RESEARCH ARTICLE

CLONING AND CHARACTERIZING THE FOLMER REGION OF *MTCOI* GENE DIAGNOSTIC FOR SUGARCANE TOP BORER, *SCIRPOPHAGA EXCERPTALIS* (WALKER) (LEPIDOPTERA: PYRALIDAE)

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Abstract

Folmer region of *mtCOI* gene from sugarcane top borer, *Scirpophaga excerptalis* (Walker) (Lepidoptera: Pyralidae) has been cloned and characterized. This would be the ideal DNA barcode as it is 658 bp in size (KJ013411) and does not have any stop codon in its amino acid sequence. The *COI* fragment cloned in this study showed less than 89% identity with other species of *Scirpophaga* having full length DNA barcodes and hence, it clearly delineates *S. excerptalis* from other closely related congenerics. The *COI* gene sequence of *S. excerptalis* would also serve as a base for identifying the cryptic species if any, in the sugarcane crop ecosystem of the Indian subcontinent.

Key words: Sugarcane top borer, *Scirpophaga excerptalis, mtCOI*, DNA barcode

Introduction

Top borer, *Scirpophaga excerptalis* (Walker) (Lepidoptera: Pyralidae) is one among the major borers of sugarcane in India, and in the subtropical cane belt, in particular. There had been a great deal of confusion in the taxonomic identity of *S. excerptalis* (Mukunthan, 1986). It was originally described under the genus *Tinea* by Fabricius. Hampson reported it as a complex of six different species under the genus *Scirpophaga* Treitschke (cited by Banerji and Butani, 1975). The six species of *Scirpophaga viz., S. excerptalis, S. intacta, S. monostigma, S. nivella, S. rhodoproctalis* and *S. xanthogastrella* were reported to constitute the top borer complex of sugarcane (Box, 1953). The above-mentioned six species were later synonymized with *S. excerptalis* (Lewanich, 1981). As of now, *S. excerptalis* is the common species of top borer infesting sugarcane in India (Mukunthan, 1986). Lack of alternative and/or complementary tools to conventional taxonomy during the twentieth century and phenotypic plasticity of key taxonomic traits could be the possible reasons for the confusion in the taxonomic position of sugarcane top borer.

It is a known fact that the identification of pest insect is a pre-requisite for the success of any pest management programme. The existence of cryptic species within the population of pest insect causes difficulties in traditional taxonomical identification. The conventional alpha taxonomy often fails to delineate the cryptic species, because the key taxonomic traits like male or female genitalia are identical among the cryptic species (Hebert *et al.,* 2004). The molecular approach with DNA barcoding has emerged as a complementary tool to the conventional taxonomy for identification of the insect species including the cryptic species without any ambiguity. A few research groups from India had made significant achievements in developing DNA barcodes for insects of important crop ecosystems. We have been successful in generating perfect DNA

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barcodes for several insect pests of sugarcane (Ramasubramanian *et al.,* 2014a; 2014b; 2015a; 2015b, 2016). Considering the economic importance and wide spread occurrence of the pest across the country, molecular identification of *S. excerptalis* with DNA barcoding approach has been successfully done.

Materials and Methods

DNA isolation

DNA was isolated from adults by adopting the method as described by Ramasubramanian *et al.* (2015b). The insect tissue was placed in a sterile 1.5 mL microcentrifuge tube and homogenized with pellet pestle in 700-1000 µL of warm CTAB buffer (Tris 100 mM, EDTA 20 mM, NaCl 1.4 M, CTAB 2%, β-mercaptoethanol 0.2%) added in three instalments. After thorough homogenization, the microcentrifuge tube was incubated in a water bath at 65°C for one hour. The tissue homogenate was centrifuged at 4°C for 10 min. at 12,000 rpm. The supernatant was added to equal volume of chloroform: isoamyl alcohol (24:1) mixture. The contents were mixed and centrifuged at 12,000 rpm for 10 min. at 4°C. The upper aqueous layer was drawn and added to equal volume of pre-chilled absolute alcohol and incubated overnight at -20°C to precipitate the DNA. The DNA was pelleted through centrifuging the contents at 12,000 rpm for 10 min. at 4°C and was washed with 70% ethanol. The DNA pellet was dried in a laminar hood and was suspended in sterile Milli-Q water (50 µL). Removal of RNA from the DNA suspension was done by addition of one µl of RNase A (10 mg mL^{-1}) and incubation at 37°C for 30 min. followed by incubation in water bath at 65°C for 10 min. to inactivate the enzyme. The quantity and quality of genomic DNA of *S. excerptalis* was finally determined in NanoDrop ND 1000 spectrophotometer (Thermo Scientific Inc., USA). The DNA was further diluted with sterile water to obtain the final working concentration of 25-50 ng µL^{-1}. This template DNA was used in the polymerase chain reaction (PCR) to amplify the target *COI* gene fragment.

