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Authors: Dong, Yaoxue, Chen, Yu, Wei, Qi, Su, Jianya, and Gao, Congfen

CLONING AND POLYMORPHISM ANALYSIS OF GLUTAMATE-GATED CHLORIDE CHANNEL GENE OF *LAODELPHAX STRIATELLUS* (HEMIPTERA: DELPHACIDAE)

Yaoxue Dong, Yu Chen, Qi Wei, Jianya Su and Congfen Gao*

Key Laboratory of Integrated Management of Crop Diseases and Pests, Ministry of Education, Department of Pesticide Science, College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China

*Corresponding author; E-mail: gaocongfen@njau.edu.cn

Abstract

A glutamate-gated chloride channel (GluCl) gene encodes a transmembrane domain protein that is an important target for insecticides. In insects, the GluCl genes of *Tribolium castaneum, Apis mellifera, Nasonia vitripennis, Drosophila melanogaster, Musca domestica, Lucilia cuprina, Aedes aegypti, Schistocerca gregaria, Plutella xylostella* and *Acyrthosiphon pisum* were cloned, but not the corresponding gene of *Laodelphax striatellus* (Fallén) (Hemiptera: Delphacidae), the small brown planthopper. In this study, the complete cDNA sequence of GluCl gene from *L. striatellus* was cloned and sequenced. The sequence includes 2,112 nucleotides, and the open reading frame ranges from 208 to 1,566 bp. The sequence encoded 452 amino acid residues. Alignment of the amino acid sequences of the GluCls between *L. striatellus* and other species indicated that the similarity of sequences was in the range of 48%-86%. The purified reverse transcription PCR product of GluCl coding sequence was cloned into a T-vector and then the sequence was analyzed. This analysis revealed polymorphism of GluCl gene among several strains, including a relatively susceptible strain, a fipronil resistant strain and a field strain of *L. striatellus*. Because the GluCls is an important target receptor, the GluCl gene in *L. striatellus* is worthy of further investigation.

Key Words: *Laodelphax striatellus*, fipronil, GluCl, cDNA clone, polymorphism

Resumen

Un gen de un canal de cloruro de apertura regulada por glutamato (GluCl) codifica una proteína de dominio transmembranal que es un objetivo importante para los insecticidas. En los insectos, se clonaron los genes de GluCl de *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis*, *Drosophila melanogaster*, *Musca domestica*, *Lucilia cuprina*, *Aedes aegypti*, *Schistocerca gregaria*, *Plutella xylostella* y *Acyrthosiphon pisum*, pero no el gen correspondiente de *Laodelphax striatellus*, un delfácido pequeño de color café. En este estudio, la secuencia completa de ADNc del gen de GluCl de *L. striatellus* fue clonado y secuenciado. La secuencia incluye 2112 nucleótidos y un rango de lectura del marco abierto de 208 a 1566 pb. La secuencia codifica 452 residuos de aminoácidos. La alineación de las secuencias de aminoácidos de los GluCls entre *L. striatellus* y otras especies indicó que la similitud de secuencias fue entre 48% -86%. Se clonó el producto purificado de la transcripción inversa de PCR GluCl secuencia de codificación en un vector-T y luego se analizó la secuencia. Este análisis reveló un polimorfismo del gen GluCl de entre las cepas, incluyendo una cepa relativamente susceptible, una cepa resistente al fipronil y una cepa de campo de *L. striatellus*. Debido a que el GluCls es un receptor objetivo importante, el gen GluCl en *L. striatellus* es digno de mayor investigación.

Palabras Clave: *Laodelphax striatellus*, fipronil, GluCl, clon de ADNc polimorfismo

The small brown planthopper (SBPH), *Laodelphax striatellus* (Fallén) (Hemiptera: Delphacidae), has a wide distribution from south-east Asia to Siberia and Europe. As a pest of rice, wheat and corn, it causes serious damage to crops by the transmission of *Rice stripe virus* (RSV), *Rice black streaked dwarf virus* (RBSDV) and *Maize rough dwarf virus* (MRDV) (Kisimoto 1967; Fang et al. 2001). In China, the SBPH has been causing serious feeding damage or crop disease since 1999 and the density of the *L. striatellus* population increased dramatically from 1999 to 2008 (Liu et al. 2006). To control the pest, neonicotinoid and phenylpyrazole insecticides, such as imidacloprid and fipronil, have been widely used. Extensive use of fipronil against the rice planthopper, *Nilaparvata lugens* (Stål), has led to insecticide resistance problems in East and Southeast Asia (Matsumura & Otuka 2009; Matsumura et al. 2009; Zhao et al. 2011).

