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ORIGINAL ARTICLE

The cys-loop ligand-gated ion channel gene superfamily of the parasitoid wasp, *Nasonia vitripennis*

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Members of the cys-loop ligand-gated ion channel (cysLGIC) superfamily mediate chemical neurotransmission and are studied extensively as potential targets of drugs used to treat neurological disorders, such as Alzheimer's disease. Insect cys-loop LGICs also have central roles in the nervous system and are targets of highly successful insecticides. Here, we describe the cysLGIC superfamily of the parasitoid wasp, *Nasonia vitripennis*, which is emerging as a highly useful model organism and is deployed as a biological control of insect pests. The wasp superfamily consists of 26 genes, which is the largest insect cysLGIC superfamily characterized, whereas *Drosophila melanogaster*, *Apis mellifera* and *Tribolium castaneum* have 23, 21 and 24, respectively. As with Apis, Drosophila and Tribolium, Nasonia possesses ion channels predicted to be gated by acetylcholine, γ-amino butyric acid, glutamate and histamine, as well as orthologues of the Drosophila pH-sensitive chloride channel (pHCl), CG8916 and CG12344. Similar to other insects, wasp cysLGIC diversity is broadened by alternative splicing and RNA A-to-I editing, which may also serve to generate species-specific receptor isoforms. These findings on *N. vitripennis* enhance our understanding of cysLGIC functional genomics and provide a useful basis for the study of their function in the wasp model, as well as for the development of improved insecticides that spare a major beneficial insect species.
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Introduction

Members of the cys-loop ligand-gated ion channel (cysLGIC) superfamily mediate both fast excitatory and inhibitory synaptic transmission in the nervous system of vertebrates and invertebrates. In insects, the superfamily includes cation-permeable nicotinic acetylcholine receptors (nAChRs) (Raymond-Delpech *et al*., 2005; Sattelle *et al*., 2005), γ-amino butyric acid (GABA)-gated anion channels (Ffrench-Constant *et al*., 1993; Buckingham *et al*., 2005), glutamate-gated chloride channels (GluCls) (Cully *et al*., 1996) and histamine-gated chloride channels (HisCls) (Gisselmann *et al*., 2002; Zheng *et al*., 2002). Studies of *Drosophila melanogaster* and *Apis mellifera* have shown that cysLGICs mediate important aspects of behaviour, such as escape response (Fayyazuddin *et al*., 2006), sleep (Agosto *et al*., 2008), learning and memory (El Hassani *et al*., 2005; Gauthier *et al*., 2006; Liu *et al*., 2007). CysLGICs are also of considerable interest as they are targets of widely used insecticides (Raymond-Delpech *et al*., 2005). For example, nAChRs are targets of neonicotinoids, which have been the fastest-growing class of insecticides in modern crop protection (Jeschke and Nauen, 2008). In addition, GABA receptors, GluCls and HisCls are targets of fipronil and avermectins (Iovchev *et al*., 2002; Bloomquist, 2003).

The parasitoid wasp, *Nasonia vitripennis*, is emerging as a useful model organism because of its ease of handling in the laboratory, as well as the possibility to apply RNA interference (Lynch and Desplan, 2006) and haplo-diploid genetics to study biological processes (Pultz and Leaf, 2003). In addition, Nasonia is used in the biological control of insects, including vectors of human, animal and plant disease, and agricultural pests (Werren *et al*., 2010). 'In the United States alone, biological control programmes using parasitoid wasps save approximately US$20 billion annually in crop loss to newly invasive species, a figure excluding the vast savings from biological control of native pests' (Werren *et al*., 2010). The recent sequencing of the *N. vitripennis* genome (Werren *et al*., 2010) will therefore likely provide a valuable basis for studying important biological processes and lead to methods for further enhancing their utility in controlling insect pests. We report here, as part of the Nasonia genome-sequencing effort, the *N. vitripennis* cysLGIC gene superfamily, which represents a critical step in identifying key components of the wasp nervous system.

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Materials and methods

Identification of cysLGIC subunits in the *N. vitripennis* genome

To identify putative cysLGIC subunits, we screened the *N. vitripennis* genome (assembly version 1.0) (http://www.hgsc.bcm.tmc.edu/projects/nasonia/) with cDNA sequences of every member of the *A. mellifera* cysLGIC superfamily using the tBLASTn algorithm (Altschul *et al*., 1990). Candidate wasp cysLGIC subunits were identified based on their considerable sequence homology with previously characterized subunits (sequence with lowest similarity had E-value 8e−8), particularly in the N-terminal ligand-binding domain and the four transmembrane regions. The highly variable N-terminal signal peptides, which are a feature of cysLGIC subunits, were identified from the GLEAN consensus set of predicted genes (Werren *et al*., 2010), the EST data available at NCBI (http://www.ncbi.nlm.nih.gov/) and by RACE PCR. Reverse transcriptase-PCRs were carried out (see Supplementary Table 1 for sequences of primers used) to verify and correct the open reading frame sequences of each subunit.

Reverse transcription and PCR

Total RNA was extracted from 12 *N. vitripennis* adult wasps homogenized in Trizol (Invitrogen, Paisley, UK) using the RNeasy Mini Kit (Qiagen, Crawley, UK). First-strand cDNA was synthesized from 1 μg total RNA using Superscript III First-Strand Synthesis Super Mix (Invitrogen). N-terminal signal peptides not identified in predicted protein sequences or EST data were obtained by 5′ RACE PCR using the 5′/3′ RACE Kit, 2nd Generation (Roche, Burgess Hill, UK). We were unable to determine the N-terminal sequences for some subunits (Figures 1 and 2). Nested reverse transcriptase-PCR reactions were carried out to amplify transcripts of wasp cysLGIC subunits and to detect transcript variants arising from alternative splicing. Primer pairs (see Supplementary Table 1) recognizing different exons were used to allow the identification of cDNA-specific products. The PCR reactions were carried out in a total volume of 50 μl containing *Taq* polymerase and 1 × PCR buffer (Sigma, Gillingham, UK), 0.2 mM dNTP mix (Roche), 0.4 μM of each primer and 2 μl first-strand cDNA template. The PCR reaction conditions were 35 cycles of 95 °C for 30 s, 50–55 °C for 30 s and 72 °C for 90 s. The first PCR was used at a final dilution of 1 in 500 as template for the second nested PCR reaction. DNA sequence chromatograms for each cysLGIC subunit were analysed using Chromas 2 (Technelysium Pty Ltd, Tewantin, Australia) to detect single nucleotide polymorphisms (SNPs) or RNA-editing sites as shown by mixed signal peaks. No SNPs were observed, and the putative RNA-editing sites detected in Nvitα6 were verified by amplifying and sequencing genomic DNA present in the extracted total RNA, which was first treated with DNase-free RNase (Roche), using primers recognizing intron DNA (see Supplementary Table 3 for primers used). Sequence chromatograms showing a defined region of mixed peaks indicated differential splicing. The corresponding cysLGIC PCR products were cloned into the pGEM-T Easy vector (Promega, Southampton, UK) and between 10 and 20 transformants were sequenced to identify individual subunit isoforms. All PCR products were analysed by electrophoresis in a TAE gel and then purified using the QIAquick Gel Extraction Kit (Qiagen), whereas subunits cloned in pGEM-T Easy were purified using the QIAprep Spin Miniprep Kit (Qiagen). Purified DNA was sequenced by the dye termination method at Cogenics (Essex, UK, http://www.cogenics.com/index.cfm).

Sequence analysis

The multiple protein sequence alignments were constructed with ClustalX (Thompson *et al*., 1997) using the slow-accurate mode with a gap-opening penalty of 10 and a gap-extension penalty of 0.1 and applying the Gonnet 250 protein weight matrix. The protein alignments were viewed using GeneDoc (http://www.nrbsc.org/gfx/genedoc/index.html). Identity values between subunit sequences were calculated using the GeneDoc program. The neighbour-joining method and bootstrap resampling, available with the ClustalX program, were used to construct a phylogenetic tree, which was then displayed using the TreeView application (Page, 1996). Signal-peptide cleavage sites were predicted using the SignalP 3.0 server (Bendtsen *et al*., 2004) and membrane-spanning regions were predicted using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). The PROSITE database (Hulo *et al*., 2006) was used to identify potential N-glycosylation and phosphorylation sites.

