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Identification of ion channel genes in the *Acyrthosiphon pisum* genome


*Syngenta, Jealotts Hill Research Centre, Bracknell, Berkshire, UK; †MRC Functional Genomics Unit, Department of Physiology Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, UK; ‡Plant Pathology and Plant-microbe Biology, Cornell University, Ithaca, NY, USA; §Department of Entomology, Texas A & M University, College Station, TX, USA; ¶Biological Chemistry Department, Rothamsted Research, Harpenden, Hertfordshire, UK; **Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath, UK; ††CSIRO Entomology, Centre for Environment and Life Sciences (CELS), Floreat Park, WA, Australia

Abstract

Aphids are major pests of crops, causing hundreds of millions of dollars worth of damage annually. Ion channel proteins are often the targets of modern insecticides and mutations in ion channel genes can lead to resistance to many leading classes of insecticides. The sequencing of the pea aphid, *Acyrthosiphon pisum*, genome has now allowed detailed *in silico* analysis of the aphid ion channels. The study has revealed significant differences in the composition of the ion channel families between the aphid and other insects. For example *A. pisum* does not appear to contain a homologue of the nACh receptor alpha 5 gene whilst the calcium channel beta subunit has been duplicated. These variations could result in differences in function or sensitivity to insecticides. The genome sequence will allow the study of aphid ion channels to be accelerated, leading to a better understanding of the function of these economically important channels. The potential for identifying novel insecticide targets within the aphid is now a step closer.

Keywords: Aphid, ion channel, genome.

Introduction

Many aphid species are important crop pests causing estimated losses worth hundreds of millions of dollars annually. For example the peach-potato aphid, *Myzus persicae*, infests a large number of commercially important food plants including sugar beet, potatoes, lettuce, sweet pepper and tomato, whereas cereal crops are attacked by other species such as the Russian wheat aphid, *Diuraphis noxia*. Aphids cause both direct feeding damage and transmit pathogenic plant viruses.

Ion channels are responsible for the movement of ions across cell membranes and are essential components of the nervous system. They are also involved in numerous critical functions within the insect such as muscle contraction and ion homeostasis. There are several families of ion channels that have different specificities for the ions transported. Ion channels can be activated by voltage-sensing or by ligand binding, allowing the essential fine control of ion channel activity. In insects, the ion channel proteins are the target of many natural venoms and toxins used by predators to render the insect immobile. Many insecticides act by targeting ion channels (Raymond-Delpech *et al.*, 2005), thereby interfering with nervous system function, leading to the death of the insect. There are relatively few chemical classes of insecticide available for use against aphids, the main ones being neonicotinoids, which target nicotinic acetylcholine (nACh) receptors, and pyrethroids, which target voltage-gated sodium channels. Aphids resistant to these classes of chemical are increasing, as seen with the spread of kdr (knockdown resistance) to pyrethroids (Martinez-Torres *et al.*, 1999), leading to an urgent need to develop new insecticides with differing modes of action. Recently, a new class of insecticides was launched, targeting ryanodine receptors of lepidopteran and sucking pests. This class includes
Cyazypyr™ and Rynaxypyr® (Sattelle et al., 2008) and these compounds will enable aphid control in previously resistant populations. The sequencing of the pea aphid, *Acyrthosiphon pisum*, genome has provided an opportunity to study the complete set of genes encoding aphid ion channels. Here we report the genes present and compare them with their counterparts in other insects. It is hoped that the identification of these genes will lead to a better understanding of aphid ion channels, and highlighting the differences between insect channels will lead to an improvement in knowledge of the differences in efficacy of current insecticides among aphids and other insects. In addition, the genome sequence may facilitate the design of aphid-specific insecticides to better control these important pests.

Herein we follow the tradition of distinguishing between voltage-gated and ligand-gated channels, although this is of course an oversimplification.

**Results and discussion**

**Voltage-gated sodium and calcium channels**

Voltage-gated sodium channel (*para*). Voltage-gated sodium channel (VGSC) α-subunits are integral membrane proteins that conduct sodium ions through a cell’s plasma membrane. In electrically excitable cells, they are responsible for the rising phase of action potentials. The genomes of insects for which sequence data is currently available contain a single identifiable VGSC gene named ‘*para*’ (Davies et al., 2007a). A putative *para* homologue has been identified in the *A. pisum* genome (Supplementary Table S1). The predicted protein has: the expected four repeat domains, each containing 6 transmembrane (TM)-spanning segments (S1–S6); positively charged S4 regions which act as the channel’s voltage sensor; and extracellular/cytoplasmic pore regions formed by the ‘P-loops’ (located between S5 and S6 of the four domains and the S5 and S6 transmembrane regions, respectively). Surprisingly, the channels sodium ‘selectivity filter’ is made up of amino acids D, E, N and S (located on the P-loops of each domain) rather than the classic DEKA filter (Catterall, 2000), which normally defines a VGSC. Point mutations within VGSCs have been implicated in kdr and super-kdr-like resistance to the pyrethroid insecticides (Kellenberger & Schild, 2002). Individual subunits assemble as homotrimers or heterotrimers to form functional channels (Jasti et al., 2007). There are more than thirty predicted ENaC/DEG subunits in *D. melanogaster* (Littleton & Ganetzky, 2000) but only a handful have been characterized (Supplementary Table S1). Pickpocket (*ENaC/DEG*) channels. The epithelial sodium channel (ENaC)/degenerin (DEG) gene family encode membrane-bound, voltage-insensitive ion channels (Kellenberger & Schild, 2002). Individual subunits assemble as homotrimers or heterotrimers to form functional channels (Jasti et al., 2007). There are more than thirty predicted ENaC/DEG subunits in *D. melanogaster* (Littleton & Ganetzky, 2000) but only a handful have been characterized (Supplementary Table S1).

