Entomon 41 (1): 21-28 (2016)
Article No. ent. 41103

Cloning Folmer region of *mtCOI* gene diagnostic for sugarcane early shoot borer, *Chilo infuscatellus* Snellen (Lepidoptera: Crambidae)

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ABSTRACT: Early shoot borer (ESB), *Chilo infuscatellus* Snellen (Lepidoptera: Crambidae) is an important pest of sugarcane and distributed across the Indian subcontinent. It was originally described as *Diatraea saccharalis* and after several revisions by many taxonomists, it was finally accepted as *C. infuscatellus*. The limitations associated with conventional alpha taxonomy such as phenotypic plasticity of key morphological traits, could be the possible reason for taxonomic uncertainty of the species. DNA barcoding has emerged as a complementary approach to conventional taxonomy and has also been proved as a powerful tool to identify cryptic species in the population. Hence, the 'Folmer region' of mitochondrial *cytochrome c oxidase I* (*mtCOI*) gene from early shoot borer has been cloned to serve as the barcode for the species. The DNA barcode developed in this study would address the anomalies that exist in the identification of early shoot borer. The barcode generated by us is the ideal one as it is exactly 658 bp in size and carries no stop codon in its amino acid sequence. KM453722 is the unique GenBank accession number for the DNA barcode of *C. infuscatellus.* The DNA barcode developed in this study would serve as an ideal molecular diagnostic kit for correct identification of *C. infuscatellus* irrespective of its sex, stage and polymorphism.

KEY WORDS: *Chilo infuscatellus, COI,* diagnostic kit, DNA barcode, sugarcane

INTRODUCTION

Early shoot borer (ESB) of sugarcane, *Chilo infuscatellus* Snellen (Lepidoptera: Crambidae) is a major pest of sugarcane and distributed across the Indian subcontinent. The annual yield loss due to this pest has been estimated to be 22-33% (Directorate of Sugarcane Development, 2016). It has become a major threat to sugarcane cultivation in the coastal cane belts of peninsular India and coastal Andhra Pradesh in particular, where it also behaves as internode borer (Bhavani, 2013). It is generally brought under check by the insecticides as the population often crosses the economic threshold level. The taxonomy of sugarcane pests including the ESB has been under constant revision over the years ever since they were originally described. ESB was originally described as *Diatraea saccharalis* Fab. by Cotes (1889) and after several revisions by many taxonomists (Lefroy, 1906; Fletcher, 1926; Kapur, 1950; Bleszynski, 1965) it was finally accepted as *Chilo infuscatellus* Snellen (Avasthy and Tiwari, 1986). It has been accepted worldwide that the conventional alpha

taxonomy suffers from its own limitations. Among the several constraints, phenotypic plasticity of key taxonomic traits employed for species discrimination might be one of the major constraints leading to wrong identification of pest insects. Besides, the conventional taxonomy relies on the morphological traits of adult insects such as the structure of genitalia and immature stages of pest insects are generally ignored for describing a species. Sometimes, the genitalia, which often distinguish the species, fails to delineate the cryptic species in the population (Hebert *et al.,* 2004).

Since Lepidoptera is the second largest order of the class Insecta with more than 1.6 lakhs described species, correct identification of Lepidopterans up to species level certainly requires unparalleled expertise in alpha taxonomy. The decreasing number of insect taxonomists worldwide and India in particular, also poses difficulty in getting the specimens identified in right time. The taxonomic identity of the pest must be ascertained to realize the success of any pest management programme irrespective of the crop under stress. The limitations associated with conventional alpha taxonomy are however, adequately addressed by the novel approach named DNA barcoding. Besides being a powerful tool to identify the cryptic species, DNA barcoding does not require the support of insect taxonomists. Once the barcode has been developed for a species in complementarity with conventional taxonomy, a non-insect taxonomist can identify the species without any ambiguity with the help of unique DNA barcode. As of now, ideal DNA barcodes are not available for many of the important cane pests including the ESB. Sugarcane entomology in India has long been dominated by the scientists specialized in the field of biological control. To the best of our knowledge, sugarcane entomologists having expertise in both insect taxonomy and insect molecular biology are very rare in the country. This could be the possible reason for lack of information on DNA barcodes for insects in sugarcane ecosystem. Hence, we have developed DNA barcode for ESB after ascertaining its taxonomic position through conventional taxonomy. The barcode developed in this study is ideal (658 bp) and best-ever DNA barcode for the species under investigation. This barcode would serve as an ideal diagnostic kit for unambiguous identification of ESB.

