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Original article

Antagonistic interactions between cadherin protein and Bt Cry1c toxin in the black cutworm, *Agrotis ipsilon* (*Hufnagel)*: implications for resistance development and genomic stability

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Abstract

The black cutworm *Agrotis ipsilon* (Hufnagel) exhibits high tolerance to the *Bacillus thuringiensis* (Bt) Cry1C toxin, posing significant challenges for effective pest management. In this study, synthetic double-stranded RNA (dsRNA) fragments targeting two regions of the cadherin gene (CAD900 and CAD1000) were assessed for their larvicidal efficacy, interaction with Cry1C, and associated genomic alterations. The *A. ipsilon* cadherin (*AiCAD)* gene cloned using the *A. ipsilon* GenBank sequence (accession no. AEB97396.1) encodes a protein with eight cadherin repeat domains and four calcium-binding sites. Bioassays revealed that CAD1000 induced the highest larval mortality (71.67%), significantly exceeding that of Cry1C (31.67%) and CAD900 (30%). Notably, combining CAD1000 with Cry1C resulted in reduced mortality (16.67%), indicating a potential antagonistic interaction. RAPD-PCR analysis revealed pronounced treatment-associated polymorphisms, with CAD1000 inducing the highest level of variation (88.31%). Unique molecular markers differentiated five genotypes, and cluster analysis separated treatments into two primary groups: a genetically distinct control cluster and a second cluster comprising Cry1C, CAD1000, and their combination. The highest genetic similarity was observed between Cry1C and CAD1000 (0.787). Phylogenetic analysis indicated that *AiCAD* is most closely related to *Sesamia inferens* among 28 lepidopteran species. Quantitative real-time PCR (qRT-PCR) revealed significant developmental stage-dependent expression of *AiCAD*. In addition, tissue-specific profiling showed predominant transcript accumulation in the midgut, with minimal expression in the foregut, hindgut, and fat body, highlighting the midgut as the primary site of cadherin activity and Bt toxin interaction.

Overall, these findings identify CAD1000 as a promising RNAi-based larvicide candidate and suggest caution in its combined use with Cry1C due to the observed antagonistic effects. In

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addition, this study provides molecular and expression insights that may support the development of targeted management strategies against this resilient pest.

Key words: *Agrotis ipsilon*, cadherin gene (*AiCAD*), RNA interference (RNAi), antagonism, Cry1C toxin

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Introduction

The black cutworm, *Agrotis ipsilon* (Hufnagel), is a highly destructive lepidopteran pest that infests numerous economically important crops worldwide, including maize, cotton, and vegetables (Gassmann and Reisig 2023). Its broad host range, high reproductive potential, and remarkable capacity to develop resistance to conventional insecticides make it a persistent threat to agricultural productivity. Prolonged and indiscriminate use of chemical insecticides has driven the evolution of resistance to multiple classes (Joshi *et al.* 2020; Ismail 2025).

Consequently, there has been an ongoing search for alternative pest management strategies, among which the use of *Bacillus thuringiensis* (Bt) toxins has gained particular prominence because of their host specificity and environmental safety (Azizoglu *et al.* 2023).

Bt is a Gram-positive, spore-forming soil bacterium that produces endospores and parasporal crystalline inclusions with potent insecticidal activity (Shahee 2023). These crystals, composed of Cry (crystal) toxins, play a central role in Bt pathogenicity against insects and some nematodes. To date, more than 200 distinct Cry proteins have been identified from various Bt strains, each exhibiting a narrow host range (Mostafa *et al.* 2013). Owing to their potency and favorable environmental profile, Bt Cry toxins are extensively utilized in transgenic crops and formulated biopesticides (Kumar *et al.* 2021).

Furthermore, Bt-based control is considered a sustainable and eco-friendly approach for managing lepidopteran pests (Kandil *et al.* 2020; Moustafa *et al*. 2022). The insecticidal action of Bt Cry proteins involves binding to specific receptors in the midgut epithelium of susceptible insects, leading to pore formation, disruption of cellular integrity, and larval death (Hu *et al*. 2025).

Among these receptors, cadherin-like proteins are critical for mediating Cry toxin binding (Li *et al.* 2022). However, the effectiveness of Bt-based control is often diminished by resistance evolution, frequently associated with mutations or modifications in cadherin receptors (Hu *et al.* 2025). The toxin-binding domains of these receptors, located within extracellular cadherin repeat (CR) regions, are key determinants of insect susceptibility and have been extensively studied in Lepidoptera (Naing *et al.* 2023).

Notably, recombinant cadherin peptides containing CRs have been shown to enhance Cry toxicity in several insect species (Moustafa *et al.* 2016). Recent studies have explored cadherin fragments not only as synergists that increase Bt efficacy but also as independent larvicidal agents (Moustafa *et al.* 2016; Gao *et al.* 2019; Li *et al.* 2022). Midgut receptors are central to the specificity and efficacy of Bt Cry toxins, with cadherin proteins historically recognized as key functional receptors for Cry1A toxins in lepidopteran larvae. Although Cry2A toxins share binding sites with Cry1A toxins, recent evidence indicates that cadherin binding to Cry2Ab is nonfunctional, as cadherin-derived peptides do not confer cellular sensitivity or significantly influence larval susceptibility to this toxin (Naing *et al.* 2023). However, the molecular and genomic consequences of such treatments, including potential genotoxic effects, remain poorly characterized in many pest species.

Molecular fingerprinting techniques, such as Random Amplified Polymorphic DNA (RAPD)-PCR, provide valuable tools for detecting treatment-induced genomic alterations and evaluating

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the effects of biopesticides at the DNA level (Huang *et al.* 2011). By identifying polymorphisms and unique genetic markers, RAPD analysis can yield important insights into the mechanisms of resistance and the genomic stability of treated populations (Bidyananda *et al.* 2024).

Given the economic significance of *A. ipsilon* and its demonstrated tolerance to Bt toxins, there is a pressing need to better understand the interactions between Bt Cry proteins, cadherin fragments, and the pest's genomic response. Therefore, this study aimed to: (1) evaluate the larvicidal activity of cadherin protein fragments, alone and in combination with Cry1C, against *A. ipsilon*; (2) assess the molecular and genomic impacts of these treatments using RAPD-PCR; and (3) investigate the evolutionary relationships of the *CAD* gene in *A. ipsilon* relative to other lepidopteran species. The results are expected to support the development of more effective and sustainable pest management strategies targeting this resilient species.

