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DNA barcoding compliments morphological identification in tomato fruit borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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ABSTRACT: Among Heliothine group of moths *viz., Helicoverpa armigera* (Hübner), *Helicoverpa assulta* (Guenée), *Heliothis peltigera* (Denis & Schiffermüller) and *Helicoverpa rama* (Bhattacharjee and Gupta), *Helicoverpa armigera*, is the most serious pest infesting many economically important crops. Though, *Helicoverpa* infesting vegetables *viz*., tomato, okra, cowpea, amaranthus, cucurbits etc in Kerala, their identity at species level is lacking. Hence, the larvae collected from tomato growing area were reared, preserved and subjected to further studies. Morphological characters *viz*, setal arrangement on prothoracic segment and genitalia structure of both the male and female adult moths were examined. Further, DNA barcoding was carried out to reveal the identity at molecular level. Both morphological characters and DNA barcoding confirmed the identity of species as *Helicoverpa armigera*.
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KEYWORDS: *Helicoverpa armigera,* setae, male and female genitalia, DNA barcoding

INTRODUCTION

Heliothine group of moths include some of the most damaging insect pests of agricultural crops throughout the world. *Helicoverpa armigera* (Hübner), *Helicoverpa assulta* (Guenée) and *Heliothis peltigera* (Denis and Schiffermüller) belong to this group have been recorded from India (Jadhav and Armes, 1996).

Earlier, *Helicoverpa rama distinguished* as a species distinct from within the commonly accepted *H. armigera* (Bhattacharjee and Gupta, 1972), but now it has been synonymyzed

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with *H. armigera*. *Helicoverpa armigera*, popularly known as cotton bollworm or tomato fruit borer or gram pod borer, is the most serious pest among the four species infesting economically important crops in India. It has been recorded on more than 181 plant species from 45 families (Manjunath *et al*., 1989) causing annual loss to the tune of Rs. 2000 crores (Ignacimuthu and Jayaraj, 2003).

In Kerala, the incidence of *H. armigera* was reported on tomato, okra, cowpea, bittergourd and amaranthus (Mathew *et al*., 1996; Levin, 2004; Levin *et al*., 2004). However, its infestation also seen in other vegetables *viz*., okra, cowpea, cucurbits, etc. Pheromone traps (Helilure) set up for monitoring the *Helicoverpa armigera* population in the tomato fields of Thrissur and Palakkad had not collected a single adult moth. Moreover, the existence of three species of Heliothine moths in India prompted us to reveal the identity of species of *Helicoverpa* infesting tomato in Kerala.

Identification of insects based on morphological characters is the core principle of classical insect taxonomy. The larva of *H. armigera* had been distinguished from other related species on the basis of setal arrangement on prothorasic segments (Amate *et al*., 1998). Whereas, both the male and female genitalia structures adult moths were well studied in species of Heliothine moths (Hardwick, 1965; Hardwick, 1970).

The phenotypic plasticity and genetic variability in the character employed for specimen recognition can lead to incorrect identification. Similarly, the morphological keys are often effective for a particular life stages or gender and the use of keys often demands high level of expertise otherwise leads to misdiagnoses. This limitation inherent in the morphology based identification system which can be overcome with the help of novel microgenomic method called DNA barcoding (Hebert *et al*., 2003). This technique involves sequencing a short fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene, the 'DNA barcode,' from a taxonomically unknown specimen and performing comparisons with a reference library of barcodes of known species origin to establish a species-level identification (Wilson, 2012).

In the present study, the morphological characters *viz*., setal arrangement (chaetotaxy) on prothoracic segment in larva and genitalia structures of adult moths were examined. Further, DNA barcoding based on mitochondrial Cytochrome Oxidase1 (mtCO1) gene was carried out to confirm the identity of *Helicoverpa* species infesting tomato.

MATERIALS AND METHODS

Field collection of larva and maintenance of culture

Helicoverpa larvae collected from tomato growing areas of Palakkad and Thrissur were brought into laboratory along with fruits in plastic boxes containing six cavities and each cavity with dimension of 6.5 cm X 6.0 cm X 3.5 cm. Larvae were fed with plant parts for two days and later transferred them into multicavity trays and fed with chickpea based semi synthetic diet (Armes *et al.*, 1992). First to third instar larvae were fed with semi synthetic diet, whereas 4th instar

onwards soaked chickpea seeds were given to them and allowed for pupation. The pupae were sexed and kept in new plastic boxes for adult emergence.

