp 3′UTR (Fig. 2). The 5′UTR contains 4

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ATGTTTACTTCCTGCCGTCACCGCGCCACCATCACAGTCGCTCTGTTCGTGGTGGCAGCAATTCAGCTGGCTGCCTGCACTGAGGACGGAAATGCCTCATGG  102
 M  F  T  S  C  R  H  R  A  T  I  T  V  A  L  F  V  V  A  A  I  Q  L  A  A  C  T  E  D  G  N  A  S  W   34

AATATCAGCACAATGGACCGTCTGCGTGTACAACTGTTTACGAACTATGATAAGAGCTCGCACCCCATGGTTACACCCACGCAGCGGACGAACATAACATTG  204
 N  I  S  T  M  D  R  L  R  V  Q  L  F  T  N  Y  D  K  S  S  H  P  M  V  T  P  T  Q  R  T  N  I  T  L   68

GGCATAGCCGTCAACTACATCGATATCGATGAATTGAAGGGCAGAATGACGCTGCATGGCTGGGTCAACATGCGCTGGCGCGATGGAGGAAGGACATGGAAG  306
 G  I  A  V  N  Y  I  D  I  D  E  L  K  G  R  M  T  L  H  G  W  V  N  M  R  W  R  D  G  G  R  T  W  K  102

CCGGAATATTTCGACAACATAACCACCTTGCATATACGTTCCAAGGAGGTATGGAAGCCGGACATTACTCTGTTCAACAGCGCCGGCAGCGAGGGCGATTAT  408
 P  E  Y  F  D  N  I  T  T  L  H  I  R  S  K  E  V  W  K  P  D  I  T  L  F  N  S  A  G  S  E  G  D  Y  136

GTTGGTGATACACAGACTATGCTCTCCTACGATGGTAGCTTCATGTGGGTGCCCCCCGGTGGTTTATACAGCCTACTGTAATCTGAATCTTAAAATGTGGCCG  510
 V  G  D  T  Q  T  M  L  S  Y  D  G  S  F  M  W  V  P  P  V  V  Y  T  A  Y  C  N  L  N  L  K  M  W  P  170

TACGACCAGCAAACGTGCAAACTCAAAGTGGGTACATGGACTTTGACTACAATCGATCCGAAATTCCTTGACTTCAAGGAAAGCATCGATTACAAGGATTTG  612
 Y  D  Q  Q  T  C  K  L  K  V  G  T  W  T  L  T  T  I  D  P  K  F  L  D  F  K  E  S  I  D  Y  K  D  L  204

ATTAAATCCACCGAATGGGACATCATCGACGCCAAGGCCACATACAACAGTGAGGAATTCTACAACTATATAGAGTACACATTCCAACTTCAACGCTACTCT  714
 I  K  S  T  E  W  D  I  I  D  A  K  A  T  Y  N  S  E  E  F  Y  N  Y  I  E  Y  T  F  Q  L  Q  R  Y  S  238

TCGATGTACACAACGGTTATTTTCACGCCAGCATCCTGCATTATACTAATGTGTCTATCCATATTCTGGCTGCCGCCCCAAATGGGAGAGAAAATTTTACTC  816
 S  M  Y  T  T  V  I  F  T  P  A  S  C  I  I  L  M  C  L  S  I  F  W  L  P  P  Q  M  G  E  K  I  L  L  272

AATGCCGTTCTGATTGTAATTATTGCCGCATTCCTGATGTACTTTGCTCAAATGCTGCCCATATTGGCGGAAAATACCCCACTAGTGGTACTCTTCTACAGC  918
 N  A  V  L  I  V  I  I  A  A  F  L  M  Y  F  A  Q  M  L  P  I  L  A  E  N  T  P  L  V  V  L  F  Y  S  306

GCCAGTTTGTTGCTTTTGAGCATATCGACAATCGTTTCGGTAACCGTCCTCTATTTGTCCACAGCCAAACATAAGCAACGTGTACCGGCATTTTTGAGAAAC 1020
 A  S  L  L  L  L  S  I  S  T  I  V  S  V  T  V  L  Y  L  S  T  A  K  H  K  Q  R  V  P  A  F  L  R  N  340

CTTCTAAATGGCGGCTTGGGTCGTGTTTTATTGCTAAGCGAGTTTTCCTTAGAAGCCGAACCGCAAACCTTGCTGAATAATGGCACCAAGGAGATGGGCGAA 1122
 L  L  N  G  G  L  G  R  V  L  L  L  S  E  F  S  L  E  A  E  P  Q  T  L  L  N  N  G  T  K  E  M  G  E  374

CACGTCTATGACGAACCAATTGAGACAACAAACGAAAATACAATGTTCAATGCATCGAATCAGACACCGAGATCATTGCAATTCGATTGGATTCTATTGGCG 1224
 H  V  Y  D  E  P  I  E  T  T  N  E  N  T  M  F  N  A  S  N  Q  T  P  R  S  L  Q  F  D  W  I  L  L  A  408

ACAGCCGTTGATCGCATCTTCTTCCTGGTGTACTGTTTTATTTTCATTATTCTGGCCATTGTCTATTCGGTGTAG                          1299
 T  A  V  D  R  I  F  F  L  V  Y  C  F  I  F  I  I  L  A  I  V  Y  S  V  *                            432
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Figure 2. Nucleotide and deduced amino acid sequences of *Mdβ3* cDNA from *Musca domestica* (accession no. EF203216). The translation start codon, ATG, is shown in bold. The stop codon, TAG, is indicated by a star. The signal peptide cleavage site is marked with a vertical arrow. Potential N-glycosylation sites are boxed. The potential phosphorylation sites are underlined with a fine line. Transmembrane domains (TM1-4) are underlined with a thick line. The Cys loop domain is underlined with a dotted line. The 5′ and 3′ UTRs are not shown.

in-frame stop codons (TAA and TGA) upstream of nt –123. The 3′UTR contains a polyadenylation signal (AATAAA) at nt 1371 and 1376 and ends with a 43 adenine tail.

