Molecular Characterization of a Ryanodine Receptor Gene in the Rice Leaffolder, *Cnaphalocrocis medinalis* (Guenée)

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**Abstract**

Ryanodine receptors (RyRs) are the targets of two novel classes of synthetic insecticidal chemicals, phthalic acid diamides and anthranilic diamides. Isolation of full-length RyR cDNAs is a critical step towards the structural and functional characterization of insect RyRs and an understanding of the molecular mechanisms underlying the species selective toxicity of diamide insecticides. However, there has been little research on the insect RyR genes due to the high molecular weight of the RyR proteins. In this study, we isolated a full-length RyR cDNA (named as CmRyR) from *Cnaphalocrocis medinalis*, an important rice pest throughout Southeast Asia. The composite CmRyR gene contains an ORF of 15264 bp encoding a protein of 5087 amino acid residues, which shares 79% overall identity with its *Drosophila melanogaster* homologue. All hallmarks of the RyR proteins are conserved in the CmRyR protein, suggesting that CmRyR is a structural and functional analogue of known RyRs. A multiple sequence alignment illustrates that the insect RyRs share high levels of amino acid sequence identity at the the COOH-terminal region. However, the amino acid residues analogous to the CmRyR residues N4922, N4924, N4935, L4950, L4981, N5013 and T5064 are unique to lepidopteran RyRs compared with non-lepidopteran insect RyRs. This finding suggests that these residues may be involved in the differences in channel properties between lepidopteran and non-lepidopteran insect RyRs and in the species selective toxicity of diamide insecticides. Furthermore, two alternative splicing sites were identified in the CmRyR gene, one of which was located in the central part of the predicted second SPRY domain. Diagnostic PCR showed that the inclusion frequencies of two mutually exclusive exons (a/b) and one optional exon (c) differed between developmental stages or adult anatomical regions. Our results imply that alternative splicing may be a major means of generating functional diversity in *C. medinalis* RyR channel.

**Introduction**

Developing insecticides that act on novel biochemical targets is important in crop protection due to the ability of insects to rapidly develop insecticide resistance. Calcium (Ca^{2+}) is a key second messenger that regulates diverse cellular processes, including muscle contraction, hormone secretion, synaptic transmission, fertilization, and gene transcription. The process of muscle contraction involves the modulation of two distinct channel types: the voltage-gated channels, which regulate the entry of external calcium, the inositol 1,4,5-triphosphate receptor (IP3Rs) and the ryanodine receptor channels (RyRs), which regulate the release of calcium from intracellular stores. It has been suggested that insect calcium channels would offer an excellent insecticide target for commercial exploitation [1–2].

Recently, the development of two new classes of synthetic insecticidal chemicals with extremely high activity against a range of lepidopteran pest species has led to a renewed interest in this field. The first chemical class is comprised of phthalic acid diamides, including the compound flubendiamide which was co-developed by Nihon Nohyaku and Bayer CropScience [3–6]. The second chemical class is comprised of the anthranilic diamides, including the compound chlorantraniliprole (Rynaxypyr™) which was discovered and developed commercially by Dupont Crop Protection [7–9]. These diamides are potent activators of insect RyRs [10] and show exceptional selectivity for insect RyRs when compared to mammalian RyRs.

As the largest ion channels currently known, RyRs are located on the sarcoplasmic reticulum of muscles and the endoplasmic reticulum of neurons and many other cell types. To date, three types of ryanodine receptor (RyR1, RyR2 and RyR3) have been identified in mammals; each is the product of a separate gene [11]. RyR1 and RyR2 are predominately found in skeletal and cardiac muscles, respectively. RyR3 is ubiquitously expressed at low levels throughout different tissues but is relatively abundant in brain and certain skeletal tissues [12–14]. There is approximately 65% amino acid sequence identity between the mammalian RyR isoforms [15], and the sequence deviations mainly stem from three...
divergent regions of the RYR genes, referred to as DR1, DR2, and DR3 [16]. The RyRs are similar in structure with a large NH2-terminal domain and a small COOH-terminal domain protruding into the cytoplasm, as well as several transmembrane regions near the COOH-terminus. Due to alternative splicing, a variety of RyR variants have been isolated, and some show differences in their channel function properties [17–24]. Despite the important role of RyRs in the development of novel and selective insecticides, there have been very few studies on insect RyR genes. In 1994, Takeshima and colleagues described a