1. Morphological characters

Setal arrangement on prothorax

Ten fifth instar larvae from above culture were selected and immobilized them by exposing to ethyl acetate (100%). The killed larvae were then transferred to glass vials containing ethyl alcohol (95%) to preserve the same for long period. The preserved specimen was transferred onto a glass slide and the position of setae on prothoracic segment was observed through stereo binocular microscope (Labomed®). The image of setae on prothoracic segment was captured using microscope with image analyser software (Leica®) and compared with setal map and diagnostic key of *Helicoverpa armigera* developed by Amate *et al*. (1998).

Adult genitalia structures

Both the male and female adult moths were selected and killed them by exposing to ethyl acetate (100%). The abdomen of adult male moth of *Helicoverpa* was detached with blunt forceps and dropped in ethyl alcohol (95%) for one minute and transferred into a labelled test tube containing 10 per cent potassium hydroxide (KOH). The test tube was kept over spirit lamp for 10-15 minutes and the boiled specimen was allowed to cool for 10-20 minutes. The softened abdomen was placed in a Petri dish containing distilled water to remove KOH.

The soft abdominal skin was cleared out with stainless steel needles. Base of the abdomen was held with straight forceps and pressed gently with round end of curve tipped forceps from base to apex and extruded the entire genitalia. This process was carried out carefully without damaging the aedeagus. The genitalia was placed on a glass slide with drops of water and the genital valves were stretched out with the help of needles, the *harpes* were opened using micro pins to obtain a full face view of inner structures. The genitalia were stained with acid fuschin for two minutes and the excess stain was removed. Drops of Canada balsm were added above the genitalia and xyline was used to remove the air bubble trapped inside the mountant. Cover slip was placed on the slide gently and the glass slide was allowed to dry for one to two days at room temperature, sealed the sides of cover slip with nail polish. The prepared glass slide of genitalia was used for microscopic study.

Compared to the dissection of male genitalia, female genitalia required a different approach in order to have the internal parts exposed. After initial procedures as that of male genitalia, female abdominal skin between 6^{th} and 7^{th} segments was removed with needles; utmost care was taken not to tear the bursae. The extruded female genitalia was then placed on a glass slide with little water, subsequently stained and slide prepared similar to that of male genitalia for comparison.

2. DNA barcoding of tomato fruit borer

Isolation of genomic DNA from tomato fruit borer larva and quality checking

The total genomic DNA of tomato fruit borer was isolated using modified CTAB method. Briefly, a single larva was ground in pre chilled mortar and pestle with the addition of 500 µl pre-warmed modified CTAB extraction buffer (2%) [CTAB (2%) 100 mM Tris-HCl [pH-8], 10 mM EDTA (pH-8), 1.5 M NaCl ,2-β mercaptoethanol (2 %)]. The homogenised sample was transferred into an autoclaved 1.5 ml Eppendorf tube. The contents were mixed well and incubated at 65^0C for 1 h with occasional mixing by gentle inversion. An equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 6000 rpm for 15 min. at 4^0C. The supernatant was collected in a fresh 1.5 ml Eppendorf tube, added 40 µl of sodium acetate (3M) and 600 µl of ethanol (95%), incubated in a deep freezer at -20^0C for 20 min. and centrifuged at 8000 rpm for 10 min. at 4^0C and the genomic DNA pellet was precipitated out. The DNA pellet was washed with ethanol (70 %) by centrifugation at 8000rpm for 10 min. The DNA pellet was air dried for 15 min, dissolved in 25 µl of autoclaved distilled water and stored in deep freezer (-80^0C) for future use. The quality of isolated DNA was assessed by 1 per cent agarose electrophoresis.