Materials and Methods

Insect rearing

Colonies of *A. ipsilon* were maintained at the Pesticide Department, Faculty of Agriculture, Cairo University, Egypt, under controlled laboratory conditions (25 ± 1 °C, 70% relative humidity, and a 16:8 h light-dark cycle). Larvae were reared on fresh castor bean leaves (*Ricinus communis* L.). To prevent cannibalism, third-instar larvae were individually placed in plastic cups (6.0 cm diameter × 3.5 cm height) containing a fresh leaf (He *et al.* 2019; Awad *et al.* 2024b). Pupae were transferred to 0.5 L glass jars until adult emergence. Adults were placed in larger jars supplied with moistened cotton pads soaked in a 10% sucrose solution, and eggs were collected daily (Kandil *et al.* 2020; Ahmed *et al.* 2024; Awad *et al.* 2024a).

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Characterization of the cadherin gene

Homologous gene sequences available in GenBank were compared with the *A. ipsilon* cadherin sequence (accession no. AEB97396.1) using MEGA version 11 to perform multiple sequence alignments and consistency analysis. A phylogenetic tree was constructed using the neighbor-joining method. The positions of cadherin repeat (CR) domains in the *A. ipsilon* cadherin protein were predicted using the ExPASy ScanProsite tool (https://prosite.expasy.org/scanprosite/) (De Castro *et al.* 2006).

Temporal and spatial expression of *AiCAD*

Expression patterns of the *AiCAD* gene were examined using quantitative real-time PCR (qRT-PCR). The *A. ipsilon* ribosomal protein L8 (*RPL8*) gene served as an internal reference for normalization (Bozzolan *et al.* 2017; Gassias *et al.* 2021). Samples were collected from larvae at six developmental stages (first to sixth instars) and from four tissues: midgut, foregut, hindgut, and fat body. qRT-PCR was used to quantify relative transcript levels across developmental stages and tissues. Primer sequences for *AiCAD* and *RPL8* amplification are listed in Table 1.

RNAi-mediated silencing of cadherin design and cloning of RNAi targets

Two regions of the cadherin gene, designated CAD900 and CAD1000, were selected as RNAi targets. Synthetic DNA fragments corresponding to the selected amino acid sequences were codon-optimized for

Escherichia *coli* expression and synthesized by Tsingke Biotechnology Co., Ltd. (full sequences retained). The fragments were flanked by XbaI and HindIII restriction sites for cloning into the pL4440 vector, which carries dual T7 promoters for double-stranded RNA (dsRNA) synthesis. Recombinant plasmids were transformed into *E. coli* HT115 (DE3) cells, which are deficient in RNase III to prevent dsRNA degradation. Transformants were selected on LB agar containing

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100 µg/mL ampicillin, screened via colony PCR, and confirmed by restriction digestion and agarose gel electrophoresis.

Expression, verification, and application of dsRNA

Confirmed *E. coli* clones were cultured in LB broth supplemented with ampicillin at 37°C until mid-log phase (OD600 = 0.6–0.8). dsRNA production was induced with 1 mM IPTG and cultures were incubated for an additional 4 h, followed by overnight growth. Bacterial pellets were collected, resuspended, and applied directly onto castor leaf discs, which were fed to larvae to induce gene silencing.

Bioassay

The bioassay evaluated the toxicity of Cry1C toxin, alone or in combination with cadherin fragments, against second-instar larvae of *A. ipsilon*. Fresh castor leaf discs (3 cm diameter) were immersed for 20 s in treatment solutions; control discs were dipped in water. Discs were air-dried before feeding. Each treatment was replicated four times, with 15 larvae per replicate. Mortality was recorded after 96 h and corrected using Abbott's formula (Abbott 1925).

Enhancement of Bt efficacy with cadherin fragments

Cadherin fragments (CAD900 and CAD1000) were combined with Cry1C protein, expressed from the pGEX-4T-1 vector in *E. coli* BL21(DE3) cells. Protein expression was induced with 0.5 mM IPTG at 28°C overnight (Mohammed *et al.* 2018). The working concentration was 250 ppm. Second-instar larvae were exposed to Cry1C alone or in combination with cadherin fragments. Mortality was recorded after 72 h to assess potential synergistic or antagonistic interactions. All treatments were conducted in triplicate.

RAPD-PCR genomic analysis

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Genomic DNA was isolated from surviving larvae using the CTAB method (Lakshita *et al.* 2024). RAPD-PCR reactions (25 µL) contained 50 ng genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq DNA polymerase, and 0.4 µM primer (Table 2). RAPD profiles were scored in binary format (presence= 1, absence= 0). Jaccard's similarity coefficient was used to calculate genetic similarity, and UPGMA clustering was performed using NTSYS-pc version v2.2 to generate dendrograms.

Results

Sequence analysis of *AiCAD*

The *A. ipsilon* CAD gene (*AiCAD*) was cloned using the *A. ipsilon* sequence (GenBank accession AEB97396.1) in FASTA format. Domain prediction revealed eight cadherin repeat (CR) domains and four calcium-binding motifs (Fig. 1).

Characterization of the cadherin gene

The *AiCAD* sequence was compared with amino acid sequences from 28 lepidopteran species representing multiple families, retrieved from NCBI (accession numbers and taxonomy are shown in Table 3). A maximum likelihood phylogenetic tree was generated to infer evolutionary relationships. As shown in Fig. 2, *AiCAD* grouped closely with other noctuid species, displaying the highest sequence homology with *Sesamia inferens*. This clustering indicates a conserved CAD sequence architecture within Noctuidae and provides insight into the evolutionary placement of *AiCAD* among lepidopterans.

Temporal and spatial expression of *AiCAD*

qRT-PCR analysis showed significant variation in *AiCAD* expression across larval instars, with the highest transcript levels detected at the second instar stage (Fig. 3A). One-way ANOVA

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indicated a highly significant difference among instars (F (5, 12) = 493.2, P < 0.0001). Means labelled with different letters are significantly different according to post hoc comparisons ($P < 0.05$). Tissue-specific profiling revealed predominant *AiCAD* expression in the midgut, with minimal expression in the foregut, hindgut, and fat body (Fig. 3B). ANOVA confirmed these differences as highly significant (F (3, 8) = 181.2, P < 0.0001, $R^2 = 0.9855$), and post hoc tests showed that tissues with different letters were significantly different (P < 0.05).

RNAi-mediated silencing

Larval mortality differed significantly among treatments (F (5, 18) = 14.14, P < 0.0001). CAD1000 produced the highest mortality (71.66%, group "d"), significantly exceeding all other treatments. The control group had the lowest mortality (1.66%, group "a"). Means labelled with different letters are significantly different according to post hoc comparisons ($P < 0.05$), as indicated in Fig. (4).

RAPD-PCR genomic analysis

RAPD-PCR using ten arbitrary primers detected marked genomic alterations in treated larvae. Controls displayed stable banding patterns, whereas CAD900 and CAD1000, (especially when combined with Cry1C) induced extensive DNA changes, including band loss, novel band appearance, and smearing. Changes were most evident with primers OPC-06, OPC-16, and OPD-05. CAD1000 alone caused the strongest genotoxic response, showing intense polymorphisms with OPA-10 and OPC-14. Combination treatments (CAD900 + Cry1C; CAD1000 + Cry1C) yielded intermediate alteration profiles with certain primers (e.g., OPD-03), indicating interactive effects on DNA stability.

