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Molecular markers to discriminate among four pest species of *Helicoverpa* (Lepidoptera: Noctuidae)

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Abstract

The four significant pest species in the *Helicoverpa* genus (*H. armigera*, *H. assulta*, *H. punctigera* and *H. zea*) are morphologically similar and can only be reliably distinguished through dissection of adult genitalia. Two partial regions of the mitochondrial DNA (mtDNA), the cytochrome oxidase subunit I (COI) and the cytochrome *b* (Cyt *b*) genes were amplified by PCR and digested with restriction endonucleases. The restriction patterns, generated by the endonucleases *BstZ*17I and *Hph*I, demonstrated reliable differentiation of the four *Helicoverpa* pest species. This technique is fast, reliable and effective at distinguishing specimens irrespective of their life stages and offers support to conventional taxonomic differentiation based on morphological characters.

Keywords: molecular species diagnosis, PCR-RFLP, mitochondrial DNA, *Helicoverpa*

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Introduction

Helicoverpa is an important lepidopteran genus containing 18 species, of which four species, *H. armigera* (Hübner), *H. zea* (Boddie), *H. punctigera* (Wallengren) and *H. assulta* (Guenée), are significant agricultural pests and collectively cause economic losses in billions of US dollars annually. Other species in this group can be pests, but they are either greatly limited in host plant range or in geographic distribution. *H. armigera* has the widest distribution, occurring throughout Asia, Africa, Europe and Australasia. The very closely related *H. zea* (Mitter *et al*., 1993; Behere *et al*., 2007) occurs in North and South America, while *H. punctigera* is endemic to Australia. Species identification based on external morphology of these noctuid moths is difficult, even using forewing morphology (Matthews, 1999). Evolutionary development within the *Helicoverpa* genus is more readily seen in changes to the morphology of genitalia (Hardwick, 1965). Various molecular and biochemical methods have been proposed for species diagnosis in this genus. *H. armigera* can be differentiated from *H. punctigera* based on a DNA-PCR of the ITS region (Pearce, 2003), allozyme electrophoresis (Daly & Gregg, 1985) and immunoassay (Trowell *et al*., 2000). Distinguishing between *H. assulta* and *H. armigera* is possible based on PCR-RFLP (Kranthi *et al*., 2005) and AFLP markers (Ming & Wang, 2006). Monoclonal antibodies raised against *H. zea* can distinguish, *inter alia*, *H. armigera* from *H. punctigera* (Greenstone *et al*., 1991). However, currently there are no uniform molecular DNA techniques that can easily separate the four pest species from each other (i.e. *H. zea* from *H. armigera* and *H. assulta*, or *H. assulta* from *H. Punctigera*).

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Table 1. Number of haplotypes and variable sites detected at partial COI (511 bp) and Cyt *b* (434 bp) in the four *Helicoverpa* pest species.

Species	Sample size	COI		Cyt *b*	
		Number of Haplotypes	Variable sites	Number of Haplotypes	Variable sites
H. armigera	249	33	32	26	22
H. zea	64	11	9	20	15
H. punctigera	30	8	9	11	10
H. assulta	5	2	1	2	2

Here, we report on a life stage-independent PCR-RFLP molecular DNA technique based on two partial regions of the mitochondrial DNA (mtDNA) genome for distinguishing between *H. armigera*, *H. zea*, *H. punctigera* and *H. assulta*.

Materials and methods

Samples and DNA extraction

Samples of *H. armigera* ($n = 249$), *H. zea* ($n = 64$), *H. punctigera* ($n = 30$) and *H. assulta* ($n = 5$) were field-collected from various continents. Collection details and DNA extraction protocols are as described in Behere *et al*. (2007). Briefly, *H. armigera* were obtained from Australia ($n = 56$; larvae and moths), China ($n = 34$; larvae), Burkina Faso ($n = 35$; larvae), Uganda ($n = 24$; larvae), India ($n = 90$; larvae, pupae and moths) and Pakistan ($n = 10$; larvae). *H. punctigera* were collected from Australia and *H. assulta* were collected from India. *H. zea* were from the USA ($n = 34$; larvae and moths) and Brazil ($n = 30$; moths). *H. punctiger*a and *H. assulta* were collected as moths and larvae, respectively. Additionally, eggs from laboratory-reared *H. armigera* (collected from Australia) at different stages of maturation (one to four days old) were placed individually in 1.7 ml sterile Eppendorf tubes, and DNA was extracted using the DNeasy® Tissue Kit (Qiagen, Cat. No 69504). DNA from eggs was eluted in 20 µl elution buffer and stored at −20°C until needed.