Polymerase Chain Reaction (PCR) with DNA barcode primer and sequencing

Good quality genomic DNA (50 ng/µl) isolated from tomato fruit borer larva was used for DNA barcoding. The universal barcode primer [Hebert *et al*. (2003)] specific to mitochondrial cytochrome oxidase I (mtCO1) was used for PCR amplification. The mtCO1 region was amplified by polymerase chain reaction from genomic DNA using the universal barcode primers (**F**: HCO - 5TAAACTTCAGGGTGACCAAAAAATCA -3', **R:** LCO - 5' - GGTCAACAAATCATAAAGATATTGG -3) in Veriti Thermal Cycler (Applied Biosystems®). The PCR reaction was performed using 5 µl template DNA (50 ng), 0.5 µl of the forward and reverse primers, 0.5 µl of 10 mM dNTP (Genei®), 0.2 µl of Taq DNA polymerase (Genei®), 2.5 µl of Taq DNA buffer B(Genei®), 0.7 µl of $MgCl_2$ and 14.5 µl of Millipore® water. The PCR conditions were programmed as, Lid temperature 98^0C, initial denaturation 94^0C for 5 min, 40 cycles each of denaturation 94^0C for 45 seconds, primer annealing 55^0C for 45 sec and primer extension 72^0C for 45 sec, followed by 10 min extension at 72^0C and storage at 4^0C. The amplified PCR product was run on agarose (1%) gel electrophoresis and the product was sequenced at SciGenom labs, Cochin. Fifty larvae collected from tomato fields of Thrissur and Palakkad and five representative samples were sent for sequencing.

Sequence analysis and submission to GenBank, NCBI and Barcode of Life Database (BOLD)

The sequence generated from this study was analyzed for sequence homology using the nucleotide BLAST at NCBI, submitted to BankIt, GenBank and the accession numbers were generated. Further the specimen details and sequences were submitted to BOLD database and barcode for *H. armigera* was generated.

RESULTS

Morphological characters

Prothoracic setal arrangement of fifth instar larvae was observed and the image was captured using microscope with image analyser software. A well developed prothoracic shield was present in larva and altogether 11 primary setae were observed on prothorax *viz*., two dorsal seta (D_1 and D_2), two additional seta (XD_1 and XD_2), two subdorsal seta (SD_1 and SD_2), two lateral seta (L_1 and L_2), two subventral seta (SV_1 and SV_2) and one ventral seta (V_1). Both the additional seate XD_1 and XD_2 lied near to the anterior margin of prothoracic shield. XD_1 was situated near the mid longitudinal line of the half of shield, while XD_2 near the lateral margin. XD_2 was slightly shorter than XD_1. The dorsal seta D_1 situated posterodorsad to XD_1whereas D_2 posterolaterad to XD_2. The sub dorsal setae SD_1 and SD_2 lied near to lateral margin of prothoracic shield. Two lateral setae L_1 and L_2 lied anterior and horizontally aligned to spiracles, among them L_1 lied laterad to SD_1 and L_2 was identical to SD_2. Subventral setae SV_1 and SV_2 lied above coxa and SV_1 situated anterior to SV_2. The ventral group consisted of a single seta V_1which was situated post coxal and most ventral in position (Fig. 1).

The male genitalia were dissected out from the adult moths and slides were prepared. The parts of male genitalia of adult moth observed were uncus-a hook like structure with hairs arise from caudal end of tegumen, socci- paired organs arising from base of the uncus above gnathos, gnathos-paired organs arise from base of uncus and normally fused at tip into a strong hook, saccus- cephalic portion of vinculum, valves- clasping organs, corona-scleotized spines (Fig. 2). In male genitalia, uncus moderately long, well developed, simple, cylindrical, hook like with narrow towards tip; tegumen inverted U shaped; vinculum V shaped, valve long, apically broadened with no projection; corona numerous closely set seta arranged in several rows; saccus short, stouter with curved apical portion. To examine the aedeagus, the valves were removed first by holding the aedeagus at the base with one set of forceps and pulled both the valves together with the other forceps, thus the valves from the aedeagus were detached. The aedeagus was elongated, simple, cylindrical, weakly sclerotised structure. The numbers of cornuti (sclerotized spine) inside the aedeagus were 12 (Fig. 3a). The long spiral tube occasionally armed with spine called vesica was extended out from the aedeagus (Fig. 3b).