| Primer name | Sequence (5’ to 3’)* | Description |
|-------------|----------------------|-------------|
| 258.RYRF13  | TGGTGACNGTNCAYCCNGC  | RT-PCR product P1 |
| 127.RYRR1   | TCCATYTTYCCTCTGCTG   | RT-PCR product P2 |
| 279.H2.2F2  | TGGGCAGAGTGAGGAGAAG   | RT-PCR product P3 |
| 280.A1.1R1  | CTCATGTCTCGAAGGCGT   | RT-PCR product P4 |
| 139.RYRF7   | GAAAYACAYAAYATNTGA    | RT-PCR product P5 |
| 141.RYRR7   | TCCARTNTNCCTTCTCAT    | RT-PCR product P6 |
| 303.A1.1F1  | GATACTCCACCGTCTG     | RT-PCR product P7 |
| 146.RYRF10  | GACAYTACGAGTGCTAGCAC | RT-PCR product P8 |
| 147.RYRR10  | GCACATGCTCTTCTTCTCT  | RT-PCR product P9 |
| 256.H2.2R1  | TGGAAGAACCTCAGGATCG   | RT-PCR product P10 |
| 257.RYRR13  | TCCCATNTNCCTTCTCAT    | RT-PCR product P11 |
| 238.A2.7F1  | CGCATCCCACTTCTCCTG    | RT-PCR product P12 |
| 235.A3.7R1  | CCCACTGATGCTCTGCTG    | RT-PCR product P13 |
| 148.RYRF11  | CCNTGAGTCAGCGNTGAGG    | RT-PCR product P14 |
| 149.RYRR11  | GCTAGACAGCTTCTTCTCT   | RT-PCR product P15 |
| 237.A3.7F2  | GCAAGACATTAGACAGAC    | 5’-RACE product R5 |
| 234.47R1    | CAAATGCGTTGCTTCTCTG   | 3’-RACE product R3 |
| 132.RYRF4   | ATGGGATTGATTGATGAGA    | diagnostic PCR for exon a |
| 133.RYRR4   | GGYTCTTTGATGATTGTCAG   | diagnostic PCR for exon b |
| 153.RYRSF1  | GGACATACCTGGAGTCTGAG   | diagnostic PCR for the absence of exon c |
| 155.RYRR2   | GGTCATCTCAGCGCCAGCA    | diagnostic PCR for the presence of exon c |
| 134.RYRF5   | GTNGAATGACCCAGATGCTG   | diagnostic PCR for exon a |
| 136.RYRR5   | GATTAGACAGCTTCTTCTCT   | diagnostic PCR for exon b |
| 276.H2.2R1  | TGGGAAGTACGAGCTTCTC    | diagnostic PCR for the absence of exon c |
| 277.H2.2R2  | ACATAAGATCGAGTCTTCCAC  | diagnostic PCR for the presence of exon c |

*Corresponding amino acid sequences for degenerate primers were shown in parentheses.