Ten RAPD primers produced 77 amplicons across treatments, of which 68 were polymorphic, yielding an overall polymorphism rate of 88.31%. Five primers (OPA-10, OPC-05, OPC-14, OPD-

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01, and OPD-03) generated 100% polymorphic bands, indicating a strong ability to detect genetic variation. The lowest polymorphism rate (50%) was recorded for OPC-16. These findings highlight both the sensitivity of the selected primers and the substantial genetic diversity induced by the treatments (Table 4).

RAPD analysis identified nine primers (OPB-06, OPC-05, OPD-03, OPC-11, OPD-01, OPC-14, OPC-16, OPE-06, and OPD-05) that produced unique markers distinguishing the six genotypes: Control, Cry1C, CAD900, CAD1000, CAD900 + Cry1C, and CAD1000 + Cry1C.

In total, 12 unique positive markers (100-1,200 bp) and seven unique negative markers (100-800 bp) were detected. The control was defined solely by six negative markers, Cry1C by four positive markers, CAD900 by one positive marker, CAD1000 by two positive markers, and CAD1000 + Cry1C by five positive and one negative marker (Table 5).

Primer-level analysis showed that OPC-11 yielded the most unique markers, differentiating five genotypes with positive markers in CAD900, CAD1000, and CAD1000 + Cry1C, and negative markers in the control. OPD-01 detected four unique markers linked to CAD1000 and CAD1000 + Cry1C (positive) and the control (negative). Cry1C-specific positive markers were generated by OPB-06, OPC-05, and OPD-03. Control-specific negative markers were detected by OPC-05, OPC-11, OPD-01, OPD-05, and OPE-06. CAD1000 + Cry1C showed unique positive markers with OPC-14 and a negative marker with OPC-16 (Table 6).

Genetic similarity values among treatments ranged from 0.281 to 0.787. The highest similarity (0.787) was observed between Cry1C and CAD1000, while the lowest (0.281) was between the control and CAD1000 + Cry1C. Intermediate similarity values included CAD900 + Cry1C with CAD1000 + Cry1C (0.747), and control with CAD900 (0.581). These results indicate that Cry1C and CAD1000 elicit closely related genomic responses, whereas CAD1000 + Cry1C diverges most

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strongly from the control profile (Table 7). Cluster analysis based on the similarity matrix produced two primary clusters. The first contained only the control, reflecting its distinct genetic profile. The second cluster split into two subclusters: (1) Cry1C and CAD1000, and (2) CAD900 + Cry1C with CAD1000 + Cry1C, and CAD900 alone. This structure shows that combination treatments form a separate group from single-agent exposures, with the control genetically isolated from all treated groups (Fig. 5).

Discussion

The increasing concerns over the environmental and health risks associated with chemical pesticides have intensified the search for safe and effective alternative control methods within Integrated Pest Management (IPM) frameworks (Deguine *et al.* 2021). In this context, the interactions between *B. thuringiensis* (Bt) toxins, insect midgut receptors, and the subsequent genomic response are critical areas of investigation. The present study elucidates these interactions in the black cutworm, *A. ipsilon*, by examining the effects of Cry1C toxin and cadherin fragments (CAD) both independently and in combination.

Our bioassay results corroborate earlier findings that *A. ipsilon* exhibits a marked tolerance to a range of Bt Cry toxins. The observed resilience to Cry1C is consistent with previous studies reporting reduced susceptibility to Cry1Ab and Cry1F, even at concentrations that are lethal to other noctuid species (Binning *et al.* 2015; Wang *et al.* 2015). For instance, Wang *et al.* (2015) demonstrated that *A. ipsilon* larvae were at least eight times more tolerant to Cry1Ab than *Mythimna separata*, showing minimal physiological disruption at high doses. Similarly, Binning *et al*. (2015) documented resistance to multiple Cry toxins across both neonate and third-instar larval stages, indicating that this tolerance is a constitutive trait not restricted to a specific

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developmental period. This inherent reduced susceptibility has been mechanistically linked to several factors, including structural differences in midgut receptors, lower binding affinity for Cry toxins, and physiological characteristics such as a reduced midgut membrane potential compared with more susceptible lepidopterans (Wang *et al.* 2015).

A pivotal finding of our research was the high larval mortality induced by the cadherin fragment CAD1000, which exceeded the mortality caused by Cry1C alone. This strongly supports the hypothesis that certain cadherin fragments can act as potent larvicidal agents. However, the central and most significant revelation of this study emerged from the combination treatments.

Contrary to a synergistic effect, a clear antagonistic interaction was observed when CAD fragments were combined with Cry1C, manifesting as a significant reduction in larval mortality compared to CAD1000 applied alone. This antagonism can be explained within the established paradigm of Bt-receptor interactions, which are known to be complex and capable of yielding either synergistic or antagonistic outcomes.

The observed reduction in toxicity in our combination treatments likely resulted from binding site competition or disruption of toxin oligomer assembly. Importantly, this antagonistic effect is best explained by nonfunctional binding rather than the loss of a functional receptor. Binding assays and functional analyses have demonstrated that cadherin can bind Cry toxins, including Cry2Ab and Cry1-family members, without mediating toxicity. Cadherin-derived peptides bind Cry toxins in vitro but fail to confer susceptibility when expressed in insect cells, whereas ATP-binding cassette transporters, such as ABCA2, act as the true functional receptors (Naing *et al*. 2023). Thus, cadherin silencing does not remove a critical receptor required for toxin action, instead, it eliminates a binding protein that may sequester the toxin and reduce its availability to the functional receptor. Consequently, the antagonism observed with CAD1000 dsRNA is unlikely to

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result from competition for cellular transporters, but rather from disruption of toxin-cadherin interactions that normally modulate toxin access to membrane-bound receptors.

This mechanistic interpretation is supported by studies in other systems. For example, truncated fragments of the Vip3Aa toxin act as antagonists by competing for membrane binding sites (Boonyos *et al.* 2021). Similarly, cadherin fragments from *Helicoverpa armigera* have been shown to diminish Cry1Ac toxicity by blocking toxin binding and preventing the formation of oligomeric pores, both of which are essential steps for toxicity (Zhang *et al.* 2017). Conversely, certain cadherin fragments, such as those from *Manduca sexta*, can enhance Cry1A toxicity by promoting oligomer formation (Xie *et al.* 2026). The specific outcome is therefore highly dependent on the insect species, the toxin, and the nature of the cadherin fragment, underscoring the high specificity of these interactions (Andrés-Garrido *et al.* 2020).