The various parts of female genitalia observed were ovipositor, a flattend sclerotized hairy lobe; anterior and posterior apophysis, setae like; ductus burase, duct connecting to bursa copulatix wherein sperms from male deposited during copulation. The ovipositor lobes were well developed, setosed, anterior and posterior apophysis almost of same length, ductus bursae sclerotized towards papilla analis, corpus bursae is oval shaped with 3 signum (Fig. 4).

DNA barcoding of tomato fruit borer

The mtCO1 region was amplified with universal DNA bacode primer in a thermal cycler and the

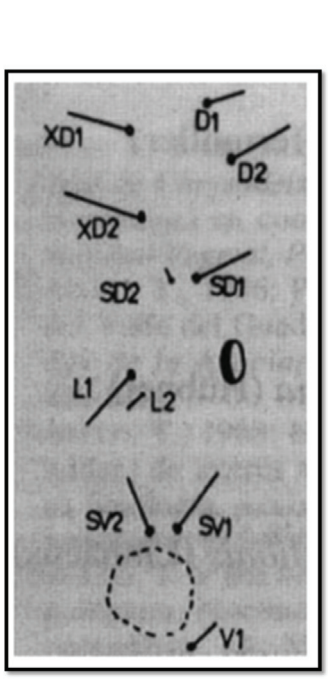
PCR product gave an intact band at 700 bp when resolved at 1 per cent agarose gel (Fig. 5). The sequence generated from tomato fruit borer consisted of 608 bp and homology of sequence with other reported sequences were analysed. The sequence showed significant homology to *H.armigera* mitochondrial Cytochrome Oxidase CO1 gene already deposited in the public domain database using 'blast n' search tool. The blast results showed 100 per cent query coverage and 99 per cent identity to *H. armigera* mtCO1gene. Then the sequence was aligned and annotated using bioinformatics tools, BioEdit and MEGA6. The sequences thus obtained were submitted to BankIt, NCBI under the accession number KM403206 and KP210095.

An account was opened in workbench session of BOLD systems v3 database and a new project 'RMTKL'was created. Specimen data *viz*., specimen identifiers, specimen taxonomy, specimen details, collection details was submitted and an auto generated process ID 'RMTKL001-14' was obtained. Further, primer details, high resolution specimen images, mitochondrial DNA sequences (fasta) and the trace files (.ab1) obtained from sequencer were uploaded to the database and the corresponding barcode of *H. armigera* (Fig. 6) was generated. Upon verification of DNA sequences submitted, the database allotted barcode index number (BIN), BOLD: AAA5223. Altogether 560 sequences of *H. armigera* were coming under the allotted BIN. Based on the distance model kimura 2 parameter, a BOLD taxon ID tree (Fig. 7) was constructed in database. It showed that the nearest neighbourhood of our sequence was *H. armigera* (Unpublished ID) sequence deposited from India. However, the sequences of *H. armigera* deposited in database from Punjab, Maharashtra, South Africa, United Kingdom, Kenya, Pakistan, Brazil, Italy and Germany also shared similarity with our sequences.