The 1296-bp *Mdβ3* ORF encodes a 432-aa proprotein that possesses a 26-aa signal peptide (predicted by SignalP 3.0). Besides the signal peptide, this protein has typical features of nAChRs including a long N-terminal extracellular domain, the Cys loop domain and four hydrophobic transmembrane domains (TM1-4). It lacks the cystein doublet in the N-terminal extracellular domain and therefore, this subunit is classified as a non-α or β subunit. Similar to Dβ3, Mdβ3 has an extremely short C-terminal extracellular domain of only one amino acid. The predicted mature Mdβ3 protein has a calculated molecular weight of 46.7 kDa and isoelectric point of 5.08, similar with those of Dβ3 (45.3 kDa and pI 5.22) (Lansdell & Millar, 2002). The mature protein has 4 potential *N*-glycosylation sites located in the N-terminal extracellular domain. There are 5 potential phosphorylation sites for protein kinase C, casein kinase II and tyrosine kinase, located in the cytoplasmic TM3-TM4 linker.

A BLASTP search of GenBank revealed that Mdβ3 is most similar to *Drosophila* Dβ3 (CAC48166), *Tribolium castaneum* XP_974495, *Ae. aegypti* EAT41882, *An. gambiae* Agamα9 (AAU12513) and the pufferfish *Takifugu rubripes* Frα4 (AAP58377), having 54%, 34%, 34%, 31% and 19% identity (based on comparison of mature protein sequences), respectively. Analysis of genomic DNA sequences revealed that *Mdβ3* contained no introns. Given that all other insect nAChR subunit genes have introns (e.g. *Dβ3* has 3), makes *Mdβ3* unique in this regard (Lansdell & Millar, 2002; Jones *et al*., 2005).

Three alleles were determined by analyzing genomic sequences. Two alleles were found (alleles A and B, accession nos EF203217 and EF203218, respectively) in aabys and two alleles (allele B and C, accession EF203219 and EF203220, respectively) in rspin. There are 27 SNPs between alleles A and B, 2 SNPs between allele B and C and 29 SNPs between allele A and C. Only one (G1231A) of them resulted in amino acid substitution (V411A). Nucleotide sequence comparison between aabys and rspin strains did not reveal any specific nucleotide difference associated with spinosad resistance, indicating that *Mdβ3* does not contribute to spinosad resistance.

Phylogenetic analysis

A phylogenetic analysis of the nAChR subunit genes with 74 proteins from other insects (which contain full or almost full sequences for mature proteins) retrieved from NCBI databases was performed using the neighbor-joining method. The tree has a good (> 436 out 1000) Boostrap support, and shows good agreement overall with assigned names. One exception is the Agamα9 clade (Fig. 3) that contains both α and β subunit genes. Other exceptions (e.g. Nlα2-4, Amelα5, etc.) suggest the current nomenclature does not provide a perfect reflection of orthologous genes.

A-to-I RNA editing

A-to-I RNA editing, catalyzed by adenosine deaminases acting on RNA (ADARs), is a pre-mRNA processing event that converts adenosine (A) to inosine (I) within short (approximately 9–15), double stranded regions of RNA molecules (Seeburg, 2002). Because the inosine is recognized as guanosine (G) by the cellular machinery, this editing functions as A-to-G conversion. Therefore, A-to-I editing may result in amino acid substitutions, changing in protein functions or altering gene expression by affecting RNA splicing, stability, and localization (Sattelle *et al*., 2005). Grauso *et al*. (2002) first investigated this phenomenon in the nAChR family and found seven edited sites in *Dα6* of *D. melanogaster.* Hoopengardner *et al*. (2003) further identified seven, four and two A-to-I RNA editing sites in *Dα5*, *Dβ1* (*ARD*) and *Dβ2* (*SBD*), respectively. We previously identified 12 and one A-to-I RNA editing sites in *Mdα6* and *Mdα2*, respectively (Gao *et al*., 2007a,b).

A-to-I RNA editing was examined in *Mdα5* and *Mdβ3* by comparing genomic and cDNA sequences. Thirteen A-to-I editing sites were identified in *Mdα5* in aabys flies, three (sites 9, 13 and 14) of which were edited in > 50% of the transcripts (Tables 2 and 3). By analyzing the *Mdα5* transcripts ($n = 14$) of rspin flies, editing frequencies were estimated as follows: 7 (site), 29% (frequency); 8, 50%; 9, 64%; 10, 0%; 11, 7%; 12, 7%, 13, 36%; 14, 100%; 15, 7%; 16, 7%; 18, 7%; 19, 100% and 20, 64%. A-to-I editing at 11 sites resulted in amino acid substitution (Table 2). These sites are situated in transmembrane domains. Two located in the TM3 domain and nine in the TM4 domain. The A-to-I RNA editing at nt 1704, 1705, 2278 and 2295 are conserved, both in terms of location and editing rate in *Mdα5* and *Dα5* (Hoopengardner *et al*., 2003), but these sites are not edited in Agamα5 of *An. gambiae* or Amelα5 of *Apis Mellifera* (Jones *et al*., 2005; Jones *et al*., 2006). No A-to-I RNA editing was found in *Mdβ3*.