Results

The *N. vitripennis* cysLGIC superfamily consists of 26 subunit members

Using tBLASTn (Altschul *et al*., 1990), 26 candidate cysLGIC subunit genes were identified in the *N. vitripennis* genome and manually annotated. This is the fourth complete insect cysLGIC superfamily to be described after those of *D. melanogaster* (Dent, 2006), *A. mellifera* (Jones and Sattelle, 2006) and *Tribolium castaneum* (Jones and Sattelle, 2007), and the largest known to date as the fruit fly, honey bee and red beetle possess 23, 21 and 24 subunits, respectively. Reverse transcriptase–PCR (see Supplementary Table 1 for primers used) was used to show that all of the Nasonia cysLGIC subunit genes are transcribed, with one exception, the nAChR subunit Nvitβ4. An alignment of their protein sequences shows that the wasp subunits possess features common to members of the cysLGIC superfamily (Sine and Engel, 2006) (Figures 1 and 2). These include: (i) an extracellular N-terminal domain containing distinct regions (loops A–F) that form the ligand-binding site (Corringer *et al*., 2000); (ii) the dicysteine loop (cys-loop) consisting of two disulphide bond-forming cysteines separated by 13 amino-acid residues; (iii) four transmembrane regions (TM1–4), the second of which (TM2) contributes most of the channel-lining residues; (iv) a highly variable intracellular loop between TM3 and TM4. As with other cysLGIC subunits, the Nasonia sequences also possess potential N-glycosylation sites in the extracellular N-terminal domain, which can affect receptor maturation, channel desensitization and conductance (Gehle *et al*., 1997; Nishizaki, 2003), and putative phosphorylation sites in the TM3–TM4

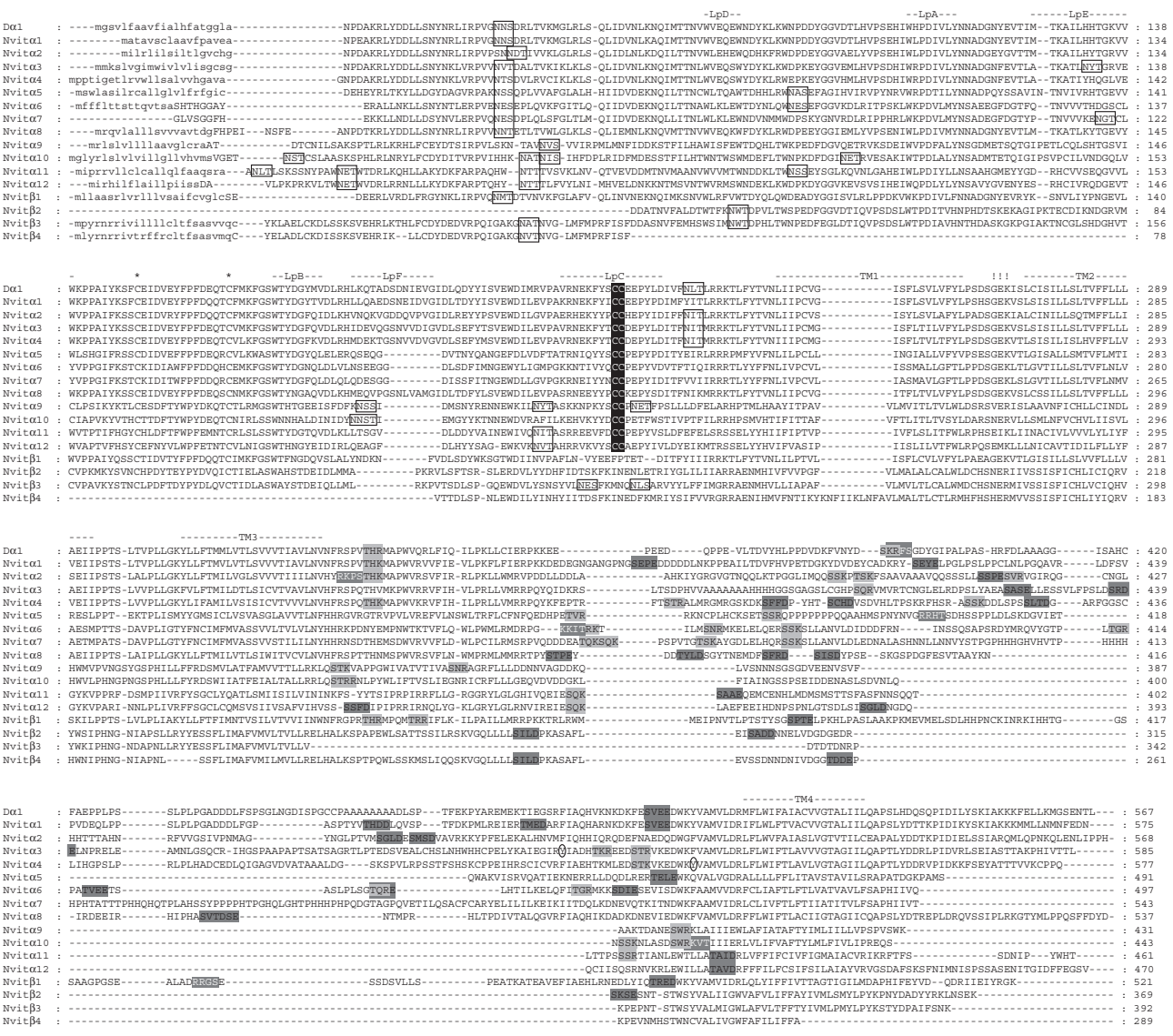

Figure 1 Protein sequence alignment of *N. vitripennis* nAChR subunits. Dα1 of *D. melanogaster* is included for comparison. N-terminal signal leader peptides are shown in lower case and the loops implicated in ligand binding (LpA-F) as well as the four transmembrane regions (TM1-4) are indicated. Residues preceding TM2, which are important for ion charge selectivity, are indicated by exclamation marks. The two cysteines forming the cys-loop are marked by asterisks and the vicinal cysteines characteristic of α subunits are highlighted by black shading. Putative N-glycosylation sites are boxed and potential phosphorylation sites are highlighted as follows: tyrosine kinase phosphorylation (encircled), protein kinase C phosphorylation (light grey shading), casein kinase II phosphorylation (dark grey shading) and cAMP- and cGMP-dependent protein kinase phosphorylation (dark grey shading with white text). nAChR, cation-permeable nicotinic acetylcholine receptor.

intracellular loop, which regulates several aspects of receptor function, such as desensitization and aggregation (Hopfield *et al*., 1988; Borges and Ferns, 2001).

A comparison of sequence identities between *N. vitripennis*, *A. mellifera* and *D. melanogaster* cys-loop LGIC subunits (Tables 1 and 2), as well as the use of a phylogenetic tree (Figure 3), indicates orthologous relationships between the wasp, honey bee and fruit fly subunits. To facilitate comparisons between the three species, Nasonia subunits were named after their Drosophila counterparts as previously done with Apis and Tribolium subunits (Jones and Sattelle, 2006, 2007). For example, the *N. vitripennis* orthologues of Drosophila Dα1, RDL and CG8916 were designated Nvitα1, Nvit_RDL and Nvit_8916, respectively.