Sodium channel 60E. Like the VGSC α-subunit genes, the *D. melanogaster* sodium channel 1 (*DSC1*) gene (Salkoff et al., 1987) and the orthologous *Blatella germanica* BSC1 gene (Liu et al., 2001) belong to the 4 × 6 TM domain family. Although originally classified as sodium channels based on their similarity to *para*, they actually encode calcium-selective channels (Zhou et al., 2004). The partially reduced expression of *DSC1* in a smell-impaired *D. melanogaster* mutant smi60E correlates with certain olfactory defects. (Kulkarni et al., 2002), suggesting a role in olfactory avoidance response. A putative homologue has been identified in the *A. pisum* genome (Supplementary Table S1).

**Voltage-gated sodium channel auxiliary subunits.** High level expression of insect VGSCs, with maximal current and optimal kinetics, requires a small auxiliary subunit, *TipE* (Warmke et al., 1997). Four additional TipE-like proteins (TEH 1-4) have been identified in *D. melanogaster* (Derst et al., 2006). We have identified genes encoding all five of these auxiliary subunits in *A. pisum* on a ~123 kb genome tract (Supplementary Table S1).

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Insect VGSCs are presumed to gain heterogeneity and functional diversity by extensive alternative splicing (Olson et al., 2008). Nine such splicing sites have been identified in *para* and its orthologues to date; seven exons, a, b, i, j, e, f, h, are optional and form parts of the intracellular linkers of mature channel proteins, whereas exons c/d and l/k are mutually exclusive and are situated in the transmembrane regions. Exons l/k have been implicated in differential sensitivity to pyrethroid insecticides (Du et al., 2006). Exons l/k are evolutionarily highly conserved, and are present in the *A. pisum* sequence, as are exons a, b. Given the absence of some splice sites in the aphid gene, it seems that fewer variants of this channel will be present in the pea aphid compared with, for example, *Drosophila melanogaster*, although there may be other unidentified splice sites.

Voltage-gated sodium channel auxiliary subunits. High level expression of insect VGSCs, with maximal current and optimal kinetics, requires a small auxiliary subunit, *TipE* (Warmke et al., 1997). Four additional TipE-like proteins (TEH 1-4) have been identified in *D. melanogaster* (Derst et al., 2006). We have identified genes encoding all five of these auxiliary subunits in *A. pisum* on a ~123 kb genome tract (Supplementary Table S1).

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Calculated channels. Calcium channels are formed from heteromeric units containing a pore-forming α subunit, a β subunit and αδ subunits, combinations of which allow distinct channel types to be formed. D. melanogaster has four α subunits, three of which correspond to the mammalian L, N and T channels and one which is related to a calcium channel found in Caenorhabditis elegans (Littleton & Ganetzky, 2000). In A. pism we have identified a single gene for the L-, N- and T-type α subunits, however, no match to the C. elegans-related CG13762 gene was found (see Supplementary Fig. S1).

The auxiliary subunits of calcium channels (β and αδ subunits) have a role in targeting the α subunit to the plasma membrane and also in aligning the α subunit into the correct conformation. We identified two orthologues of the β subunit in A. pism using the D. melanogaster β subunit gene as the target. These proteins, ACYPI005770 and ACYPI008838, show 71% and 47% similarity to the D. melanogaster orthologue, respectively. D. melanogaster also has three αδ subunits, which are post-translationally cleaved into two peptides (αδ1 and δ) (Dickman et al., 2008), and the analysis of the A. pism genome reveals four orthologues of these genes with the αδ1 1 subunit being duplicated (Supplementary Table S2).

Narrow abdomen or Dma1U. Dma1U in D. melanogaster (Nash et al., 2002) belongs to a unique family of putative 4 × 6 TM ion channels related to voltage-gated sodium and calcium channels. These channels diverge from voltage-gated sodium/calcium channels by virtue of a unique selectivity filter sequence and a reduced number of positively charged amino acids (arginine or lysine) in the voltage-sensing transmembrane domains, suggesting that they may form channels with distinctive properties. Dma1U corresponds to the narrow abdomen (na) gene, a locus for point mutations associated with changes in abdominal morphology, locomotor behaviour and day/night circadian rhythms (Lear et al., 2005). We have identified a gene corresponding to na in A. pism (Supplementary Table S1); its function is likely to be similar to that of the D. melanogaster channel since the sequence is highly conserved (≏80% identity).

Potassium channels

Potassium channels are the largest family of ion channels in invertebrates and have a wide range of roles, including the maintenance of the membrane potential in cells, maintenance of the circadian rhythm and potassium homeostasis. There are several distinct families of potassium channels, both voltage-gated and non-voltage-gated and each family is responsible for various different currents. In this study, we have identified thirty-two potassium channel genes in A. pism (Supplementary Fig. S2 and Supplementary Table S3).