The glutamate-gated chloride channel (GluCl) is a member of the "Cys-loop" superfamily of

ligand-gated ion channels. GluCls mediate synaptic inhibition in neurons and are expressed extra-junctionally in striated muscles. They may play key roles in regulating swallowing, locomotion, sensing, memory and juvenile hormone biosynthesis (Yates et al. 2003; Liu et al. 2005). Until now GluCl channels have been found only in invertebrates, and therefore they are ideal insecticide targets with high selectivity (Janssen et al. 2007). Most of the information about the GluCl channel has come from studies on model nematodes and model insects. All together several α subunits and β subunits of the channel are found in nematodes. One α subunit has been cloned from insects. The GluCl channels are most similar to GABA (γ-amino butyric acid) receptors with respect to their physiology and pharmacology. However, the amino acid sequences of GluCl channels are most similar to those of glycine receptor (Cully et al. 1996). The insecticides acting on GluCl channels are avermectin/milbemycin, fipronil, nodulisporic acid, a novelindole diterpene (Kane et al. 2000; Smith et al. 2000; Janssen et al. 2007).

Fipronil is a phenyl pyrazole insecticide introduced for pest control, and it is highly effective against both piercing sucking and chewing insects (Colliot et al. 1992; Moffat 1993). Fipronil is a potent blocker of the insect GABA-gated chloride channel or GABA receptor (Cole et al. 1993; Buckingham et al. 1994), and therefore it belongs to IRAC (Insecticide Resistance Action Committee) mode of action group 2B (IRAC 2006). Growing evidence indicates that fipronil may inhibit GluCls of invertebrates generally, because it does so in the nematode, *Caenorhabditis elegans* Maupas (Horoszok et al. 2001), in the American cockroach, *Periplaneta americana* (L.) (Ikeda et al. 2003) and the migratory locust, *Locusta migratoria* (L.) (Janssen et al. 2007).

Polymorphisms, abundant in insect genes, result in a permanent changes in gene activity that determine amino acid substitutions. Three amino acid (AA) substitutions, E114G, V235A and L256F, in the AVR-14B in *C. oncophora* have been associated with resistance to ivermectin (IVM), whose main site of action is glutamate-gated chloride (GluCl) channels (Njue & Prichard 2004; Njue et al. 2004). In this study, the cDNA sequence of glutamate-gated chloride channel gene from *L. Striatellus* was cloned by molecular technology and then analysed. Subsequently, single nucleotide polymorphisms sites were detected by direct sequencing. Our purpose was to provide a foundation of theory and findings for the further study of the mechanism of Fipronil resistance.

MATERIALS AND METHODS

Insects

The relatively susceptible strain (WX) of *L. striatellus*, which had been maintained in the laboratory for 8 years without exposure to insecticides, was collected from Wuxi, Jiangsu, China in 2005. A fipronil resistant strain (WX-F) was derived from the field-collected strain (WX) by 90 generations of continuous selection with fipronil using the rice seedling dip method. A field-collected *L. striatellus* strain (CX) collected from Changxing, Zhejiang, China in 2013, which since 2010 was exposed several insecticides but not to fipronil. All strains were reared on rice seedlings, seedlings were kept at 25 ± 1 °C, 70 ± 10% RH and 16:8 (L: D) h.

RNA Preparation and cDNA Synthesis

Total cellular RNA was isolated from 30 mg of *L. striatellus* adult tissue using the RNeasy Midi kit (QIAGEN, Hilden, Germany) by following the manufacturer's instructions. Single-stranded cDNA was synthesized from the total RNA with reverse transcriptase M-MLV and oligo (dT) 18. 5'-and 3'-RACE-ready cDNA were prepared according to the instructions of the Gene Racer Kit (Invitrogen).