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25.7 kb genomic DNA fragment that contained a gene for a
*Drosophila melanogaster* RyR homologue (DmRyR) [23]. In all, 26 exons were found to encode the open reading frame and a
predicted protein of 5216 or 5112 amino acids was reported. This
receptor showed 45–47% identity with the three mammalian
RyRs. Functional expression of *Drosophila* RyR has subsequently
been reported in CHO cells [26] and a *Spodoptera frugiperda* cell
line (SF9) [7,27], respectively. In addition to the *D. melanogaster*
RyR, cDNAs encoding the COOH-terminal 1172 amino acids of
a RyR gene (Hv-RyR) have been cloned and characterized from the
lepidopteran pest *Heliothis virescens* [28]. Recently, cDNAs
encoding two novel lepidopterous RyRs were cloned from the
silkworm, *Bombyx mori* (sRyR), and the diamondback moth,
*Plutella xylostella* (Px-RyR), respectively [29–30]. In the present
study, we isolated a full-length RyR cDNA (named CmRyR)
from the rice leaffolder, *Cnaphalocrocis medinalis* (Lepidoptera: 
Pyralidae), an important rice insect pest throughout Southeast
Asia. We report the structural features of CmRyR and document
the developmentally regulated alternative splicing of CmRyR
transcripts.

**Materials and Methods**

**Insects**

Adult rice leaffolders were collected in 2008 in the paddy field of
the experimental farm at Yangzhou University and cultured in
potted rice plants covered with an 80-mesh cage.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA from eggs (0–12 h old), first instar larvae (0–6 h old),
third instar larvae, fifth instar larvae, pupae, and adult heads and
bodies (0–24 h old), was extracted using an SV total RNA isolation
system (Promega, Madison, WI), according to the manufacturer’s
instructions. First-strand cDNA was synthesized from 5 μg of total
RNA using the Primescript™ First-Strand cDNA Synthesis kit
(TaKaRa, Dalian, China) according to the manufacturer’s
instructions. Degenerate primer pairs were designed against the
amino acid residues that were conserved between insects,
other invertebrates, and vertebrate RyR isoforms (Table 1). PCR
was performed in a 25 μℓ reaction volume containing 20–50 ng cDNA,
0.8 μM of each degenerate primer, 0.2 mM of each dNTP, 2 mM
of MgCl2, 1.25 U Ex Taq™ polymerase (TaKaRa, Dalian, China)
and 2.5 μL Ex Taq™ buffer (TaKaRa, Dalian, China). A
touchdown PCR protocol was used, which consisted of 1 cycle at 94°C for
5 min, 12 cycles at 94°C for 30 s, 52–41°C (decreasing by −1°C/
cycle) for 30 s, and 72°C for 2 min, followed by 25 cycles of 94°C
for 30 s, 40°C for 30 s, and 72°C for 2 min, and a final extension at
72°C for 10 min. Furthermore, specific primers were designed to
amplify across the gaps (Table 1). A total of 14 RT-PCR products
were sequenced to confirm the reliability of the diagnostic
PCR reactions. Briefly, fragments containing the alternative exons were amplified by a set of primers flanking the
alternative exon region. The PCR products were cloned into the
pMD18-T vector (TaKaRa, Dalian, China), and a 2.5 μℓ aliquot of the overnight culture of each fragment clone was used as a template for diagnostic PCR. Mutually exclusive exons were
identified using two exon-specific primers, one based on the sense
strand sequence of one exon and the other on the antisense strand
of the other exon. Optional exons were identified using a primer
spanning the exon and flanking region or a primer spanning the
flanking region excluding the exon sequence. These exon-specific primes were paired with counterpart primers located on either
cide of the alternatively spliced segment to generate unique PCR
products representing the presence or absence of alternative exon
in each clone. Amplicons were resolved on a 1% agarose gel,
stained with ethidium bromide, and visualized with an UV
transilluminator. Representative clones exhibiting unique banding
patterns were sequenced to confirm the reliability of the diagnostic
PCR assay.

**Figure 1. Schematic diagram of clones used to compile the complete CmRyR cDNA sequence.** The top line indicates the full-length
CmRyR cDNA in kilobases. Thin lines (P1–P14) represent CmRyR cDNA clones obtained by RT-PCR. Solid lines (R5 and R3) represent CmRyR cDNA clones obtained by RACE.
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Database entries

The entire coding sequence of CmRyR has been deposited in the GenBank and the accession number is JQ799046.