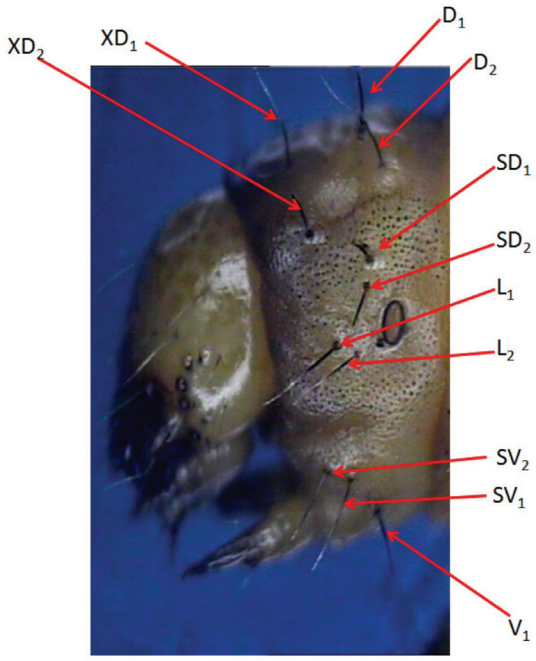
DISCUSSION

Morphological characters

The identification of pests in larval stage is difficult when compared to the adult stage, hence formal keys for the identification of caterpillar heavily depends on chaetotaxy, particularly primary setae (Waganer, 2005). The basic number of primary setae on body segment was of lepidopteran larva was11 and had a specific name based on position, it included two dorsal, two subdorsal, three lateral, three sub ventral and one ventral, and sometimes the prothorax bear two additional setae also (Stehr, 1987). However, in the present study altogether 11 primary setae were observed on prothoracic segments, it included two each of dorsal, subdorsal, lateral, subventral and additional setae, one ventral seta and the lateral setae aligned horizontally with prothoracic spiracle. This is in accordance with Amate *et al*. (1998) who prepared diagnostic key based on above characters to distinguish *H. armigera* from related species. The position of each seta on prothoracic segment was recorded and that was in consonance with Goel (2003) who described the taxonomy of Noctuidae with special reference to immature stages. Whereas, according to Sri *et al*. (1998) *H. armigera* larve were greenish in colour with dark coloured longitudinal stripes and had a dark prothoracic shield extended up to the margin of subdorsal seta (SD_1).

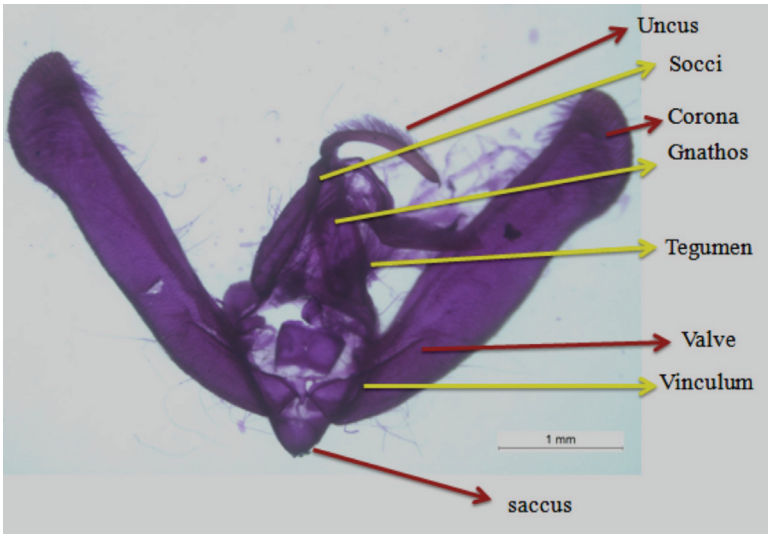


(Setal map, Amate *et al*., 1998)



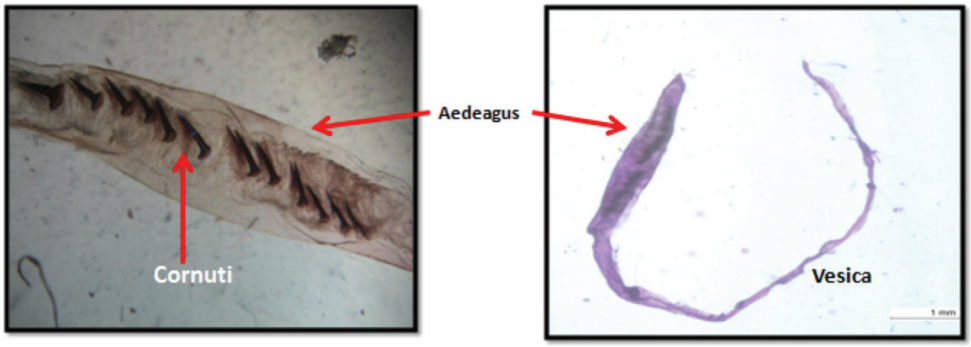
(Magnification 25x)