Degenerate PCR Amplification

Degenerate primers targeting conserved gene regions were determined by alignment of published GluCl sequences from related species. Primers were synthesized by Invitrogen (Shanghai, China). The 3 degenerate primers used are shown in Table 1. Polymerase chain reaction (PCR) contained 2 μL of cDNA, 5 μL of 10 × standard PCR buffer, 4 μL of 25 mM Mg^{2+}, 10 pmol of each primer, 1 μL of 10 mM dNTP, and 1 U Taq DNA polymerase. The initial step of the amplification reaction denaturation at 94 °C for 3min, followed by 30 cycles of 94 ºC for 30 s, 55 °C for 30 s, 72 °C for 1 kb/min, and a final extension at

TABLE 1. THE DEGENERATE PRIMERS FOR TARGETED FRAGEMENTS OF THE GLUCL SUBUNIT GENE.

Primer	Sequences of the primer
Degenerate Forward Primer(F)	5'-AAYGARAADGARGGNCAYTTYCAYAA-3'
Degenerate Reverse Primer(R1)	5'-CRAABGTBARVCANACVCCNGTCCA-3'
Degenerate Half-nest Reverse Primer(R2)	5'-TGRTCNARCCARAABGABACCCA-3'

72 °C for 10 min. PCR products of the expected size were excised and purified by using the DNA agarose Gel Pur Kit (BIOMIGA, Nanjing, China) and cloned by using the pUCm-T vector system (Sangon, Shanghai, China). The second round of polymerase chain reaction was the same as the above. Primer-F and primer-R2 were used.

Cloning of the Full Length Fragment of the GluCl Gene by the RACE Technique

In order to amplify the GluCl gene of *L. striatellus* by means of degenerate primers, specific primers were designed and used to amplify the full length sequence. Primers used are shown in Table 2.

Analysis of the GluCl Gene Sequence with Online Tools

The cDNA sequences were analyzed with BioEdit software (Thompson et al. 1997). Sequences were aligned with ClustalX software, and then viewed with the Bioedit software (Thompson et al. 1997). The identity of the cDNA as GluCl was confirmed by similarity searches of GenBank using Blastx NR (http://www.ncbi.nlm. nih.gov/blast/). Multiple Sequences Alignment of the cDNA was accomplished by using bioedit and ClustalX softwares (Thompson et al. 1997). The trans-membrane helix was predicted using TMpred on-line tools (http://www.cbs.dtu.dk/ services/ TMHMM-2.0/). A phylogenetic tree was constructed by the neighbor-joining method (1000 replicates; seed = 64,238) for 14 amino acid sequences of the GluCls of the various species using the MEGA 4.0 program.

Analysis of cDNA Polymorphism of GluCl from 3 Strains of *L. striatellus*

There is a phenomenon of single nucleotide polymorphism (SNP) in the open reading frame of GluClα of *L. striatellus*, moreover, some animo acids are changed because of SNP. To explore this phenomenon in-depth, the full-length GluCl coding sequence of individual *L. striatellus* adults from the original WX strain, the fipronil resistant

WX-F strain and the field CX strain were cloned by the RNA prep MicroKit (BIOTEKE) in accordance with its instructions. Thereafter we used Phanta Super Fidelity DNA Polymerase (Vazyme) to minimize the probability of mismatches during PCR. We used 5'-CAGCGCAGCCCAACTAAC-CACC-3' as the Specific Forward Primer (F5) and 5'-AGCTGTTGTGTTGCGAGTCGCCC-3' as the Specific Reverse Primer (R5).

RESULTS

Cloning the Full Length cDNA of the GluCl Gene of *L. striatellus*

The full-length cDNA of *L. striatellus* was amplified with the degenerate primers (Table 1) and the specific primers (Table 2). The cloned sequence included 2,112 nucleotides (GenBank accession JF430868), and the open reading frame ranged from 208 to 1566 bp, which encoded 452 amino acid residues. This gene was designated as *LsGluCl*. This *L. striatellus* transcript was the most similar to the reported GluCl-like transcript of *Lucilia cuprina* (81%), followed by those of *Musca domestica* (80%), *Drosophila melanogaster* (80%-83%), *Aedes aegypti* (86%),*Tribolium castaneum* (84%- 86%), *Plutella xylostella* (84%), *Nasonia. vitripennis* (83%-84%), *Apis mellifera* (82%), *Acyrthosiphon pisum* (79%), *Tetranychus urticae* (64%), *Riphicephalus sanguineus* (68%), *Haemonchus contortus* (45%-46%) and *Caenorhabditis elegans* (48%-51%) (Table 3). Furthermore, according to the analysis with TMpred on-line tools, the GluCl gene included 2 pairs of cysteine residues (enclosed in the black boxes Fig. 1) within the N-terminal region and the 4 proposed trans-membrane regions (M1-4: underlined in Fig. 1) in the C-terminal region.