Results

Cloning of CmRyR cDNA

RT-PCR and RACE were used to amplify the entire coding sequence of the ryanodine receptor cDNA from C. medinalis. A total of 16 overlapping cDNA fragments were obtained (Figure 1). Compilation of the cDNA clones resulted in a 15,773-bp contiguous sequence containing a 15,264 bp ORF. The 289 bp of 5′-untranslated sequence upstream of the initiation codon is 41% GC and lacks a TATA box. The 220 bp of 3′-untranslated sequence downstream of the termination codon (TGA) is 65% AT but does not contain the canonical polyadenylation signal AAAA. The encoded 5,087 amino acid residues predict a protein with molecular mass of 574,339 Da, including the initiator methionine. An amino acid sequence alignment shows that CmRyR shares the greatest amino acid identity with sRyR (94%) and is 92% and 79% identical in pairwise comparisons with the PxRyR and DmRyR amino acid sequences, respectively. Identities of CmRyR with the three rabbit RyR isoforms are similar (45–46%). Phylogenetic analysis also reveals that CmRyR is grouped with sRyR and PxRyR, with high bootstrapping support in 1000 replications (Figure 2).

During the sequencing of these clones, a number of nucleotide differences were observed between overlapping clones. A total of 35 nucleotide substitution sites were found in the ORF, of which 20 nucleotide substitutions resulted in amino acid substitution, and 15 nucleotide substitutions were silent (Table 2). These polymorphisms were located both in the NH2- and COOH-terminal parts of CmRyR, and may represent different alleles or errors committed during the PCR procedure.

Predicted domain structure of CmRyR

The CmRyR amino acid sequence was analyzed for putative regulatory domains (Figure 3). The NH2-terminal region of CmRyR contains an MIR (Mannosyltransferase, IP3R and RyR) domain at positions 212–393, a RIH (RyR and IP3R Homology) domain at positions 440–649, and a suppressor-domain-like domain (SD) at positions 12–201, which showed high levels of homology to the suppressor domain of IP3Rs [33]. As with the mammalian RyRs, three copies of a SPRY (SPla and RyR) domain (666–803, 1092–1213, 1518–1659) and four copies of a RyR domain (854–948, 967–1061, 2002–2095, and 2923–3016) were also predicted. The RIH-associated domain preceding the C-terminal transmembrane regions was conserved and can be found at position 3973–4098. Six transmembrane helices (TM1 to TM6) were predicted at positions 4334–4456, 4615–4637, 4697–4719, 4839–4961, 4987–4909, and 4967–4986, respectively. Additionally, a consensus sequence for the ATP/GTP-binding motif (P-loop), [GA]XXXXGK[ST] [34], was found in the NH2-terminal region (1088–1095).

Analysis of COOH-terminal region of CmRyR and comparison with known RyRs

The COOH-terminal region of RyR has been shown to be functionally important [35], and the insecticide flubendiamide is mainly incorporated into the transmembrane domains (amino acids 4111–5084) of the B. mori sRyR [29]. Therefore, the COOH-terminal region of CmRyR was compared with other reported insect RyRs, including sRyR from B. mori, PxRyR from P. xylostella, HsRyR from Harpegnathos saltator, DmRyR from D. melanogaster, and AaRyR from Aedes aegypti (Figure 4). The alignment showed that the six transmembrane regions in CmRyR exhibited high sequence identity to other insect RyRs. The pore helix, which is analogous to the P loop of the voltage-activated Ca2+, Na+ and K+ channels [36], was predicted to be in the loop region between the putative fifth and sixth transmembrane helices of the receptors. The sequence motif, GXRXGGGXGD, which constitutes part of the pore-forming segments of the Ca2+ release channels [37], was also highly conserved in CmRyR (4939–4948) and other insect RyRs. Two consensus Ca2+ binding EF-hand