Polymerase chain reaction

PCR was performed in 20 µl reaction mixture containing 25-30 ng of template DNA, 0.2 µM each of forward (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer (HCO2198: 5'-TAA ACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.,* 1994), 0.2 mM each of dNTPs (Thermo Scientific Inc., USA), one unit of Taq DNA polymerase (Merck Biosciences, Mumbai), 1x Taq buffer and sterile Milli-Q water. The S-1000 PCR Touch Cycler (BioRad, USA) was programmed for initial denaturation step at 94°C for 4 min. followed by 35 cycles of denaturation step at 94°C for 30 sec., annealing at 47°C for 45 sec. and extension at 72°C for 45 sec. The final extension step was performed at 72°C for 20 min. The PCR amplified products were electrophoresed in 1.5% agarose gel.

Cloning and characterization

The amplified PCR products were purified by using PCR product purification kit (Bio Basic Canada Inc., Canada). The purified PCR products were quantified in the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific Inc., USA). After quantification, the purified PCR products were cloned into pTZ57R/T plasmid vector using InsTAclone PCR cloning kit as per the manufacturer's instruction (Thermo Scientific Inc., USA). This was followed by transformation in *Escherichia coli* (strain DH5α) competent cells. The transformed colonies were identified by colony PCR and then confirmed by restriction-digestion of plasmids isolated from the recombinant colonies. Plasmid DNA was isolated from single recombinant colony by using plasmid DNA mini

prep kit (Bio Basic Canada Inc., Canada) as per the manufacturer's instruction. Restriction-digestion of plasmid DNA was done in 20 µL reaction mixture with 4 µL of recombinant plasmid, 2 µL of NE buffer, 2 µL each of *Eco*R1 and *Hin*dIII, 1 µL of 1x BSA and 8 µL of nuclease-free water. The release of insert (~700 bp) from the recombinant plasmid confirmed the success of cloning. The purified recombinant plasmids were sent for sequencing. Sequencing was done at Chromos Biotech India Pvt. Ltd., Bengaluru in both forward and reverse directions to find out the mismatch if any, in the target sequence. BioEdit sequence alignment editor (Version 7.0.4.1) was used to edit the nucleotide sequences received from the firm. Homology between the *cytochrome c oxidase I* (*COI*) fragment of *S. excerptalis* sequenced in this study and those already deposited in the GenBank of NCBI was deduced by using the Blast algorithm of NCBI. The nucleotide sequences were translated into amino acid sequences using ExPASy (Expert Protein Analysis System) translate tool of Swiss Institute of Bioinformatics and the open reading frames (ORF) were obtained using invertebrate mitochondrial genetic code. The well-characterized barcode of sugarcane top borer was submitted in the GenBank of NCBI and unique accession number for the sequence was obtained.