Nasonia nicotinic acetylcholine receptor subunits

We identified 16 candidate nAChR subunit-encoding genes in the *N. vitripennis* genome. This is larger than other insect nAChR gene families described, which are of *D. melanogaster* (10 subunits), *Anopheles gambiae* (10), *A. mellifera* (11), *Bombyx mori* (12) and *T. castaneum* (12) (Sattelle *et al*., 2005; Jones *et al*., 2005a, 2006; Jones and Sattelle, 2007; Shao *et al*., 2007). Twelve of the Nasonia nAChR subunits possess the two adjacent cysteine

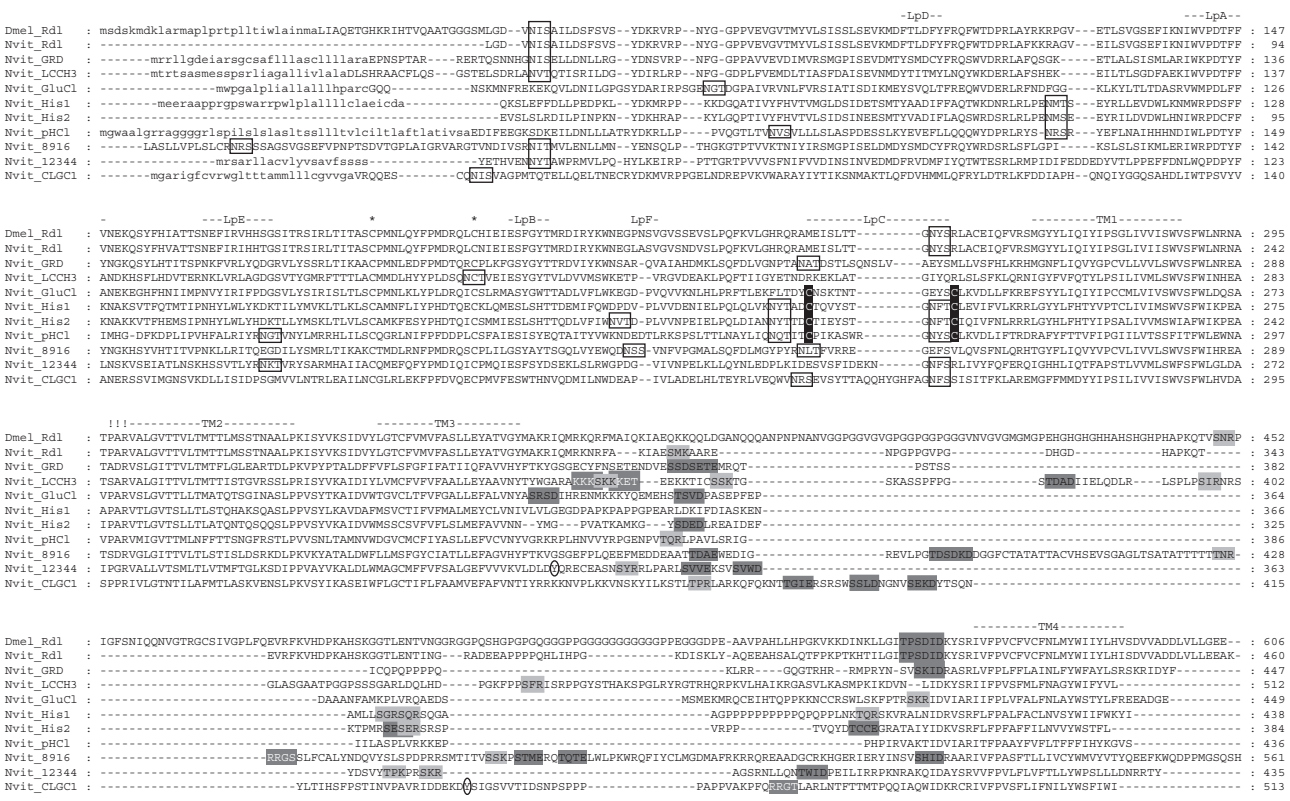

Figure 2 Protein sequence alignment of *N. vitripennis* cysLGIC subunits other than nAChRs. Rdl of *D. melanogaster* is included for comparison. N-terminal signal leader peptides are shown in lower case and the loops implicated in ligand binding (LpA–F) as well as the four transmembrane regions (TM1–4) are indicated. Residues preceding TM2, which are important for ion charge selectivity, are indicated by exclamation marks. The two cysteines forming the cys-loop are marked by asterisks, whereas the putative second cys-loop in LpC is highlighted by black shading. Putative N-glycosylation sites are boxed and potential phosphorylation sites are highlighted as follows: tyrosine kinase phosphorylation (encircled), protein kinase C phosphorylation (light grey shading), casein kinase II phosphorylation (dark grey shading) and cAMP- and cGMP-dependent protein kinase phosphorylation (dark grey shading with white text). HisCl1 and HisCl2 have been abbreviated to His1 and His2, respectively. cysLGIC, cys-loop ligand-gated ion channel; HisCl, histamine-gated chloride channel; nAChR, cation-permeable nicotinic acetylcholine receptor.

Table 1 Percentage identity/similarity between *N. vitripennis* and *A. mellifera* nAChR subunit protein sequences

Subunit	*Nvα1*	*Nvα2*	*Nvα3*	*Nvα4*	*Nvα5*	*Nvα6*	*Nvα7*	*Nvα8*	*Nvα9*	*Nvα10*	*Nvα11*	*Nvα12*	*Nvβ1*	*Nvβ2*	*Nvβ3*	*Nvβ4*
Amelα1	**78/83**	49/63	53/65	51/63	28/42	31/45	30/45	50/61	15/27	13/26	18/33	18/33	37/52	8/20	11/22	6/13
Amelα2	50/66	**82/88**	48/63	48/64	27/45	32/49	31/47	50/66	16/32	14/30	18/36	19/36	39/56	10/23	11/25	6/15
Amelα3	54/65	50/65	**87/91**	67/76	29/45	32/49	32/47	56/68	15/29	15/29	18/36	20/35	41/57	10/22	12/25	6/16
Amelα4	51/64	49/65	65/75	**89/93**	28/45	33/48	30/46	53/67	14/29	13/28	18/36	19/35	40/55	10/22	11/24	6/16
Amelα5	29/45	27/45	29/45	28/44	**76/84**	35/54	30/49	29/47	17/35	17/33	20/40	20/38	31/49	11/23	14/28	7/16
Amelα6	31/47	31/48	33/50	32/47	35/53	**81/89**	56/68	35/51	15/32	14/31	21/40	19/38	33/51	10/25	12/28	6/17
Amelα7	31/47	31/47	33/49	32/47	31/50	58/70	**85/90**	32/47	13/28	12/29	18/35	17/34	32/51	10/22	11/24	6/15
Amelα8	52/65	50/64	55/67	53/67	29/47	34/51	32/46	**85/92**	16/31	14/30	21/38	19/37	41/59	10/22	12/27	6/15
Amelα9	12/28	12/29	12/27	12/29	16/32	14/31	13/28	14/31	44/63	36/55	19/39	17/35	13/30	20/39	27/47	13/27
Amelβ1	38/52	36/54	38/56	39/55	31/50	33/52	31/49	41/57	14/30	14/30	19/36	18/35	**92/96**	10/24	13/28	5/16
Amelβ2	10/26	9/26	9/25	9/26	13/32	14/32	12/28	10/27	26/46	26/46	17/37	14/34	11/30	25/43	11/25	18/31

Proposed orthologues are shown in bold.

residues in loop C (Figure 1), which are important for acetylcholine (ACh) binding (Corringer *et al*., 2000), defining them as α subunits. The remaining four subunits were designated β as they lack the vicinal cysteines. For Nvitβ4, only a partial sequence was found, possibly because of incomplete genome information, with loops D to F missing (Figure 1). Nvitβ4 is likely to be a pseudogene as a frameshift in exon 1 (Supplementary Figure 1) results in a premature stop codon and its peptide sequence is likely to be spread over two reading frames. As PCR with primers specific to different exons failed to amplify Nvitβ4 cDNA products, we conclude that the subunit is unlikely to be transcribed. To enable Nvitβ4 to be included in the protein sequence analysis, we assumed that the subunit was encoded by a single reading frame (Figure 1, Supplementary Figure 1).