Gene expression

Expression of *Mdα5* and *Mdβ3* in the head, thorax and abdomen of aabys adult flies was investigated using quantitative real-time PCR. The number of transcripts (per 360 ng total RNA) of *Mdα5* was 18 636 ± 3286 (mean ± SE), 1250 ± 222 and 53 ± 9.7 in the head, thorax and abdomen, respectively (Fig. 4A). *Mdα5* expression was 340- and 23.0-fold higher in the head and thorax, respectively, relative to the abdomen. The number of transcripts (per 360 ng total RNA) of *Mdβ3* was 14 600 ± 4300, 4550 ± 1070 and 6030 ± 1680 in the head, thorax and abdomen, respectively (Fig. 4A). *Mdβ3* expression was 2.4-higher in the head but 0.24-fold lower in the thorax, respectively, relative to the abdomen. The expression of these two genes

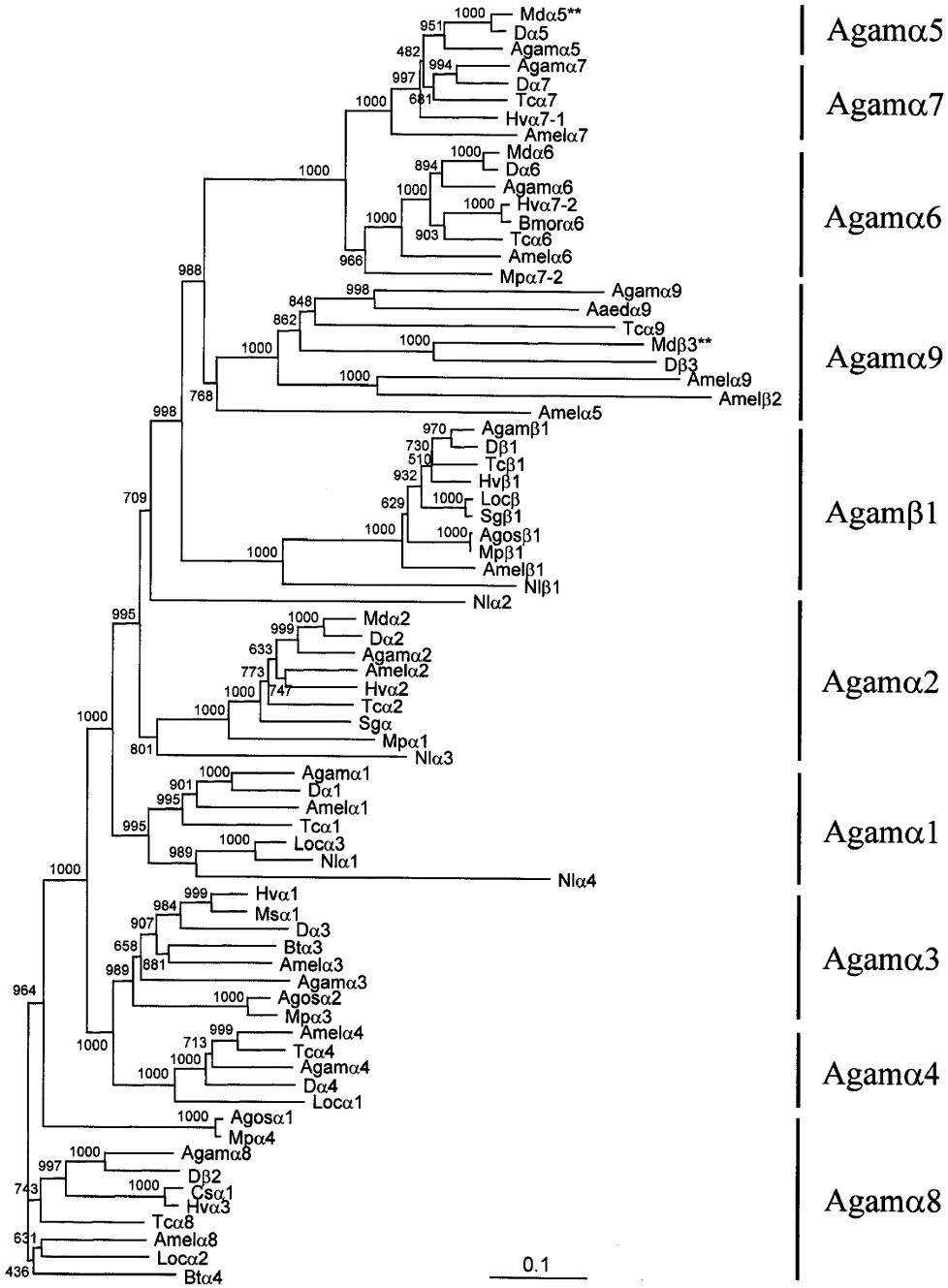

Figure 3. Evolutionary relationships of Mdα5 and Mdβ3 with other insect nAChR subunits constructed using the neighbor-joining method. Bootstrap values with 1000 trials are indicated on branches. The scale bar represents substitutions per site. Sequences (GenBank accession numbers) used: *Aedes aegypti* Aaegα9 (EAT41882), *Anopheles gambiae* Agamα1 (AAU12503), Agamα2 (AAU12504), Agamα3 (AAU12505), Agamα4 (AAU12506), Agamα5 (AAU12508), Agamα6 (AAU12509), Agamα7 (AAU12511), Agamα8 (AAU12512), Agamα9 (AAU12513), Agamβ1 (AAU12514); *Aphis gossypii* Agosα1 (AAM94383), Agosα2 (AAM94382), Agosβ1 (AAM94385); *Apis mellifera Amelα1* (*AAY87890*), Amelα2 (AAS48080), Amelα3 (AAY87891), Amelα4 (AAY87892), Amelα5 (AAS75781), Amelα6 (AAY87894), Amelα7 (AAR92109), Amelα8 (AAM51823), Amelα9 (AAY87896), Amelβ1 (AAY87897), Amelβ2 (AAY87898); *Bemisia tabaci* Btα3 (CAI54098), Btα4 (CAI54099); *Bombyx mori* Bmorα6 (ABL67935); *Chilo suppressalis* Csα1 (AAL40742); *Drosophila melanogaster* Dα1 (CAA30172), Dα2 (CAA36517), Dα3 (CAA75688), Dα4 (CAB77445), Dα5 (AAM13390), Dα6 (AAM13393), Dα7 (CAD86936), Dβ1 (CAA27641), Dβ2 (CAA39211), Dβ3 (CAC48166); *Heliothis virescens* Hvα1 (CAA04056), Hvα2 (AAD09808), Hvα3 (AAD09809), Hvα7-1 (AAD32697), Hvα7-2 (AAD32698), Hvβ1 (AAD09810); *Locusta migratoria* Locα1 (CAA04052), Locα2 (CAA04053), Locα3 (CAA04054), Locβ (CAA04055); *Myzus persicae* Mpα1 (CAA57476), Mpα3 (CAB52297), Mpα4 (CAB52298), Mpα7-2 (CAI54103), Mpβ1 (CAB87995); *Manduca sexta* Msα1 (CAA70928); *Musca domestica* Mdα2 (DQ372062), Mdα5 (EF203213), Mdα6 (DQ498130), Mdβ3 (EF203216); *Nilaparvata lugens* Nlα1 (AAQ75737), Nlα2 (AAQ75741), Nlα3 (AAQ75739), Nlα4 (AAQ75738), Nlβ1 (AAQ75742); *Schistocerca gregaria* Sgα (CAA39081), Sgβ1 (ABA39253); *Tribolium castaneum* Tcα1 (XP_973899), Tcα2 (XP_972094), Tcα4 (XP-974090), Tcα6 (XP_967171), Tcα7 (XP_969669), Tcα8 (XP_969525), Tcα9 (XP_974495) and Tcβ1 (XP_ 969511).