Phylogenetic Relations of Various Invertebrates Based on Their GluCl Genes

A phylogenetic tree was generated using 14 amino acid sequences of GluCl genes from 14 invertebrate species (Fig. 2). From the phylogenetic

TABLE 2. THE SPECIFIC PRIMERS FOR TARGETED FRAGMENTS OF THE GLUCL SUBUNIT GENE.

Primer	Sequences of primer
3'RACE Forward Primer(F3)	5'-CCACTTGATCGTCAAGTCTGCTC-3'
3'RACE Half-nest ForwardPrimer(F4)	5'-GTCCCATGAACCTCAAACTCTATCC-3'
3'RACE ReversePrimer(3'end)	5'-GACTCGAGTCGACATCGA-3'
5'RACE Forward Primer(GSP1)	5'-CCAGAACGAGACCCACGACACGATCAC-3'
5'RACE Forward Primer(GSP2)	5'-GGACACTGACATGGACTGAAGGAGTA-3'
5'RACE Reverse Primer(R3)	5'-CCAGAACGAGACCCACGACACGATCAC-3'
5'RACE Nest Reverse Primer(R4)	5'-GGATAGAGTTTGAGGTTCATGGGACACG-3'

TABLE 3. SIMILARITY ANALYSIS OF THE GLUCL GENE OF *LAODELPHAX STRIATELLUS* WITH THAT OF OTHER SPECIES.

Percent similarity of the GluCl gene in *Laodelphax striatellus* to those of other species	
Tribolium castaneum	84%-86%
Nasonia vitripennis	83%-84%
Drosophila melanogaster	80%-83%
Musca domestica	80%
Lucilia cuprina	81%
Apis mellifera	82%
Plutella xylostella	84%
Aedes aegypti	86%
Acyrthosiphon pisum	79%
Rhipicephalus sanguineus	68%
Tetranychus urticae	64%
Caenorhabditis elegans	48%-51%
Haemonchus contortus	45%-46%

tree branches, we may find that Class Insecta, Class Arachnida (mites. ticks, spiders, etc.) and Class Secernetea (nematodes) belong to 1 group, and that the GluCl genes of the various different species in these Classes share a high degree of similarity. This suggests that GluCl genes have had a long common evolutionary history. Thus, it is reasonable to deduce that the GluCl genes might have evolved from an ancient ancestral gene, and might be generally distributed in a wide range of organisms.

Analysis of cDNA Polymorphism of GluCl from 3 Strains of *L. striatellus*

The ORF of the GluCl gene was cloned from the DNA of 10 *L. striatellus* individuals of each strain, i.e., the susceptible strain in laboratory, the fipronil-resistant strain, and the field strain . Upon comparing 30 sequences of the SS (the relatively fipronil-susceptible strain), the RS (the fipronil-resistant strain) and the field strain, we discovered notable polymorphisms involving 80 base substitutions, and these were predominantly C-T nucleotide substitutions. All single nucleotide substitutions (SNPs) led to 20 amino acid replacements within the GluCl subunit. These amino acid substitutions involved 20 loci of GluCl gene of the 3 strains, i.e., I31K, D63A, V68I, I73L, S79A, K80T, etc. By comparing and analyzing cDNA sequences of the GluCl gene of the 3 strains, we found 2 loci with amino acid substitutions (A294D ,G298D) located between the M2-M3 trans membrane domain, 3 loci with amino acid substitutions (R352C,A364V, K378S) located between the M3-M4 trans membrane domain, the remaining loci determining amino acid substitutions at the hydrophilic N-terminus extracellular region (Table 4).

