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Molecular identification of *Helicoverpa armigera* (Hubner) associated with tomatoes (*Solanum lycopersicum* L.) in Ogun State

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Abstract

The **tomato fruit worm *Helicoverpa armigera* is a key polyphagous agricultural pest with a worldwide distribution. Apart from tomatoes, *H. armigera* also infests cotton, maize, chickpea and pigeon-pea. Its larvae affect almost all the aerial parts of tomato plants from the vegetative stage to the fruit maturation stage. Due to its high mobility, it causes a huge damage on crops and, as such, early detection of *H. armigera* from different agro-ecosystems is crucial for implementing control measures. Herein, we used a molecular marker approach to identify *Helicoverpa spp.* collected from twelve different locations in Ogun State. The *H. armigera* larvae used for the molecular identification were collected on tomato fruits from the 12 different locations from three agro-ecological zones in Ogun State. Genomic DNA of *Helicoverpa* was extracted and its purity was checked using Nanodrop spectrophotometer. Cytochrome Oxidase-I (CO-I) region of the DNA was subjected to Polymerase Chain Reaction (PCR) amplification. The PCR amplification of the CO-I gene after gel electrophoresis of PCR products of *H. armigera* showed a similar banding pattern at 700 base pairs which confirmed the insect as *H. armigera*.**

Keywords: *Helicoverpa armigera*, Deoxyribonucleic acid, Cytochrome Oxidase-I, Polymerase Chain Reaction, Electrophoresis.
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Introduction

Tomato fruit worm *Helicoverpa armigera* is one of the most damaging and hard to control

bollworm insect pests. It feeds on important agricultural crops such as tomato, maize, chickpea, pigeon pea, cotton, sorghum, sunflower, soybean and groundnut (Fitt, 1989, Regupathy et al., 1997). The average crop damages due to this polyphagous pest are valued to be US $350 million annually (Lammer and Macleod, 2007). The insect was reported to cause estimated yield loss of over US$ 5 billion annually in Africa even with application of pesticides (Sharma, 2005). The larvae of the insect affect almost all parts of the tomato plant from the vegetative stage to the reproductive stage (Tripathy et al., 1999). *H. armigera* damage varies among different varieties of tomato, suggesting that some tomato varieties could be highly damaged while others could resist the effect of the pest (Osipitan et al., 2017). It has been reported that *H. armigera* has high movement and productiveness and it also revealed a great capability to develop resistance to insecticides used for its control (Ramasubramaniam and Regupathy, 2004). The cosmopolitan and polyphagous nature of the insect makes it have a wide host range and geographical distribution with the ability to adapt to new environments (Djihinto, 2012). *H. armigera* was reported to possess the ability to migrate from one fruit to the other without fully consuming it; and this explains the widespread damage of the insect to crops even when the larvae population is low (Zalucki et al., 1986). Molecular markers are a convenient tool to reveal population genetic structure, and to fingerprint the individuals and the populations by eliminating the anomaly related to the standard morphology based markers (Heckel, 2003, Fakruddin et al., 2006). Molecular tools find variation at DNA sequence level and are evidenced to be an effective tool for identifying closely related genotypes. The performance of this insect could be due to the presence of a strong genetic makeup that makes it a serious pest on several crops. (Zhou et al., 2000, Scott et al., 2003) There is the need to understand the genetic variations of polyphagous pests like *H. armigera*. The knowledge of the genetic variation can be very helpful in determining the population dynamics, mode of action and control measures for the insect. The early detection of *H. armigera* infestation in different agro-ecosystem is crucial to implement control measures. The observation of morphological characteristics alone (e.g., wing pattern design) may not be adequate to identify *Helicoverpa* species, especially when the integrity of pheromone-trapped specimens cannot be assured. This is particularly true for *H. armigera* and *H. zea*, which, besides their similar morphology and overlapping host range, are attracted by the same volatile compounds (Pogue, 2004). Increase in the development of DNA based markers has reduced the use of morphological, physical and cytological markers for evaluating genetic diversity and population dynamics (Cruickshank, 2002).

Information on molecular identification of *H. armigera* in Ogun State is scarce. This study is therefore tailored towards identifying *H. armigera* molecularly in Ogun state.

Materials and methods

Collection of larva

Different larvae of *Helicoverpa* ranging from green to dark brown used for molecular identification were collected from tomato fruits from 12 different farms at three agricultural zones namely; Ijebu-Imushin, Imodi-Imosan, Ipebi and Ijebu-Ode (Ijebu zone), Anigbajo, Ayetoro, and Abule Oje (Ilaro zone), Sogbade, Odeda, Olugbo, Latemo and FUNAAB (Abeokuta zone) Ogun State. Each collected larva was placed in a 1.5 ml Eppendorf tube , labelled and kept in a Haier Thermocool freezer at -20^{o}c.