Figure 2. Phylogenetic tree of the RyR family. The CmRyR amino acid sequence was aligned to 9 representative RyR isoforms from 7 species and used for phylogenetic analysis. The Neighbor-joining tree was generated in MEGA5 with 1000 bootstrap replicates. RyR sequences are obtained from the following GenBank entries: DJ085056 for Bombyx mori (sRyR); AET09964 for Plutella xylostella (PxRyR); EFN78897 for Harpegnathos saltator (HsRyR); BAA41471 for Dro sophila melanogaster (DmRyR); EAT44864 for Aedes aegypti (AaRyR); BAA08309 for Caenorhabditis elegans (CeRyR); CAA33279 for Ortygolagus cuniculus (OcRyR1); NP_001076226 for Ortygolagus cuniculus (OcRyR2); NP_001076231 for Ortygolagus cuniculus (OcRyR3). doi:10.1371/journal.pone.0036623.g002
motifs originally reported in the lobster RyR [38] were also present in tandem at positions 4175–4202 and 4210–4237. A glutamate residue proposed to be involved in the Ca\(^{2+}\) sensitivity in rabbit RyR3 (E\(^{327}\)) [39] and RyR1 (E\(^{413}\)) [40] was also detected in CmRyR (E\(^{413}\)) and other insect RyRs. Additionally, residues corresponding to I\(^{3957}\), R\(^{4913}\), and D\(^{4917}\) of rabbit RyR1, which were recently shown to play an important role in the activity and conductance of the Ca\(^{2+}\) release channel [41], were conserved in CmRyR (I\(^{3957}\), R\(^{4913}\), and D\(^{4917}\)) and other insect RyR isoforms.

Despite the significant similarity, CmRyR and other lepidopteran homologues show some potentially important sequence divergence from the non-lepidopteran insects in the COOH-terminal region, especially in close proximity to the pore-forming segment. For example, the lepidopteran homologues differ from the non-lepidopteran RyRs at positions analogous to CmRyR residues N\(^{4922}\), N\(^{4924}\), N\(^{4935}\), L\(^{4950}\), L\(^{4981}\), N\(^{5013}\), and T\(^{5064}\) (Figure 4). To verify this finding, a total of 31 RyR isoform sequences from 26 species were aligned, including 5 lepidopteran homologues, 13 non-lepidopteran insect homologues, 5 other invertebrate homologues, 2 non-mammalian vertebrate homologues, and 6 mammalian homologues. The alignment showed that seven CmRyR residues (N\(^{4922}\), N\(^{4924}\), N\(^{4935}\), L\(^{4950}\), I\(^{4961}\), N\(^{5013}\), and T\(^{5064}\)) were highly conserved in the lepidopteran homologues, while different residues at the corresponding positions (D\(^{4942}\), K\(^{4944}\), H\(^{4955}\), I\(^{4970}\), L\(^{4971}\), G\(^{5001}\), and Q\(^{5061}\), respectively, in DmRyR) were shared by all the 13 non-lepidopteran insect RyRs (Figure S1). Surprisingly, residues at these positions are highly conserved in the non-lepidopteran insect, other invertebrate, and vertebrate RyRs, except that one residue in *Tetranychus urticae* (N\(^{5026}\)) and one residue in *Caenorhabditis elegans* (L\(^{5033}\)) were identical to lepidopteran homologues (N\(^{4935}\) and L\(^{4950}\) in CmRyR), respectively (Figure S1). Our results suggest that these residues might be involved in the differences in channel properties between lepidopteran and non-lepidopteran insect RyRs and in the species selective toxicity of diamide insecticides.