Table 2. Single nucleotide polymorphisms and the corresponding deduced amino acids found in *Mdα5* of insecticide susceptible (aabys) and spinosad (rspin) resistant strains of *Musca domestica*

		Site																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
aabys																											
nt		G/A	C/T	A/G	A/G	A/G	T/C	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	
		253	589	1019	1262	1367	1531	1660	1716	1717	1749	2275	2277	2284	2290	2293	2307	2328	2330	2332	2335						
aa		A/T	Q/Stop	D/V	E/G	E/G	F/L	T/A	L	I/V	T	I/V	I/M	M/V	T/A	I/V	I/M	P	H/R	I/V	I/V						
		85	197	340	421	456	511	454	572	573	583	759	759	762	764	765	769	776	777	778	779						
rspin																											
nt		G/A	A/G	T/C	C/T	A/G	A/G	T/C	T/C	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G
		253	297	476	589	905	909	1405	1635	1660	1716	1717	1768	1770	2057	2063	2150	2275	2277	2284	2290	2293	2307	2328	2330	2332	2335
aa		A/T	A	L/S	Q/Stop	N/S	E	Y/H	T	T/A	L	I/V	I/V	I/M or V	Q/R	D/G	E/G	I/V	I/M	M/V	T/A	I/V	I/M	P	H/R	I/V	I/V
		85	99	159	197	302	301	469	545	454	572	573	590	590	686	688	717	759	759	762	764	765	769	776	777	778	779

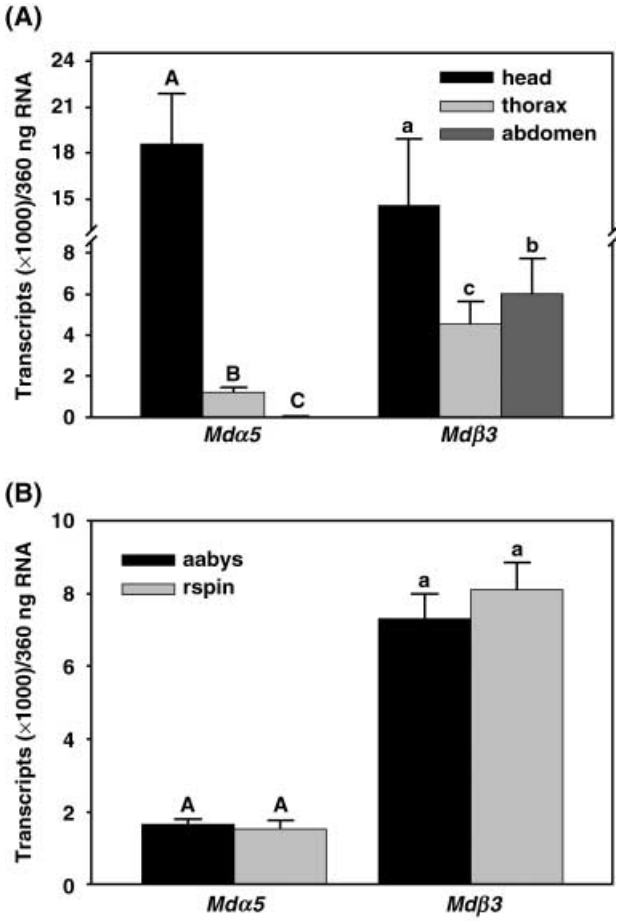

Figure 4. Expression of *Mdα5* and *Mdβ3* in different tagmata (A) and whole body (B) of adult houseflies measured by quantitative real-time PCR. Copy number is per 360 ng of total RNA (amount used in each reaction for the qRT-PCR). Error bars represent standard error of the means of three replicates. Different letters with the bars for each gene indicate that the means are significantly different ($P < 0.05$) in Tukey's test.

(Fig. 4B) was not significantly different ($P > 0.05$) in aabys and rspin flies.

The central nervous system (CNS) of the adult house flies is highly specialized, consisting of a cephalic ganglion (a complex of brain and suboesophageal ganglia) in the head, and a thoracic compound ganglion (a fusion of all the thoracic and abdominal ganglia) (Hewitt, 1914). The expression of *Mdα5* is consistent with expression in the CNS of house flies and is quite similar to the expression patterns of *Mdα2* (Gao *et al.*, 2007a) and *Mda6* (Gao *et al.*, 2007b). However, the relative high expression level of *Mdβ3* in the abdomen was unique (relative to *Mdα2*, *Mdα5* and *Mdα6*), and its significance will require further investigation.

Linkage analysis

Analysis of the partial *Mdα5* genomic sequences of the parental (aabys and OCR) and F_1 (aabys × OCR) flies indicated the aabys strain had allele A (accession no.

