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Comparison of Amino Acid Sequences of Halloween Genes in *Spodoptera litura* and *Spodoptera littoralis*

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Abstract

20-Hydroxyecdyone (20E), an active form of ecdysteroid, is the key hormone in insect growth and development. To date five P450 enzymes involved in the last four steps of ecdysteroid biosynthesis from ketodiol to 20E namely Spook (Spo), Phantom (Phm), Disembodied (Dib), Shadow (Sad) and Shade (Shd) related to ecdysteroid biosynthesis, are identified and the character of last four enzymes is well studied in *Drosophila melanogaster*, *Bombyx mori* and *Manduca sexta*. All of these genes are called Halloween genes. In this study, we extended these works to a major pest insect in agriculture in India, the cotton leafworm *Spodoptera litura* (Lepidoptera: Noctuidae). We identified the sequence of five Halloween genes partially and converted into amino acid sequences and then three of them were compared with a very near African species of *Spodoptera*, *Spodoptera littoralis*. The results suggest amino acid substitution in open reading frame of phantom, shade and disembodied protein in *Spodoptera litura*.

Keywords

Halloween Genes, Amino Acid

1. Introduction

Halloween genes are a set of genes, Spook (Spo), Phantom (Phm), Disembodied (Dib), Shadow (Sad) and Shade (Shd) identified in *Drosophila melanogaster* that influence embryonic development. All the above genes code for cytochrome P450 enzymes. These are strongly implicated in the biosynthesis of ecdysone (E) from cholesterol (ecdysteroidogenic pathway). Ecdysteroids such as 20-hydroxyecdysone and ecdysone influence many of the important morphological, physiological, biochemical changes that occur during molting in insects. Identifi-

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cation and expression profile of Halloween genes involved in ecdysteroid biosynthesis in *Spodoptera littoralis* was studied by Iga *et al.* [1]. In addition to insects, the Halloween genes are also identified in the crustacean genome of *Daphnia pulex* suggesting a high conservation for ecdysteroid biosynthesis in the Arthropoda phylum. A phylogenetic tree is displayed in **Figure 1** which shows the conserved nature of Halloween genes of insects of different orders. A high similarity can be traced within order Lepidoptera. In this paper, I will focus on the Halloween genes that control the ecdysteroid biosynthesis pathway to build up a peak titer of 20E hormone. These genes were selected for amplification in *Spodoptera litura* and then converted into protein sequences. Then the protein sequences of *Spodoptera litura* were compared with near African species of *Spodoptera*, *Spodoptera littoralis* to find if any changes occur in amino acids in these conserved regions of Halloween genes. These genes are functionally conserved across the different insect orders. So within same genus, it is not expected to have changes in amino acids in their protein sequences.

2. Materials and Methods

Spodoptera litura (Tobacco caterpillar) was cultured in the lab in special cylindrical mating chambers. The average life cycle of *Spodoptera litura* is 25 days and they need small place for mating. The young caterpillars from hatched eggs were fed with artificial diet in all the months except winter. The artificial diet was standardized in the lab. In winter they were fed with cabbage leaves. When they were transformed into adult moth, both the males and females were fed with dilute honey and kept in the mating chamber for mating. After 7 - 10 days, the female hatched plenty of eggs in small cut tissue paper kept inside the mating chamber. The hatched larvae were reared and allowed to feed in small petri-plate and were grown inside temperature maintained BOD.

Isolation of RNA: RNA was extracted from *Spodoptera litura* by using pure link RNA mini kit (Ambion) and first-strand cDNA was reverse transcribed using 1 µg of total RNA by Reverse aid first strand cDNA synthesis kit (Thermo-scientific) and further processed for Halloween gene amplification (**Table 1**).

