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Molecular identification of Heliothine (Lep. Noctuidae) pest species by nuclear and mitochondrial PCR-RFLP profile

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Abstract. The taxonomy of the Heliothinae subfamily is complex, often becoming an obstacle in the identification of these species, especially during the larval stage. An alternative is the use of molecular methods such as the PCR-RFLP technique (or Restriction Fragment Length Polymorphism), which helps to aid and complement the identification of some Heliothine species. The aim of this study was to establish the PCR-RFLP profile using molecular markers for the identification of four Heliothine species. Specific primers were constructed for the amplification of the cytochrome oxidase subunit I (COI) and elongation factor 1 alpha (EF-1α) genes for identifying *Helicoverpa armigera*, *H. zea*, *H. gelotopoeon*, and *Chloridea virescens* species. In the digestion of the COI gene with the *Bfa*I enzyme, fragments of 294 bp and 321 bp were obtained for *H. armigera*, while there was no digestion for *H. zea*, *H. gelotopoeon*, and *C. virescens*. The *Hpa*I enzyme produced fragments of 58 bp and 557 bp for *H. gelotopoeon*, while there was no digestion for *H. armigera*, *H. zea*, and *C. virescens*. The digestion of the EF-1α gene with the *Eco*RV enzyme produced fragments of 142 bp and 964 bp for *C. virescens*, while there was no cleavage for the other heliothines. These molecular markers can help the entomologists, aiding in the discrimination between *H. zea*, *H. armigera*, *H. gelotopoeon*, and *C. virescens*, and can also be used as a tool for monitoring the spread of these pests in agricultural regions.

Keywords: *Chloridea*, Cytochrome oxidase subunit I, Genetic diversity, *Helicoverpa*, Nuclear elongation factor 1 alpha

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Introduction

The subfamily Heliothinae (Noctuidae) includes significant agricultural pests, causing annual losses estimated at US$ 5 billion (Lammers & MacLeod, 2007). *H. armigera* alone results in around US$ 2 billion in losses annually

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(Tay *et al*., 2013). Key pest species are *Helicoverpa* spp. and *Chloridea* (=*Heliothis*) *virescens* (Fabricius, 1777), reclassified due to genetic and morphological research (Pogue *et al*., 2013). *C. virescens* attacks cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), and tomato (*Solanum lycopersicum* L.) in Brazil (Ávila *et al*., 2013). *H. armigera* and *H. zea* (corn earworm or tomato fruitworm) are highly destructive due to their polyphagy, high fecundity, mobility, and migration (Ferrelli *et al*., 2015). Though *H. gelotopoeon* is not reported in Brazil, it occurs in Argentina, Chile, Uruguay, and Paraguay and is often confused with *H. armigera* due to their similarities (Walsh *et al*., 2019; Czepak *et al*., 2013). The geographical distribution of the genus *Helicoverpa* is broad, with *H. armigera* found in Europe, Africa, Asia, Australia, and recently in South America, while *H. zea* is predominantly found in North and South America. In Brazil, *H. armigera* impacts corn (*Zea mays* L.), soybean, cotton, millet (*Pennisetum glaucum* L), sorghum (*Sorghum bicolor* L.), wheat (*Triticum aestivum* L.), tomato, pepper (*Capsicum annuum* L.), lettuce (*Lactuca sativa* L.), watermelon (*Citrullus lanatus* L.), coffee (*Coffea arabica* L.), citrus, sunflower (*Helianthus annuus* L.), tobacco (*Nicotiana tabacum* L.), and weeds (Albernaz *et al*., 2014). *H. armigera* caterpillars in the Cerrado region were observed from February 2012, causing significant economic losses to corn, cotton, and soybean (Bueno *et al*., 2014; Czepak, 2013). Subsequent reports indicated its spread to the North, South, and Southeast regions (Specht et al., 2013; Mastrangelo *et al*., 2014). The taxonomy of *Helicoverpa* is complex, requiring specific knowledge of morphological and reproductive structures, posing challenges in identifying species, especially during the larval stage (Pogue *et al*., 2004; Bueno *et al*., 2014; Leite *et al*., 2013).