Fig.1 Setal arrangement on prothorax



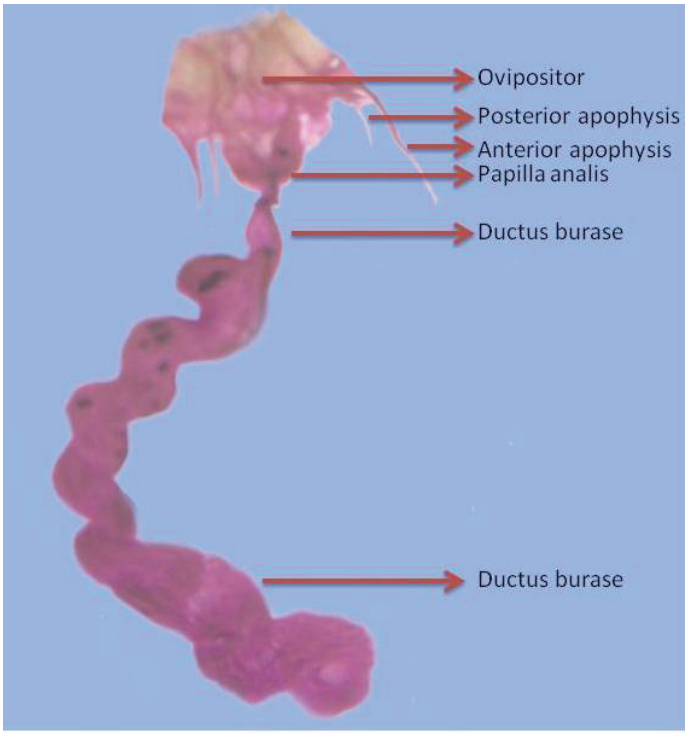
(Magnification 25x)

Fig 2. Male genitalia of *Helicoverpa armigera*



(Dissected male genitalia - magnification 100x) (Dissected male genitalia - magnification 25x)

Fig 3a. Aedeagus and Cornuti **Fig 3b. Aedeagus and Vesica**



(Magnification 25x)

Fig. 4 Female genitalia of *Helicoverpa armigera*

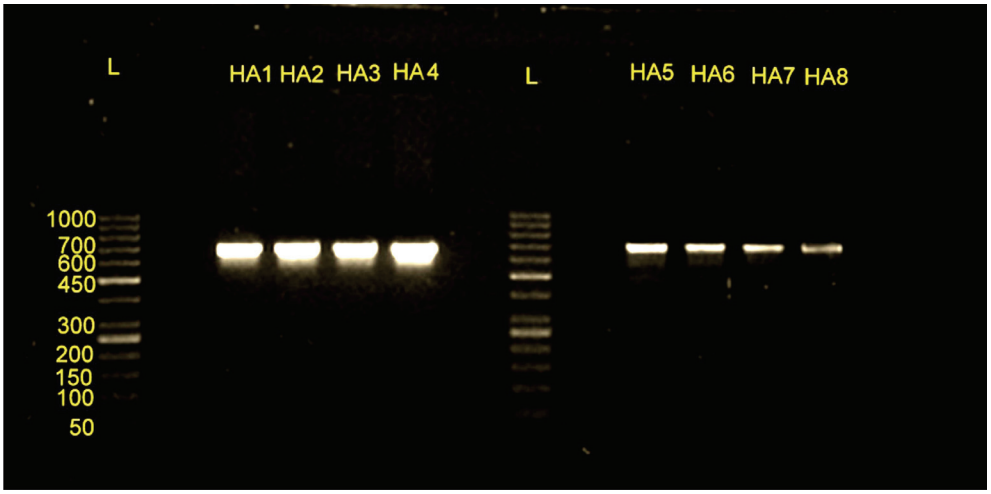


Fig. 5 Agarose gel electrophoresis of genomic DNA isolated from *Helicoverpa* upon amplification with mtCO1 primer. (L: 50 bp ladder, H1-H8: *Helicoverpa* DNA samples)