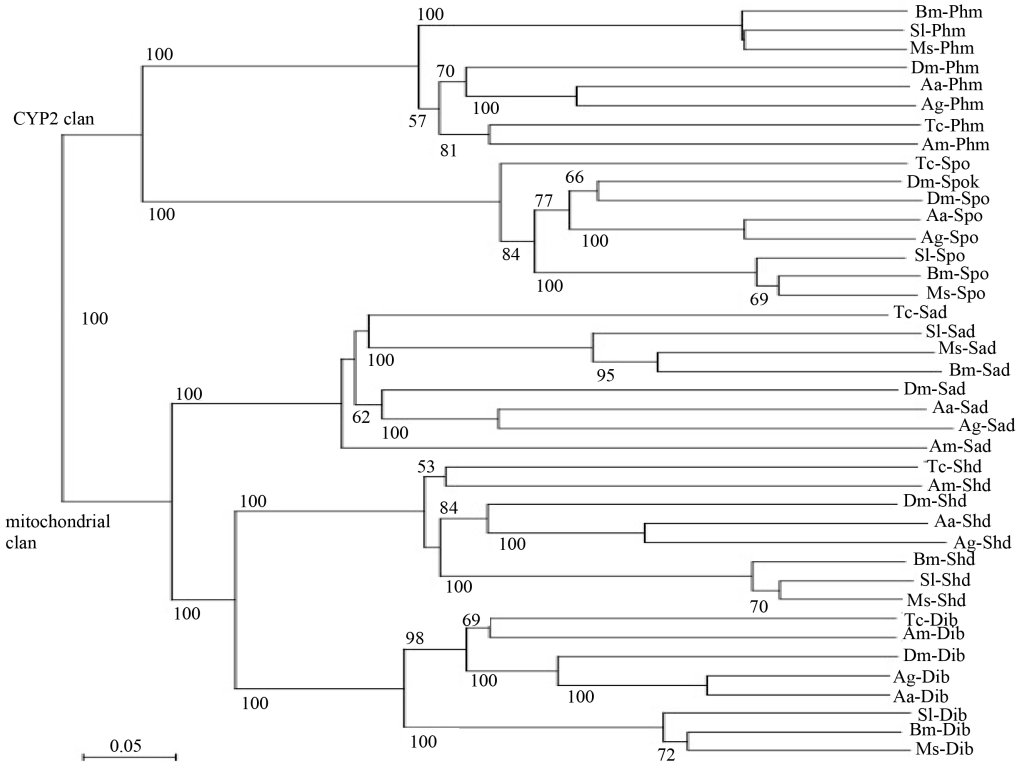

Figure 1. Phylogenetic tree of Halloween genes. The tree was generated based on the whole amino acid sequences by neighbor-joining method using ClustalX multiple alignment program with a bootstrap value of 1000 trials for each branch position by Iga *et al.*, 2010. Aa: *Aedes aegypti*, Ag: *Anopheles gambiae*, Am: *Apis mellifera*, Bm: *Bombyx mori*, Dm: *Drosophila melanogaster*, Ms: *Manduca sexta*, Sl: *Spodoptera littoralis*, Tc: *Tribolium castaneum*.

Table 1. Halloween genes.

Gene name	Primer name	Primer sequence (5' - 3')	Targeted region (bp)
Spook (Spo)	Forward	TTGGCGGTCACTCTTCTGTC	613
	Reverse	TGGCGCAAATCTTCCAGACT	
Phantom (Phm)	Forward	ATCATCGATCGGCGACGAAA	857
	Reverse	TATGAAACGGTTGGCGGTGA	
Disembodied (Dib)	Forward	ATGGGAGTGGCAAGACACAG	409
	Reverse	TTAGCACTTTTCGGGCTGGT	
Shadow (Sad)	Forward	CAGTTTGTTTGGGGACGCAG	1000
	Reverse	ATCACCAGCAGCGATGACAA	
Shade (Shd)	Forward	ATTGTGCCTGGCTTCGATCA	594
	Reverse	TAGCCTGGCCAAGAAAGGTG	

The NCBI database was extensively searched and *Spodoptera littoralis* cytochrome P450 mRNA, complete cds for all the genes were obtained. Gene-specific primers used for amplification of the target genes were prepared by primer designing software available in NCBI. The sequence specificities of the primer sequence so designed were verified using the BLAST program available at the NCBI website (www.ncbi.nlm.nih.gov/BLAST/). The parameters chosen for the BLAST search include sequence database "blastn". The primer sequence was also evaluated for various other characteristics like melting point, presence of secondary structure formations like hairpins and propensity for dimer formation using software available in internet, Oligonucleotide Properties Calculator (http://www.unc.edu/~cail/biotool/oligo/).