Table 3. A-to-I RNA editing efficiency at different sites and the corresponding amino acid substitution in *Mdα5* from aabys strain of *Musca domestica*

Site*	7	8	9	10	11	12	13	14	15	16	18	19	20
nucleotide	1660	1716	1717	1749	2275	2277	2284	2290	2293	2307	2330	2332	2335
aa substitution	T/A	L	I/V	T	I/V	I/M	M/V	T/A	I/V	I/M	H/R	I/V	I/V
	454	572	573	583	759	759	762	764	765	769	777	778	779
%†	12	42	58	6	3	6	22	100	3	18	13	100	34

*Sites were numbered as indicated in Table 2.
†Three individual flies were used. Ten or 11 clones were analyzed per fly.

Table 4. Patterns of A-to-I RNA editing in *Mdα5* of aabys houseflies

Type	N	Sites edited*
1	6	13, 16
2	3	13, 16, 18
3	1	9, 13, 16, 18
4	3	8, 9, 13, 14, 16
5	1	9, 12, 13, 16, 18
6	1	8, 9, 12, 13, 16, 18
7	1	13, 15, 16
8	1	9, 13, 16
9	1	11, 13, 14, 16,
10	1	8, 9, 13, 16,
11	1	9, 10, 13, 16
12	1	8, 9, 13, 14, 16, 18
13	1	8, 9, 10, 12, 13, 16, 18
14	1	13, 14, 15, 16
15	1	7, 8, 9, 13, 16
16	1	8, 9, 12, 13, 16
17	1	7, 8, 9, 12, 13, 16
18	1	8, 9, 13, 15, 16, 18
19	1	9, 13, 15, 16, 17
20	1	7, 13, 16
21	1	8, 9, 11, 12, 13, 16
22	1	8, 9, 13, 16, 18

*See Table 2 for numbering.

EF203214) and the OCR strain had allele B (accession no. EF203215). Detection of individuals homozygous for the allele A in one of the five genotypes isolated from the backcross would indicate that *Mdα5* was not linked to the autosome having the wild type trait. For example, if an individual homozygous for the allele A had the genotype *ac/ac;+/ar;bwb/bwb;ye/ye;snp/snp*, we would conclude that *Mdα5* is not on autosome 2. Our analyses were made on three individuals for each of the backcross genotypes (having mutant markers for four of the five autosomes). Results showed that heterozygotes were only detected in individuals that were wild type for autosome 1 (*+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp*) (Table 4). The lack of homozygotes of allele A exclusivity in *+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp* individuals indicates that *Mdα5* is on autosome 1. This result is in agreement with the *Drosophila/Musca* homology map (Foster *et al*., 1981).

Analysis of *Mdβ3* genomic sequences of the parental (aabys and OCR) and F_1 (aabys × OCR) flies revealed two alleles, allele A (EF203217) and B (EF203218) in the aabys strain and one allele (allele B, accession no. EF203221) in the OCR. The lack of a unique allele prevented us from determining the linkage of this gene. Given that the *Mdβ3* ortholog in *Drosophila (Dβ3) is located on chromosome 2L, the Drosophila/Musca homology map suggests Mdβ3* should reside on autosome 1 (Foster *et al*., 1981).

Mechanism of spinosad resistance in house flies

Spinosad is thought to exert its toxicity primarily by activating a nAChR (nAChN subtype) (Salgado & Sparks, 2005). Studies showing that functional deletion of *Dα6* results in *s*trains of *D. melanogaster* that are highly resistant to spinosad (Perry *et al*., 2007) indicates the importance of nAChRs in spinosad toxicity. Spinosad resistance in house fly is a recessive trait that could not be overcome with insecticide synergists, suggesting resistance was caused by an altered target site encoded by a locus on autosome 1 (Shono & Scott, 2003). *Drosophila Dα5*, *Dα6* and *Dβ3* homologs were assumed to reside on the autosome 1 according to the *D. melanogaster/M. domestica* homology map (Foster *et al*., 1981). *Mdα5*, *Mdα6* and *Mdβ3* have been cloned and analyzed from spinosad susceptible and resistant strains. Contrary to our expectation, none of the nAChR subunit genes on autosome 1 have any difference between insecticide susceptible and spinosad resistant strains, suggesting that spinosad resistance is not due to a mutation in these nAChR subunits. Identification of the gene responsible for spinosad resistance in house flies will require further study.

Experimental procedures

House flies

Three strains of house flies were used: aabys (insecticide susceptible, with the recessive morphological markers *ali-curve (ac)*, *aristapedia (ar), brown body (bwb)*, *yellow eyes (ye)* and *snipped wings* (*snp*) on autosomes 1, 2, 3, 4 and 5, respectively, (Hamm *et al*., 2005)), OCR (cyclodiene resistant (*Rdl*) (Gao *et al*., 2007c)) and rspin (spinosad resistant) (Shono & Scott, 2003). Flies were maintained in the laboratory as previously described (Scott *et al*., 2000).

Messenger RNA extraction, cDNA synthesis and PCR reagents

Flies (< 1-d old) of aabys and rspin strains were used. Messenger RNA was isolated from ~100 heads of mixed sexes using

Table 5. Linkage analysis of *Mdα5* of in the backcrosses of *Musca domestica*

Genotype	*Mdα5* heterozygous individuals /total*
+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp	3/3
ac/ac;+/ar;bwb/bwb;ye/ye;snp/snp	0/3
ac/ac;ar/ar;+/bwb;ye/ye;snp/snp	0/3
ac/ac;ar/ar;bwb/bwb;+/ye;snp/snp	0/3
ac/ac;ar/ar;bwb/bwb;ye/ye;+/snp	0/3

*The recessive markers *ac*, *ar*, *bwb*, *ye* and *snp* are on autosomes 1, 2, 3, 4 and 5, respectively. The *Mdα5* allele A was found in aabys and allele B in the OCR strain.