Voltage-gated potassium channels: Shaker potassium channels

The Shaker family of potassium channels control membrane potential in a variety of cells and have six transmembrane domains, S1–S6. Mutations in the potassium channel Shaker in D. melanogaster give rise to disturbances of the transient outward potassium (iA) current. Other members of the Shaker family include Shal, Shab, Shal and Shal, which contribute to the transient and delayed rectifier currents in neurons and muscles. D. melanogaster has a single gene for Shab and Shal, whilst Shal is encoded by two genes, Shal and Shal, which are highly divergent in the C-terminal regions and are again shorter in length than the D. melanogaster protein.

KCNQ channels

The KCNQ potassium channel is encoded by a single gene in D. melanogaster and is responsible for the M-current (Littleton & Ganetzky, 2000). P-element insertion lines in the KCNQ gene have shown that the KCNQ channel is involved in control of heart rhythm and the knockout line showed increased arrhythmias compared with wild-type controls (Ocorr et al., 2007). A. pism contains a single KCNQ gene, hm111514, which is currently modelled as a pseudogene as it contains a premature stop codon. The aphid protein model shows low homology, 24%, to the D. melanogaster protein and is especially divergent at the C-terminal end.

Eag potassium channels

There are three sub-families of the Eaq potassium channels; Eag, Egl and Erg. Although their basic structure is similar to the voltage-gated potassium channels with six transmembrane domains and a pore domain, the Eaq channels also encode a C-terminal sequence with a cyclic-nucleotide binding domain (Guy et al., 1991). D. melanogaster Eaq mutants display a phenotype of leg-shaking, caused by repetitive firing and enhanced neurotransmitter release in the motor neurons, whereas homozygous mutations in the D. melanogaster eag (Seizure) gene result in paralysis (Warmke et al., 1994; Wang et al., 1997a). We have identified three Eaq-type channels in A. pism. The aphid Eaq and Egl proteins are shorter than the D. melanogaster orthologues, having much shorter N- and C-terminal sequence. The aphid Erg protein, however, has a longer C terminus but shorter N-terminus.
Non-voltage gated potassium channels: Calcium-activated potassium channels

This group of potassium channels are heavily involved in the control of cell excitability (Derst et al., 2003). The group is divided into large (BK), intermediate (IK) and small-conductance (SK) channels that are regulated by both voltage gating and calcium ions. A C-terminal calcium-binding motif known as the ‘calcium bowl’ is encoded by the amino acid sequence DDDDD (Bian et al., 2001). In D. melanogaster a single gene, Slowpoke (pSlo), is responsible for the large-conductance current and associates with a second gene, Slack, to form the IK channel. The SK channel is also encoded by a single gene. We have a single homologue of each of these genes in A. pisum. The pSlo protein is especially well conserved with 82% identity between the aphid and D. melanogaster sequences. The current aphid Slack protein model shows good conservation with D. melanogaster, with the exception of two large deletions (250aa and 200aa) in the central section of the protein.

Inward-rectifying potassium channels

Three inward-rectifying potassium (irk) channel genes have been identified in D. melanogaster, dir, dirk2 and dirk3. These are involved in neuronal excitability and maintenance of potassium levels. It has been shown that renal tissues have high expression of all three irk genes, indicating a role in fluid secretion (Evans et al., 2005). We have identified two genes in A. pisum, which show close homology to dir and dirk2.

Two-pore, weakly inward rectifying potassium channels (TWIK)

TWIK channels, characterized by four transmembrane domains and two potassium selective pores, are involved in a diverse range of processes including mediating resting potential and mechanosensation (Buckingham et al., 2005). We have identified 16 putative TWIK genes in A. pisum, all containing the potassium ion selectivity motif GYGD. One of these genes, ACYPIG684244, is currently modelled as a pseudogene.

Chloride channels

Voltage-gated chloride channels Voltage-gated chloride channels are involved in several biological and physiological functions such as pH regulation, volume homeostasis and organic solute transport. The channels are characterized by the presence of twelve transmembrane domains. Mammals have ten genes encoding voltage-gated chloride channels whereas only three were found in D. melanogaster (Littleton & Ganetzky, 2000). We have found five in A. pisum (Supplementary Table S4), with three of them being similar to D. melanogaster CG8594 gene.

Ionotropic glycin and γ-aminobutyric acid and glycin receptors. Ionotropic glycine and γ-aminobutyric acid (GABA) receptors are chloride channels mediating rapid, mostly inhibitory, synaptic transmission. Both GABA and glycine receptors belong to the dicysteine-loop (‘Cys-loop’) superfamily of neurotransmitter receptors (Grenningloh et al., 1987; Schofield et al., 1987). These chloride channels are pentameric (Nayeem et al., 1994) and they are the target of cyclodiene and phenylpyrazole insecticides.

In vertebrates, these GABA receptors have been divided into two families: GABAA, characterized by bicuculline sensitivity (Sieghart, 1995), and bicuculline-insensitive GABAC receptors (Woodward et al., 1993). Several GABAA (α 1-6, β 1-4, γ 1-4, δ, ε, θ and π 1-3) and five GABAC (p1-5) genes have been identified with the GABAA receptors having two α, two β and one of the other types of subunits (δ, ε, θ or π) (reviewed in Kash et al., 2004). The glycine receptors are composed of three α and two β subunits and in humans, four α (α1–α4) and one β genes have been identified (Bowery & Smart, 2006).