PCR and sequencing

PCR and sequencing primers for cytochrome oxidase I (COI) and cytochrome *b* (Cyt *b*) were designed from GeneBank sequences (accession numbers, AY437834, AY437835, DQ059302 and AF467260) using the primer designing program Oligo 6.4 (Molecular Biology Insights, Inc.). Two mtDNA primer sets were designed for COI and Cyt *b*. The primer set COI-F02 (5′ CTC AAA TTA ATT ACT CCC CAT C 3′) and COI-R02 (5′ GGA GGT AAG TTT TGG TAT CAT T 3′) was used to amplify 511 base pairs (bp) of partial COI. The primer set Cytb-F02 (5′ GAA TCC TTT AAT TTA AAA TAT AC 3′) and Cytb-R02 (5′ AAA TAT GGG TTA GTT AAA GTT AA 3′) was used to amplify 434 bp of partial Cyt *b*. PCR conditions had the following profile: 94°C for 4 min (one cycle), 30 s each of 94°C, 50°C and 1 min at 72°C (35 cycles) followed by a final extension cycle of 72°C for 10 min. The proof of DNA amplification was confirmed by running out 5 µl of the post-PCR volume on 1.5% agarose gels. PCR amplification of individual DNA samples was carried out in a total of 25 µl reaction volume and contained approximately 25 ng of genomic DNA, 0.2 µM each of forward and reverse primers, 0.2 mM of dNTP's, 1 × PCR reaction buffer (Promega), 1.5 mM Mg^{2+} and one unit of *Taq* DNA polymerase (Promega). We directly sequenced all 348 individuals for Cyt *b* from both ends for accuracy. The sequencing protocol was as reported in Behere *et al*. (2007). COI sequences (GenBank Accession numbers: EF116226-EF116274) of all samples were as reported in Behere *et al*. (2007) with the exception of *H. punctigera* (accession numbers EF410014-EF410019) because these represented additional six haplotypes from 25 new samples detected in this study. All Cyt *b* nucleotide sequences obtained were submitted to the National Centre for Biotechnology Information (NCBI; GenBank Accession numbers EF410020–EF410078). Partial COI and Cyt *b* DNA fragments of the *H. armigera* egg samples were not sequenced.

Restriction fragment length polymorphism analysis

Restriction sites were predicted from the COI and Cyt *b* sequence data using NEBcutter V2.0 <http://tools.neb.com/NEBcutter2/index.php>. For each sample, 5 µl of PCR product was digested with two units of restriction enzyme (*Bst*Z17I or *Hph*I) in a 20 µl reaction volume according to the manufacture's instructions (New England Biolabs). Following incubation at 37°C for 6 h, the digested products were separated by electrophoresis on 2.5% 1 × TAE agarose gels at 180V for 30 min and stained with ethidium bromide. In order to confirm restriction band sizes accurately, the digested products were also tested on 6% polyacrylamide gels. The size of restriction fragments was estimated using a Kodak® Electrophoresis Documentation and Analysis System (EDAS 290) by comparison with the BenchTop pGEM® DNA Ladder molecular weight standard (Promega).

Results

A total of 348 individuals from the four agriculturally significant *Helicoverpa* pest species were sequence-analysed. For each individual, partial COI (511 bp) and Cyt *b* (434 bp) genes were obtained from PCR amplification. DNA from all individuals tested in this study was amplified successfully, including the DNA extracted from single eggs. The numbers of haplotypes and of variable sites identified at COI and Cyt *b* in all species are presented in table 1. Within partial COI sequences, a single base pair mutation was found at the *Bst*Z17I recognition site (GAATAC) in *H. zea* and *H. assulta* but not in *H. armigera* and *H. punctigera* (GTATAC). Restriction endonuclease digest of the 511 bp COI PCR product, therefore, gave two bands of 318 bp and 193 bp in *H. armigera* and *H. punctigera* but not in *H. zea* and *H. assulta* (fig. 1).

Within the 434 bp of Cyt *b* sequences from the four pest species, the recognition site for *Hph*I (GGTGA $(N)_8$) was present in *H. armigera* and *H. zea* but absent in *H. assulta*

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Table 2. Restriction fragment length polymorphism (RFLP) patterns at COI (511 bp) and Cyt *b* (434 bp) in pest species of *Helicoverpa*.