Our results align with the findings of Andrés-Garrido *et al.* (2020), who reported that cadherin fragments from various Lepidopteran and Coleopteran species failed to enhance the toxicity of Cry1Ca or Vip3Aa in *Spodoptera exigua*. Consistent with this model, reduced toxicity has been observed only when functional receptors such as ABCA2 are disrupted, whereas cadherin knockdown alone has little or no effect on mortality.

To gain deeper insight into the genomic-level effects of these treatments, RAPD-PCR analysis was employed as a preliminary screening tool. The results revealed substantial DNA polymorphism and treatment-specific molecular markers. The CAD1000 treatment induced the most pronounced genotoxic effects, as evidenced by the highest polymorphism rate (88.31%) and a unique profile of positive and negative markers. Cluster analysis clearly differentiated the control group from all treated larvae, with the Cry1C and CAD1000 treatments showing the closest genetic similarity. Notably, the combination treatments formed a separate cluster, a genetic profile that corresponded

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directly to their lower phenotypic toxicity. This strong concordance between molecular markers and bioassay mortality data provides compelling evidence for the biological relevance of the antagonistic interaction in *A. ipsilon* and confirms the utility of RAPD as a sensitive tool for detecting genomic stress and alterations in lepidopteran pests (Huang *et al.* 2011).

The antagonistic effect identified in this study has important implications for resistance management and the development of Bt-based bioinsecticides. The presence of such interactions in field populations may facilitate adaptive responses, thereby compromising the long-term effectiveness of Bt technologies. Our molecular data further suggest that the cadherin fragment involved in this interaction may influence gene regulatory pathways related to detoxification, stress response, or cellular repair, ultimately affecting the genomic integrity and adaptive potential of *A. ipsilon*.

In conclusion, this study demonstrates a clear antagonistic interaction between a cadherin protein fragment and the Bt Cry1C toxin in *A. ipsilon*. This finding underscores the intricate complexity of bioinsecticide interactions and highlights the critical need for comprehensive evaluations of potential antagonistic effects when designing new Bt toxin formulations or combination strategies for IPM programs. Ultimately, acknowledging and understanding these complex interactions is paramount for optimizing Bt application strategies and proactively delaying the evolution of resistance in pest populations.

Conclusion

Overall, our findings highlight the potential of cadherin fragments, particularly CAD1000, to counter Bt tolerance in *A. ipsilon*. RNAi-based and receptor-mimicking approaches may enhance Cry1C performance while also triggering genomic stress responses. However, careful optimization

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of combination strategies is essential to avoid antagonistic interactions. This study provides a framework for integrated biocontrol programs targeting difficult-to-manage lepidopteran pests.

Future perspective

RAPD-PCR provided a useful preliminary screening tool to detect treatment-associated DNA polymorphisms in *A. ipsilon*. However, to achieve higher resolution and confirm the genomic effects observed, future research should incorporate more robust techniques such as real-time PCR -based analysis of stress-, detoxification-, and DNA repair-related genes. Future studies should also aim to validate and expand the molecular insights using more advanced and quantitative approaches such as NGS and real-time PCR. In addition, a semi-field investigation should be conducted.

References

Abbott W.S. 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18(2): 265-267. https://doi.org/10.1093/jee/18.2.265a

Ahmed F.S., Helmy W.S., Alfuhaid N.A., Moustafa M.A. 2024. Target enzymes of *Origanum majorana* and *Rosmarinus officinalis* essential oils in black cutworm (*Agrotis ipsilon)*: *in vitro* and *in silico* studies. Insects 15(7): 483. https://doi.org/10.3390/insects15070483

Andrés-Garrido A., González-Martínez R.M., Ramos S., Escriche B. 2020. Cadherin fragments of Lepidopteran and Coleopteran species do not enhance toxicity of Cry1Ca and Vip3Aa proteins to *Spodoptera exigua* (Hübner)(Lepidoptera: Noctuidae). Biocontrol Science and Technology 30(9): 941-950. https://doi.org/10.1080/09583157.2020.1772960

Awad M., Alfuhaid N.A., Amer A., Hassan N.N., Moustafa M.A. 2024a. Towards sustainable pest management: Toxicity, biochemical effects, and molecular docking analysis of *Ocimum basilicum* (Lamiaceae) essential oil on *Agrotis ipsilon* and *Spodoptera littoralis* (Lepidoptera: Noctuidae). Neotropical Entomology 53(3): 669-681. https://doi.org/10.1007/s13744-024-01137-6

PRE-PROOF PUBLICATION

PRE-PROOF PUBLICATION JOURNAL OF PLANT PROTECTION RESEARCH

Awad M., El Kenawy A.H., Alfuhaid N.A., Ibrahim E.-D.S., Jósvai J.K., Fónagy A., Moustafa, M.A., 2024b. Lethal and sublethal effects of cyantraniliprole on the Biology and Metabolic enzyme activities of two lepidopteran Pests, *Spodoptera littoralis* and *Agrotis ipsilon*, and A *generalist predator*, *Chrysoperla carnea* (Neuroptera: Chrysopidae). Insects 15(6): 450. https://doi.org/10.3390/insects15060450

Azizoglu U., Salehi Jouzani G., Sansinenea E., Sanchis-Borja, V. 2023. Biotechnological advances in *Bacillus thuringiensis* and its toxins: Recent updates. Reviews in Environmental Science and Bio/Technology 22(2): 319-348. https://doi.org/10.1007/s11157-023-09652-5

Bidyananda N., Jamir I., Nowakowska K., Varte V., Vendrame W.A., Devi R.S., Nongdam P., 2024. Plant genetic diversity studies: Insights from DNA marker analyses. International Journal of Plant Biology 15(3): 607-640. https://doi.org/10.3390/ijpb15030046

Binning R.R., Coats J., Kong X., Hellmich R.L. 2015. Susceptibility to *Bt* proteins is not required for *Agrotis ipsilon* aversion to *Bt* maize. Pest Management Science 71(4): 601-606. https://doi.org/10.1002/ps.3901

Boonyos P., Trakulnalueamsai C., Rungrod A., Chongthammakun S., Promdonkoy B. 2021. Antagonistic effect of truncated fragments of *Bacillus thuringiensis* Vip3Aa on the larvicidal activity of its full-length protein. Protein and Peptide Letters 28(2): 131-139. https://doi.org/10.2174/0929866527666200625205846

Bozzolan F., Durand N., Demondion E., Bourgeois T., Gassias E., Debernard, S. 2017. Evidence for a role of oestrogen receptor-related receptor in the regulation of male sexual behaviour in the moth *Agrotis ipsilon*. Insect Molecular Biology 26(4): 403-413. https://doi.org/10.1111/imb.12303

De Castro E., Sigrist C.J.A., Gattiker A., Bulliard V., Langendijk-Genevaux P.S., Gasteiger E., Bairoch A., Hulo, N. 2006. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Research 34(suppl_2): W362-W365. https://doi.org/10.1093/nar/gkl124