DNA extraction

Total genomic DNA was extracted using a Qiagen DNeasy insect Blood and Tissue Kit (Qiagen, Valencia, Calif.). The larva was macerated using mortar and pestle; the macerated larva was placed in a 1.5 ml microcentrifuge tube, 180 µl of tissue lysis buffer (sodium dodecyl sulfate) and 20 µl Proteinase K enzyme were added and mixed by vortexing and incubated at 56°C (in a dry bath) for 1 hour to denature the proteins. About 200µl lysis buffer was added and mixed thoroughly by vortexing and incubated at 56°C for 10 minutes to isolate the DNA ; 200 µl ethanol was added and mixed thoroughly by

vortexing. The mixture was then pipetted into DNeasy mini spin column placed in a 2 ml collection tube and centrifuged at ≥ 6000g for 1 minute. The collection tube and the flow through were discarded, and the spin column was placed in another 2 ml collection tube. 500µl wash buffer (AW1) was added and centrifuged at ≥ 6000g for 1 minute and the collection tube and the flow through were also discarded. The spin column was placed in another 2 ml collection tube, 500µl wash buffer (AW 2) was added again and centrifuged at ≥ 20,000g for 3 minutes to remove protein residues. The collection tube and the flow through were discarded and the spin column was placed in a new 1.5 ml micro centrifuge tube. The DNA was eluted in 200 µl elution buffer (AE buffer), incubated for 1 minute at room temperature (15°C - 25°C) and centrifuged for 1 minute at ≥ 6000g. The spin column was discarded and the DNA was stored in a refrigerator at 4°c. (DNeasy Blood & Tissue Handbook, 2006).

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using an electrophoresis set-up (Bio-Rad®) and the steps were as follows. Agarose (2% - 2 g in 100ml) solution was prepared in a conical flask with the addition of 49 ml 1X TAE buffer. It was kept in a micro wave oven for 60 seconds until the agarose was completely dissolved and the solution was clear. The solution was allowed to cool and 5 µl ethidium bromide solution was added and mixed well. The solution was poured into a gel casting tray (which was placed on a horizontal surface and comb was placed properly in the gel caster) and allowed to solidify for 45 minutes at room temperature. The comb was removed gently and the gel casting tray was placed in an electrophoresis tank and submerged (just until wells were submerged) with electrophoresis buffer (1X TAE).

The DNA sample was prepared by mixing 3 µl of tracking dye (loading buffer) with 10µl of the DNA solution. The samples were mixed well and loaded at 13µl per well. The set-up was connected to the power source and the gel was run at constant voltage of 80 volts for 30 minutes. The gel containing the electrophoresed DNA was viewed photographed in a UV transilluminator.

Purity and concentration of DNA

The purity and concentration of the DNA was checked using a Nanodrop spectrophotometer model NanoDrop-1000 (Thermo Scientific TM). Absorbance was recorded at both wavelengths and the purity was indicated by the ratio OD260/OD280. A value between 1.8 and 2.0 indicates that the DNA was pure and free from proteins and RNA. When the ratio is <1.8, the sample is contaminated with RNA, but when the ratio is >2.0 the sample is protein contaminated.

Polymerase Chain Reaction (PCR) amplification

Good quality genomic DNA isolated from *Helicoverpa* larva was used for PCR amplification of the cytochrome oxidase subunit - 1 (CO1) locus using the LCO52 (GGTCAACAAATCATAAAGATATTGG) Forward primer and HCO52 (TAAACTTCAGGGTGACCAAAAAATCA) Reverse primer. The amplification was carried out in Verti Thermal Cycler (Applied Biosystems®). PCR amplification was performed in a 20 µl reaction mixture which consisted of

a) Genomic DNA - 2.0 µl
b) Fire pol master mix - 4.0 µl
c) Forward Primer - 0.5 µl
f) Reverse Primer - 0.5 µl
g) Nuclease Free Water - 13.0 µl
Total volume - 20.0µl

Several PCR conditions were tested in the experiments to determine the effects of annealing temperature, annealing time, and cycle number on the marker. The PCR conditions were programmed as; Lid temperature 98 °C, initial denaturation 94 °C for 5 minutes, 40 cycles each of denaturation at 94 °C for 45 seconds, primer annealing at 55.8 °C for 45 seconds and primer extension 72 °C for 45 seconds, followed by 7 minutes' extension at 72 °C. The second round of amplification (i.e. re-amplification) was undertaken as follows: 2µl of each of the above

PCR products was used as a template. The reaction was carried out using the procedure of the first amplification.