Helicoverpa species	COI (511 bp) fragment digested with restriction enzyme *Bst*Z17I		Cyt *b* (434 bp) fragment digested with restriction enzyme *Hph*I	
	318 bp & 193 bp	511 bp	280 bp & 154 bp	434 bp
H. armigera	✓		✓	
H. zea		✓	✓	
H. punctigera	✓			✓
H. assulta		✓		✓

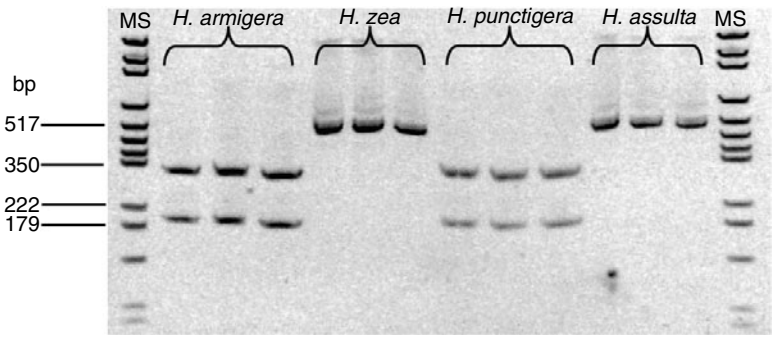

Fig. 1. Partial COI (511 bp) RFLP pattern (digested with *Bst*Z17I) of *H. armigera*, *H. zea*, *H. punctigera* and *H. assulta*, with bands separated on 6% polyacrylamide gel. Lanes labeled as MS are pGEM molecular weight standard DNA ladders.

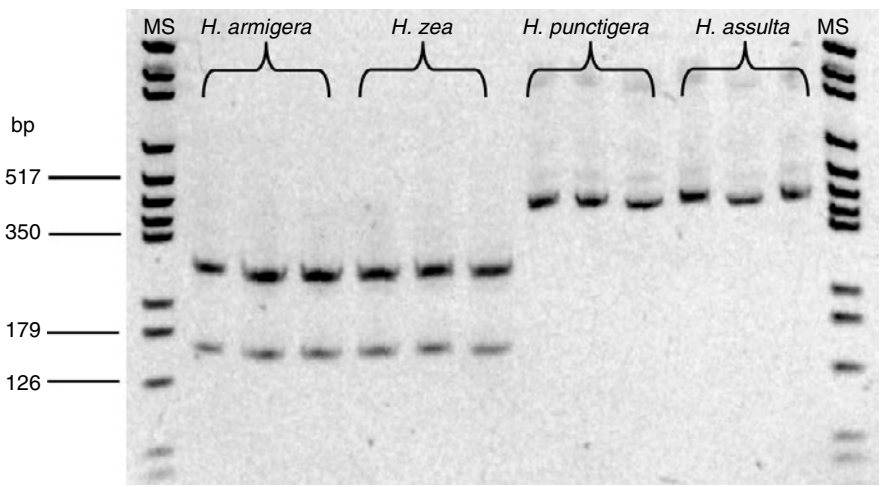

Fig. 2. Partial Cyt *b* (434 bp) gene amplified from *H. armigera*, *H. zea*, *H. punctigera* and *H. assulta* and restriction digested with *Hph* I, with bands separated on 6% polyacrylamide gel. Lanes labeled as MS are pGEM molecular weight standard DNA ladders.

(GATGG) and *H. punctigera* (GATGT). Restriction digestion of the 434 bp Cyt *b* PCR product, therefore, resulted in two bands of 280 bp and 154 bp in *H. armigera* and *H. zea* (fig. 2, table 2). Restriction banding patterns generated were easily visualised on 2.5% agarose gels. Digested PCR amplicons fragment sizes estimated from 6% polyacrylamide gels were also confirmed by DNA sequencing data.