DISCUSSION

GluCls were first identified in arthropods as extra-junctional glutamate receptors (H-receptors) that hyperpolarized the membrane potential of locust (*S. gregaria*) leg muscle (Gration et al. 1979; Patlak et al. 1979; Dudel et al. 1989), and later they were cloned from the soil nematode, *Caenorhabditis elegans* (Cully et al. 1994). GluCls are activated by the glutamate analog, ibotenic acid, and are inhibited weakly by the ligand-gated chloride channel blocker, picrotoxin (Smith et al. 1999; Raymond et al. 2000; Horoszok et al. 2001). Until now the glutamate-gated chloride channel has been described only in invertebrates, and it is an important target for the development of insecticides with high selective toxicity to arthropods (Cully et al. 1996; Raymond & Sattelle, 2002).

Insect glutamate-gated chloride channels, along with GABA-gated chloride channel, are important targets for the action of certain insecticides (Bloomquist 2001). In this study, the GluCl gene in the *L. striatellus* was cloned successfully, then submitted to GenBank and given the registration number, JF430868. Thus the GluCl gene of the *L. striatellus* probably will be of great fundamental importance for further research.

Fipronil was designated as a new GABA-gated chloride channel blocker and introduced into pest control, for instance against the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Chrysomelidae), and against important crop and stored product pests in various Orders and families (Moffat 1993; Smith & Lockwood 2003). The blocking action of fipronil on GluCl channels was demonstrated in *Xenopus* sp. (Anura: Pipidae) oocytes transfected with GluCls (Horoszok et al. 2001), and a similar degree of blocking was observed on chloride currents induced by glutamate and ibotenate in the dorsal unpaired median (DUM) neurons of the cockroach, *Periplaneta americana*, and the grasshopper, *Melanoplus sanguinipes* (F.) (Smith et al. 1999; Raymond et al. 2000; Ikeda et al. 2003). The blocking effect of *fipronil* on GluCls was previously proposed to explain part of the toxicity of this insecticide, which is widely used in pest control to eradicate dieldrin-resistant insects (Horoszok et al. 2001; Smith & Lockwood 2003; Tingle et al. 2003; Zhao et al. 2004).

This study revealed substantial polymorphism of GluCl gene in *L. striatellus*. The relatively fipronil-susceptible strain (WX) of *L. striatellus* and the fipronil-resistant strain (WX-F), which had been continuously selected with fipronil for 90 generations, were collected in Wuxi, Jiangsu, China, they each have a uniform genetic background. By comparing and analyzing amino acid sequences of GluClα, we discovered 7 loci determining amino acid substitutions in the WX and WX-F strains, 2 loci unique to the WX-F strain, and 5 loci unique to the WX strain. In addition,

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LcGluCl  ---MGTGHYFWAIFYFASLCSASLAN-----------------NAKINFREKEKKVLDQILGAGKYDARI  50
MdGluCl  ---MGTGHFFWVIFYFASLCSASLAN-----------------NAKVNFREKEKKVLDQILGAGKYDARI  50
DmGluCl  ---MGSGHYFWAILYFASLCSASLAN-----------------NAKVNFREKEKKVLDQILGAGKYDARV  50
AaGluCl  ---MRK--------YLPN--HPNCTN-----------------APKTDKCKTKNKILDG-----------  29
PtGluCl  ---MDVLRPSCALFVLFLLYCAHLTEC----------------VNAKINFREKEKQILGPGPYDARI  52
TcGluCl  ---MYTHT---LIALVHHVTVCT-------------------NVKINFREKEKEVLDQILGQGMYDARI  47
AmGluCl  ---MWPGVLK-LLVLLTFLLHPSRCTQ----------------G--KVNYREKEKEVLDNILG--GYDARI  47
NvGluCl  ---MWPGALP-LIALLALLLHPARCGQ----------------QNSKMNFREKEKQVLDNILGPGSYDARI  51
LtGluCl  ---MASAIHTAIIPLLLYFAQCWCNQPS-------------STIKINYREKEKEVLDQILGPSSYDARI  54
ApGluCl  MASFKEWPSFLMVFLFAHFAQYCCTRIDDDGCNNHKGNSQHRCVPKVNYRHKEKEVLDQILGPGSYDARI  70