PCR: The PCRs were carried out using *Taq* DNA polymerase (Fermentas) with the following general conditions: 15 - 20 ng of genomic DNA was used in a 20 µl reaction with 5 U/µl of *Taq* DNA polymerase, 2.5 mM each dNTP mix and 10 Pm/µl l of each primer. For Spo and Phm, initial denaturation was at 94˚C for 5 min followed by 94˚C for 30 sec, 35 cycles, annealing at 55˚C for 45 sec, extension at 72˚C for 3 min and final extension was at 72˚C for 10 min. Similarly for Shd, Dib and Sad, initial denaturation was at 95˚C for 10 min followed by 95˚C for 15 sec, 45 cycles, annealing at 45˚C for 1 min, extension at 60˚C for 15 sec and final extension was at 72˚C for 15 sec.

The PCR products (5 µl) were resolved in 1.5% agarose gel which was run at 80 V for 45 minutes. Negative PCR control was also run with double distilled water instead of DNA to eliminate doubts. The amplified products were eluted from the gel and purified by gel purification kit before sending for sequencing. The sequencing both through forward and reverse primers were done by Sanger method through outsourcing.

3. Results and Discussions

Nucleotide sequences of 600 bp, 800 bp, 450 bp, 1100 bp, 600 bp region of the spook, phm, Dib, Sad, Shd respectively of Halloween genes were obtained, blasted against nr sequences in NCBI and confirmed to be halloween genes against *Spodoptera littoralis*, then annotated and submitted to NCBI and reported first time in *Spodoptera litura*. Protein sequences were obtained by ExPASy translational tool in both *Spodoptera litura* and *Spodoptera littoralis.* Open reading frame with maximum length was selected. The sequences for both *Spodoptera litura* and *Spodoptera littoralis* of phantom, shade and disembodied partial gene proteins were aligned in protein alignment tool, SIM alignment tool-ExPASy.

I got accession numbers for phantom and shade from NCBI. They are KP212132 and KP212133. The rest are in progress. The alignment results of the above mentioned three partial gene protein sequences are represented in **Figure 2**.

The alignment results indicate substitution of amino acids in *Spodoptera littoralis* vis *Spodoptera litura*. **Table 2** depicts the substitution of the depicted amino acids in *Spodoptera littoralis* vis *Spodoptera litura* as studied.

In phantom gene protein, glutamine and proline in *Spodoptera litura* are replaced by proline and threonine respectively in *Spodoptera littoralis.* In shade gene protein, phenylalanine, lysine and iso-leucine in *Spodoptera*

PHANTOM:

UserSeq1: ***Spodoptera litura***

UserSeq2: ***Spodoptera littoralis***

```
99.0% identity in 199 residues overlap; Score: 1037.0; Gap frequency: 0.0%

UserSeq1       1 MTDEQMLYLLADMFGAGLDTTSVTLSWFLLYMALYPEEQEIVRKEILSVYPEDGEVDGSR
UserSeq2     334 MTDEQMLYLLADMFGAGLDTTSVTLSWFLLYMALYPEEQEIVRKEILSVYPEDGEVDGSR
                 ************************************************************

UserSeq1      61 LPHLMAAICETQRIRSIVPVGIPHGCLQDTFIGNYRIPKGAMVIPLQWALHMDPDVWEDP
UserSeq2     394 LPHLMAAICETQRIRSIVPVGIPHGCLQDTFIGNYRIPKGAMVIPLQWALHMDPDVWEDP
                 ************************************************************

UserSeq1     121 EVFRPQRFLAEDGSLLKPQEFIPFQTGKRMCPGDELSRMLACGLVARLFRRRRVRLATDP
UserSeq2     454 EVFRPQRFLAEDGSLLKPQEFIPFQTGKRMCPGDELSRMLACGLVARLFRRRRVRLATDP
                 ************************************************************

UserSeq1     181 PSTKDMQGTVGVTLSPQPV
UserSeq2     514 PSTKDMQGTVGVTLSPPTV
                 ***************  *
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SHADE:

UserSeq1: ***Spodoptera littoralis***

UserSeq2: ***Spodoptera litura***