Table 2 Percentage identity/similarity between *N. vitripennis* and *A. mellifera* non-nAChR subunit protein sequences

Amel	*Nvit_RDL*	*Nvit_GRD*	*Nvit_LCCH3*	*Nvit_GluCl*	*Nvit_HisCl1*	*Nvit_HisCl2*	*Nvit_pHCl*	*Nvit_8916*	*Nvit_12344*	*NvitCLGC1*
RDL	**90/92**	31/45	34/50	30/45	23/40	25/42	21/39	24/39	22/38	21/38
GRD	31/48	**72/76**	29/44	27/44	24/40	22/40	18/31	38/51	22/38	20/34
LCCH3	35/54	29/44	**84/91**	30/48	25/41	27/43	19/36	27/40	22/42	21/39
GluCl	28/43	29/44	27/43	**83/89**	28/47	28/45	24/44	21/38	23/40	20/38
HisCl1	23/40	25/41	23/39	29/47	**86/90**	57/71	22/40	19/33	26/48	19/36
HisCl2	25/42	24/43	25/40	30/48	55/70	**95/98**	22/40	19/33	29/49	20/38
pHCl	22/38	19/34	19/36	26/47	21/40	22/40	**87/89**	15/29	21/40	19/36
CG8916	21/35	40/53	28/43	23/39	22/36	22/37	16/30	**69/76**	19/33	17/31
CG12344	22/39	22/38	22/37	24/42	25/47	29/50	19/36	18/30	**67/77**	20/38
CG6927	21/38	19/35	22/39	21/37	21/37	21/38	18/35	17/31	20/39	**77/87**

Proposed orthologues are shown in bold.

Nasonia possesses the typical core groups of nAChR subunits that are highly conserved between different insect species (Jones *et al*., 2007). Thus, subunit equivalents of Dα1–7, Dβ1 and Dβ2, are evident in the wasp genome (Figure 3). As is the case for other non-drosophilid insects (Jones *et al*., 2005b), the Nasonia orthologue of Dβ2 is of the α type (Nvitα8). Nvitα5, along with its orthologues in Apis, Bombyx and Tribolium, depart strongly from the Dipteran orthologues, Dα5 (Figure 3), Agamα5 and Mdα5, of *Musca domestica* (Gao *et al*., 2007b). As is the case for Dα1, Dα2, Dα3, Dα4, Dβ2 and their orthologues in other insect species, the corresponding Nasonia subunits (Nvitα1–4 and Nvitα8) have an insertion in loop F (Figure 1), which may contribute to interactions with the neonicotinoid, imidacloprid (Shimomura *et al*., 2004). The Dα1, Dα2 and Dβ2 genes, as well as their Anopheles orthologues, Agamα1, Agamα2 and Agamα8, are similarly arranged and tightly clustered within 200 and 220 kb, respectively (Jones *et al*., 2005a). Immunohistochemical and coimmunoprecipitation studies show that Dα1, Dα2 and Dβ2 are integral components of certain nAChR subtypes, leading to the suggestion that gene clustering may favour coordinated expression and co-assembly of these nAChR subunits (Chamaon *et al*., 2002). In Nasonia, as with Apis, Nvitα1 and Nvitα2 are clustered on scaffold 1 but are separated from the wasp Dβ2 orthologue, Nvitα8, which is located on scaffold 9. The separation of these genes may thus result in diversification of receptor expression and co-assembly. Another subunit, Nvitα5, is however clustered with Nvitα1 and Nvitα2, being located within 220 kb of both subunits. The clustering of the honey bee subunits Amelα7 and Amelβ1 (Jones *et al*., 2006), which is also seen in the genomes of Anopheles and Tribolium (Jones *et al*., 2005a; Jones and Sattelle, 2007), is not conserved in Nasonia, as Nvitα7 and Nvitβ1 are located on scaffolds 31 and 8, respectively.

Insect nAChR gene families possess at least one divergent subunit that shares relatively low sequence identity with other nAChR subunits (Jones *et al*., 2007). The five insect nAChR gene families previously described (those of *A. gambiae*, *A. mellifera*, *B. mori*, *D. melanogaster* and *T. castaneum*) contain up to three divergent subunits, each with a different complement of α and β subunits (Lansdell and Millar, 2002; Sattelle *et al*., 2005; Jones *et al*., 2005a, 2006; Jones and Sattelle, 2007; Shao *et al*., 2007). We found a considerably larger number of divergent subunits in the Nasonia genome consisting of four α and three β subunits (Figure 3). As with one of the Tribolium divergent subunits, Tcasα9, Nvitα11 possesses an atypical FxCC amino-acid motif (Figure 1), instead of the highly conserved YxCC found in loop C, which may result in unusual ligand-binding properties (Galzi *et al*., 1991). In addition, the Nasonia divergent subunits lack the GEK motif characteristic of nAChR subunits, which precedes TM2 (Figure 1), and has an important role in ion permeation and selectivity (Jensen *et al*., 2005). This raises the possibility that subunits of this type may form nAChRs with distinct ion channel characteristics. Nvitα11 and Nvitα12 are present within 300 kb of each other on scaffold 4 of the Nasonia genome, whereas Nvitβ2 and Nvitβ3 are clustered within 15 kb on scaffold 164, indicating that both pairs of subunits arose from a recent duplication event from a common gene. Nvitα9 and Nvitβ4 are also clustered together within 10 kb on scaffold 69. In the case of these two subunits, a duplication event may have caused a frameshift, resulting in Nvitβ4 being a pseudogene (Supplementary Figure 1).

Two Nasonia nAChR subunits, Nvitα4 and Nvitα6, have alternatively spliced exons most likely arising from tandem exon duplication (Kondrashov and Koonin, 2001). Thus, as with Dα4 and orthologues of several other insect species (Lansdell and Millar, 2000; Jones *et al*., 2005a, 2006; Jones and Sattelle, 2007; Shao *et al*., 2007), Nvitα4 possesses two alternatives for exon 4 (denoted exon4 and exon4′) (Figure 4a), and similar to Amelα6 (Jin *et al*., 2007), Nvitα6 has three alternatives for exon 8 (Figure 4b). Analysis of sequence chromatograms shows that both alternatives for Nvitα4 exon4 are transcribed and reverse transcriptase–PCR (see Supplementary Table 2 for primers used) detected all three possible combinations of alternate exons for Nvitα6. Alternative splicing of Nvitα4 exon4 substitutes residues in loop E, which may affect ligand binding (Corringer *et al*., 2000; Amiri *et al*., 2008), as well as residues in the vicinity of the cys-loop, which may affect receptor assembly (Lansdell and Millar, 2000). For Nvitα6, alternative splicing of exon 8 changes residues in the TM2 domain, which may alter the ion channel properties of the receptor (Grauso *et al*., 2002). The alternative exons of both Apis and Nasonia α4 and α6 subunits are identical, with the only exception that an alanine residue in Nvitα6 exon 8c is replaced by an asparagine in the equivalent honey bee exon (Figure 4b).

In analysing the cDNA sequences of the Nasonia cysLGICs, we detected potential RNA A-to-I editing (Jepson and Reenan, 2007) in one subunit, Nvitα6