Deguine J.-P., Aubertot J.-N., Flor R.J., Lescourret F., Wyckhuys K.A., Ratnadass, A. 2021. Integrated pest management: good intentions, hard realities. A review. Agronomy for Sustainable Development 41(3): 38. https://doi.org/10.1007/s13593-021-00689-w

Gao M., Dong S., Hu X., Zhang X., Liu Y., Zhong J., Lu L., Wang Y., Chen L., Liu, X. 2019. Roles of midgut cadherin from two moths in different *Bacillus thuringiensis* action mechanisms:

PRE-PROOF PUBLICATION

PRE-PROOF PUBLICATION JOURNAL OF PLANT PROTECTION RESEARCH

correlation among toxin binding, cellular toxicity, and synergism. Journal of Agricultural and Food Chemistry 67(48): 13237-13246. https://doi.org/10.1021/acs.jafc.9b04563

Gassias E., Maria A., Couzi P., Demondion E., Durand N., Bozzolan F., Aguilar P., Debernard, S. 2021. Involvement of methoprene-tolerant and Krüppel homolog 1 in juvenile hormone-signaling regulating the maturation of male accessory glands in the moth *Agrotis ipsilon*. Insect Biochemistry and Molecular Biology 132: 103566. https://doi.org/10.1016/j.ibmb.2021.103566

Gassmann A.J., Reisig D.D. 2023. Management of insect pests with *Bt* crops in the United States. Annual Review of Entomology 68(1): 31-49. https://doi.org/10.1146/annurev-ento-120220-105502

He F., Sun S., Tan H., Sun X., Qin C., Ji S., Li X., Zhang J., Jiang X. 2019. Chlorantraniliprole against the black cutworm *Agrotis ipsilon* (Lepidoptera: Noctuidae): From biochemical/physiological to demographic responses. Scientific Reports 9(1): 10328. https://doi.org/10.1038/s41598-019-46915-0

Hu D., Wang D., Pan H., Liu X. 2025. Molecular mechanisms underlying resistance to *Bacillus thuringiensis* Cry toxins in Lepidopteran pests: An Updated Research Perspective. Agronomy 15(1): 155. https://doi.org/10.3390/agronomy15010155

Huang F., Andow D.A., Buschman L.L. 2011. Success of the high-dose/refuge resistance management strategy after 15 years of *Bt* crop use in North America. Entomologia Experimentalis et Applicata 140(1): 1-16. https://doi.org/10.1111/j.1570-7458.2011.01138.x

Ismail S.M. 2025. Resistance level and enzymatic detoxification responses in field populations *Agrotis ipsilon* (Hufnagel) to commercial insecticides. International Journal of Advanced Biological and Biomedical Research 13: 219-227: https://doi.org/10.48309/IJABBR.2025.2039962.1540

Joshi M., Rana A., Prithiv Raj V., Kaushal S., Inamdar A., Verma K., Chandel, R. 2020. The potency of chemical insecticides in management of cutworm, *Agrotis ipsilon Hufnagel* (Noctuidae: Lepidoptera): A review. Journal of Entomology and Zoology Studies 8(3): 307-311.

Kandil M.A., Abdel-Kerim R.N. and Moustafa M.A. 2020. Lethal and sub-lethal effects of bio- and chemical insecticides on the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera:

PRE-PROOF PUBLICATION

PRE-PROOF PUBLICATION JOURNAL OF PLANT PROTECTION RESEARCH

Gelechiidae). Egyptian Journal of Biological Pest Control 30(1): 76. https://doi.org/10.1186/s41938-020-00278-1

Kumar P., Kamle M., Borah R., Mahato D.K., Sharma B. 2021. *Bacillus thuringiensis* as microbial Biopesticide: uses and application for sustainable agriculture. Egyptian Journal of Biological Pest Control 31(1): 95. https://doi.org/10.1186/s41938-021-00440-3

Li Y., Wang C., Ge L., Hu C., Wu G., Sun Y., Song L., Wu X., Pan A., Xu Q. 2022. Environmental behaviors of *Bacillus thuringiensis* (*Bt*) insecticidal proteins and their effects on microbial ecology. Plants 11(9): 1212. https://doi.org/10.3390/plants11091212

Mohammed A.M., Diab M.R., Khalil S.M. 2018. Synergistic activity of *Bacillus thuringiensis* δ-endotoxin and TMOF against Culex pipiens and *Spodoptera littoralis* larvae. Entomological Research 48(3): 156-164. https://doi.org/10.1111/1748-5967.12257

Mostafa M., Vlasák J., Sehnal F. 2013. Activities of modified Cry3A-type toxins on the red flour beetle, *Tribolium castaneum* (Herbst). Journal of Applied Entomology 137(9): 684-692. https://doi.org/10.1111/jen.12054

Moustafa M., Vlasák J., Sehnal F. 2016. Fragments of *Tenebrio molitor* cadherin enhance Cry3Aa toxicity for the red flour beetle, *Tribolium castaneum* (Herbst). Journal of Applied Entomology 140(4): 277-286. https://doi.org/10.1111/jen.12255

Moustafa M.A., Elmenofy W.H., Osman E.A., El-Said N.A., Awad M. 2022. Biological impact, oxidative stress and adipokinetic hormone activities of *Agrotis ipsilon* in response to bioinsecticides. Plant Protection Science 58(4): 326–337. https://doi.org/10.17221/46/2022-pps

Naing Z.L., Soe E.T., Zhang C., Niu L., Tang J., Ding Z., Yu S., Lu J., Fang F., Liang G. 2023. Cadherin is a binding protein but not a functional receptor of *Bacillus thuringiensis* Cry2Ab in *Helicoverpa armigera*. Applied and Environmental Microbiology 89(7): e00625-00623. https://doi.org/10.1128/aem.00625-23

Shahee C. 2023. Biocontrol against inset pests by *Bacillus thuringiensis* isolated from organic agricultural farm. PhD Thesis. Department of Microbiology Central Campus of Technology, Dharan, Nepal. pp. 1-92.