LcGluCl  RPSGINGTENLPTYVYVNMFLRSISKIDDYKMEYSVQLTFREQWTDERLKFDDIQGRLKYLTLTEANRVW 120
MdGluCl  RPSGINGTDG-PAVRNLFVRSIMTISDIKMEYSVQLTFREQWTDERLKFDDMQGRLKYLTLTEANRVW 119
DmGluCl  RPSGINGTDG-PAVVRVNFVRSISKIDDVTMEYSVQLTFREQWLDERLKFDDIQGRLKYLTLTEANRVW 119
AaGluCl  ----------PAVVRVNFVRSISKIDDVTMEYSVQLTFREQWLDERLKFNNLGGRLKYLTLTEANRVW  88
PtGluCl  RPSGINGTDG-PAVVSVNFVRSISKIDDVTMEYSVQLTFREQWLDERLKFNNLGGRLKYLTLTEANRVW 121
TcGluCl  RPSGVNGTDG-PTVVNNFFLRSISKIDDYKMEYSVQLTFREQWLDERLKFNDFGGRLKYLTLTEASRVW 116
AmGluCl  RPSGENATDG-PAVVRVNFVRSISKIDDVTMEYSVQLTFREQWLDERLKFNDFGGRLKYLTLTDASRVW 116
NvGluCl  RPSGENGTDG-PAVRVNLFVRSIATISDIKMEYSVQLTFREQWVDERLRFNDFGGKLKYLTLTDASRVW 120
LtGluCl  RPSGVNQTDG-PAVVRVNFVRSISKIDDYKMEYSVQLTFREQWLDERLKFNDFGGKIKYLTLTEANRVW 123
ApGluCl  RPSGVNGTGDSPCVYVNMLVRSISRIDDYKMEYSVQLTFREQWMDERLKFNDYNGKMKYLTLTEANRVW 140

LcGluCl  MPDLFFSNEKEGHFHNMPNVYIRIFPNGSVLYSIRISLTLA[C]PMNLKLYPLDRQI[C]SLRMASYGWTTN 190
MdGluCl  MPDLFFSNEKEGHFHNMPNVYIRIFPNGSVLYSIRISLTLA[C]PMNLKLYPLDRQI[C]SLRMASYGWTTN 189
DmGluCl  MPDLFFSNEKEGHFHNMPNVYIRIFPNGSVLYSIRISLTLA[C]PMNLKLYPLDRQI[C]SLRMASYGWTTN 189
AaGluCl  MPDLFFSNEKEGHFHNMPNVYIRIFPYGSVLYSIRISLTLA[C]PMNLKLYPLDRQV[C]SLRMASYGWTTA 158
PtGluCl  MPDLFFSNEKEGHFHNMPNVYIRIFPNGNVLYSIRISLTLS[C]PMNLKLYPLDKQT[C]SLRMASCGWTTD 191
TcGluCl  MPDLFFSNEKEGHFHNMPNVYIRIFPYSGVLYSIRISLTLS[C]PMNLKLYPLDRQI[C]SLRMASYGWTTD 186
AmGluCl  MPDLFFANEKEGHFHNMPNVYIRIFPNGSVLYSIRISLTLS[C]PMNLKLYPLDRQV[C]SLRMASYGWTTD 186
NvGluCl  MPDLFFNEKEGHFHNMPNVYIRIFPDGSVLYSIRISLTLS[C]PMNLKLYPLDRQI[C]SLRMASYGWTTA 190
LtGluCl  MPDLFFSNEKEGRFHNMPNVYIRIFPYGSVLYSIRISLTLS[C]PMNLKLYPLDRQV[C]SLRMASYGWTTD 193
ApGluCl  MPDLFFSNEKEGHFHNMPNVYIRIFPNGLVLYSIRISLTLS[C]PMNLKLYPLDRQI[C]SLRMVSYGWTTD 210

                                                                     M1
LcGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 260
MdGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 259
DmGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFRREFSYYLIQIYIPCCMLV 259
AaGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 228
PtGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 261
TcGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 256
AmGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFFTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 256
NvGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 260
LtGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 263
ApGluCl  DLVFLWKEGDPVQVVKNLHLPRFTLEKFLTDY[C]NSKTNTGEYS[C]LKVDLLFKREFSYYLIQIYIPCCMLV 280