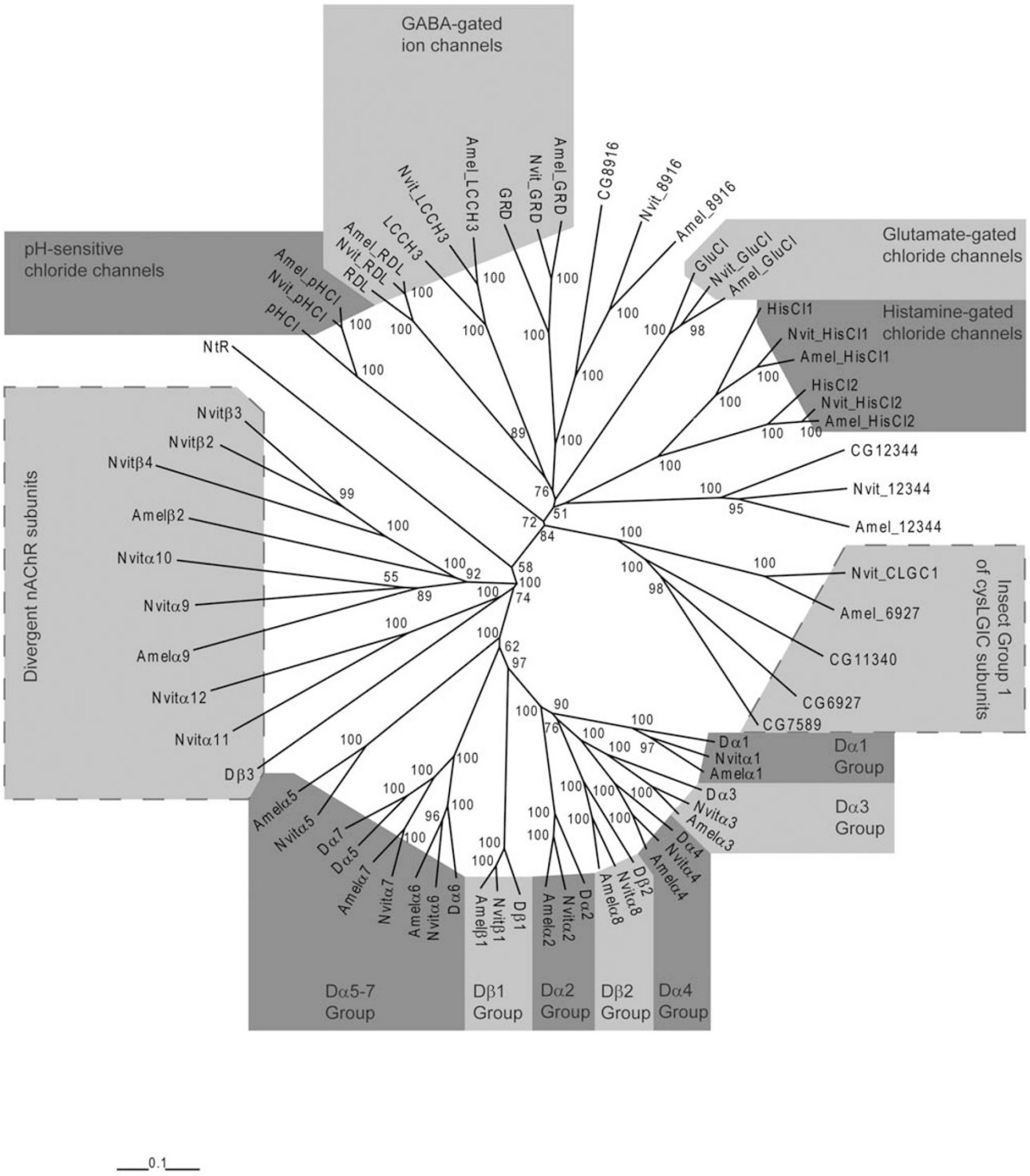

Figure 3 Unrooted tree showing relationships of *N. vitripennis*, *A. mellifera* and *D. melanogaster* cys-loop LGIC subunit protein sequences. Numbers at each node signify bootstrap values with 100 replicates and the scale bar represents substitutions per site. The subunits shown in the tree are as follows: *A. mellifera* Amelα1 (DQ026031), Amelα2 (AY540846), Amelα3 (DQ026032), Amelα4 (DQ026033), Amelα5 (AY569781), Amelα6 (DQ026035), Amelα7 (AY500239), Amelα8 (AF514804), Amelα9 (DQ026037), Amelβ1 (DQ026038), Amelβ2 (DQ026039), Amel_GluCl (DQ667185), Amel_GRD (DQ667183), Amel_HisCl1 (DQ667187), Amel_HisCl2 (DQ667188), Amel_LCCH3 (DQ667184), Amel_pHCl (DQ667189), Amel_RDL (DQ667182), Amel_6927 (DQ667195), Amel_8916 (DQ667193), Amel_12344 (DQ667194); *D. melanogaster* Dα1 (CAA30172), Dα2 (CAA36517), Dα3 (CAA75688), Dα4 (CAB77445), Dα5 (AAM13390), Dα6 (AAM13392), Dα7 (AAK67257), Dβ1 (CAA27641), Dβ2 (CAA39211), Dβ3 (CAC48166), GluCl (AAG40735), GRD (Q24352), HisCl1 (AAL74413), HisCl2 (AAL74414), LCCH3 (AAB27090), the putative cysLGIC subunit Ntr (AF045471), pHCl (NP_001034025), RDL (AAA28556), CG6927 (AAF45992), CG7589 (AAF49337), CG8916 (BT022901), CG11340 (AAF57144), CG12344 (AAF58743); *N. vitripennis* Nvitα1 (FJ821429), Nvitα2 (FJ821430), Nvitα3 (FJ821431), Nvitα4 (FJ821432), Nvitα5 (FJ821434), Nvitα6 (FJ821435), Nvitα7 (FJ821438), Nvitα8 (FJ821439), Nvitα9 (FJ821440), Nvitα10 (FJ821441), Nvitα11 (FJ821442), Nvitα12 (FJ821443), Nvitβ1 (FJ821444), Nvitβ2 (FJ821445), Nvitβ3 (FJ821446), Nvitβ4 (FJ821447), Nvit_HisCl1 (FJ851089), Nvit_HisCl2 (FJ851090), Nvit_pHCl (FJ851091), Nvit_GRD (FJ851092), Nvit_8916 (FJ851093), Nvit_12344 (FJ851094), Nvit_CLGC1 (FJ851095), Nvit_LCCH3 (FJ851096), Nvit_RDL (FJ851097), Nvit_GluCl (FJ851099). cysLGIC, cys-loop ligand-gated ion channel; GluCl, glutamate-gated chloride channel; HisCl, histamine-gated chloride channel.

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                          ------LpE-------        *             *      --LpB--
Nvitα4 exon 4    ADGNFEVTLATKATIYHQGLVEWKPPAIYKSSCEIDVEYFPFDEQTCVLKFGSWTYDGFK
Amelα4 exon 4    ADGNFEVTLATKATIYHQGLVEWKPPAIYKSSCEIDVEYFPFDEQTCVLKFGSWTYDGFK

Nvitα4 exon 4'   ADGNYEVTLMTKATVYYSGLVVWQPPAVYKSSCSIDVEFFPYDVQTCVLKLGSWTYDGFK
Amelα4 exon 4'   ADGNYEVTLMTKATVYYSGLVVWQPPAVYKSSCSIDVEFFPYDVQTCVLKLGSWTYDGFK
```

b

```
                              LpD-                    -------TM2-------
Nvitα6 exon 3a DEKNQILTTNAWLKL   Nvitα6 exon 8a  GVTILLSLTVFLNLVAESMPTTSDAVPLI
Amelα6 exon 3a DEKNQILTTNAWLKL   Amelα6 exon 8a  GVTILLSLTVFLNLVAESMPTTSDAVPLI

                                 Nvitα6 exon 8b  GVTILLSLTVFLNLVAETLPQVSDAIPLL
                                 Amelα6 exon 8b  GVTILLSLTVFLNLVAETLPQVSDAIPLL

                                 Nvitα6 exon 8c  GVTILLSQTVFSLLVAHVLTRTSEAVPLI
                                 Amelα6 exon 8c  GVTILLSQTVFSLLVNHVLTRTSEAVPLI
```

c

```
Nvit_RDL exon 3a GPPVEVGVTMYVLSISSVSEVLM
Amel RDL exon 3a GPPVEVGVTMYVLSISSVSEVLM

Nvit_RDL exon 3b GPPVEVGVTMYVLSISSLSEVKM
Amel RDL exon 3b GPPVEVGVTMYVLSISSLSEVKM
```

```
                                LpF-                      --LpC---
Nvit_RDL exon 6a GYTMRDIRYKWNSGLQSVGISSEVELPQFRVLGHRQRQTTIHLSTG
Amel RDL exon 6a GYTMRDIRYKWNAGLQSVGISNEVELPQFRVLGHRQRHSTIHLSTG

Nvit_RDL exon 6b GYTMRDIRYKWNEGLASVGVSNDVSLPQFKVLGHRQRAMEISLTTG
Amel RDL exon 6b GYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTG
```

d