The limited number of trained taxonomists in Brazil and the difficulty in morphology-based taxonomy for the larval stage of *Helicoverpa* species create a need for new identification alternatives, feasible at any life stage (Mastrangelo *et al*., 2014). Various molecular (PCR, qPCR, STR, VNTR) and biochemical (isoenzymes) methods have been proposed for identifying *Helicoverpa* species (Pearce, 2003; Daly & Gregg, 1985; Trowell *et al*., 2000; Kranthi *et al*., 2005; Ming & Wang, 2006). The PCR-RFLP technique is one such method, discriminating insect species based on small DNA sequence differences that affect restriction sites. This low-cost technique requires only a thermocycler for PCR and a gel electrophoresis system. Elongation factor 1 alpha EF-1α, a conserved protein-encoding nuclear gene, is useful at higher taxonomic levels but shows low genetic diversity in populations of Heliothinae (Krishnarao *et al*., 2019). The mtDNA Cytochrome Oxidase subunit I (COI) gene is widely used in entomological studies due to its rapid evolution rate, enabling species-level identification (Behere *et al*., 2007). All the described techniques mainly target *H. armigera* and *H. zea*. and, in our study, we propose to extend to *C. virescens* and *H. gelotopoeon*, as the latter is an exotic pest for Brazil and can somehow be confused with *H. armigera*. The aim of this work was to establish the PCR-RFLP profile using molecular markers for the identification of four Heliothine species.

Materials and methods

In silico design and validation of markers

The selected genes were the mitochondrial cytochrome oxidase subunit I (COI) gene and the nuclear elongation factor 1 alpha (EF-1α) gene (Table 1). Several sequences of these genes from Heliothinae species were obtained from NCBI (https://www.ncbi.nlm.nih.gov/), with the exception of *H. gelotopoeon*, which had few sequences available at the time of the study. The gene sequences were aligned using the Geneious program (Kearse *et al*., 2012), and a scan of the consensus sequences of both COI and EF-1α genes to search for SNPs (Single Nucleotide Polymorphisms) containing the probable amplification region to identify possible restriction enzymes for the discrimination by PCR-RFLP of the four species was performed using the NEBcutter program (Vincze *et al*., 2003). Specific primers (Table 2) were then designed for the amplification of the target SNPs using the Primer3 program (Koressaar & Remm, 2007; Untergrasser *et al*., 2012).

Marker validation by PCR-RFLP

For the experiment, 20 third instar larvae from each species, *H. zea*, *H. armigera*, and *C. virescens*, from the company BUG Agentes Biológicos, along with 6 adult moths (1:1 female and male proportion) of *H. gelotopoeon* kindly provided by Dr. Specht, Embrapa – Genetic Resources and Biotechnology, were used. These samples underwent a DNA extraction procedure (Monnerat *et al*., 2006) and were stored at -20 °C until used. Subsequently, this DNA was used for PCR amplification with the COI and EF-1α gene primers (Table 2).

Table 1. GenBank accession numbers for the COI and EF-1α genes of *H. armigera*, *H. zea*, *H. gelotopoeon*, and *C. virescens* species.

Species	Genbank access number	
	COI gene	**EF-1 gene**
H. armigera	EU768936, HQ132369, JF776377, JX156325, JX156326	U20124, U20128, U20129, FJ768770
H. zea	EU768942, HQ177289, JX156328, JX156329, KJ390233	KF624903, KF624906, KF624907, KF624908, U20136
H. gelotopoeon	EU768938	U20132
C. virescens	GU336570, KF492648, KJ389774, JQ604277, JN799042	U20135

The PCR temperature cycles consisted of an initial denaturation step at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 5 minutes. The same conditions were used for both pairs of primers. Amplifications were carried out in a TC-412 thermocycler (TECHNE) and the successful DNA amplification was confirmed by agarose gel electrophoresis at 1.5%, stained with ethidium bromide, visualized, and photographed using a Gel Logic 212 PRO photodocumenter (Carestream).