```
98.4% identity in 183 residues overlap; Score: 899.0; Gap frequency: 0.0%

UserSeq1     220 SVCALMLGSRLGFLERWMSGRAATLASAVKAHFRAQRDSFYGAPLWKFAPTTLYRTFAKS
UserSeq2       5 SVCALMLGSRLGFLERWMSGRAATLASAVKAHFRAQRDSFYGAPLWKFAPTTLYRTFAKS
                 ************************************************************

UserSeq1     280 EDTIHTIVSDLMEEAKLKTQKNASDEAMREIFMRILENPALDMRDKKAAVIDFITAGIET
UserSeq2      65 EDTIHTIVSDLMEEAKLKTQKNASDEAMREIFMRILENPALDMRDKKAAFIDFITAGIET
                 ********************************************** **********

UserSeq1     340 LANSLVFLLYLLSVRPDWQRTIRSELPSCSTLTVEDLAAAPSVRAAISEAFRLLPTAPFL
UserSeq2     125 LANSLVFLLYLLSVRPDWQRTIRSKLPSCITLTVEDLAAAPSVRAAISEAFRLLPTAPFL
                 ********************** **** ******************************

UserSeq1     400 ARL
UserSeq2     185 ARL
                 ***
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DISEMBODIED:

UserSeq1: ***Spodoptera littoralis***

UserSeq2: ***Spodoptera litura***

```
95.0% identity in 60 residues overlap; Score: 301.0; Gap frequency: 0.0%

UserSeq1       1 MYSLVSTILHRLCSVIHQFYTHQVCSYDSGTSQYGFSLLGTDDLDGILFLYLLDHKHAGR
UserSeq2       1 MYSLVSTILHRLCSVIHQFYTHQACSYDSGTSQYGFSLLGTDDLVGILFLYLLDHKHARR
                 ********************** ******************** ************* *
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Figure 2. Alignment of amino acid sequences of three partial gene proteins.

Table 2. The differences of amino acids in *Spodoptera litura* and *Spodoptera littoralis*.

Spodoptera litura	Gene protein	*Spodoptera littoralis*
	Phantom	
Glutamine		Proline
Proline		Threonine
	Shade	
Phenylalanine		Valine
Lysine		Glutamic acid
Iso-leucine		Serine
	Disembodied	
Alanine		Valine
Valine		Aspartic acid
Arginine		Glycine

litura are replaced by valine, glutamic acid and serine in *Spodoptera littoralis*. In disembodied gene protein, alanine, valine and arginine in *Spodoptera litura* are replaced by valine, aspartic acid and glycine in *Spodoptera littoralis* respectively.

I got amino-acid substitution in open reading frame which may lead to functional change in proteins and it indicates a change in conserved halloween gene proteins in very close related species of same genus *Spodoptera*. Halloween genes are known for their important function, ecdysteroid biosynthesis which is required in developmental pathway (metamorphosis) of insects. So the changed amino-acids in two species may indicate changes in developmental pathway which is significant in evolutionary point of view. Though evolutional conservation in halloween genes with a high similarity was found in Lepidoptera by Masatoshi Iga *et al.*, change in amino-acid signifies change in protein in very close species of *Spodoptera*.

Similar reports were obtained for accelerated rate of amino acid change in halloween gene proteins in different species of Drosophila [2].

There is also genetic evidence that phantom (Cyp306a1) has been the target of recent natural selection in the *Drosophila melanogaster* [3].

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