Both ionotropic GABA and glycine receptor subunits have large N-terminal and short C-terminal extracellular domains and four hydrophobic transmembrane domains (M1–M4) with a large intracellular loop of variable length between M3 and M4. In D. melanogaster, ten different genes encoding proteins with similarity to GABA/glycine subunits have been identified (Littleton & Ganetzky, 2000). The GABA receptors are bicuculline-insensitive but are regulated by some though not all of the allosteric modulators of GABAA receptors. Splice variants have been identified in some of these genes (Hosie et al., 2001). In A. pisum, we have identified ten genes encoding proteins with similarity to GABA/glycine subunits (Supplementary Table S4) and interestingly, two of the genes show similarity to the D. melanogaster Resistant to diedrin (Rdl) gene and two to D. melanogaster pH-sensitive chloride Channel (pHCl).

There are five genes in D. melanogaster, present in other insect species, with no identifiable orthologues in A. pisum (Supplementary Table S4). Two of the genes identified in A. pisum encode subunits not present in D. melanogaster and are most similar to human Glycine receptor α3.

Two other putative chloride receptors genes have been identified in A. pisum, a histamine-gated chloride channel subunit 1 (HisC1) and glutamate-gated chloride channel (GluC1o) (Supplementary Table S4).

Cyclic nucleotide-gated ion channels

Cyclic nucleotide-gated (CNG) ion channels are activated by cyclic nucleotides, cAMP or cGMP, and play an impor-
Glutamate receptors

The ionotropic glutamate receptor family mediates most forms of excitatory synaptic transmission in the mammalian CNS and play an important role in learning and memory. In mammals, four categories have been identified according to the agonist response: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate, N-Methyl-D-Aspartate (NMDA) and δ. All of the receptor subunits are thought to adopt the same membrane topology and each subunit is composed of an extracellular N-terminal domain, three transmembrane domains, a single ‘P-loop’ membrane domain and an intracellular C-terminal domain that interacts with scaffolding proteins (Hollmann et al., 1994; Roche et al., 1994; Bennett & Dingledine, 1995). Approximately 30 ionotropic glutamate receptor genes were identified in D. melangaster (Littleton & Ganetzky, 2000). Invertebrates have additional subtypes, absent in mammals, which have low similarity to the Arabidopsis thaliana Glu5 glutamate receptor. Some glutamate receptors are the target of the avermectin family of insecticides.

In mammals, NMDA receptors are heterotetramers and seven subunits have been identified encoded by the genes NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B. A functioning receptor requires at least two subunits: NR1 and NR2 (Stephenson et al., 2008) and diversity is generated by the association of the obligatory NR1 subunit with different NR2 subunits.

In D. melangaster, two NMDA receptor genes have been identified (NMDAR1 and NMDAR2) and our analysis of the A. pisum genome shows the presence of one copy of each one of these genes. One copy of NMDAR3 was also identified (Supplementary Table S6 and Supplementary Fig. S3), a gene which is absent from D. melangaster but present in other insects such as Anopheles gambiae, Tribolium castaneum, Apis mellifera and Nasonia vitripennis. Two other genes identified in D. melangaster and described as showing NMDA selective glutamate activity in Flybase, CG3814 and CG30379, could not be identified in A. pisum; these genes have only been identified in other Drosophila species.

AMPA and kainate receptors mediate fast synaptic transmission in the CNS and both are described as ionotropic, although there is evidence that they can be metabotropic, inducing the activation of G proteins and signalling cascades (Wang et al., 1997b; Huettner, 2003; Lerma, 2003). Kainate receptors are tetramers. Five different subunits have been identified, GLU_K5, GLU_K6, GLU_K7, and GLU_K8 (Hollmann & Heinemann, 1994), divided into two families based on sequence similarity.

Ten genes encoding kainate selective glutamate receptors were identified in D. melangaster. We have identified seven putative genes in the A. pisum genome (Supplementary Fig. S4 and Supplementary Table S6) but were unable to identify genes homologous to KaiRI, GluRIFI, GluRIFII, GluRIIC and GluRIFIE, present in members of the Drosophilidae family. The gene CG5621 also seems to be absent from A. pisum despite being present in Aedes aegypti, An. gambiae and Culex quinquefasciatus. Interestingly in A. pisum, there is a cluster of kainate receptor genes located in the same scaffold, with three genes with similarity to CG3822 and one gene similar to clumsy. There is another gene similar to CG3822 located in another cluster.

The AMPA receptors, have four types of subunits, GluR1, GluR2, GluR3, and GluR4 (also called GluRA-D), and are usually heterotetrameric, consisting of symmetric ‘dimer of dimers’ of GluR2 and either GluR1, GluR3 or GluR4. Only two AMPA receptors have been described in D. melangaster, GluRI and GluRIB. We have found two genes in the A. pisum genome, both similar to GluRIB that might be two halves of the same gene (Supplementary Fig. S4 and Supplementary Table S6). In addition to these receptors, several novel subtypes have been identified in D. melangaster with no mammalian orthologues: CG32704, CG15627, CG7385, CG6185, CG2657 and CG42315. One orthologue of each has been identified in A. pisum (Supplementary Fig. S5 and Supplementary Table S6). Another ionotropic channel, CG14076, has six copies in A. pisum, two located in the same scaffold (Supplementary Fig. S5 and Supplementary Table S6). Several other ionotropic receptors present in D. melangaster are not found in A. pisum.