Amplification products generated from Heliothine DNA using COI gene primers were digested with *Bfa*I and *Hpa*II enzymes. Meanwhile, amplification products obtained with EF-1α gene primers were digested with the EcoRV enzyme. All digestion reactions were incubated at 37 °C during 2 h and the digested products were visualized by agarose gel electrophoresis at 1.5%, stained with ethidium bromide, visualized, and photographed using a Gel Logic 212 PRO photodocumenter (Carestream).

In addition, insects from the species *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae), *Hypsipyla grandella* (Zeller, 1848) (Lepidoptera: Piralidae), *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae), *Chrysodeixis includens* (Walker, 1858) (Lepidoptera: Noctuidae), and *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Noctuidae) from the insect breeding program at Embrapa Recursos Genéticos e Biotecnologia were also used as controls for marker validation.

Results

The alignment of the 16 sequences corresponding to the COI gene selected in this study generated a consensus sequence of 658 bp, and the alignment of the 11 sequences corresponding to the EF-1α gene generated a consensus sequence of 1240 bp. The designed primers for the COI gene annealed between nucleotides 27 to 47 at the 5' end for primer COIF and between nucleotides 622 to 641 at the 3' end for primer COIR. The analysis of restriction sites that cleaved the COI sequence once indicated a restriction site for the *Hpa*I endonuclease in the sequence corresponding to the species *H. gelotopoeon* and a restriction site for the *Bfa*I endonuclease in *H. armigera*, with no restriction sites for these two enzymes found in the other two species. For the EF-1α gene, the designed primers annealed between nucleotides 40 to 59 at the 5' end for primer EF1F and between nucleotides 1127 to 1146 at the 3' end for primer EF1R. The analysis of restriction sites that cleaved the EF-1α sequence once indicated a restriction site for the *Eco*RV endonuclease in the sequence corresponding to the species *C. virescens*, with no restriction sites for this enzyme found in the *Helicoverpa* species. The application of both set of primers in the same PCR conditions with the designed primers (Table 2) produced amplification fragments of 615 bp for the cytochrome oxidase subunit I gene and 1,106 bp for the nuclear elongation factor Iα gene (Fig. 1) for the four species. It was observed that the applied methodology allowed the use of both larvae and adults, regardless of the insect's gender, for obtaining molecular profiles.

Table 2. Characteristics of primers for the identification of four Heliothine species.

Gene	Primers sequences (5' ➔ 3')	Tm (°C)	Length (pb)
Cytochrome oxidase subunit I (COI)	COIF: GAG CWG GAA TAG GAA CTT COIR: AAA TAG GAT CWC CTC CTC CA	55	615
Nuclear elongation factor I (EF-1α)	EF1F: GAT CTA CAA ATG CGG TGG TA EF1R: GAC TTG ATG GAC TTA GGG YY	55	1106

W = T (Thymine) or A (Adenine).

The other Lepidoptera, i.e., *A. gemmatalis*, *C. includens*, *H. grandella*, *P. xylostella*, and *S. frugiperda* species used in this study did not produce any PCR amplification products for the two molecular markers, demonstrating the specificity of these PCR primer markers.

Following PCR, the amplification products were subjected to digestion with the restriction enzymes *Bfa*I and *Hpa*II for the COI gene. In the *Bfa*I digestion, the results obtained were fragments of 294 bp and 321 bp for *H. armigera*, while *H. zea* and *C. virescens* maintained the same size as the original PCR product. The *H. gelotopoeon* produced a PCR fragment with approximate size of 615 bp. As for the *Hpa*II enzyme, fragments of 58 bp and 557 bp were obtained for *H. gelotopoeon*, but *H. armigera*, *H. zea*, and *C. virescens* maintained the total size of the original fragment. The PCR product of the EF-1α gene was cleaved using the *Eco*RV enzyme, resulting in fragments of 142 bp and 964 bp for *C. virescens*, and no cleavage of the fragments from the other heliothines (Fig. 2).