Wang Y., Hu Z., Wu W. 2015. Different effects of *Bacillus thuringiensis* toxin Cry1Ab on midgut cell transmembrane potential of *Mythimna separata* and *Agrotis ipsilon* larvae. Toxins 7(12): 5448-5458. https://doi.org/10.3390/toxins7124894

PRE-PROOF PUBLICATION

PRE-PROOF PUBLICATION JOURNAL OF PLANT PROTECTION RESEARCH

Xie J., He W., Qiu M., Lin J., Shu H., Wang J., Liu L. 2026. Field-Evolved resistance to Bt Cry toxins in Lepidopteran Pests: Insights into multilayered regulatory mechanisms and next-generation management strategies. Toxins 18(2): 60. https://doi.org/10.3390/toxins18020060

Zhang H., Yu S., Shi Y., Yang Y., Fabrick J.A., Wu Y. 2017. Intra-and extracellular domains of the *Helicoverpa armigera* cadherin mediate Cry1Ac cytotoxicity. Insect Biochemistry and Molecular Biology 86: 41-49. https://doi.org/10.1016/j.ibmb.2017.05.004

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Fig. 1. Conserved domains of the *A. ipsilon* cadherin (*AiCAD*) protein, showing Ca^{2+}-binding sites within the cadherin repeat domains. Conserved domains were predicted using the NCBI Conserved Domain Database (CDD) search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

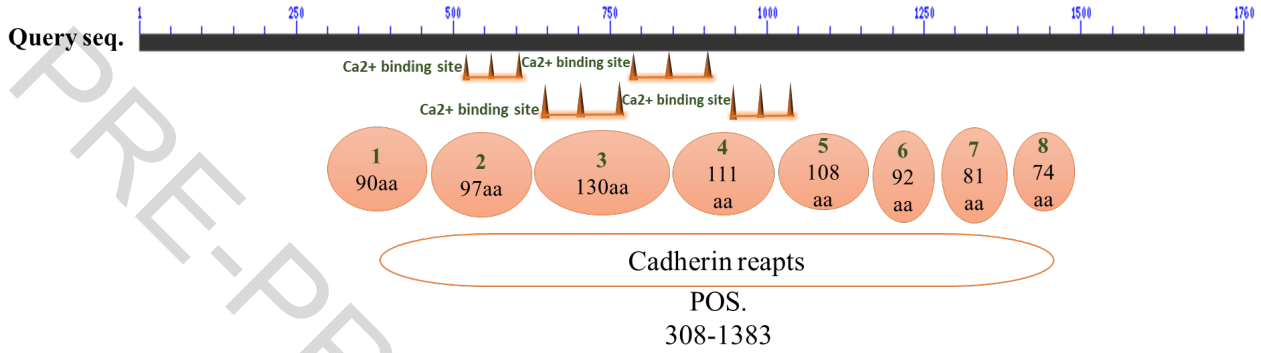

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Fig. 2. Neighbor-joining phylogenetic tree of 29 cadherin amino acid sequences from diverse insect species, generated using MEGA-11. Bootstrap support values (percentages from 500 replications) are indicated at branch nodes, with a cut-off applied for condensed clustering. *A. ipsilon* CAD proteins are highlighted in gray, and sequences from different insect families are color-coded. Species names are abbreviated by the first letter of the genus followed by the first one or two letters of the species name.

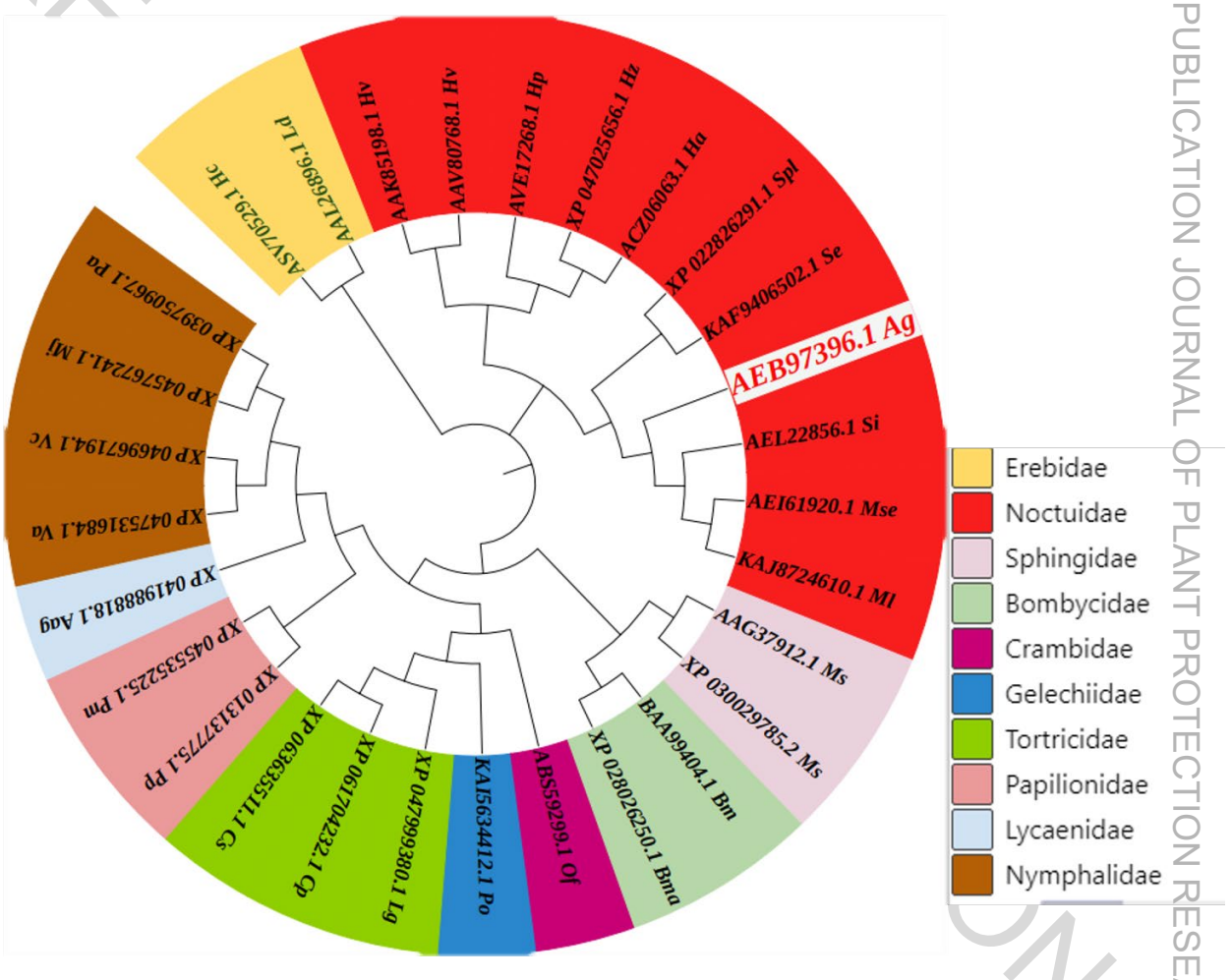

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Fig. 3. Relative expression of *AiCAD* mRNA in *A. ipsilon*. (A) Expression across larval instars, normalized to the first instar. (B) Expression across different tissues, normalized to the foregut. Relative transcript levels were quantified using qRT-PCR.