MATERIALS AND METHODS

DNA isolation, PCR and cloning

Larvae of ESB collected from the research farm at ICAR-Sugarcane Breeding Institute, Coimbatore were cultured in the laboratory till the emergence of adults. The adults (F_0) were allowed to lay eggs and the first generation adults (F_1) obtained from the egg mass of a pair were used for identification through conventional taxonomy and for developing the DNA barcodes. The taxonomic identity of *C. infuscatellus* was ascertained by running the keys developed by Sallam and Allsopp (2008). This was followed by the development of barcode.

DNA isolation, polymerase chain reaction (PCR) and cloning were performed by adopting the protocols as described by Ramasubramanian *et al.* (2015d). The protocol followed to isolate DNA from ESB is briefed here. The body tissues of a de-winged adult insect were homogenized in warm CTAB buffer (Tris 100 mM, EDTA 20 mM, NaCl 1.4 M, CTAB 2%, β-mercaptoethanol 0.2%). The well-homogenized sample was kept in water bath at 65°C for 1 hr. This was followed by centrifugation at 12,000 rpm for 10 min. at 4°C. The supernatant was pipetted out and equal volume of chloroform: isoamyl alcohol (24:1) mixture was added. The contents were shaken vigorously and again centrifuged at 12,000 rpm for 10 min at 4°C. The top layer was collected and 0.7 volume of ice-cold isopropanol was added to the recovered layer. The supernatant was drained after brief centrifugation and the DNA pellet settled at the bottom of the tube was washed with 70% ethanol. The dried DNA pellet was dissolved in 30-50 µL of sterile water. This was followed by RNaseA treatment. The resultant RNA-free DNA was stored at -80°C until further analysis. The quantity and quality of the DNA were determined in NanoDrop ND 1000 spectrophotometer (Thermo Scientific Inc., USA).

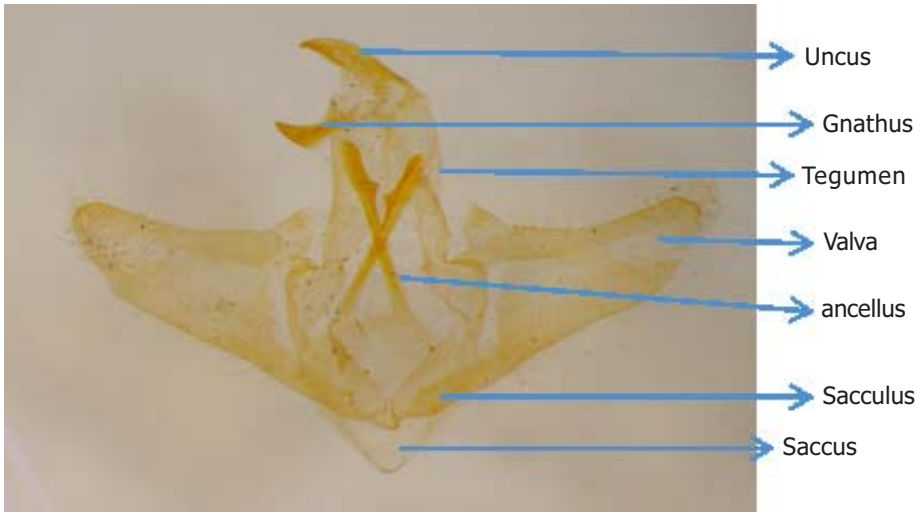

Fig 1. Male genetalia (Ventral view)