Nicotinic acetylcholine receptors

Nicotinic acetylcholine (nACh) receptors are prototypical members of the Cys-loop ligand-gated ion channel (cysLGIC) superfamily, consisting of five subunits arranged around a central ion channel. In insects, nACh receptors mediate the fast actions of acetylcholine in the nervous system and are of particular interest as they are targets of a major group of insecticides, the neonicoti-
noids, which have been the fastest-growing class of insecticides in modern crop protection (Jeschke & Nauen, 2008). The A. pisum genome contains 11 genes encoding putative nACh receptor subunits (Fig. 1), a similar number to that found in An. gambiae, Ap. mellifera, Bombyx mori, D. melanogaster and T. castaneum, all with between 10 and 12 subunits. Eight of the aphid subunits fall into core groups (Da1 Group, Da2 Group, etc.), which are highly conserved (over 60% amino acid identity) between different insect species (Jones et al., 2007). As in other insects, A. pisum also has divergent subunits, which show particularly low sequence identity (20% or less) with other nACh receptors and may perform species-specific roles. The complement of A. pisum divergent subunits, comprising one β (Apisumβ2) and two α (Apisum α9 and Apisum α10) subunits, is currently unique to the aphid. Apisumα4 and Apisumα6 have alternative splicing of exons 4 and 8, respectively, which is also seen in orthologous subunits of other insect species (Supplementary Fig. S6). Such splicing effectively diversifies receptor function and generates species-specific isoforms by introducing variations in (1) the Cys-loop that is involved in receptor assembly (Landsell & Millar, 2000), (2) loops E and B that contribute to ligand binding, and (3) the second transmembrane domain that forms the ion channel (Grauso et al., 2002).

We have found features particular to the A. pisum nACh receptor gene family. For example, the aphid is the first insect that appears not to possess a Da5 orthologue (Fig. 1). In this regard, it is worth noting that the closely related Apisumα7 subunit has unusual tandem duplications of exons 6 and 7, which likely represent alternatively spliced exons (Supplementary Fig. S6). It is interesting to speculate that the genome duplication events seen in Apisumα7 may represent the early steps of diversification leading to the generation of a separate gene identified as α5 in more highly evolved insects. This finding would

Figure 1. Tree showing relationships of Acyrthosiphon pisum, Apis mellifera and Drosophila melanogaster nAChR subunit protein sequences. Numbers at each node signify bootstrap values with 1000 replicates and the scale bar represents substitutions per site. The nAChR subunits shown in the tree (as well as GenBank accession numbers) are as follows: Da1 (CAA30172), Da2 (CAA36517), Da3 (CAA75688), Da4 (CAB77445), Da5 (AAM13390), Da6 (AAM13392), Da7 (AAK67257), Db1 (CAA27641), Db2 (CAA39211), Db3 (CAC48166), Amela1 (DQ026031), Amela2 (AY540846), Amela3 (DQ026032), Amela4 (DQ026033), Amela5 (AY569781), Amela6 (DQ026035), Amela7 (AY500239), Amela8 (AF514804), Amela9 (DQ026037), Amelb1 (DQ026038), Amelb2 (DQ026039). The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) available with the CLUSTALX program (Thompson et al., 1997) and displayed using TreeView (Page, 1996).
The cysteine residues forming the cys-loop are highlighted in black shading, while grey shading indicates conserved residues.

imply that $\alpha 5$ is the newest member of the insect core group of subunits which is perhaps subjected to fewer functional constraints, because Amelu5, Tcaso5 and Bmo5 show considerably more divergence in amino acid sequence than any other core subunit orthologues (Jones et al., 2006; Jones & Sattelle, 2007; Shao et al., 2007). The two aphid divergent subunits, Apisum9 and Apisum10, are the first examples of insect nACh receptor subunits without both cysteine residues of the characteristic Cys-loop motif (Table 1). Instead, both subunits have the first cysteine while the second is replaced by a glycine residue. The Cys-loop plays a key role in receptor assembly (Green & Wanamaker, 1997) and removal of one cysteine from the Torpedo californica $\alpha$ subunit altered the response to ACh (Sumikawa & Gehle, 1992). Thus, Apisum9 and Apisum10 may show distinct functional properties. As neonicotinoid insecticides are frequently used to combat outbreaks of aphid infestation of crops (Jeschke & Nauen, 2008), knowledge of the receptor family targeted by such chemicals will accelerate our understanding of the molecular basis of aphid control.