Electrophoresis of PCR products

PCR products were analyzed by electrophoresis in 1% agarose gel electrophoresis run at 80 W for 30 min in 1x TAE buffer. The molecular size of the amplified products was estimated using a 100 bp DNA marker (Norgen Biotek Corp).

Results

Concentrations of DNA extracted from Helicoverpa larva using Nanodrop spectrophotometer model NanoDrop-1000 (Thermo Scientific TM)

Concentrations of DNA extracted from *Helicoverpa* larva is shown in Table 1. Imodi - imosan had the highest (189.3 ng/µl) DNA quantity among the DNA extracted from the 12 locations while Funaab had the lowest (4.80 ng/µl) DNA. High absorbance ratio was recorded on DNA extracted from Olugbo (2.02) while Latemo had the lowest absorbance ratio (1.08)

Agarose gel electrophoresis of the PCR product at the 12 locations

The a garose gel electrophoresis of the PCR products to identify the species of *Helicoverpa* collected from tomato fields at 12 locations in ogun state is shown in Plate 2. The electrophoresed PCR products of eleven out of the twelve DNA extracted revealed 700 bp for the amplified cytochrome oxidase subunit - I region.

Agarose gel electrophoresis of the PCR products of samples collected from the three agro ecological zones

The a garose gel electrophoresis of the PCR products to identify the species of *Helicoverpa* collected on tomatoes from the three agro ecological zones in ogun state is shown in Plate 3. The electrophoresed PCR products of the DNA extracted revealed 700 bp for the amplified cytochrome oxidase subunit - I region.

Table 1: Concentrations of DNA extracted from *Helicoverpa armigera larva* using Nanodrop Spectrophotometer Model NanoDrop-1000 (Thermo Scientific TM)

Location	**A260/A280**	**Quantity (ng/µl)**
Anigbajo	1.80	66.3
Ayetoro	1.82	55.5
Abule oje	1.93	30.7
Sogbade	1.97	131.2
Odeda	1.72	27.7
Olugbo	2.02	75.3
Imodi –Imosan	1.99	189.3
Ipebi	1.83	85.1