QuickPrep™ Micro mRNA purification kit (Amersham Biosciences, Piscataway, NJ, USA). First strand cDNA was synthesized from 450 ng of mRNA by priming with oligo(dT) using SuperScript™ III first strand synthesis system for RT-PCR kit (Invitrogen, Carlbad, CA, USA). The 3′- and 5′-RACE-ready cDNA was synthesized with 1 μg of mRNA using SMART™ RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA, USA). All procedures were carried according to the manufacturer's instructions.

Primer sequences are presented in Table 1 and PCR reactions were performed using Advantage® 2 polymerase mix (BD Biosciences Clontech Lab, Palo Alto, CA, USA) in a total volume of 25 μl, unless otherwise specified and thermal cycler programs all included an initial denaturation at 95 °C for 1 min and a final extension at 72 °C for 10 min in addition to the cycles mentioned below.

Cloning of Mdα5

Degenerate primers MaF2 and MaR1 were designed based on the conserved sequences (MKFGSWTYD and FNCIMFMVA, respectively) of previously reported insect nAChR α subunit genes. A 428-bp fragment was amplified from aabys flies. The PCR amplification program consisted of 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min. PCR products were directly cloned into pCR®2.1-TOPO® vector and transformed into TOP10 cells using TOPO-TA Cloning® kit (Invitrogen). DNA sequencing was performed using ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster, CA, USA) at the Biotechnology Resource Center, Cornell University, Ithaca, NY.

3′ and 5′ RACE was performed with a gene specific primer and a UPM primer provided with SMART™ RACE cDNA amplification kit (BD Biosciences). 3′ RACE was performed using primer 3′M5Race1 and an amplification program of 95 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min for 30 cycles. 5′RACE was completed by four RACE reactions some of which were coupled with semi-nested PCR. The first reaction was performed with primer 5′ M5Race3 and an amplification program of 95 °C for 30 s, 66 °C for 30 s, 72 °C for 4 min for 30 cycles. Its purified product was used in a semi-nested PCR, using 2X Reddy Mix™ PCR master mix (ABgene House, Surrey, UK), with primer 5′M5Race4 and an amplification program of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 3 min for 30 cycles. The second RACE was performed with primer 5′M5Race6 and an amplification program of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min for 30 cycles. Its purified product was used in a semi-nested PCR, using 2X Reddy Mix™ PCR master mix (ABgene House), with 5′M5Race7 and amplification program of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 2 min for 35 cycles. The third 5′RACE was performed with primer 5′M5Race10 and an amplification program of 95 °C for 30 s, 64 °C for 30 s and 68 °C for 3 min for 30 cycles. Its purified product was used in a semi-nested PCR, using 2X Reddy Mix™ PCR master mix, with primer 5′M5Race11 and an amplification program of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min for 30 cycles. The fourth 5′RACE was performed with primer 5′M5Race15 and an amplification program of 95 °C for 30 s, 64 °C for 30 s and 68 °C for 3 min for 30 cycles. All PCR products were analyzed on 1% agarose gels. The target bands were excised and DNA was purified with QIAEX®II gel extraction kit (QIAGEN Sciences, MD, USA). Cloning, transformation and sequencing were performed as above.

A fragment containing the ORF of *Mdα5* was amplified from 5′-RACE-ready cDNA with a forward primer (M5F1) and a reverse primer (M5R1) using 32 cycles of 95 °C for 30 s, 64 °C for 30 s and 68 °C for 4 min. PCR products were directly cloned into the pCR®2.1-TOPO® vector. Transformation and sequencing were performed as above.

Cloning of Mdβ3

A 293-bp fragment was amplified with degenerate primers MBF1 and MBR2, based on the sequence WMPPAVYT and MYTAVIYTP of *Dβ3* of *Drosophila melangoster*, using 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. PCR products were separated on 1% agarose gels. The expected size fragment was excised, purified using QIAEXII gel purification kit (Qiagen Sciences), cloned into pCR®2.1-TOPO® vector, and transformed into TOPO10 cells using TOPO TA Cloning® kit (Invitrogen).

A 5′ and 3′ RACE was performed with primers 5′MBRace1 and 3′MBRace1, respectively, coupled with a UPM primer provided with SMART™ RACE cDNA amplification kit (BD Biosciences), using the following program: 30 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 2 min. PCR products were purified and cloned as described above.

A 1492 bp-fragment containing ORF of *Mdβ3* was amplified from the 5′-RACE-ready cDNA of aabys and rspin flies with MB3F1 and MB3R1 and the following program: 30 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 2 min. PCR product purification and cloning were as described above.

Sequence and phylogenetic analysis

Nucleotide polymorphisms were analyzed based on the available transcripts from both cDNA cloning and A-to-I RNA editing studies. The signal peptide was predicted by SignalP 3.0 (Bendtsen *et al.*, 2004). N-glycosylation and phosphorylation sites were determined using Prosite (Hulo *et al.*, 2006). Transmembrane domains were predicted by TMHMM v 2.0 (Krogh *et al.*, 2001). The phylogenetic analysis was performed using the PHYLIP software package (Felsenstein, 1993). The amino acid sequences were aligned using CLUSTAL X. The phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei, 1987). Bootstrap values were calculated with SEQBOOT program (Felsenstein, 1985) on 1000 replications. The phylogenetic tree was drawn by the TreeView program (Page, 1996). The cDNA sequences of *Mdα5* and *Mdβ3* were deposited in Genbank (accession nos: EF203213 and EF203216).

A- to-I RNA editing

Total RNA and genomic DNA extraction from a head was performed using TRIzol® reagent (Invitrogen). DNA contamination in

the total RNA was eliminated by treating the samples with TURBO DNA-*free*™ (Ambion, Austin, TX, USA) and confirmed by PCR. All procedures were carried out according to the manufacturer's instructions. RNA pellets were dissolved in 22 μl water and genomic DNA in 50 μl Tris-Cl Buffer (10 mM, pH 8.5).