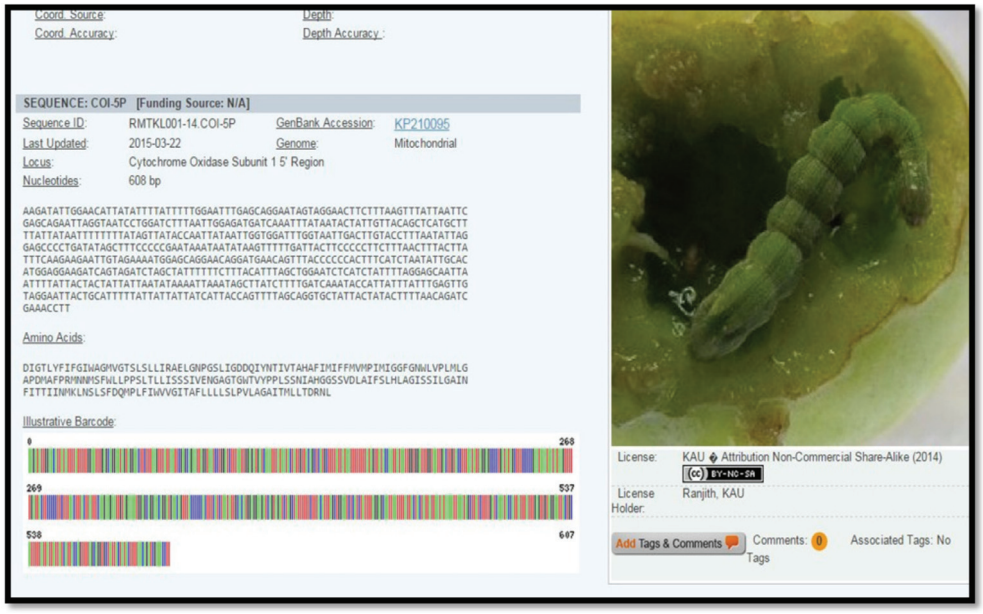


Fig. 6 DNA barcode of *H. armigera* generated by BOLD systems v3

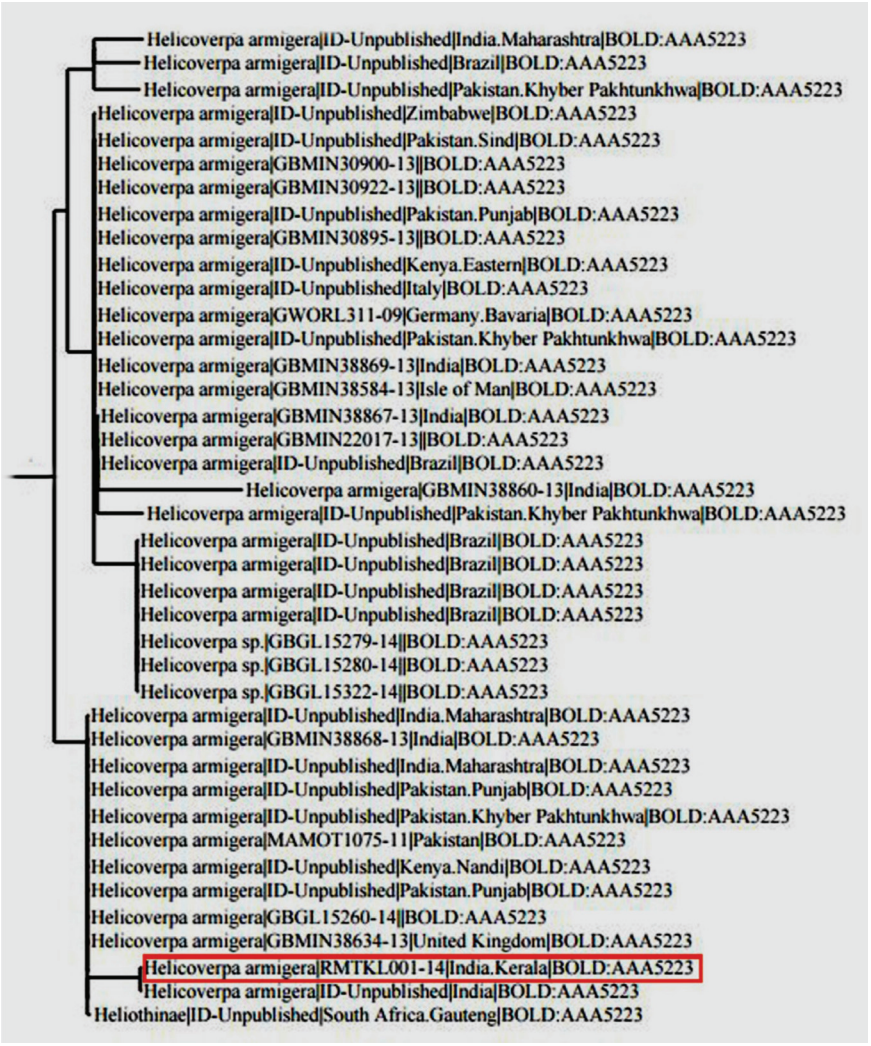


Fig. 7 A section of BOLD ID tree constructed in database

The consistency in genital character is universally recognised and as in any other taxonomic study, identification of species by means of genitalia depends upon a valid recognition of morphological characters (Siverly, 1947). The observation made on male genitalia structure was in accordance with previous workers. According to Hardwick (1970) male genitalia of *H. armigera* consisted of long to moderately long valves with broadened apical possed numerous coronas. Whereas, Brambila (2009) distinguished the species of *H. armigera* based on cornuti count. If the count of cornuti sets were equal to or less than 12, it could be *H. armigera*, and

if the count of cornuti sets exceeds more than 12 sets, it might probably be *H. zea*. If the aedeagus had no cornuti, or very few, the specimen was probably aberrant and sterile.

The adult female genitalia were dissected out and the various parts were recorded. The observation made are in agreement with Hardwick (1965), who reported that in female genitalia of *H. armigera*, dorsal sclerotization at the base of appendix bursae was restricted and it terminated apically in a normal dilation and lumen surface of appendix bursae clothed with spicules. Whereas, ductus bursae and appendix bursae in female in *H. armigera* were observed for possible variation in adult female moth infesting different host plants. Among the population collected from different crops, significantly the highest length of ductus bursae and appendix bursae was recorded in *H. armigera* population collected from chickpea (Patil *et al*., 2012).

DNA barcoding of tomato fruit borer

The barcode involves DNA sequence analysis of a portion (typically between 600- 900 bp) of the mitochondrial gene cytochrome c oxidase subunit I (COI). In the present study, we used the mtCO1 gene of *Helicoverpa* to reveal its species identity. The gene sequences were submitted to BOLD and corresponding barcode for *Helicoverpa armigera* was generated. Possibly we have been made the pioneer attempt to barcode *H. armigera* infesting tomato in Kerala.

We isolated DNA from larval stage of the insect and through barcoding techniques revealed the identity of specimen. The result envisaged the feasibility of using DNA barcode to rapidly assign the unknown specimen at different developmental stages either as a complement to morphological analysis or as the primary diagnostic indicator in cases where the requisite morphological keys are unavailable (Hebert *et al*., 2003).