**Table 1. Cys-loop sequences of divergent insect nAChR subunits**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Cys-loop sequence</th>
<th>Reference</th>
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Transient Receptor Potential channels

Transient Receptor Potential (TRP) proteins have six transmembrane domains and form calcium-permeable cation channels. The first description of a TRP gene was in *D. melanogaster* but there have been relatively few comparative studies on the TRP genes in insects. *D. melanogaster* has 13 well described genes, in seven sub-families (Supplementary Table S7) (Montell, 2005). Most were identified from mutant flies which display aberrant sensory perception phenotypes and many TRP channels do seem to be involved in various forms of sensation. In contrast, *A. pisum* seems to have only eight or nine $\text{trp}$ genes, with no representatives of the TRPML and TRPP sub-families (Supplementary Fig. S7 and Supplementary Table S7). Interestingly, these two sub-families are the most distantly related members of the whole gene superfamily and have been referred to as the group-2 subfamilies. As aphids are primarily parthenogenetic, it is perhaps not surprising that a gene, the TRPP *Pkd-2*, with a role in sperm motility, is absent. The aphid genome, then, only has representatives of the group 1 trp sub-families.

The best characterized of these subfamilies is TRPC, or classical TRP, which is involved in photoreception (Hardie & Minke, 1992). TRPL (TRP-like) and TRP-$\gamma$ form heteromultimeric complexes with TRP (Phillips et al., 1992; Xu et al., 2000). Together, these three genes make up the TRPC subfamily. In *D. melanogaster*, these channels are held in a signalling complex involving the scaffolding protein InaD (Georgiev et al., 2005). Only one member of the TRPC sub-family was found in the aphid genome and InaD is not obviously present, suggesting that the physiology of visual signalling is markedly different in the aphid.

As in *D. melanogaster*, there are two members of the TRPV subfamily, one each related to *nanchung* and *inactive*, though the aphid equivalent of *inactive* is more similar to that of *A. mellifera* than to *D. melanogaster*. In flies, these genes are involved in sound perception (Kim et al., 2003) and the responses to humidity (Liu et al., 2007) and heat (Chentsova et al., 2002). The single insect member of the TRPM family is also conserved in the aphid. The function of this gene has not been determined, although it is known not to play a role in larval thermotaxis (Rosenzweig et al., 2005). *Nompc* is the sole member of the TRPN subfamily and mutations in this gene produce a phenotype of defective mechanoreception (Kernan et al., 1994; Walker et al., 2000). There is a clear orthologue of *Nompc* in the aphid genome (ACYCIT949557).

The TRPA subfamily is the largest and most diverse in both *D. melanogaster* and *A. pisum*. Though the genomes of both species seem to contain four members of this subfamily, it has proved very difficult, based on sequence data, to deduce a gene-for-gene relationship between the two TRPA subfamilies. The one exception is *TrpA1*, which has a clear aphid equivalent (ACYCIT433926). In flies, this gene is involved in larval thermotaxis (Rosenzweig et al., 2005). The other three Drosophila TRPA genes, *pyrexia*, *waterwitch* (CG31284) and *painless* (*painless* is somewhat divergent) are involved in responses to heat (Lee et al., 2005), humidity (Liu et al., 2007) and in mechanosensation/nociception (Goodman, 2003; Tracey et al., 2003), respectively. The best candidate for an orthologue to any of these genes in the aphid is for *waterwitch* where a putative orthologue seems to have been duplicated, with two copies adjacent to each other in the inverse orientation. There is no obvious aphid equivalent of *pyrexia*. There is a gene distantly related to *painless*, ACYCIT907328, but the sequence divergence is sufficient to suggest that this may be a novel member of the TRP family, rather than an orthologue.
Ryanodine receptors

Ryanodine receptors (so named due to their interaction with an insecticidal alkaloid ryanodine) are ligand-gated calcium channels that mediate the release of calcium ions from intracellular stores. They are located in the sarcoplasmic reticulum of skeletal muscle, where calcium release is important in muscle contraction, and also in the endoplasmic reticulum of neurons and other cell types, where they are heavily involved in calcium signalling (Sattelle et al., 2008). Each receptor is composed of a large cytoplasmic domain at the N terminus and a small transmembrane domain at the C terminus (Hamilton, 2005). Functional channels present as large homomeric tetramers. The A. pisum receptor (Supplementary Table S2) is highly conserved with 77.7% identity/85.8% similarity with the D. melanogaster receptor. The sequence conservation within aphids is even more striking, with >98% amino acid identity between A. pisum, M. persicae and Aphis gossypii channels. Compared with the D. melanogaster gene which is composed of twenty-six exons, with exons 9a/b being mutually exclusive and exon 13 having an optional splice site, the A. pisum receptor has an estimated 98 exons and no equivalent splice sites. The recently commercialized synthetic insecticide Cyazypyr™, which targets the ryanodine receptors of aphids, acts as a channel activator and binds at a site distinct from the ryanodine binding site (Sattelle et al., 2008).

Inositol 1,4,5-triphosphate (IP3) receptor

A second receptor that also releases calcium from intracellular stores is the inositol 1,4,5-triphosphate (IP3) receptor. The receptor is activated by binding of the IP3 ligand and is involved in numerous cellular response pathways and sensory mechanisms (Hasan and Rosbash, 1992). The IP3 receptor protein is highly conserved between D. melanogaster and A. pisum with amino acid identity of 62% and similarity of 72% (Supplementary Table S2).