PCR fragments were amplified from RNA using Titanium™ one-step RT-PCR kit (BD Biosciences Clontech Lab.). For *Mdα5*, primers *M5F4* and M5R1 and 2 μl RNA were used in a total volume of 25 μl with the following thermal program: 50 °C for 60 min followed by 94 °C 5 min, 32 cycles of 94 °C for 30 s, 64 °C for 30 s and 68 °C for 2.5 min and a final extension at 68 °C for 10 min. For *Mdβ3*, the same PCR was performed, except the primers were MB3F2 and MB3R1a, the annealing temperature was 62 °C and 32 cycles were used. The PCR products were purified and cloned as described above. Ten clones were sequenced for each fly and 3 flies were used for each gene.

Genomic DNA fragments were amplified from the same head that was used for total RNA (above). For *Mdα5*, three PCR reactions were conducted using primer pairs gM5I7F2+gM5VIIIR3, gM5F1+gM5I11R, and gM5XIF+M5R1, with the following thermal cycler program: 32 cycles of 95 °C for 30 s, 63 °C /64 °C/62 °C for 30 s and 72 °C for 30 s/60 s/30 s, respectively. For *Mdβ3*, a PCR was conducted with primer pair MB3F2 and MB3R1a and a thermal cycler program of 32 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 2 min. PCR products were purified and sequenced directly. Three flies were used for each gene.

Real time PCR quantification

Total RNA was isolated from heads, thoraces and abdomens, respectively, of aabys flies (< 1-d old), or from whole bodies of aabys and rspin strains (< 1d old) using TRIzol® reagent (Invitrogen) and treated with DNase (Ambion). First strand cDNA was synthesized in a total 100 μl reaction volume using 18 μg of the DNase-treated RNA with TaqMan Reverse transcription reagents using random hexamers (Applied Biosystems). PCR (20 μl per reaction) was performed using 2 μl of the cDNA samples, power SYBR® Green PCR master mix (Applied Biosystems) and specific primer pairs qMa5F2 and qMa5R2 for *Mdα5*, *qMBF1* and qMBR1 for *Mdβ3* using an ABI PRISM 7900 HT Sequence Detection System with Sequence Detection Software (version 2.1) (Applied Biosystems). All the procedures were conducted according to the manufacturer's instructions. The PCR program consisted of 50 °C for 2 min and 95 °C for 10 min for initiation, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s for melting curve analysis. The PCR specific amplification was assessed by the melting curve analysis and electrophoresis of the PCR products on 1.8% agarose gel. External standard curves were constructed using 6 serial 10-fold dilutions of plasmids (pCR®2.1 containing *Mdα5* or *Mdβ3* ORF) starting from 0.04 ng/μl. This analysis was replicated three times. The gene expression levels were analyzed with a randomized complete block design using a one-way ANOVA. The number of transcripts were transformed to log values and significant differences were determined using Tukey's test at $P = 0.05$ (PROC GLM, (SASInstitute, 2001).

Linkage analysis

Linkage analysis was performed by the association of *Mdα5* polymorphisms (between aabys and OCR) with the five recessive markers of the aabys strain (Kozaki *et al*., 2002). Female aabys were crossed with male OCR to produce F_1 flies heterozygous for all five autosomes. The F_1 males were then backcrossed to aabys females. The offspring were sorted according to phenotype. Five phenotypes were used to conduct the linkage analysis, being heterozygous at only one chromosome, as indicated by the absence of a recessive morphological marker. Flies that were heterozygous for each of autosomes one through five were denoted as *+/ac;ar/ar;bwb/bwb;ye/ye;snp/snp*, *ac/ac;+/ar;bwb/bwb;ye/ye;snp/snp*, *ac/ac;ar/ar;+/bwb;ye/ye;snp/snp*, *ac/ac;ar/ar;bwb/bwb;+/ye;snp/snp*, *ac/ac;ar/ar;bwb/bwb;ye/ye;+/snp*, respectively.

Genomic DNA was extracted as previously described (Gao *et al*., 2007a) from 10 aabys and nine OCR parental individuals, three F_1, and three individuals from each previously stated backcross phenotype. A genomic DNA fragment (613-bp in aabys and 625-bp in OCR) was PCR amplified with a forward primer gM5IF1 and a reverse primer 5′M5Race8 using the following thermal cycler program: 30 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s. The PCR product was purified using QIAquick PCR purification kit (Qiagen) and then sequenced as described above.

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References

Bendtsen, J.D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**: 783–795.

Charpantier, E., Wiesner, A., Huh, K.H., Ogier, R., Hoda, J.C., Allaman, G., *et al*. (2005) Alpha7 neuronal nicotinic acetylcholine receptors are negatively regulated by tyrosine phosphorylation and Src-family kinases. *J Neurosci* **25**: 9836–9849.

Chen, D., Dang, H. and Patrick, J.W. (1998) Contributions of N-linked glycosylation to the expression of a functional alpha7-nicotinic receptor in *Xenopus* oocytes. *J Neurochem* **70**: 349–357.

Couturier, S., Bertrand, D., Matter, J.-M., Hernandez, M.-C., Bertrand, S., Millar, N., *et al*. (1990) A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron* **5**: 847–856.

Deacutis, J.M., Leichter, C.A., Alec C. Gerry, A.C., Rutz, D.A., Watson, W.D., *et al*. (2007) Susceptibility of field collected house flies to spinosad before and after a season of use. *J Agric Urban Entomol* **23**: 105–110.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Felsenstein, J. (1993) PHYLIP (phylogeny inference package) version 3.5c.

Fenster, C.P., Beckman, M.L., Parker, J.C., Sheffield, E.B., Whitworth, T.L., Quick, M.W., *et al*. (1999) Regulation of α4β2 nicotinic receptor desensitization by calcium and protein kinase C. *Mol Pharmacol* **55**: 432–443.

Foster, G.G., Whitten, M.J., Konovalov, C., Arnold, J.T.A. and Maffi, G. (1981) Autosomal genetic maps of the Australian sheep blowfly, *Lucilia cuprina dorsalis* R.-D. (Diptera: Calliphoridae), and possible correlations with the linkage maps of *Musca*

domestica L. and *Drosophila melanogaster* (Mg.). *Genet Res Camb* **37**: 55–69.