The data base comparing the unknown barcode sequence using pairwise sequence divergence calculations (e.g., the Kimura 2-parameter model) as visualized using a neighbor-joining (NJ) tree. Based on the distance model kimura 2 parameter analysis, the nearest neighbourhood of our specimen was from India. The similar methodology was used effectively in a elucidating the cryptic aphid species in India (Rebijith *et al*, 2003).

Genetic diversity analysis of tomato fruit borer, *H. armigera* based on mitochondrial cytochrome oxidase-I (mtCO-I) showed that there was no significant variations in the CO-I sequences of *H. armigera* collected on various hosts and geographical locations. However, the phylogenetic tree constructed from the CO1 sequences indicated the possibility of emerging host associated genetic differences in *H. armigera* populations (Asokan *et al*., 2012).

DNA barcoding had been successfully applied in studying the lepidopteran specimens and correctly assigned them in taxonomic category. The barcode comparisons were successfully

applied to distinguish between closely related *Helicoverpa* species, *H. armigera* and *H. zea* (Behere *et al*. 2007). Studies indicated that the successful application of barcoding for species assignment might be taxa-dependent, but with poorly studied or recently diverging groups it became problematic. However, this method has potential for facilitating the identification of invasive insect pests (Floyd *et al*., 2010). DNA barcoding supplemented the morphological methods for identifying the invasive armyworm, *Spodoptera* species in Florida (Nagoshi *et al*., 2011).

In the present study the morphological characters *viz*., setal arrangement on prothorax of larva, genitalia structure of both male and female adult moths were explored and further DNA based identification system, DNA barcoding based on mitochondrial cytochrome oxidase 1 gene confirmed the species level of identity of tomato fruit borer as *H. armigera.* The identification of insects' pest based on both the morphological and molecular traits might be helpful in revealing the divergence of species, evolution of biotypes and species complex existing in the crop ecosystem, thus helps to evolve insect specific management strategies to reduce their menace in economically important crops.

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