### Developmental regulation of alternative exon usage

The alignment of multiple cDNA clone sequences revealed two putative alternative splice sites in CmRyR, named insect alternative splicing I (IASI) and insect alternative splicing II (IASII). IASI is located between amino acid residues 1136–1168 and forms one pair of mutually exclusive exons (a/b), which was also reported in *Drosophila* [25]. IASII is located between amino acid residues 2915–2920 and forms the optional exon c. Figure 5 shows the nucleotide and inferred amino acid sequences of the three alternative exons identified in the present study. Interestingly, IASI corresponds to the central part of the predicted second SPRY domain, while IASII is located between the predicted third and fourth RyR domains.

Diagnostic PCR was used to determine the usage of each putative alternative exon in seven discrete mRNA pools: eggs, first instar larvae (0–6 h old), third instar larvae, fifth instar larvae, pupae, 0–24 h adult heads, and 0–24 h adult bodies. Data were collected from sets of 18–27 clones for each fragment and developmental stage. The usage frequencies of each putative alternative exon for the seven mRNA pools are summarized in Figure 6. The results show that the usage of the mutually exclusive exons (a/b) exhibited marked developmental and anatomical regulation. Exon a was present in all cDNA clones examined from the egg and adult head cDNA pools, but only in 16 of the 23 clones and 11 of the 21 clones analyzed from the pupal and adult body cDNA pools, respectively. In contrast, exon b was not detected in the egg and adult head cDNA pools, but was present at low to moderate frequencies (30% and 48%) in the pupal and adult body cDNA pools. Developmental regulation of exon inclusion was also observed for exon c. It was present at low frequencies (0%, 17%, and 10%) in eggs, third instar larvae and fifth instar larvae, respectively, but at moderate to high frequencies (55% and 71%) in first instar larvae and pupae.

### Discussion

RyR is known to release Ca\(^{2+}\) from the sarcoplasmic reticulum to induce muscle contraction in various vertebrate and invertebrates.
brane muscles. The structural divergence between the mammalian and the insect RyR isoforms implies that RyRs may serve as potential targets for potent insecticides with low mammalian toxicity. Recently, two new classes of synthetic insecticidal chemicals, phthalic acid diamides and anthranilic diamides, have been developed. Treatment with both phthalic and anthranilic diamides were reported to elicit intracellular Ca\textsuperscript{2+} release in isolated insect neurons [3–4,7]. These RyR modulators exhibit exceptional insecticidal activity against lepidopteran insect pests and show a high selectivity for insect RyRs compared to mammalian RyRs. However, the basis of this selectivity is not yet understood.

Isolating the full-length RyR cDNA is a critical step towards a comprehensive structural and functional characterization of insect RyRs and could lead to an understanding of the molecular mechanisms that underlie the species selective toxicity of diamide insecticides. However, this approach has been hampered by the high molecular weight of the RyR isoforms. To the best of our knowledge, full-length RyR homologues have been cloned and characterized in only three insect species: *D. melanogaster*, *B. mori*, and *P. xylostella*. In the present study, we cloned and sequenced the full-length cDNA encoding the RyR from *C. medinalis*. The deduced amino acid sequence of CmRyR shows a reasonable degree of identity with known RyRs throughout the entire molecule and also shares several common structural features. Two consensus Ca\textsuperscript{2+}-binding EF-hands are present in the COOH-terminus of the CmRyR at positions 4183–4194 and 4285–4296, suggesting that this channel, similar to mammalian RyRs, may be regulated by cytosolic Ca\textsuperscript{2+}. The close similarity of the pore regions of CmRyR and mammalian RyRs, including the conserved GGGXGD selectivity filter motif, suggests that CmRyR likely forms functional cation channels with a high single-channel conductance and permeability to Ca\textsuperscript{2+}. Molec-

ular phylogenetic analyses also confirm that the cloned cDNA encodes a RyR isoform that is most closely related to the sRyR isoform (Figure 2). Taken together, these results suggest that CmRyR is a structural and functional analogue of other known RyRs.