Based on the data presented, it was possible to formulate a dichotomous key for distinguishing the main heliothine pests of Brazilian agriculture through molecular techniques (Table 3).

Table 3. PCR-RFLP patterns for the identification of four Heliothine pest species in Brazil.

Species	EF-1α (1,106 bp)	EF-1α + *Eco*RV (142 bp and 964 bp)	COI (615 bp)	COI + *Hpa*II (557 bp and 58 bp)	COI + *Bfa*I (294 bp and 321 bp)
H. armigera	√		√		√
H. zea	√		√		
H. gelotopoeon	√		√	√	
C. virescens	√	√	√		

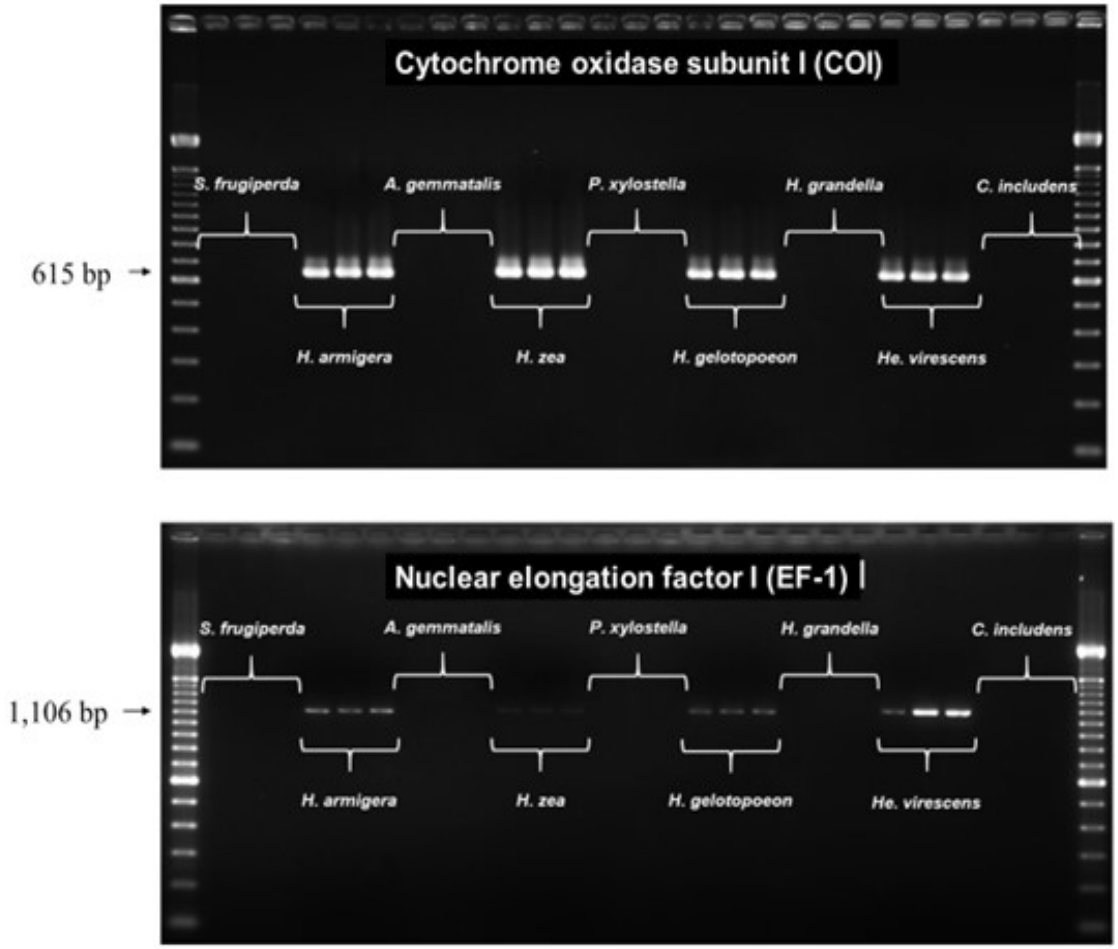

Fig. 1. 1.5% agarose gel showing the 1,106 bp amplification product corresponding to the EF-1α gene (top) and the 615 bp product for the COI gene (bottom). The DNA marker used was the 100 bp ladder (Invitrogen).

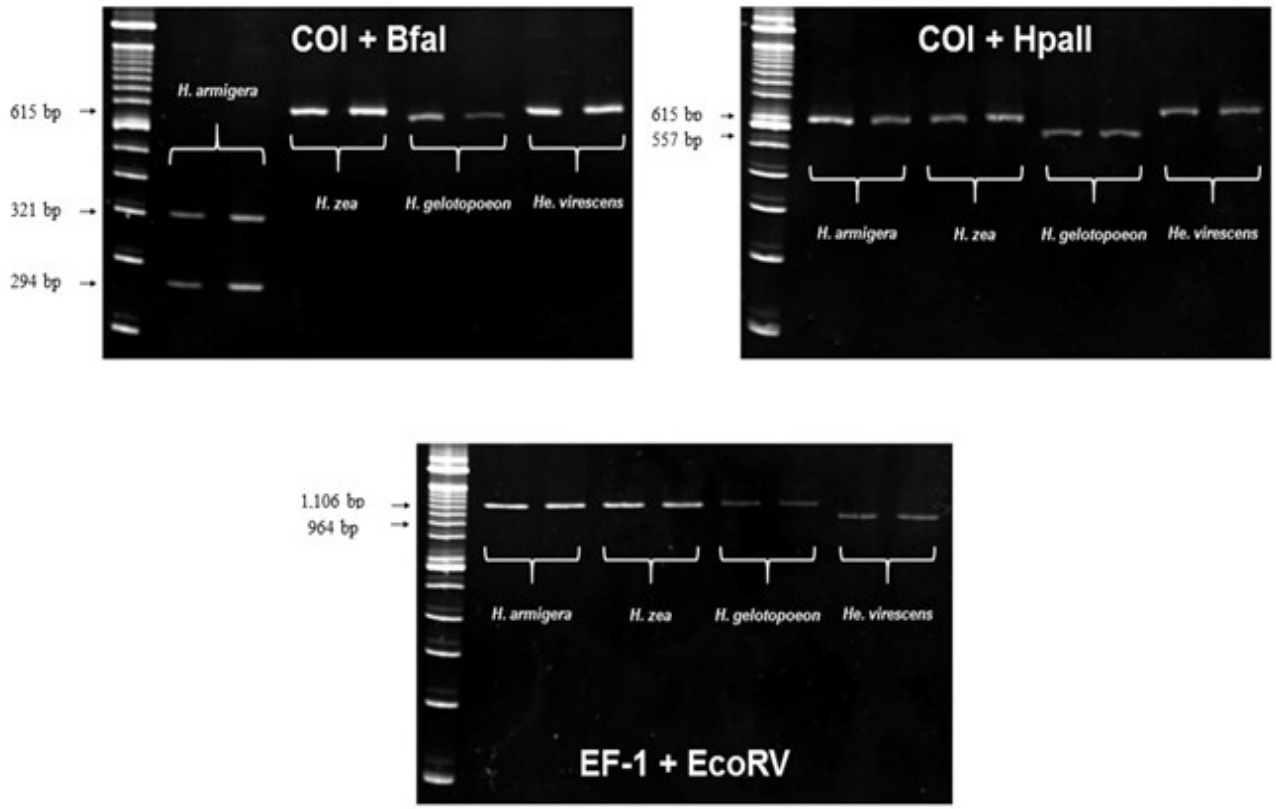

Fig. 2. PCR-RFLP restriction profiles for the COI gene (top) and EF-1 gene (bottom) in individuals belonging to the heliothine group. Visualization of digestion products was done on a 12% SDS-PAGE gel.