General discussion

The sequencing of the A. pisum genome is an important milestone in basic insect and insecticide research. This sequencing effort and the uncovering of ion channel genes will play an important part in the development of the next generation of chemicals to control these pests in three ways. Firstly, the genome will allow an understanding of the broad architecture of aphid ion channels and will reveal the role of these genes in aphid biology. Important differences in ion channel composition between aphids and other insect species have already been identified in this study. A major finding is that aphids are the only insect to date without an orthologue of the Drosophila Dα5 subunit. Interestingly, it does have orthologues of insect subunits shown to be targeted by imidacloprid (Apisumα2) (Matsuda et al., 1998) and spinosad (Apisumα6) (Perry et al., 2007). Also, of considerable interest is the finding that two of the divergent nACh receptor subunits Apisumα9 and Apisumα10 are the only insect nAChRs known to have incomplete signature Cys-loop sequences, likely to profoundly modify their assembly and functional properties. Another example of the role of ion channels in differences in aphid biology is seen with the TRP genes. The TRP channels that are involved in vision are reduced in A. pisum when compared with D. melanogaster. In terms of sensory process, the best conserved genes between the two species appear to be those involved in detecting humidity and sound/mechanical stimuli. The genes involved in larval thermotaxis and heat avoidance are less well conserved. It is tempting to speculate that these differences reflect the relative importance of the senses in the two species.

This study has shown that the pea aphid is divergent from other insects in the number of auxiliary calcium channel subunits it encodes. The aphid has a second beta subunit and a duplicated α5δ subunit 1. These genes may act in an aphid-specific manner or be expressed in alternative tissues to give rise to novel calcium currents (Grabner et al., 1994). It is also seen that the pea aphid contains a duplicated GABA αδ subunit and the duplicated gene contains the A302S allele as is the case with Myzus persicae. The presence of this allele, however, does not confer resistance to insecticides that act at GABA chloride channels, such as cyclodienes (Anthony et al., 1998). The presence of the second gene in A. pisum would indicate that the duplication is likely to be present throughout aphid species.

The second important role of the genomic information will be in the design and development of new insecticides to control aphid infestations. The genome data is likely to include novel target proteins that insecticides can act upon either as agonists or antagonists. The ion channel amino acid sequences will also be of use to researchers trying to identify how chemicals bind to these receptors and what domains are involved in the binding. The genome data will allow the development of better tools such as specific cell lines for binding studies and in this way accelerate research into new chemistries. An important example of this will be in the better understanding of how the new ryanodine receptor-inhibiting chemicals bind to the target and what motifs in the protein sequence are responsible for the aphid selectivity.

The third use of the genomic data will be in the area of insecticide resistance research. Through knowledge of the sequences of the target genes of insecticides, gene sequencing of suspected resistant aphids can proceed rapidly without the complication of lengthy gene cloning.
steps. This will allow novel mutations causing insecticide resistance to be identified and quickly characterized, and diagnostic tests to be developed to enable early screening of aphid populations. In this way, the problem of resistance will be easier to contain and manage in resistance management programs.

Some of the current gene models produced through the in silico analysis of the genome will need to be confirmed through expressed sequence tag (EST) data. Currently, this level of EST coverage is not available for A. pismut but it is expected to be generated within 12 months. Once this information is available then the current models will no doubt be refined and extra detail added such as splice variation. Future research on the aphid ion channels may focus on understanding which subunits combine together to form the channels, and in which tissues or developmental stages these are expressed. The roles of the novel aphid ion channel proteins will also need to be addressed.

**Experimental procedures**

To identify putative nAChR subunits, the A. pismut genome was screened with every member of the Ap. mellifera, D. melanogaster and T. castaneum nAChR gene families, using the TBLASTN algorithm. Candidate aphid nAChR subunits were identified based on their considerable sequence homology with previously characterized subunits, particularly in the N-terminal ligand-binding domain and the four transmembrane regions.

Sequences of the TRP gene family from D. melanogaster were used to perform reciprocal BLAST searches of the genome sequence database of A. pismut. Translated Drosophila sequences were used to search the aphid genome sequences using the TBLASTN algorithm. Sequences which showed a significant match to the Drosophila sequence used in the initial query were then used to search the NCBI sequence database, using the TBLASTX algorithm, in order to ascertain whether they were likely to be true orthologues of the Drosophila gene in question. In cases where the reciprocal BLAST criteria were not fully met, further searches of the aphid genome were performed using the most similar sequence found in the database, in order to assemble fragments of any aphid TRP gene sequences with no clear Drosophila orthologue.

For sodium channels, potassium channels, calcium channels and the ryanodine receptor, TBLASTN and BlastP searches of the A. pismut genome were carried out using D. melanogaster orthologues. The Genome Informatics Laboratory of Indiana University Biology Department, which provides computed annotations for the first draft genome sequence release (Acyr_1.0) of A. pismut (http://insects.eugenes.org/aphid), was also queried. Soft-berry protein-based gene predictions software FGENESH+ (http://linux1.softberry.com/berry.phtml) was used to refine gene sequence data.

Previously identified members of the Glutamate receptor, Cyclic nucleotide-gated ion channel and chloride receptor families were retrieved for D. melanogaster and Homo sapiens. These sequences were uploaded into TBLASTN with A. pismut specified as the search set. High probability hits were compared with the Gnomon prediction in AphidBase (http://www.aphidbase.com, 2008 release). Where available, EST data were used to verify the coding sequence of each candidate. To control for possible erroneous annotation, multiple sequence alignments were performed and clustering patterns on individual phylogenetic trees were noted. Gene names were assigned following the closest Drosophila orthologue.