```
Nvit_GluCl exon3a DGPAIVRVNLFVRSIATISDIKM
Amel GluCl exon3a DGPAIVRVNLFVRSIATISDIKM

Nvit_GluCl exon3b DGPAVVRVNIFVRSISKIDDVTM
Amel GluCl exon3b DGPAVVRVNIFVRSISKIDDVTM
```

Figure 4 Alternative splicing of exons in Nasonia cys-loop LGIC subunits. Equivalent alternate exons of *N. vitripennis* and *A. mellifera* cys-loop LGIC subunits are aligned. (**a**) Exon 4 splice variants in Nvitα4 and Amelα4. The cysteine residues forming the cys-loop are marked by asterisks. (**b**) Splice variants of exon 8 in Nvitα6 and Amelα6. Similar to Apis, but unlike Drosophila, no alternatives for exon 3 were found. (**c**) Splice variants of exons 3 and 6 in both Nvit_RDL and Amel_RDL. (**d**) Exon 3 splice variants in Nvit_GluCl and Amel_GluCl. Throughout the figure, Nasonia residues, which differ from those of the orthologous Apis exon, are highlighted in bold, and loops B–F, as well as TM2, are indicated. GluCl, glutamate-gated chloride channel.

(Figure 5). Sequencing of the corresponding genomic DNA using primers listed in Supplementary Table 3 confirmed that the nucleotide changes occur at the RNA level. Nvitα6 is edited at five sites, three of which correspond to sites 4, 5 and 6 in Dα6 (Grauso *et al*., 2002), which are conserved in several diverse insect species such as *A. mellifera* (Jones *et al*., 2006), *B. mori* (Jin *et al*., 2007), *D. melanogaster* (Grauso *et al*., 2002), *Heliothis virescens* (Grauso *et al*., 2002), *M. domestica* (Gao *et al*., 2007a) and *T. castaneum* (Jones and Sattelle, 2007). The remaining two editing sites have so far been observed only in Nasonia and they may generate receptor isoforms with characteristics particular to the wasp as both sites alter the amino-acid residues in the vicinity of the ligand-binding domain and the cys-loop.

A comparison of Nasonia and Apis nAChR gene structures shows that four orthologue pairs (Nvitα1 and Amelα1, Nvitα4 and Amelα4, Nvitα8 and Amelα8, Nvitβ1 and Amelβ1) share identical sets of exon–intron boundaries (Figure 6). As noted for Amelα5, Amelα6 and Amelα7 (Jones *et al*., 2006), the orthologous Nasonia subunits, Nvitα5, Nvitα6 and Nvitα7, in addition to closely resembling the vertebrate's α7 subunits (up to 48% identity), possess exon–intron boundaries found in mammalian, bird and fish α7 and the closely related α8 subunits.

Nasonia GABA-gated anion and cation channels

The Nasonia genome contains orthologues of the three known *D. melanogaster* GABA-gated ion channels (Figure 3 and Table 2), RDL, GRD and LCCH3 (Buckingham *et al*., 2005). As is the case for Drosophila, Apis and Tribolium (Jones and Sattelle, 2006, 2007),

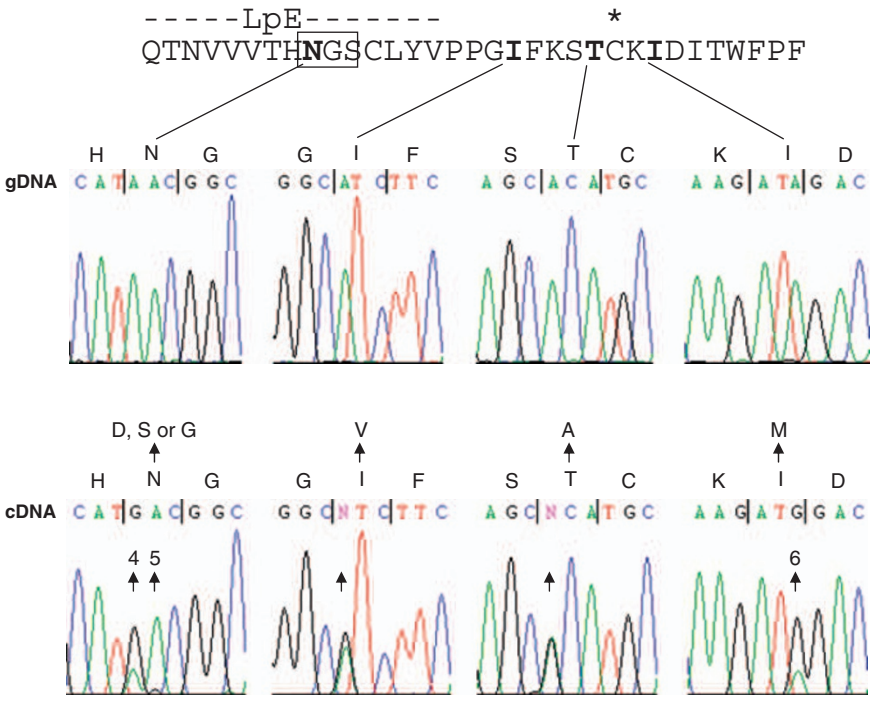

Figure 5 RNA A-to-I editing in Nvitα6. Arrows highlight the mixed adenosine/guanosine peak in the cDNA sequence indicating RNA editing and the resulting amino-acid change. The corresponding genomic DNA (gDNA) sequence, which lacks this mixed A/G signal, is also shown. Editing sites 4, 5 and 6 (Grauso *et al*., 2002), are indicated. The edited region is included with loop E indicated and a cysteine, which is part of the cys-loop, marked by an asterisk. An N-glycosylation site, which is removed by editing at sites 4 and 5, is boxed. Amino acids targeted by editing are highlighted in bold.

Nvit_RDL possesses a PAR sequence before TM2 (Figure 2), which is important for forming anion channels (Jensen *et al*., 2005), whereas Nvit_GRD and Nvit_LCCH3 lack this sequence, and thus may form cation-permeable channels as shown for Drosophila GRD and LCCH3 expressed in oocytes of *Xenopus laevis* (Gisselmann *et al*., 2004). However, whereas electrophysiology has clearly shown that GABA induces inhibitory chloride channels in insects, it remains to be established whether GABA-gated cation channels function *in vivo* (Buckingham *et al*., 2005).