Ijebu-Ode	1.89	143.5
Ijebu-Imushin	1.90	79.3
Funaab	1.56	4.80
Latemo	1.08	31.1

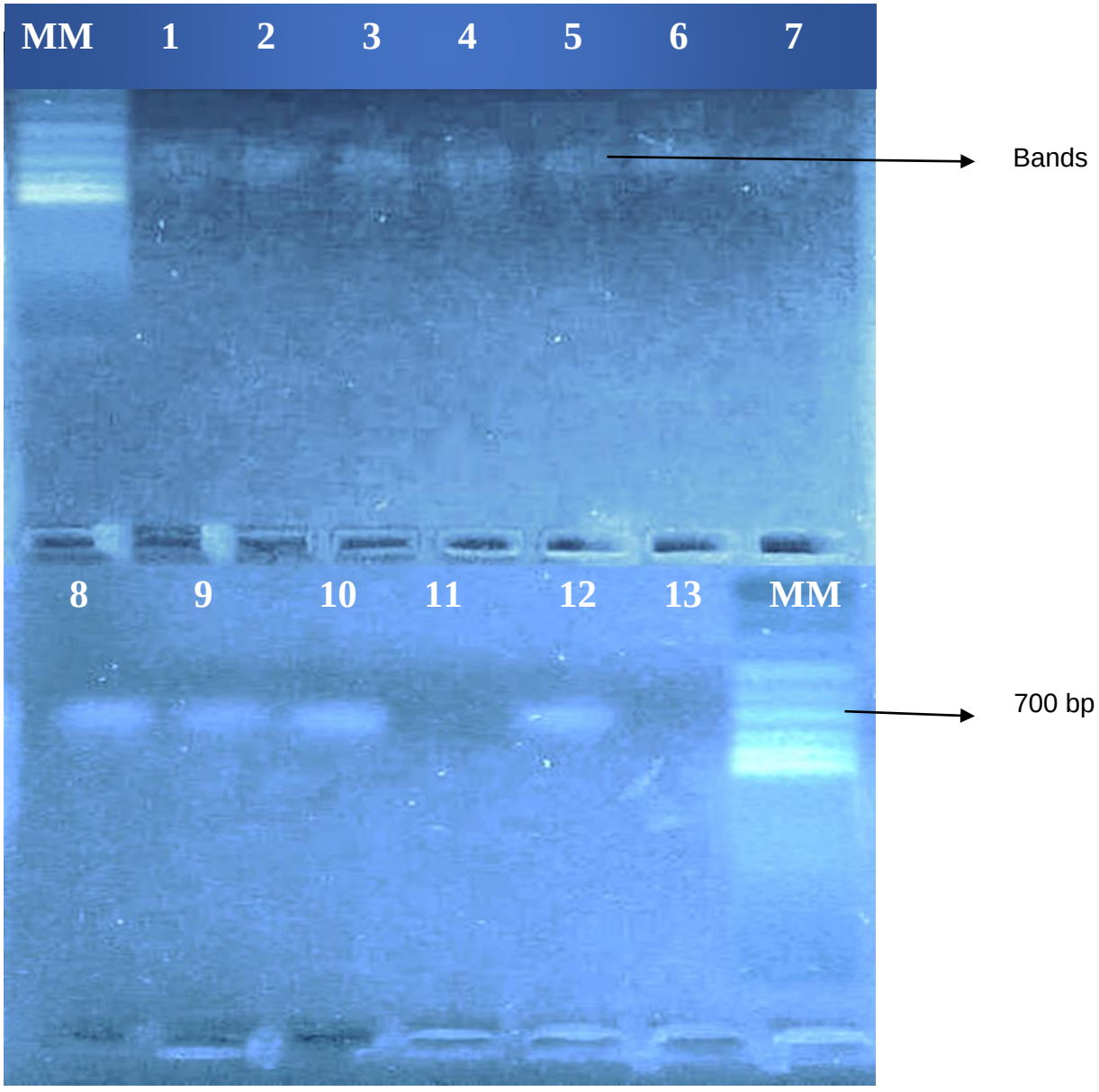

Plate 1: Agarose Gel Electrophoresis of the PCR Products Showing the Bands: 1- Funaab, 2 – Imodi imosan, 3 – Sogbade, 4 – Oje, 5 – Ijebu – Imushin, 6 – Anigbajo, 7- Ipebi, 8- Ayetoro, 9- Olugbo, 10 – Latemo, 11- Odeda, 12 – Ijebu ode, 13- negative control and MM- Molecular marker