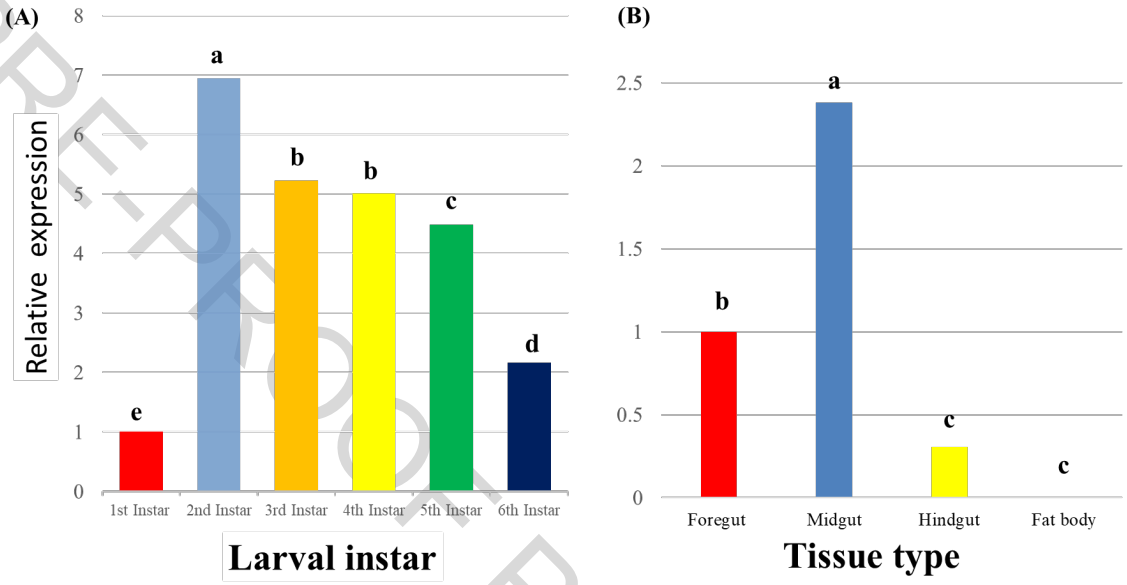

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Fig. 4. Larval mortality (%) of *A. ipsilon* was recorded following exposure to RNAi-induced cadherin fragments (CAD900 and CAD1000), Cry1C alone, and combined treatments of *AiCAD* with Cry1C.

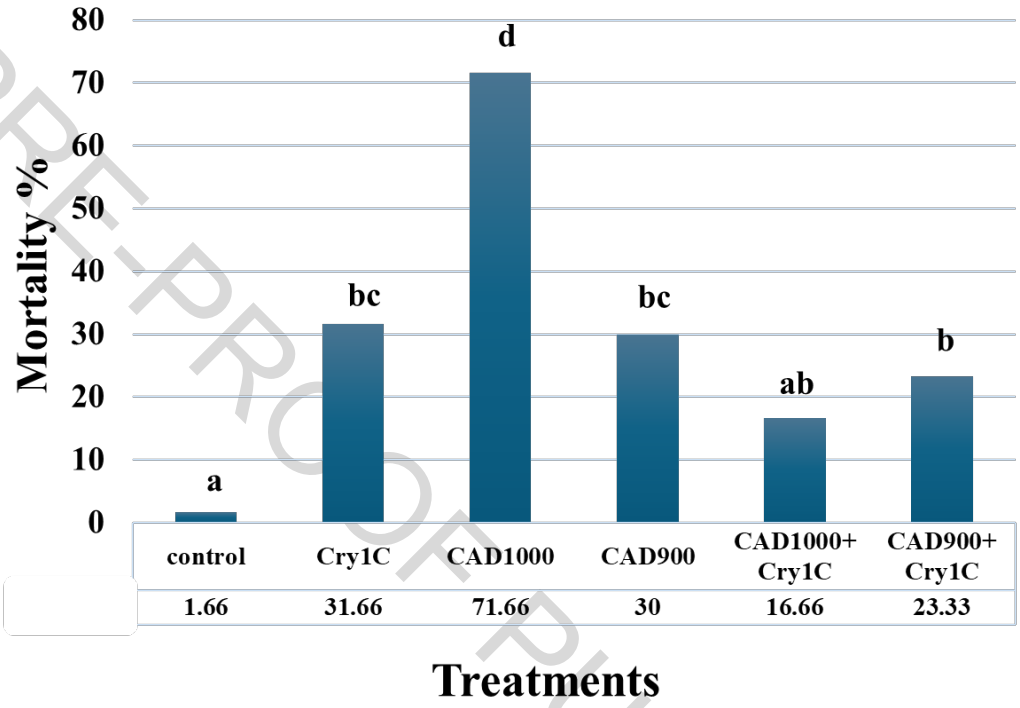

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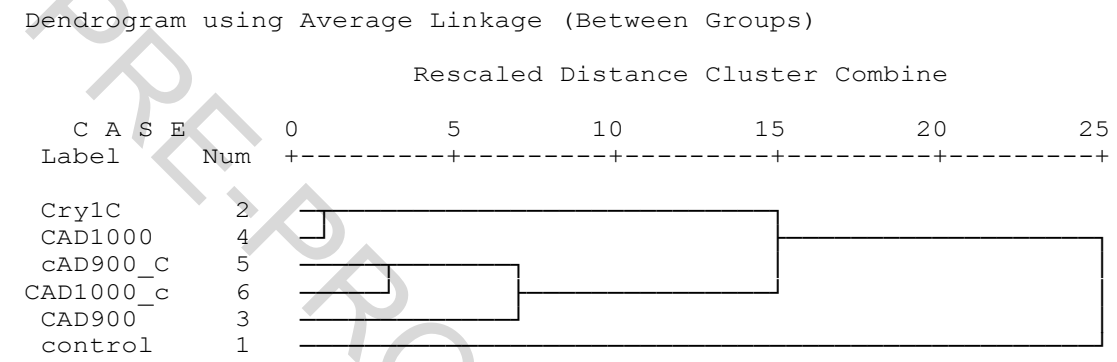
Fig. 5. Dendrogram of *A. ipsilon* treatments constructed from RAPD data. Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), and similarity matrices were computed based on the Dice coefficient.



Abbreviated Name	Extended Name
CAD1000_C	CAD1000+Cry1c
CAD900_c	CAD900+Cry1c

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Table 1. Primer sequences used for quantitative real-time PCR analysis of *A. ipsilon* cadherin (*AiCAD*) and ribosomal protein L8 (*RPL8*) genes.

Primer name	Forward (5' -3')	Reverse (5' -3')	Expected size
AiRT-qPCR	CATCCCGCAACAGAGTCTA	ATGGGTTTCGGTAGTCAGAT	170
AiRpL8	CCAGTTTGTCTACTGCGGCAA	GCTTAACCCTAGTACGCTTGGCA	Housekeeping

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Table 2. Random amplified polymorphic DNA (RAPD) primers used for genomic DNA analysis in *A. ipsilon*.