         M2                                                     M3
LcGluCl  IVSWVSFWLDQGAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 330
MdGluCl  IVSWVSFWLDQGAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 329
DmGluCl  IVSWVSFWLDQGAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 329
AaGluCl  IVSWVSFWLDQGAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 298
PtGluCl  IVSWVSFWLDQGAVPARVSLGVTTLLTMATQSSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 331
TcGluCl  IVSWVSFWLDQGAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 326
AmGluCl  IVSWVSFWLDQGAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDIWTGVCLTFVFGALLEFALVN 326
NvGluCl  IVSWVSFWLDQSAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 330
LtGluCl  IVSWVSFWLDQSAVPARVSLGVTTLLTMATQTSGIDASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 333
ApGluCl  IVSWVSFWLDQAVPARVSLGVTTLLTMATQTSGINASLPPVSYTKAIDVWTGVCLTFVFGALLEFALVN 350

LcGluCl  YASRS-----DMHKENMKKKRR--DLEQAS-IDAASDLLDTDSNATFAMKPLVRHPGDPLALEKLRQCEV 392
MdGluCl  YASRSGSNKANMHKENMKKKRR--DLEQAS-IDAASDLLDTDSNATFAMKPLVRHPGDPLALEKLRQCEV 396
DmGluCl  YASRS-----DVHKENMKKKRR--DLEQAS-LDAASDLLDTDSNATFAMKPLARHPGDPLALEKRLQCEV 391
AaGluCl  YASRSADRAADIQRENMKKKRR--EMEQVS-LDAASDLLDTDSNATFAMKPLVRHPGDPMAMEKLRQCEV 365
PtGluCl  YASRP-----DMHKENMKKTRR--EMEAAASMDAASDLLDTDSNATFAMKPLMRG----AVLDSKMRRCEV 391
TcGluCl  YASRS-----DMHRENMKKQRRQCELEHAASMDATSDLIDTDSNATFAMKPLVRHPGDPMSLEKVRQCEI 391
AmGluCl  YASRS-----DMHSDNEKKYPPSETEQSTSVKPSDQVEPDNSSNFAMKPLVRQPEDTMSVDRMQHCEL 391
NvGluCl  YASRS-----DIHRENMKKRR--QEMEHSTSVDPASEPFEPDAAANFAMKPLVRQAEDSMSMEKMQRCEI 393
LtGluCl  YASRS-----DMHRDQMQKQRRQCELEHAASLEAAADLLQ-DGGTNFPMKPLVRS--GDQTLEMKMRQCEV 395
ApGluCl  YASRS-----DTHRDMSKKCD---LERTVSMDVGD---YEDGPNSFNMV----RS----NSTTSRMSFA 401