As is the case for Drosophila and Apis RDL, exons 3 and 6 are alternatively spliced in Nvit_RDL (Figure 4c) with two alternatives for each exon. Exons 3a and 3b are completely conserved in both the Hymenoptera species. In contrast, exons 6a and 6b of the wasp and honey bee have different amino-acid residues in loops C and F, which may give rise to receptor variants that have distinct ligand-binding characteristics in the two insect species. The gene structures of Nasonia and Apis GABA subunits (Figure 7) differ in the Nvit_RDL and Nvit_LCCH3; each possess one more exon than their honey bee orthologues, whereas Nvit_GRD has no introns from TM3 to its C-terminus, and Amel_GRD has one in the TM3–TM4 intracellular loop. All three subunits possess up to four of the five exon–intron boundaries (labelled a, b, c, d and e) observed to be highly conserved in the invertebrate and vertebrate GABA receptor-like genes (Tsang *et al*., 2007) (Figure 7). In fact, boundaries b and d are present in all Apis and Nasonia non-nAChR cysLGIC subunits (Figure 7).

Nasonia glutamate and HisCls

As with *D. melanogaster*, *A. mellifera* and *T. castaneum*, *N. vitripennis* has one glutamate-gated chloride channel (Nvit_GluCl) and two histamine-gated chloride channel (Nvit_HisCl1 and Nvit_HisCl2) orthologues (Figure 3). Consistent with their putative function as anion channels, Nvit_GluCl, Nvit_HisCl1 and Nvit_HisCl2 all have the PAR motif preceding TM2 (Figure 2) (Jensen *et al*., 2005). Out of the ligand-gated anion channels, HisCl2 is the most highly conserved between Nasonia and Apis, sharing 95% identity (Table 2).

Nvit_GluCl has two alternatives for exon 3 (Figure 4d), as is the case for Amel_GluCl (Jones and Sattelle, 2006), with both exons being identical in the honey bee and wasp. The gene structures of Nvit_GluCl and Amel_GluCl are also the same, as are the gene structures of Nvit_HisCl1 and Amel_HisCl (Figure 7), whereas Nvit_HisCl2 possesses an additional exon to Amel_HisCl2 in the N-terminal extracellular domain.

Nasonia pHCl and uncharacterized cys-loop LGIC subunits

The *N. vitripennis* genome possesses an orthologue of the pH-sensitive chloride channel (Nvit_pHCl, Figures 2 and 3), which was first identified in Drosophila (Schnizler *et al*., 2005). The Drosophila pHCl has several splice variants, of which Variant 3 introduces an insertion and a protein kinase C phosphorylation site in the intracellular region between TM3 and TM4. We detected a similar variant in Nvit_pHCl transcripts (Figure 8a), where the

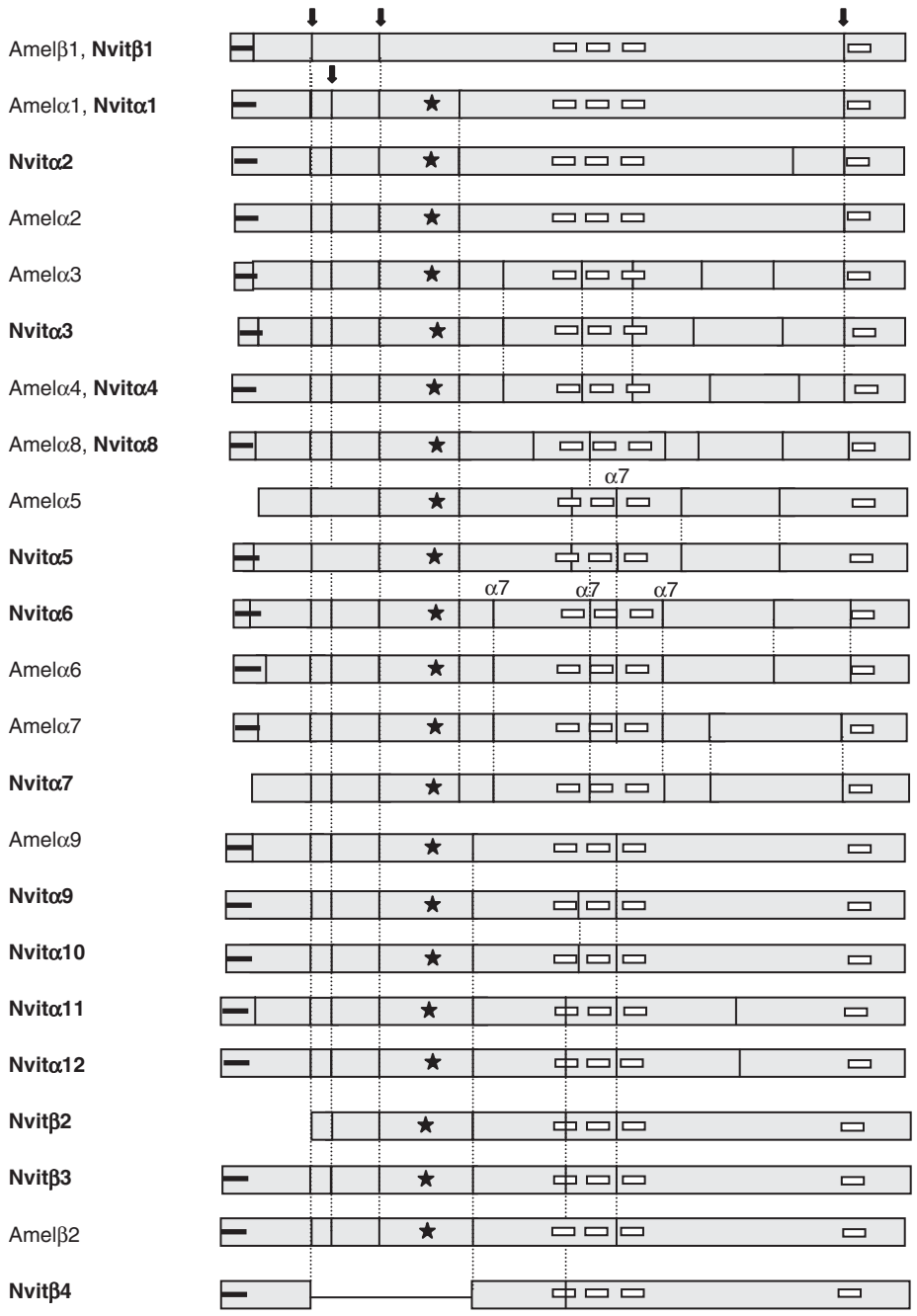

Figure 6 Exon composition of *N. vitripennis* and *A. mellifera* nAChR subunits. The N-terminal signal peptide is shown as a bar, the cys-loop is denoted as a star, and the four transmembrane regions are marked as white boxes. Conserved exon–intron boundaries are indicated by broken lines. Boundaries highly conserved in *nAChR* genes of invertebrates and vertebrates are highlighted by arrows, and boundaries particular to the vertebrate α7 subunits are also indicated. nAChR, cation-permeable nicotinic acetylcholine receptor.

peptide insertion differs by only one amino-acid residue from that of Drosophila pHCl and is completely identical to the equivalent insertion in Amel_pHCl (Jones and Sattelle, 2006). The novel insertion in loop C found in Amel_pHCl (Variant 4) (Jones and Sattelle, 2006) is also present in Nvit_pHCl, which differs from the bee insertion by four amino-acid residues (Figure 8b). The gene structures of Amel_pHCl and Nvit_pHCl differ, with the Nasonia gene having two extra exons near the N-terminus and an extra exon in the TM3–TM4 intracellular loop (Figure 7).

Five Drosophila cysLGIC subunits have yet to be functionally characterized. These are CG6927, CG7589, CG8916, CG11340 and CG12344. CG8916 and CG12344 appear to be closely related to GRD and HisCls, respectively (Figure 3), whereas CG6927, CG7589 and CG11340 form a distinct subfamily of cysLGIC subunits, which we have denoted Insect Group 1 of cysLGIC subunits (Jones and Sattelle, 2007). Nasonia possesses candidate orthologues of CG8916 and CG12344 and only one member (Nvit_CLGC1) of Insect Group 1 (Figure 3). *A. mellifera* also has one Insect Group 1 subunit called

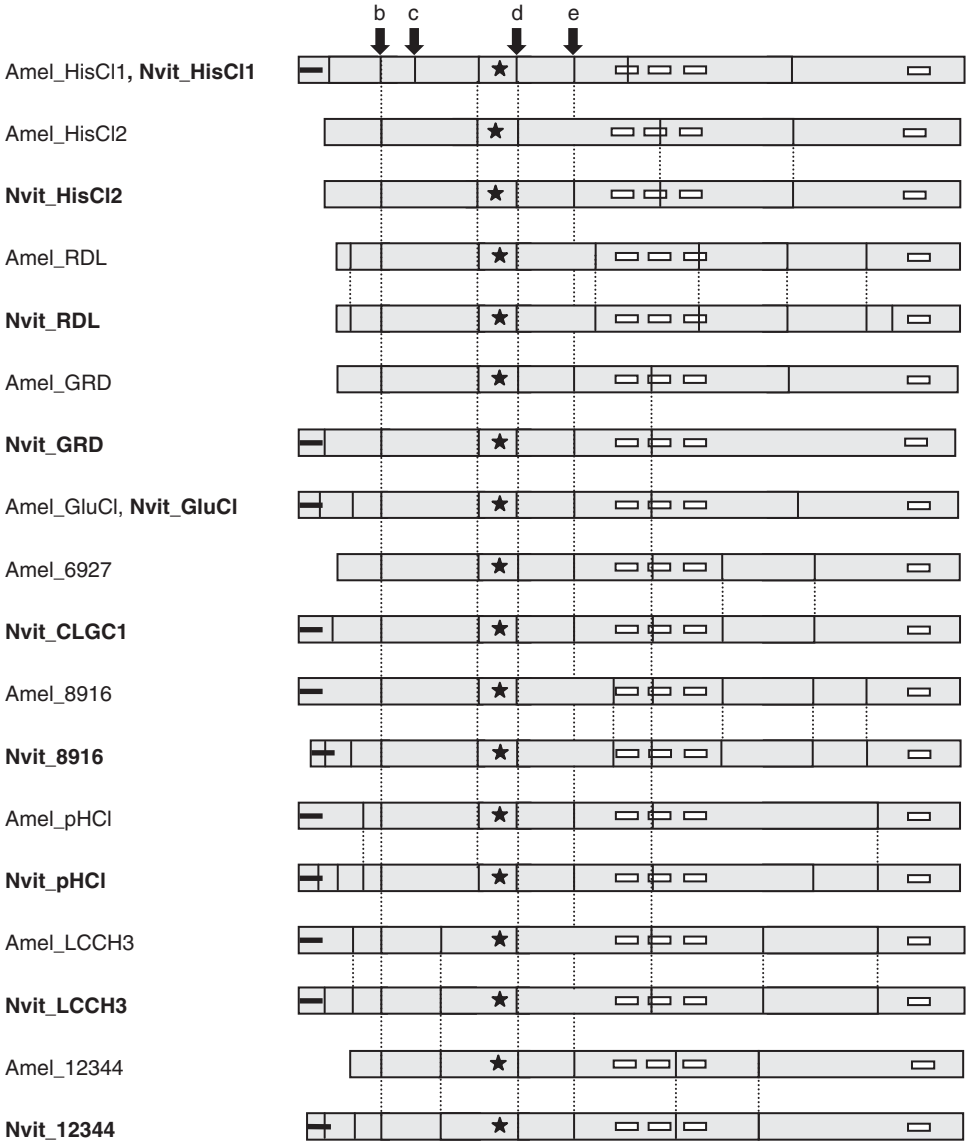

Figure 7 Exon composition of *N. vitripennis* and *A. mellifera* non-nAChR subunits. The N-terminal signal peptide is shown as a bar, the cys-loop is denoted as a star, and the four transmembrane regions are marked as white boxes. Conserved exon–intron boundaries are indicated by broken lines. Boundaries b–e, which are highly conserved in GABA receptor-like genes of invertebrates and vertebrates (Tsang *et al*., 2007) are highlighted by arrows. GABA, γ-amino butyric acid.

a