While the exact binding sites for flubendiamide or chloran-traniliprole have not yet been identified, binding studies on the microsomal membranes from insect muscles suggest that flubendiamide and chlorantraniliprole act at a site distinct from the ryanodine binding site localized in the pore of the insect RyR complex [4,7]. Recently, it was found that flubendiamide mainly incorporates into the transmembrane domain (amino acids 4111–5084) of sRyR [29]. In this study, alignment of the deduced amino acid sequences of the COOH-terminal region of six insect RyRs illustrates that insect RyRs share high levels of sequence identity, especially surrounding the TM5 and TM6 regions. Sequence analysis, however, uncovered amino acid residues with marked uniqueness in this region in lepidopteran insects. For example, amino acid residues at positions analogous to the CmRyR residues N\textsuperscript{4922} (N\textsuperscript{4922}–N\textsuperscript{4924}), N\textsuperscript{4953}, L\textsuperscript{4955}, L\textsuperscript{4961}, N\textsuperscript{5013}, and T\textsuperscript{5064} are unique to lepidopteran RyR homologues, whereas the corresponding residues in non-lepidopteran insect RyRs are highly conserved with other invertebrate and vertebrate RyRs. Therefore, it is thought that these residues might be involved in the differences in channel properties between lepidopteran and non-lepidopteran insect RyRs and in the species selective toxicity of diamide insecticides. Further studies, including mutagenesis and a heterologous expression of various mutagenized RyRs, are needed to address this interesting hypothesis.

Alternative splicing is a key posttranscriptional processing mechanism that generates structural and functional diversity, leading to the specialization of many membrane proteins, including sodium and calcium channels. A total of nine alternative splice sites (a, b, c/d, i, j, c, f, h, l/k) have been identified in the para sodium channel in *D. melanogaster* [42]. In mammalians, more than 10 distinct splice variants have been identified in RyR isoforms from human, rabbit, mouse, mink, and dog. Some splice variants of RyR isoforms were found to predominantly suppress Ca\textsuperscript{2+} release or to contribute to distinct Ca\textsuperscript{2+} releasing patterns [22–24,43–45]. For example, one smooth muscle-specific human RyR3 splice variant, which lacks a 29 amino acid fragment (His\textsuperscript{4106}–Lys\textsuperscript{4134}) encompassing a predicted transmembrane helix, acts as a tissue-specific dominant negative regulator of RyR2 channels via the formation of heteromeric channel complexes [22]. Similarly, one human RyR2 splice variant, which includes a 24 bp exon, protected cells from caffeine-evoked apoptosis through its negative effects on intracellular Ca\textsuperscript{2+} release [24]. Recent studies with peptides corresponding to the RyR1 splice variants ASI (+) or ASI (−) suggest that the ASI region (Ala\textsuperscript{3481}–Gln\textsuperscript{3485}) in the RyR1 channel contributes to an inhibitory module in RyR1 that influences EC coupling [44].

In contrast to the three RyR genes in mammals, only one gene (Rya-r44F) encoding a ryanodine receptor is present in *D. melanogaster*, and it is likely that the major means of generating diversity in insect RyR channels involves alternative splicing. In fact, alternative splicing of the RyR transcript has been reported in *D. melanogaster* [25]. In this study, two putative alternative splice sites were identified in the CmRyR gene. One of the two
Figure 4. Alignment of COOH-terminal region of the insect RyR isoforms. Identical amino acids are shown in black boxes and similar amino acids are highlighted in gray boxes. Gaps have been introduced to permit alignment. Asterisks below the alignment indicate the position of residues discussed in the text (E4134, I4946, R4962, and D4966 of CmRyR). Triangles below the alignment indicate unique residues (N4922, N4924, N4935, T4950, L4981, N5013, and T5064 of CmRyR) in the lepidopteran homologues. Abbreviations and GenBank entries for the RyR isoforms are described in Figure 2.