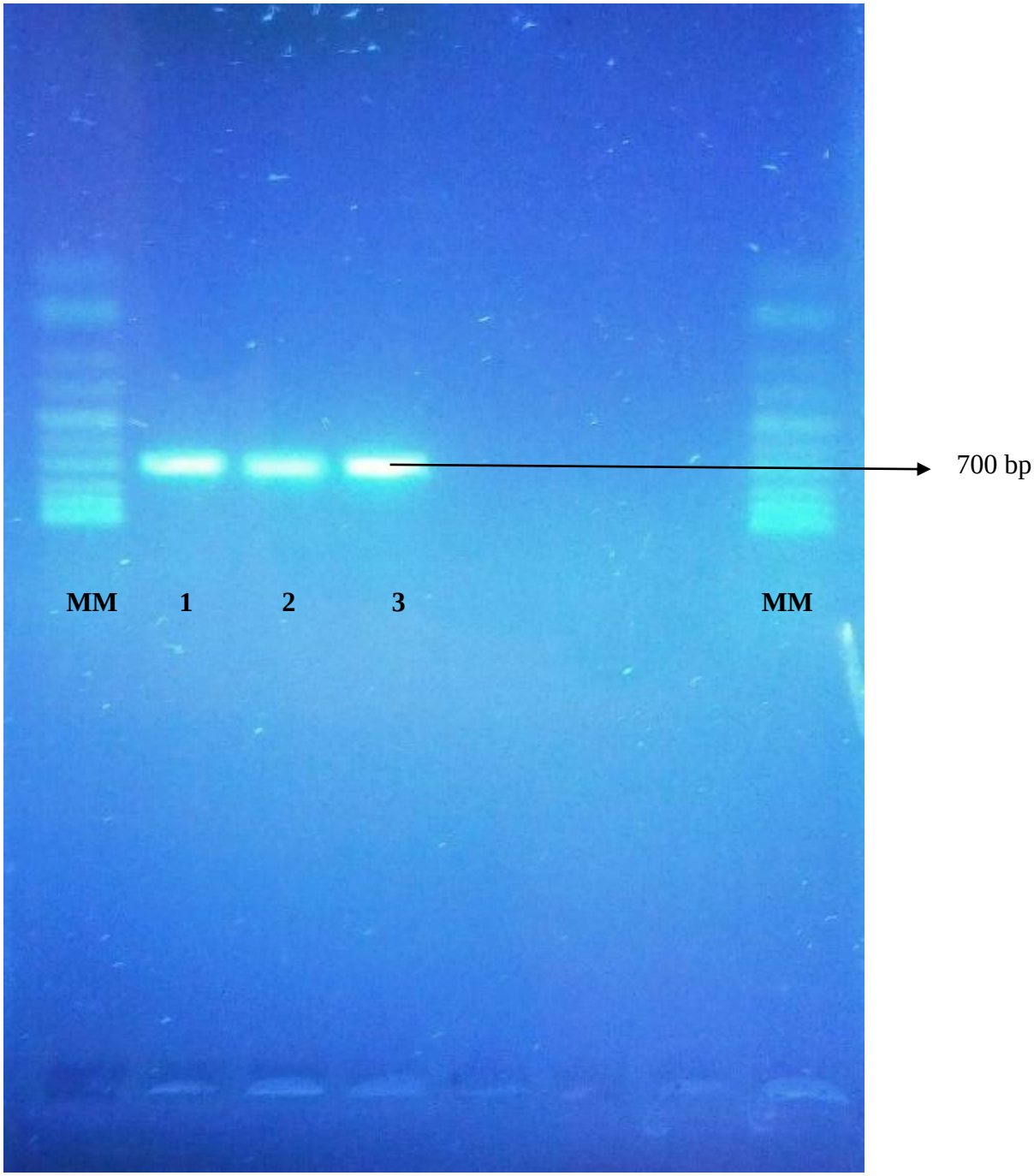

Plate 2: Agarose Gel Electrophoresis of the PCR Products Showing the bands for the three agro-ecological zones: 1- Ijebu, 2 – Ilaro, 3 – Abeokuta, MM- Molecular marker

Discussion

DNA barcode involves analysis of a portion of the mitochondrial gene cytochrome oxidase subunit I

(COI). This study used the cytochrome Oxidase-I (mtCO-I) gene of *Helicoverpa* to reveal species identity of the tomato fruit borer *H. armigera*. The mitochondrial cytochrome oxidase 1 region of the DNA obtained after amplification with universal DNA – based markers in a thermal cycler gave an intact band of 700 base pairs when resolved at 1 per cent agarose gel. Gel electrophoresis of the PCR products of 11 *Helicoverpa* out of the 12 *Helicoverpa* CO-I gene amplified showed a similar banding pattern. The DNA from one of the larvae could not be amplified ; this failure could have been due to poor gDNA and/or the fact that it belonged to other lepidopteran species. There was variation in the quantity of DNA extracted from the 12 locations; this could be due to variation in the larva color which ranges from green color to dark brown.

The results showed that there was no significant variation in the CO-I sequences of *H. armigera* from the eleven geographical locations, suggesting that the larvae are the same molecularly, though they differ in color morphologically . The result is similar to an earlier report by Asokan et al. (2012) which indicated that *H. armigera* sourced from various hosts and from different geographical locations in India has no significant variation when mtCO1 gene of *Helicoverpa* was used to reveal their species identity. Behere et al. (2007) reported that DNA barcoding has been successfully used to assign lepidopteran specimens into taxonomic category and to distinguish between closely related *Helicoverpa* species, *H. armigera* and *H. zea*. Floyd et al. (2010) reported that DNA barcoding has the potential for easing the identification of aggressive insect pests affecting crops. Nagoshi et al. (2011) reported that DNA barcoding supplemented the morphological methods for identifying the invasive armyworm, *Spodoptera* species in Florida. In the present study, DNA barcoding based on mitochondrial cytochrome oxidase 1 gene confirmed the species level of identity of tomato fruit borer as *H. armigera*. *H. armigera* identification based on molecular traits could be helpful in revealing the divergence of species, evolution of biotypes and species complex. Arnemann et al. (2016) reported that knowledge of mtDNA genes such as the widely used cytochrome oxidase subunit I will greatly assist in the confirmation of species apart from providing an opportunity to ascertain the population diversity and to infer possible patterns associated with widespread infestation of the insect. Omaththage et al. (2015) reported that diversity of morphologically similar species using standard taxonomic features is challenging, time wasting, and needs technical know-how.

H. armigera larvae identification based on molecular traits could be used as the primary diagnostic tool to reveal the divergence of species, evolution of biotypes and species complex where the requisite morphological keys are unavailable.

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