```
Dmel_pHCl Variant 3   PVTQRLPAVLSRIGVILASPLPGEK
Nvit_pHCl Variant 3   PVTQRLPAVLSRIGIILASPLRKKE
Amel_pHCl Variant 3   PVTQRLPAVLSRIGIILASPLKREG
```

b

```
                      ---LpC---------------------------------------
Nvit_pHCl Variant 4   CPIKASWRADGQLIVDYEDEYDDFGDSKCSLCQRRFEEQGNYS
Amel_pHCl Variant 4   CPIKVSWRADGQIMVDYEDEFDEFGDSKCSLCQRRFEEQGNYS
```

Figure 8 Differential splicing in Nvit_pHCl. (**a**) Alignment of Variant 3 of Drosophila pHCl with the equivalent Nasonia and Apis variants. The variants are caused by the differential use of splice sites, which inserts stretches of amino acids (underlined). A potential kinase C phosphorylation site is highlighted in grey shading. (**b**) Alignment of loop C (LpC) sequences of Nvit_pHCl Variant 4 and a similar variant in Apis (Amel_pHCl Variant 4) where use of differential splice sites introduces an insertion (underlined). Residues that differ between the equivalent splice variants of the two species are highlighted in bold. The N-glycosylation sites are boxed.

Amel_6927 (Jones and Sattelle, 2006), which shares high sequence identity with Nvit_CLGC1 of 77% (Table 2) as well as a similar gene structure (Figure 7). The finding of a single Group 1 subunit in both these Hymenoptera species is consistent with the suggestion that 'gene duplication occurred after the emergence of the

Hymenoptera to give rise to the three subunits present in both Tribolium (Coleoptera) and Drosophila (Diptera)' (Jones and Sattelle, 2007).

Discussion

As part of the Nasonia genome-sequencing project (Werren *et al*., 2010), we have described the wasp cysLGIC gene superfamily, which encodes for receptors that have major roles in the nervous system and are also targets of highly successful insecticides. This is the fourth complete insect cysLGIC superfamily to be described after those of the Dipteran *D. melanogaster* (Littleton and Ganetzky, 2000), the Coleopteran *T. castaneum* (Jones and Sattelle, 2007) and the Hymenopteran *A. mellifera* (Jones and Sattelle, 2006). Thus, we report here the first comparison of two cysLGIC superfamilies from insects of the same order. Considering that the cysLGIC superfamilies of Drosophila, Apis and Tribolium, which represent diverse insect species of different orders, are compact with only minor changes in gene numbers (Jones and Sattelle, 2007), we find in the Nasonia genome a striking expansion in the number of nAChR subunits. Although *A. gambiae* (Jones *et al*., 2005a), *A. mellifera* (Jones *et al*., 2006), *B. mori* (Shao *et al*., 2007), *D. melanogaster* (Lansdell and Millar, 2002) and *T. castaneum* (Jones and Sattelle, 2007) possess one, two, three, one and two divergent subunits, respectively, *N. vitripennis* has seven (Figures 1 and 3). One of these is a pseudogene (Supplementary Figure 1), whereas the remaining six are potentially functional. It will be of interest to determine the functional roles of insect divergent nAChR subunits and why different species possess their own distinct complement. The non-nAChR subunits of Apis and Nasonia appear to be under a higher degree of evolutionary constraint with both species possessing an equal number of subunits, each with obvious orthologous relationships (Figure 3, Table 2).

Nasonia wasps are of agricultural importance as they control crop-damaging insect pests. The characterization of their cysLGICs provides an important basis for the future rational design of insecticides that control pests though sparing beneficial species. The use of heterologous expression systems such as *X. laevis* oocytes (Buckingham *et al*., 2006) has allowed the functional characterization of several Drosophila cysLGICs, such as RDL (Ffrench-Constant *et al*., 1993), GRD and LCCH3 (Gisselmann *et al*., 2004), GluCl (Cully *et al*., 1996), HisCl1 and HisCl2 (Gisselmann *et al*., 2002; Zheng *et al*., 2002) and pHCl (Schnizler *et al*., 2005). Similar studies of heterologously expressed ion channels from other insect species, including *N. vitripennis*, in combination with the use of three-dimensional models such as those based on the crystal structure of the molluscan acetylcholine-binding protein (Smit *et al*., 2003), will likely prove useful in the search for novel compounds that show selectivity for receptors of certain insect species as well as in determining the mechanisms of insecticide interaction with cysLGICs. For insect nAChRs, functional expression in heterologous systems has so far proven elusive, although α subunits of Drosophila and other insect species, such as *Nilaparvata lugens*, can form robust functional channels when coexpressed with a vertebrate β2 subunit and studies on such hybrid receptors have provided insights into the selectivity of neonicotinoids for insect nAChRs over those of vertebrates, regions of subunit proteins involved in imidacloprid interactions and the actions of different neonicotinoids (Matsuda *et al*., 2005; Liu *et al*., 2009).

Nasonia provides a powerful tool for the study of cysLGIC function. The possibility to use haplo-diploid genetics, according to which unfertilized eggs develop as haploid males, facilitates screening for recessive mutations affecting a biological process of interest, which may include cysLGIC physiology, pharmacology, trafficking or assembly. To add to the utility of Nasonia as a model organism, parental RNA interference, as a result of which RNA interference arising from double-stranded RNA introduced into pupae or adults also spreads to their progeny, is effective in *N. vitripennis* (Lynch and Desplan, 2006). Thus, RNA interference could be used to elucidate the roles of Nasonia cysLGIC subunits in various stages of development, different behaviours and responses to insecticides.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)