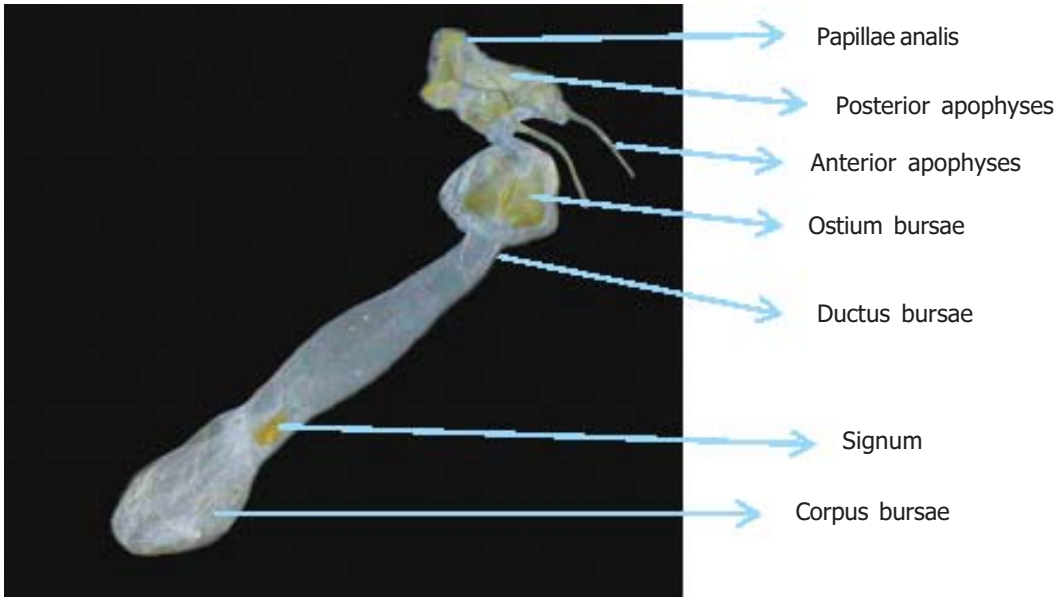

Fig 2. Female genetalia

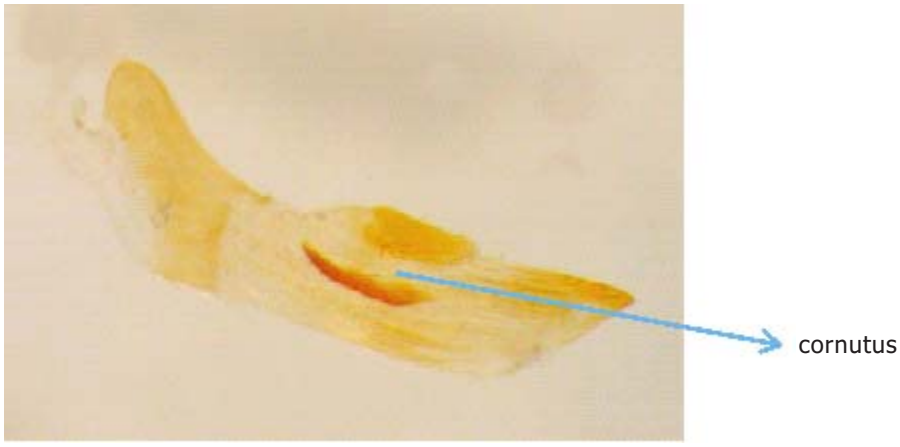

Fig 3. Aedeagus

AACTTTATATTTTATTTTTGGAATTTGAGCAGGAATAATTGGAACTTCCCTTAGACTTTTAATT
CGAGCTGAATTAGGGACTCCAGGATCTTTAATTGGAGATGATCAAATTTATAATACTATTGTT
ACAGCTCATGCATTTATTATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGATTTGGA
AATTGATTAGTTCCTTTAATACTAGGGGCACCTGGTATAGCTTTTCCACGAATAAATAATATA
AGCTTTTGATTATTACCACCATCATTAACATTATTAATTTCTAGAAGAATTGTTGAAAATGGG
GCAGGGACTGGTTGAACTGTTTATCCCCCTTTATCCTCAAATATTGCTCATGGGGGAAGCTC
TGTAGATTTAGCAATTTTTTCCCTTCACTTAGCGGGTATTTCATCAATTTTAGGAGCTATTAA
TTTTATTACAACAATTATTAATATACGAGTTAATGGTCTATCATTTGATCAAATACCTTTATTT
GTTTGATCTGTAGGTATTACAGCACTTTTATTATTACTTTCTCTACCAGTATTAGCAGGTGCT
ATTACTATACTACTAACTGATCGAAATTTAAATACATCTTTTTTTGACCCTGCTGGAGGGGGG
GATCCAATCCTTTATCAACATTTATTT

Fig 4. DNA barcode of sugarcane early shoot borer, *C. infuscatellus*

TLYFIFGIWAGMIGTSLSLLIRAELGTPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGGFGNWLV
PLMLGAPGMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTVYPPLSSNIAHGGSSVDLAIFS
LHLAGISSILGAINFITTIINMRVNGLSFDQMPLFVWSVGITALLLLLSLPVLAGAITMLLTDRNLN
TSFFDPAGGGDPILYQHLF