Primer name	Primer sequence (5'-3')
OPA-10	GTGATCGCAG
OPB-06	TGCTCTGCCC
OPC-05	GATGACCGCC
OPC-11	AAAGCTGCGG
OPC-14	TGCGTGCTTG
OPC-16	CACACTCCAG
OPD-01	ACCGCGAAGG
OPD-03	GTCGCCGTCA
OPD-05	TGAGCGGACA
OPE-06	AAGACCCCTC

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Table 3. GenBank accession numbers, scientific names, and taxonomic classifications of sequences used for construction of the neighbor-joining phylogenetic tree.

Accession No.	Scientific name	Abbreviation	Order	Family
AEB97396.1	*Agrotis ipsilon*	*Ag*		*Noctuidae*
AEL22856.1	*Sesamia inferens*	*Si*		*Noctuidae*
KAJ8724610.1	*Mythimna loreyi*	*Ml*		*Noctuidae*
AEI61920.1	*Mythimna separata*	*Mse*		*Noctuidae*
AVE17268.1	*Helicoverpa punctigera*	*Hp*		*Noctuidae*
ACZ06063.1	*Helicoverpa armigera*	*Ha*		*Noctuidae*
AAV80768.1	*Heliothis virescens*	*Hv*		*Noctuidae*
AAK85198.1	*Heliothis virescens*	*Hv*		*Noctuidae*
XP_047025656.1	*Helicoverpa zea*	*Hz*		*Noctuidae*
AAL26896.1	*Lymantria dispar*	*Ld*		*Erebidae*
XP_028026250.1	*Bombyx mandarina*	*Bma*		*Bombycidae*
BAA99404.1	*Bombyx mori*	*Bm*		*Bombycidae*
KAF9406502.1	*Spodoptera exigua*	*Se*		*Noctuidae*
XP_041988818.1	*Aricia agestis*	*Aag*		*Lycaenidae*
XP_030029785.2	*Manduca sexta*	*Ms*	Lepidoptera	*Sphingidae*
AAG37912.1	*Manduca sexta*	*Ms*		*Sphingidae*
XP_045535225.1	*Papilio machaon*	*Pm*		*Papilionidae*
XP_013137775.1	*Papilio polytes*	*Pp*		*Papilionidae*
XP_046967194.1	*Vanessa cardui*	*Vc*		*Nymphalidae*
XP_063635511.1	*Cydia splendana*	*Cs*		*Tortricidae*
ABS59299.1	*Ostrinia furnacalis*	*Of*		*Crambidae*
XP_022826291.1	*Spodoptera litura*	*Spl*		*Noctuidae*
XP_047999380.1	*Leguminivora glycinivorella*	*Lg*		*Tortricidae*
XP_061704232.1	*Cydia pomonella*	*Cp*		*Tortricidae*
KAI5634412.1	*Phthorimaea operculella*	*Po*		*Gelechiidae*
XP_039750967.1	*Pararge aegeria*	*Pa*		*Nymphalidae*
XP_047531684.1	*Vanessa atalanta*	*Va*		*Nymphalidae*
ASV70529.1	*Hyphantria cunea*	*Hc*		*Erebidae*
XP_045767241.1	*Maniola jurtina*	*Mj*		*Nymphalidae*

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Table 4. Primer codes, total number of amplified products, monomorphic and polymorphic bands, and percentage of polymorphism detected by RAPD analysis in *A. ipsilon* larvae.

Primers	Total no. of amplicons	Monomorphic amplicons	Polymorphic amplicons	% of polymorphism
OPA-10	9	0	9	100%
OPB-06	7	1	6	85.7%
OPC-05	10	0	10	100%
OPC-11	10	1	9	90%
OPC-14	6	0	6	100%
OPC-16	6	3	3	50%
OPD-01	8	0	8	100%
OPD-03	8	0	8	100%
OPD-05	5	2	3	60%
OPE-06	8	2	6	75%
Total	77	9	68	88.31%
Average	7.7	0.9	6.8	88.31%

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Table 5. Unique RAPD markers detected in *A. ipsilon* larvae. The table presents positive and negative genotype-specific RAPD markers identified across six genotypes, including marker sizes (bp), corresponding primers, and the number of markers characterizing each genotype.

Genotype	Unique positive marker			Genotype	Unique negative marker		
	Marker size (bp)	primers	#of markers per genotype		Marker size	Primers	# of markers per genotype
Cry1C	500	OP-B06	4	Control	800	OPC_05	6
Cry1C	400	OP-C05		Control	700	OP-C11	
Cry1C	250	OP-D03		Control	400	OPD-01	
Cry1C	700	OP-D03		Control	450	OPD-05	
				Control	500	OP-E06	
CAD900	100	OP-C11	1	Control	700	OP-E06	
CAD1000	200	OP-C11	2	CAD1000+ Cry1C	100	OPC-16	1
CAD1000	200	OP-D01					
CAD1000+ Cry1C	300	OP-C11	5				
CAD1000+ Cry1C	1100	OP-C11					
CAD1000+ Cry1C	1100	OP-C14					
CAD1000+ Cry1C	1000	OP-D01					
CAD1000+ Cry1C	1200	OP-D01					

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Table 6. Distribution of unique RAPD markers across *A. ipsilon* genotypes under different treatments. The table summarizes positive and negative markers revealed by individual primers, along with the total number of genotypes identified per primer.

Primer	Treatment showing positive unique marker	Treatment showing negative unique marker	Total
OPA-10	0	0	0
OPB-06	Cry1C	0	1
OPC-05	Cry1C	Control	2
OPC-11	CAD900, CAD1000, CAD1000+Cry1C (2)	Control	5
OPC-14	CAD1000+Cry1C	0	1
OPC-16	0	CAD1000+Cry1C	1
OPD-01	CAD1000, CAD1000+Cry1C (2)	Control	4
OPD-03	Cry1C (2)	0	2
OPD-05	0	Control	1
OPE-06	0	Control (2)	2

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Table 7. Genetic similarity matrix among six *A. ipsilon* treatments based on RAPD analysis. Pairwise similarity values were calculated using the Dice coefficient, where a value of 1.000 indicates identical genomic profiles.

Proximity Matrix

Case	Matrix File Input					
	control	Cry1C	CAD900	CAD1000	CAD900 + Cry1C	CAD1000 + Cry1C
Control	1.000	0.464	0.581	0.462	0.407	0.281
Cry1C	0.464	1.000	0.571	0.787	0.659	0.581
CAD900	0.581	0.571	1.000	0.529	0.716	0.674
CAD1000	0.462	0.787	0.529	1.000	0.571	0.607
CAD900+Cry1C	0.407	0.659	0.716	0.571	1.000	0.747
CAD1000+Cry1C	0.281	0.581	0.674	0.607	0.747	1.000

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