Discussion

Species separation based on PCR-RFLP patterns at a different partial mtDNA COI region has previously been demonstrated for *H. armigera* and *H. assulta* (Kranthi *et al*., 2005), although the sample sizes tested for *H. armigera* and *H. assulta* were smaller than in this study. In China, AFLP markers have been proposed for identification of these two species (Ming & Wang, 2006). Combining restriction digest patterns of partial mtDNA COI and Cyt *b* genes with the two endonucleases allowed differentiation of the four pests *Helicoverpa* species and the tested life stages (egg, larva, pupae and adults) in *H. armigera*. We have not specifically tested all four life stages in the other three *Helicoverpa* pest species, although results from *H. armigera* indicated this to unlikely represent significant problems.

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The accuracy of RFLP band sizes was initially determined on 6% polyacrylamide gel electrophoresis (PAGE) and confirmed by sequencing data. All remaining samples were screened on 2.5% agarose gels. Although 6% PAGE provided better resolution for smaller fragments (i.e. less than 200 bp), (figs 1 and 2), we were able to easily score the 154 bp fragment in *H. armigera* and *H. zea* as digested by *Hph*I on 2.5% agarose gels (figures not shown). For routine identification, the use of agarose gels is preferred over PAGE, as this considerably reduces chemical and reagent costs and is non-toxic. The PCR amplification protocol and primer sets described in this study are very robust, as have been demonstrated in cross-species PCR amplification. Although PCR amplification can facilitate the use of minute quantities of DNA template from samples that have not been optimally preserved (Taylor & Szalanski, 1999), we recommend only using well-preserved samples (i.e. consistently stored at between −20°C to −70°C or kept in a sufficient volume of ≥95% ethanol) in PCR-RFLP analyses of these four pest *Helicoverpa* species. The procedures described in this study, from DNA extraction, digestion, gel electrophoresis to species identification, can be accomplished within a single working day. The use of more than one restriction endonuclease will further minimise species misidentification.

In this study, we have sequenced only five *H. assulta* individuals and identified only two mtDNA haplotypes. The PCR-RFLP patterns for *H. armigera*, *H. zea* and *H. punctigera* based on the partial COI and Cyt *b* gene regions were clearly distinguishable from those of *H. assulta*, while phylogenetic analysis based on the COI region (Behere *et al*., 2007) clustered the two *H. assulta* mtDNA haplotypes together after 1000 bootstrap re-sampling analyses with a very high support value (98%). Nevertheless, it would be desirable to increase our *H. assulta* sample size, especially from other parts of Asia (e.g. China) and from Australia and Africa. Performing a nucleotide–nucleotide BLAST search (BLASTN: Altschul *et al*., 1997), using the two *H. assulta* mtDNA COI haplotypes detected in this study (EF116270, EF116271) against the non-redundant (nr) DNA database, showed no overlapping with the *H. assulta* partial COI sequence (AY264943) submitted by Kranthi *et al*. (2005) at the *Bst*Z17I restriction enzyme recognition site; while the *H. armigera Bst*Z17I restriction site identified in this study from all 33 haplotypes further matched an additional *H. armigera* sequence available in GenBank (AY437834).

Given the differential response in tolerance to insecticides among these pest species, the ability to distinguish pest species at the egg and early instar larval life-stages has important implication for pest management because insecticides are usually most effective at these life-stages. Air-freight transportation of agricultural commodities between continents is an ever-increasing activity, especially with vegetables and ornamentals. Almost every year, *H. zea* caterpillars are intercepted on agricultural and horticultural produce to the UK (Seymour, 1978). Regular interception of *H. armigera* or *Helicoverpa* species in the USA have been commonly reported, with Venette *et al*. (2003) reporting 4431 interceptions in the USA since 1985 on fruits, vegetables, ornamentals and other miscellaneous plants and Pouge (2004) adding a further 20. The risk assessment study conducted by Venette *et al*. (2003) gave a high potential entry and establishment rating for *H. armigera* into the North American continent. Due to the extreme similarity of *H. zea* and *H. armigera* at larval stages, it is extremely difficult to separate these pest species without rearing larvae to adulthood followed by examining genitalia of adults through dissection (<http://www.eppo.org/QUARANTINE/insects/Helicoverpa_zea/HELIZE_ds.pdf>; EPPO data sheets on Quarantine Pests, *Helicoverpa zea*). For quarantine purposes, PCR primers must faithfully amplify samples stored under a variety of conditions. The mtDNA diagnostic primers reported in this study have been designed, based on highly conserved mtDNA regions, to amplify short fragments of COI and Cyt *b* genes and, hence, to take advantage of greater efficiencies of PCR at amplification of short DNA fragments (Muraji & Nakahara, 2002).

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