Fig 5. Uninterrupted ORF of *COI gene* fragment from *C. infuscatellus*

PCR was performed in S-1000 PCR Touch Cycler (BioRad, USA) and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') were the primers used to amplify the target fragment (Folmer *et al.* 1994). After amplification, the PCR products were purified using GenElute Gel Extraction Kit (Sigma-Aldrich India Private Ltd., Bengaluru) by following the manufacturer's instruction. The purified PCR products were cloned into pTZ57R/T plasmid vector as per the manufacturer's instruction (Thermo Scientific Inc., USA). This was followed by transformation of *Escherichia coli* (strain DH5α) competent cells. The recombinant clones were confirmed by colony PCR and also by restriction digestion of plasmids isolated from the recombinant colonies. Plasmid DNA was isolated from single recombinant colony using Plasmid DNA MiniPreps Kit (Bio Basic Canada Inc.) as per the instruction given by the manufacturer. Sequencing of purified recombinant plasmids was done by Chromous Biotech India Private Ltd., Bengaluru. The sequencing was done in both forward and reverse directions to find out mismatches if any, in the target sequence. Homology search was made in the National Centre for Biotechnology Information (NCBI) using blast algorithm. The nucleotide sequence was translated into amino acid sequence using ExPASy (Expert Protein Analysis System) translate tool of Swiss Institute of Bioinformatics and the open reading frame (ORF) was obtained using invertebrate mitochondrial genetic code. The well-characterized barcode was submitted in the GenBank of NCBI and unique accession number was obtained.

RESULTS AND DISCUSSION

The taxonomic identity of *C. infuscatellus* was ascertained by running the keys developed by Sallam and Allsopp (2008). The male and female genitalia were dissected and studied for the key characters specific to *C. infuscatellus* (Figs. 1-3). The voucher specimens were deposited at the Division of Crop Protection, ICAR-Sugarcane Breeding Institute, Coimbatore, India. DNA barcode was developed only after confirming the identity of the species by conventional alpha taxonomy.

In the present study, we could isolate DNA of better quality and yield from ESB of sugarcane. Quantity of the DNA extracted varied between 900.7 ng/µL and 1090.6 ng/µL across the individuals. The 260/280 ratio of the DNA was in the range of 1.77-1.83. The intact genomic DNA as visualized in 0.8% agarose gel also indicated its suitability for PCR. A 658 bp DNA fragment (Folmer region) that lies in the 5' end of *mtCOI* gene has been designated as the standard DNA barcode for identification of species in the animal kingdom (Hajibabaei *et al.,* 2005; Floyd *et al.,* 2009) including insects. The primers designed by Folmer *et al.* (1994) could amplify the target fragment from *mtCOI* gene of ESB. DNA fragment of ~700 bp in size was amplified from all the positive colonies after transformation. The recombinant clones were further confirmed by restriction-digestion of the recombinant plasmids. DNA barcode developed for *C. infuscatellus* is a complete barcode of 658 bp in size (Fig. 4). The amino acid sequence of the barcode does not have any stop codon (Fig. 5). The uninterrupted open reading frame (ORF) indicates the flawlessness of the *COI* sequence. The occurrence of mismatches was eliminated by performing the sequencing in both directions. The barcode generated in this study was submitted in the GenBank of NCBI with accession number KM453722. As of now, we could retrieve only one *COI* gene fragment of *C. infuscatellus* from NCBI. The barcode sequence retrieved from the public domain was 678 bp in size (Accession No. JQ066747) and may not be considered as an ideal one. Conversely, the barcode generated by us is the ideal one as it is exactly 658 bp in size. The ESB *COI* sequence cloned in the present study showed 96.16% identity with the one retrieved from the public domain. The *COI* sequence of *C. infuscatellus* developed in the study was aligned with the barcode fragment of *Chilo auricilius* Dudgeon (KR153874) developed by us earlier. The extent of identity between these two *COI* sequences was 89.21%. The inter-specific sequence variation of more than 10% would undoubtedly delineate these two species without any ambiguity, which were once considered as single species.

Lepidoptera is one of the taxonomically most diverse orders of the class Insecta with low sequence divergences. However, DNA barcoding with *COI* gene fragment has been shown to be highly successful in correct identification of closely related species of lepidoptera. The *COI* fragment could successfully delineate 196 out of 200 lepidopteran species (98%) prior to their morphological studies with minimal interspecific genetic divergence of 3%. The *COI* fragments of only four congeneric species of very recent in origin showed less than 3% (0.6-2.0%) interspecific genetic variation (Hebert *et al.,* 2003). Since the species involved in the present study (*C. infuscatellus*) is a member of lepidoptera, we have employed the most reliable *COI* gene fragment as DNA barcode. It is an indisputable fact that the barcode generated in the present study is the best one for the ESB of sugarcane. The 658 bp *COI* fragment was reported to be quite successful in identifying insects irrespective of the orders they belong to. The *COI* fragment could delineate most (96%) of the aphid species (335 species from 134 genera) belonging to the subfamily Aphidinae with intra-specific sequence divergence of 0.2% (Foottit *et al.,* 2008). Besides, the sequence divergence among the individuals of *Aphis gossypii,* which has several host-associated genotypic lineages, was less than 0.62% (Foottit *et al.,* 2008). Low level of intra-specific sequence divergence and significantly high level of inter-specific sequence divergence make this DNA fragment as an ideal DNA barcode (Savolainen *et al.,* 2005; Simon *et al.,* 1994) for delineating closely related species.