Gao, J.-R., Deacutis, J.M. and Scott, J.G. (2007a) Characterization of the nicotinic acetylcholine receptor subunit gene *Mdα2* from the housefly, *Musca domestica*. *Arch Insect Biochem Physiol* **64**: 30–42.

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 Mol Biol* **16**: 325–334.

Gao, J.-R., Kozaki, T., Leichter, C.A., Rinkevich, F.D., Shono, T. and Scott, J.G. (2007c) The A302S mutation in *Rdl* that confers resistance to cyclodienes and limited cross-resistance to fipronil is undectable in field populations of house flies from the USA. *Pestic Biochem Physiol* **88**: 66–70.

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.

Grutter, T. and Changeux, J.P. (2001) Nicotinic receptors in wonderland. *Trends Biochem Sci* **26**: 459–463.

Hamm, R., Shono, T. and Scott, J.G. (2005) A cline in frequency of autosomal males is not associated with insecticide resistance in house fly (Diptera: Muscidae). *J Econ Entomol* **98**: 171–176.

Hewitt, C.G. (1914) *The Housefly Musca domestica Lnn. Its Structure, Habits, Development, Relation to Disease and Control*, University Press, Cambridge, London.

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

Hsu, J.-C. and Feng, H.-T. (2006) Development of resistance to spinosad in oriental fruit fly (Diptera: Tephritidae) in laboratory selection and cross-resistance. *J Econ Entomol* **99**: 931–936.

Hulo, N., Bairoch, A., Bulliard, V., Cerutti, L., De Castro, E., Langendijk-Genevaux, P., *et al*. (2006) The PROSITE database. *Nucleic Acides Res* **34**, D227–D230.

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**: 1422–1430.

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

Kozaki, T., Shono, T., Tomita, T., Taylor, D. and Kono, Y. (2002) Linkage analysis of an acetylcholinesterase gene in the house fly *Musca domestica* (Diptera: Muscidae). *J Econ Entomol* **95**: 129–133.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. (2001) Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. *J Mol Biol* **305**: 567–580.

Lansdell, S. and Millar, N. (2002) Dβ3, an atypical nicotinic acetylcholine receptor subunit from *Drosophila*: molecular cloning, heterologous expression and coassembly. *J Neurochem* **80**: 1009–1018.

Lester, H.A., Dibas, M.I., Dahan, D.S., Leite, J.F. and Dougherty, D.A. (2004) Cys-loop receptors: new twists and turns. *Trends Neurosci* **127**: 329–336.

Marshall, J., Buckingham, S.D., Shingai, R., Lunt, G.G., Goosey, M.W., Darlison, M.G., *et al*. (1990) Sequencing and functional expression of a single alpha subunit of an insect nicotinic acetylcholine receptor. *EMBO J* **9**: 4391–4398.

Moulton, J.K., Pepper, D.A. and Dennehy, T.J. (2000) Beet armyworm (*Spodoptera exigua*) resistance to spinosad. *Pest Management Science* **56**: 842–848.

Nishizaki, T. (2003) N-glycosylation sites on the nicotinic ACh receptor subunits regulate receptor channel desensitization and conductance. *Brain Res Mol Brain Res* **114**: 172–176.

Page, R.M.W. (1996) TREEVIEW: an application to display phylogenetic tree on personal computers. *Comput Appl BioSci* **12**: 357–358.

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.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Salgado, V.L. and Sparks, T.C. (2005) The spinosyns: chemistry, biochemistry, mode of action, and resistance. In *Comprehensive Molecular Insect Science* (Gilbert, L.I., Iatrou, K. and Gill, S. S., eds), pp. 137–173. Elsevier, Boston.

SAS Institute (2001) *SAS/STAT Guide For Personal Computers*, version 8.2, SAS Institute, Cary, NC.

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.

Scott, J.G., Alefantis, T.G., Kaufman, P.E. and Rutz, D.A. (2000) Insecticide resistance in house flies from caged-layer poultry facilities. *Pest Manag Sci* **56**: 147–153.

Seeburg, P.H. (2002) A-to-I editing: new and old sites, functions and speculations. *Neuron* **35**: 17–20.

Shono, T. and Scott, J.G. (2003) Spinosad resistance in the house fly, *Musca domestica*, is due to a recessive factor on autosome 1. *Pestic Biochem Physiol* **75**: 1–7.

Swope, S.L., Moss, S.J., Blackstone, C.D. and Huganir, R.L. (1992) Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. *FASEB J* **6**: 2514–2523.

Wanamaker, C.P. and Green, W.N. (2005) N-linked glycosylation is required for nicotinic receptor assembly but not for subunit associations with calnexin. *J Biol Chem* **280**: 33800–33810.

Wecker, L., Guo, X., Rycerz, A.M. and Edwards, S.C. (2001) Cyclic AMP-dependent protein kinase (PKA) and protein kinase C phosphorylate sites in the amino acid sequence corresponding to the M3/M4 cytoplasmic domain of alpha4 neuronal nicotinic receptor subunits. *J Neurochem* **76**: 711–720.

Wu, P., Ma, D., Pierzchala, M., Wu, J., Yang, L.-C., Mai, X., Chang, X. and schmidt-Glenewinkel (2005) The Drosophila acetylcholine receptor subunit Dα5 is part of an α-Bungarotoxin binding acetylcholine receptor. *J Biol Chem* **280**: 20987–20994.

Zhao, J., Li, Y., Collins, H., Gusukuma-minuto, L., Mau, R., Thompson, G. and Shelton, A. (2002) Monitoring and characterization of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad. *J Econ Entomol* **95**: 430–436.

Zhao, J.Z., Collins, H.L., Li, Y.X., Mau, R.F., Thompson, G.D., Hertlein, M., Andaloro, J.T., Boykin, R. and Shelton, A.M. (2006) Monitoring of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad, indoxacarb, and emamectin benzoate. *J Econ Entomol* **99**: 176–81.