Discussion

DNA-based strategies are becoming increasingly necessary and prevalent in everyday agriculture (Behere *et al.*, 2008; Tsai *et al.*, 2020). The difficulty in distinguishing some agricultural pests, especially in their larval stage, coupled with the limited availability of taxonomic experts for certain lepidopteran species, necessitates the use of molecular tools to aid in morphological characterization and in cases where morphology alone may not be sufficient to differentiate individuals from different species. The molecular techniques applied in our study using larvae and adult female and male moths proved to be suitable for obtaining electrophoretic profiles. Our results were similar to those found by Behere *et al.* (2006), who used larvae, adults, and pupae for his molecular analyses of the mitochondrial DNA of *H. armigera* and *H. zea*, demonstrating that the molecular techniques adopted in this study are applicable to any stage of insect growth.

Among the Heliothinae subfamily, such as *C. virescens*, nuclear genes can be used for species discrimination (Mitchell & Gopurenko, 2016), as demonstrated in this study. The use of the nuclear gene EF-1α made it possible to differentiate *C. virescens* from the species within the *Helicoverpa* genus that were studied. These findings are supported by the study of Che *et al.* (2017), who demonstrated that nuclear molecular markers applied to beetle studies are easier to use and provide more informative phylogenetic information than other types of markers, showing great potential to expedite resolution of taxonomic issues. In our study, the EF-1 marker allowed us to discriminate *C. virescens* from other species of *Helicoverpa*. This is important in field situations where pupae or fragmented insects found may not allow for complete identification of the insect immediately. Thus, we applied a strategy that enables differentiation of a specimen at the genus level.

For the molecular differentiation of the three species within the *Helicoverpa* genus, it was necessary to use the mitochondrial gene COI (Walsh *et al.*, 2019), which, through SNPs among the studied species, allowed for the development of an easily usable and efficient molecular marker for distinguishing *Helicoverpa* species. These

molecular markers represent a complementary tool for entomologists, aiding in the discrimination between *H. zea*, *H. armigera*, *H. gelotopoeon*, and *C. virescens*.

They can also be used as a tool for monitoring the spread of these pests in agricultural regions, including border areas (Walsh *et al.*, 2019). They can be used preventively for the identification of exotic pests, such as *H. gelotopoeon*, which is prevalent in Argentina (Murúa *et al.*, 2014).

Furthermore, in cases where intact larvae and/or adults are not available for morphological identification, the use of this dichotomous key (Table 3) allows for the differentiation of Heliothine species occurring in major agricultural crops, providing essential support for the establishment of sampling systems and decision-making within the framework of integrated pest management (Dean *et al.*, 2021). It is worth noting that in our work, we optimized the PCR reactions so that we can use both sets of primers under the same reaction and amplification conditions, ensuring rapid acquisition of the PCR products. Additionally, in our study, we were able to differentiate *H. gelotopoeon* from the other species through a single digestion reaction using the restriction enzyme *Hpa*II. Arneodo *et al*. (2015), applied the concept of Behere *et al*. (2007) and developed an RFLP method to assist with the rapid differentiation between New World *H. zea*, *H. gelotopoeon*, and *H. armigera*. However, both Behere *et al*. (2008) and Arneodo *et al*. (2015) used different mtDNA COI gene regions, and identification by PCR-RFLP between these *Helicoverpa* pest species would therefore require different PCR amplicons.