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alternative splice sites, IASI, is conserved in *D. melanogaster* [25], and the other alternative splice site, IASII, is also found in *Helicoverpa armigera* (Wang et al., unpublished data). The expression profiles of the CmRyR splice variants exhibit marked developmental or anatomical regulation. Interestingly, IASI was located in the central part of the predicted second SPRY domain (SPRY2). The SPRY domain structure was first described in the *Dictyostelium discoideum* tyrosine kinase spore lysis A (SplA) and the mammalian RyRs [46]. SPRY domains are widely regarded to be protein-protein interaction domains that are involved in a wide spectrum of biological functions, including the regulation of cytokine signaling and innate retroviral restriction [47]. Recently, the SPRY2 domain in RyR1 has been identified as an in vitro binding partner for the II–III loop of the α1S subunit of the skeletal muscle dihydropyridine receptor (DHPR) [48]. Because the sequences of the mutually exclusive exons a and b in CmRyR were highly divergent, differing at 13 of 33 amino acid residues (Figure 5), it is quite likely that the CmRyR splicing variants generated by IASI may have different protein-protein interactions. In summary, while the properties and functions of the CmRyR splice variants remain to be studied, our results imply that alternative splicing may play an important role in the spatial and temporal coding of Ca^{2+} signals in insects, and this signaling may greatly influence cellular function and phenotype.

Figure 5. Nucleotide and inferred amino acid sequences of alternative exons in the CmRyR gene.
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Figure 6. Relative frequencies of individual CmRyR alternative exon usage in eggs (E), first instar larvae (L1), third instar larvae (L3), fifth instar larvae (L5), pupae (P), adult heads (AH), and adult bodies (AB). Mutually exclusive exons are represented by hatched bars (exon a) and open bars (exon b). Optional exon c is indicated by black bars. Numbers in parentheses represent the total numbers of clones investigated.
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Supporting Information

Figure S1 Alignment of amino acid sequence of partial COOH-terminal region in 31 RyR isoforms from 26 species. Identical amino acids are shown in black boxes and similar amino acids are highlighted in gray boxes. Gaps have been introduced to permit alignment. Triangles below the alignment indicate unique residues (N^{4922}, N^{4924}, N^{4935}, T^{4950}, L^{5017}, N^{5013} and T^{5004}) of CmRyR) for lepidopteran homologues. Abbreviations and GenBank entries for sRyR, PxRyR, HsRyR, DmRyR, AaRyR, CeRyR, OcRyR2, OcRyR3 isoforms are described in Fig. 2. The other RyR sequences are obtained from the following GenBank entries: AAD01425 for Heliotis virescens (HvRyR); EH77857 for Danaus plexiptus (DpRyR); EEB11809 for Pediculus humanus corporis (PhRyR); EEZ99829 for Tribolium castaneum (TcRyR); AF483192 for Periplaneta americana (PaRyR); EAA13701 for Anopheles gambiae (AgRyR); XP_392217 for Apis mellifera (AmRyR); XP_003484552 for Bombus impatiens (BiRyR); XP_000339394 for Bombus terrestris (BrRyR); EFN67324 for Camponotus floridanus (CfRyR); XP_001842971 for Anopheles gambiae (AgRyR); BAK26392 for Tetanychus urticae (TuRyR); XP_002578660 for Schistosoma mansoni (SmRyR); CAX95439 for Schistosoma japonicum (SjRyR); BAB04714 for Hemicentrotus pulcherrimus (HpRyR); BAA04646 for Rana catesbeiana RyR2 (RcRyR2); BAA04647 for Rana catesbeiana RyR3 (RcRyR3); P21817 for human RyR1 (hRyR1); Q52736 for human RyR2 (hRyR2); Q15413 for human RyR3 (hRyR3). (DOC)

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Author Contributions

Conceived and designed the experiments: JW ZJH XCL. Performed the experiments: JW YQL YLZ JX JYL. Analyzed the data: JW YQL. Wrote the paper: JW ZJH XCL.

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