**Acknowledgements**

We thank the International Aphid Genomics Consortium and the Baylor College of Medicine Human Genome Sequencing Centre for making the A. pismut genome sequences publicly available prior to publication. Rothamsted Research receives grant-aided support from the BBSRC of the UK. We would also like to acknowledge the USDA grant (number 2005-35604-15446).

**References**


Zhou, W., Chung, I., Liu, Z., Goldin, A. and Dong, K. (2004) A phylogenetic tree showing relationships of calcium channel protein sequences. Numbers at each node signify bootstrap values with 1000 replicates and the scale bar represents substitutions per site. The proteins shown in the tree (as well as GenBank accession numbers) are as follows: DmelCG5621 (NP_650927), Dmel KainRA (NP_651982), Dmel GluRIIE (NP_001036733), DmelCG3822 (NP_650925), Dmel CG9935 (NP_651831), Dmel CG11155 (NP_651941), Dmel Clumsy (NM_078866), Dmel GluRIIC (NP_608557), Dmel GluRIIE (NP_523484), Dmel GluRIIB (NP_523485), AmelCG3822-3 (XP_392185), AmelCG3822-1 (XP_394265), AmelCG3822-2 (XP_394264). The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) available with the CLUSTALX program (Thompson et al., 1997) and displayed using MEGA.

**Figure S4.** Phylogenetic tree of the kainate receptors in *Drosophila melanogaster* and *Acyrthosiphon pisum*. Tree showing relationships of *A. pisum*, *Apis mellifera* and *D. melanogaster* kainate receptors protein sequences. Numbers at each node signify bootstrap values with 1000 replicates and the scale bar represents substitutions per site. The proteins shown in the tree (as well as GenBank accession numbers) are as follows: DmelCG5621 (NP_650927), Dmel KainRA (NP_651982), Dmel GluRIIE (NP_001036733), DmelCG3822 (NP_650925), Dmel CG9935 (NP_651831), Dmel CG11155 (NP_651941), Dmel Clumsy (NM_078866), Dmel GluRIIC (NP_608557), Dmel GluRIIB (NP_523484), Dmel GluRIIE (NP_523485), AmelCG3822-3 (XP_392185), AmelCG3822-1 (XP_394265), AmelCG3822-2 (XP_394264). The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) available with the CLUSTALX program (Thompson et al., 1997) and displayed using MEGA.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI 10.1111/j.1365-2583.2009.00975.x

**Figure S1.** Phylogenetic tree showing relationships of *Apis* *pisum* and *Drosophila melanogaster* calcium channel protein sequences. Numbers at each node signify bootstrap values with 1000 replicates and the scale bar represents substitutions per site. The proteins shown in the tree (as well as GenBank accession numbers) are as follows: Dmel Alpha 1T (NP_572296), Dmel Alpha 1D (NP_602305), Dmel Alpha Cacophony (NP_511133), Dmel Ca Beta 1 (NP_523546), Dmel CG2295 (NP_610902), Dmel CG4587 (NP_001097163), Dmel CG12455 (NP_609779) and Dmel CG13762 (NP_570013). The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) available with the CLUSTALX program (Thompson et al., 1997) and displayed using TreeView (Page, 1996).

**Figure S2.** Phylogenetic tree showing relationships of *Acyrthosiphon pisum* and *Drosophila melanogaster* potassium channel protein sequences. Numbers at each node signify bootstrap values with 1000 replicates and the scale bar represents substitutions per site. The proteins shown in the tree (as well as GenBank accession numbers) are as follows: Dmel Slo (NP_524486), Dmel Slack (NP_00107259), Dmel KCNQ (NP_786300), Dmel Shail (NP_001097131), Dmel ShaiW (NP_476721), Dmel ShaiB (NP_728783), Dmel Shaker (NP_728124), Dmel Shai (NP_524159), Dmel It (NP_001033947), Dmel Seizure (NP_476713), Dmel Eag (NP_511158), Dmel Egl (NP_477009), Dmel SK (NP_729688), Dmel Irkl1 (NP_651131), Dmel Irk2 (NP_651149) and Dmel Irk3 (NP_609903). The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) available with the CLUSTALX program (Thompson et al., 1997) and displayed using TreeView (Page, 1996).

**Figure S3.** Phylogenetic tree of the NMDA receptors in *Drosophila melanogaster* and *Acyrthosiphon pisum*. Tree showing relationships of *A. pisum*, *Apis mellifera* and *D. melanogaster* NMDA receptors protein sequences. Numbers at each node signify bootstrap values with 1000 replicates and the scale bar represents substitutions per site. The proteins shown in the tree (as well as GenBank accession numbers) are as follows: DmelNmrdar1 (NP_730940), DmelNmrdar2 (NP_001014716), DmelCG3814 (NP_610824), DmelCG30379 (NP_724626), AmelNmrdar1 (NP_001015173), AmelNmrdar2 (XP_396217), AmelCG3814 (XP_391854), AmelNmrdar3 (XP_624931). The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) available with the CLUSTALX program (Thompson et al., 1997) and displayed using MEGA.
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