The molecular markers can promote a deeper understanding of insect diversity and ecology (Avise, 2004), and the information provided in this work will be useful for analyzing the dispersion of the *Helicoverpa* complex in the core agricultural lands of Brazil.

This study demonstrates the efficacy of using molecular markers in the field of entomological taxonomy, particularly within the Heliothinae subfamily. Employing techniques such as PCR-RFLP for the identification of *Helicoverpa* and *Chloridea* species, especially during their larval stages, represents a notable advancement.

The construction of specific primers for amplifying the COI and EF-1α genes and their subsequent digestion with enzymes like *Bfa*I, *Hpa*II, and *Eco*RV, yielded distinct DNA fragment profiles, enabling precise identification of species such as *H. armigera*, *H. zea*, *H. gelotopoeon*, and *C. virescens*. The research also paves the way for future investigations, where these molecular markers can be used to enhance the field of entomology and crop protection.

Author's Contributions

Paulo Roberto Martins Queiroz: Conceptualization, Methodology, Investigation, Draft Preparation, Final Review, and Editing; **Elias Ferreira Sabiá Júnior**: Methodology, Formal Analysis, Investigation, Draft Preparation, and Visualization. **Érica Soares Martins**: Supervision and Project Administration. **Rose Gomes Monnerat**: Supervision, Funding Acquisition, and Project Administration.

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Data Availability Statement

All data supporting the findings of this study are available within the paper.

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Ethics Approval

Only insects were used in this study. All applicable international, national, and institutional guide lines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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شناسایی مولکولی گونه های آفت Heliothine (Lep. Noctuidae) با مشخصات PCR-RFLP هسته‌ای و میتوکندریایی

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چکیده

آرایه‌بندی زیرخانواده Heliothinae پیچیده است، به‌ویژه شناسایی این گونه‌ها در مرحله لاروی با مانع مواجه می‌شود. روش جایگزین، استفاده از روش‌های مولکولی مانند تکنیک PCR-RFLP (یا پلی‌مورفیسم طول قطعه محدود) است که به کمک و تکمیل شناسایی برخی از گونه‌های Heliothinae کمک می‌کند. هدف از این مطالعه ایجاد مشخصات PCR-RFLP با استفاده از نشانگرهای مولکولی برای شناسایی چهار گونه Heliothinae بود. آغازگرهای اختصاصی برای تکثیر ژن‌های سیتوکروم اکسیداز زیر واحد یک (COI) و فاکتور افزایش طول ۱ آلفا (EF-1α) برای شناسایی گونه‌های *Helicoverpa armigera*، *H. zea*، *H. gelotopoeon* و *Chloridea virescens* ساخته شدند. در هضم ژن COI با آنزیم *Bfa*I، قطعات ۲۹۴ جفت باز و ۳۲۱ جفت باز برای *H. armigera* به دست آمد، در حالی که برای *H. zea*، *H. gelotopoeon* و *C. virescens* هیچ هضمی وجود نداشت. آنزیم *Hpa*I قطعات ۵۸ جفت باز و ۵۵۷ جفت باز برای *H. gelotopoeon* تولید کرد، در حالی که هیچ هضمی برای *H. armigera*، *H. zea* و *C. virescens* وجود نداشت. هضم ژن EF-1α با آنزیم *Eco*RV قطعات ۱۴۲ جفت باز و ۹۶۴ جفت باز برای *C. virescens* تولید کرد، در حالی که برای سایر گونه‌ها هیچ شکافی وجود نداشت. این نشانگرهای مولکولی می توانند به حشره شناسان برای تمایز بین گونه‌های *H. zea*، *H. armigera*، *H. gelotopoeon* و *C. virescens* کمک کنند و همچنین می‌توانند به عنوان ابزاری برای نظارت بر گسترش این آفات در مناطق کشاورزی استفاده شوند.

کلمات کلیدی: *Chloridea*، سیتوکروم اکسیداز زیرواحد I، تنوع ژنتیکی، *Helicoverpa*، ژن EF-1α

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