As many as 212 insects were recorded as pests of sugarcane (David and Nandagopal, 1986). Although sugarcane entomologists are competent enough in identifying the pests of regular occurrence, they are often approaching insect taxonomists for unambiguous identification of occasional pests and biocontrol agents of rare in occurrence. Being a major pest of sugarcane, ESB can be identified easily by observing key morphological traits. However, the existence of cryptic species among the diverse populations of ESB cannot be ignored

due to its pan-India distribution. The conventional alpha taxonomy often fails to identify the morphologically identical yet reproductively isolated cryptic species in the population. Some of the species-specific parasitoids will miserably fail to parasitize the host when the target species is wrongly identified. Hence, correct identification of cryptic species is inevitable to achieve desired level of control of target pests. This can be achieved only by DNA barcoding approach. Neotropical skipper butterfly *Astraptes fulgerator* (Lepidoptera: Hesperiidae), which was originally described in 1775, has long been considered as single species for more than two centuries until 2004. Hebert *et al.* (2004) were the first to identify ten cryptic species in the population of *A. fulgerator* with the help of DNA barcoding approach. Hence, an ideal DNA barcode was generated for *C. infuscatellus.* The barcodes to be developed for diverse populations of ESB will be compared with the barcode generated in the study to fish out the cryptic species if any, in the sugarcane ecosystem.

Worldwide development of DNA barcodes for insects is at its rapid pace. The development and use of DNA barcodes for correct identification of pests and beneficial insects in agro ecosystems need to be intensified in India. In India, *COI*-based DNA barcoding approach has been employed to differentiate closely related species form across the orders of class Insecta (Asokan *et al.,* 2012; Rebijith *et al.,* 2012; 2013; Ojha *et al.,* 2014; Tembe *et al.,* 2014; Jalali *et al.* 2015). Rebijith *et al.* (2013) could identify three cryptic aphid species for the first time from India with the help of *COI* gene sequences. Only few attempts were made to generate DNA barcodes for insects in sugarcane ecosystem. Rakshit *et al.* (unpublished) have submitted partial CDs of *COI* gene pertaining to sugarcane borers collected from Indian subcontinent. The *COI* sequences of internode borer *Chilo sacchariphagous indicus* Kapur (KC306951), stalk borer *C. auricilius* (KC306949) and top borer *Scirpophaga excerptalis* Walker (KC306948) were observed to be 611 bp in size. The *COI* gene fragment of *Sesamia inferens* Walker (KC911715) was found to be of 659 bp in size. However, Ramasubramanian and Ramaraju (2014) and Ramasubramanian *et al.,* (2014, 2015a, 2015b, 2015c, and 2015d) have been consistent in cloning ideal DNA barcodes (658/ 649 bp) for sugarcane pests. They have cloned and characterized the *mtCOI* fragments from *S. inferens* (KJ013410), *Melanaspis glomerata* Green (KR153875), *Melanaphis sacchari* Zehntner (KM453722),*Tetraneura javensis* van der Goot (KM453723), *Aleurolobus barodensis* Maskell (KF986269), *Neomaskellia bergii* Signoret (KF986270) and *Pyrilla perpusilla* Walker (KJ013412). The *COI* fragments of *M. glomerata* and *A. barodensis* are of 649 bp in sizes and the loss of 9 bases (3 amino acids) has been reported as real deletion from the COI enzyme of insects (Ramasubramanian et al., 2015b and 2015d). The barcodes developed for *P. perpusilla*, *M. glomerata*, *T. javensis* and *N. bergii* were the firsts and no other barcodes are available as on date for these species in the public domain. Since the Folmer region of *mtCOI* gene cloned from *C. infuscatellus* is exactly 658 bp in size, it would certainly serve as an ideal species diagnostic kit for unambiguous identification of ESB irrespective of its sex, stage and polymorphism.

ACKNOWLEDGEMENTS

The first author is grateful to Dr. Bakshi Ram, Director, ICAR-Sugarcane Breeding Institute; Dr. R. Viswanathan, Head, Division of Crop Protection and Dr. R. Jayanthi, Principal Scientist for their constant encouragement and support in carrying out this research.

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(Received 11 January 2016; Accepted 19 February 2016; Published 31 March 2016)