              M4
LcGluCl  HMQPPKRPNCCKTWLSKFPTRQCSRSKRIDVISRITFPLVFALFNLVYWSTYLFREEEDE-- 452
MdGluCl  HMQAPKRPNCCKTWLSKFPTRQCSRSKRIDVISRITFPLVFALFNLVYWSTYLFREEEDETF 458
DmGluCl  HMQAPKRPNCCKTWLSKFPTRQCSRSKRIDVISRITFPLVFALFNLVYWSTYLFREEEDETF 453
AaGluCl  HMQAPKRPNCCRTWMSRFPTRQCSRSKRIDVISRITFPLVFALFNLVYWSTYLFREEEDE-- 425
PtGluCl  HMAPPRK-NCCRLWLSKFPT----RSKRIDVISRITFPLVFALFNLAYWSTYLFRDEDEEN- 447
TcGluCl  HMQ-PARPNCCRSWLSKFPT----RSKRIDVISRITFPLVFALFNIVYWSTYLFREEAEES- 447
AmGluCl  HMQPRKK-NCCRSWLSKFPT----RSKRIDVISRITFPLVFAFFNLAYWSTYLFREEEQNE- 447
NvGluCl  HTQPPKK-NCCRSWLSKFPT----RSKRIDVIARITFPLVFALFNLTYWSTYLFREEADGE- 449
LtGluCl  HMQQPHRPNCCRSWLSKFPT----RSKRIDVISRITFPLVFALFNLTYWSTYLFRDESDGE- 452
ApGluCl  WGSARVTVTCRGCGLSLTS----LADLNQHGLKLSFEVSIILFNMTQLCTILGMHL----- 454
```

Fig. 1. Alignment of the GluClα subunit amino acid sequences from *Lucilia cuprina*, *Musca domestica*, *Drosophila melanogaster*, *Aedes aegypti*, *Plutella xylostella*, *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis*, *Acyrthosiphon pisum* and *Laodelphax striatellus*. These include the signature pairs of cysteine residues (enclosed in the black boxes at the third row) within the N-terminal region and the 4 proposed transmembrane regions (M1–4: underlined) in the C-terminal region. A second pair of N-terminal cysteine residues is enclosed by the black boxes at the fourth row.

we analyzed polymorphism in the *L. striatellus* field strain, which had a genetic background different from the SS (the relatively fipronil-susceptible) strain and the RS (fipronil-resistant) strain. There were 6 other amino acid replacements in the field strain besides the above loci, and no sharp contrast between the WX and the CX strains. Most of these polymorphism sites exist in an extracellular region. It was reported that 3 amino acid (AA) substitutions, E114G, V235A and L256F in the AVR-14B of GluClα of *C. on-cophora*, which is associated with IVM resistance, also exist in an extracellular region (Njue & Prichard 2004; Njue et al. 2004). There is no published report linking fipronil-resistance to specific polymorphisms in the glutamate-gated chloride channel. Our comparison of the full-length GluCl coding sequence in the susceptible and resistant *L. striatellus* strains did not show definitive evidence linking nucleic acid sequences to fipronil-resistance in this study. This important problem awaits further study.

TABLE 4. POLYMORPHISM SITES OF THREE STRAINS OF *LAODELPHAX STRIATELLUS*.

Strain	1	2	3	4	5	6	7	8	9	10	11	12
						Sites of polymorphism						
WX												
Base	G\A	A\T	A\C	A\C	T\C	T\C	G\C	T\C	A\G	C\A	G\A	C\T
Position	202	219	237	239	246	252	255	344	427	560	893	1092
Amino acid	V\I	I\L	S\A	K\T	D\S	V\I	K\T	S\L	V\M	A\D	D\G	A\V
Position	68	73	79	80	82	84	85	115	143	187	298	364
WX-F												
Base	T\A	G\A	A\T	A\C	A\C	T\C	T\C	G\C	A\C			
Position	92	202	219	237	239	246	252	255	1132			
Amino acid	I\K	V\I	I\L	S\A	K\T	D\S	V\I	K\T	K\S			
Position	31	68	73	79	80	82	84	85	378			
CX												
Base	T\A	G\C	G\A	A\T	A\C	A\C	T\C	T\C	G\C	A\G	T\C	T\A
Position	188	198	202	219	237	239	246	252	255	677	776	1054
Amino acid	D\A	P\H	A\T	I\L	S\A	K\T	D\S	V\I	K\T	C\Y	P\L	R\C
Position	63	66	67	73	79	80	82	84	85	226	259	352

Abbreviations: A: Alanine, C: Cysteine, D: Aspartic acid, G: Glycine, H: Histidine, I: Isoleucine, K: Lysine, L: Leucine, M: Methionine, P: Proline, R: Arginine, S: Serine, T: Threonine, V: Valine, Y: Tyrosine
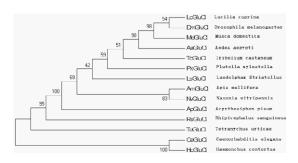

Fig. 2. A phylogenetic tree constructed by neighbor-joining method (1000 replicates; seed = 64238) for amino acid sequences of GluCls from 14 species using MEGA 4.0 program. Bootstrap values are shown at the branching points. Scale bar indicates the number of changes inferred as having occurred along each branch. GenBank accession codes: LcGluCl (*Lucilia cuprina*; AF081674); MdGluCl (*Musca domestica*; AB177546); DmGluCl (*Drosophila melanogaster*, DQ665648); AaGluCl (*Aedes aegypti*, XM001662847); TcGluCl (*Tribolium castaneum*, EF545123); PxGluCl (*Plutella xylostella*, GQ221939); LsGluCl (*Laodelphax Striatellus*, JF430868); NvGluCl (*Nasonia vitripennis*, FJ851100), AmGluCl (*Apis mellifera*, DQ667186); ApGluCl (*Acyrthosiphon pisum*, XM 001943378); TuGluCl (*Tetranychus urticae*, JQ738193); RsGluCl (*Rhipicephalus sanguineus*, GQ215234); CeGluCl (*Caenorhabditis elegans*, CEU14524); HcGluCl (*Haemonchus contortus*, AF119791).

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