Results and Discussion

Populations of *S. excerptalis* collected from the research farm of ICAR-Sugarcane Breeding Institute, Coimbatore were used for species identification through conventional taxonomy and for developing DNA barcodes. Taxonomic identity of *S. excerptalis* was confirmed (Fig. 1) by running the keys developed by Sohn *et al.* (2015). We could clone and characterize the 658 bp Folmer region from the mitochondrial *cytochrome c oxidase I* (*COI*) gene of *S. excerptalis* (Figs. 2 and 3). Earlier, Ramasubramanian *et al.* (2014b) were successful in cloning the 658 bp *COI* gene fragment (KJ013410) from sugarcane pink borer, *Sesamia inferens* (Walker) (Lepidoptera: Noctuidae). The *COI* sequences of *S. inferens* cloned by other workers (KJ500027-635 bp, KJ635882-638 bp, KC911715-659 bp, HM160126-652 bp, and HM160124-703 bp) were found to vary in their sizes (either <658 bp or >658 bp in size). We could also retrieve *COI* gene sequences for some of the major lepidopteran pests of sugarcane *viz., Chilo infuscatellus* (JQ066747-678 bp, JQ066748-551 bp), *Chilo auricilius* (KC306949-611 bp) and
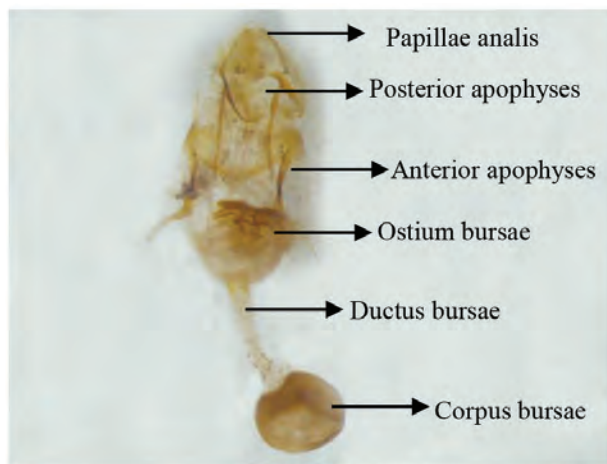
Fig. 1a. Female genitalia
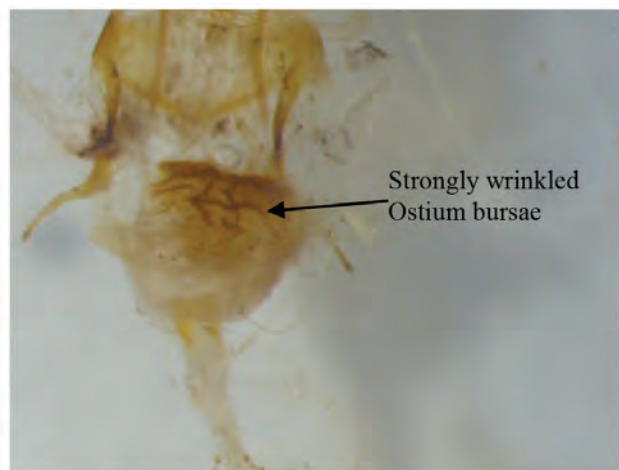
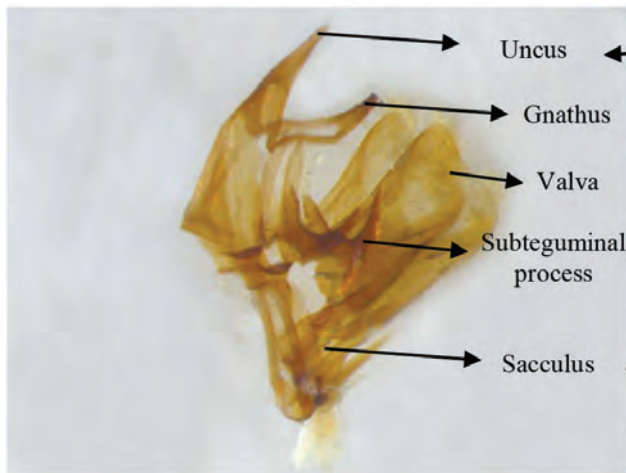**Fig. 1b.** Female genitalia (Close-up view)
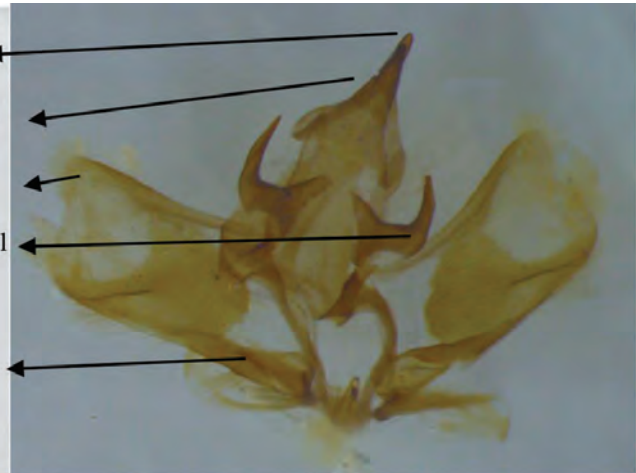
Fig. 1c. Male genitalia (Lateral view)

Fig. 1d. Male genitalia (Ventral view)

Fig. 1e. Aedeagus
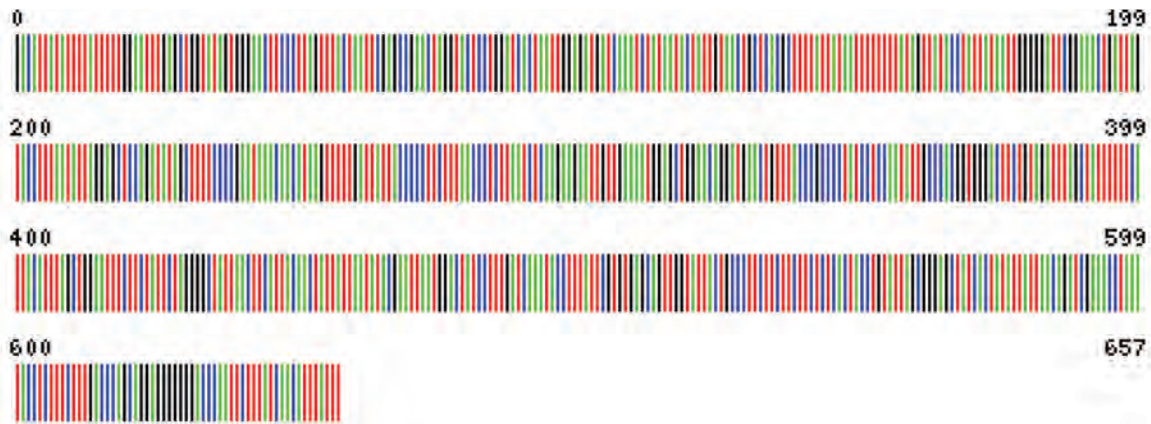
Fig. 1. Male and female genitalia

Chilo sacchariphagus indicus (KC306951-611 bp) from NCBI. These partial CDs of *COI* gene are however, not exactly 658 bp in size.

The barcode generated by us for *S. excerptalis* may be considered as the most appropriate one for its unambiguous identification as the *COI* sequence cloned in this study is of 658 bp in size (i.e., ideal DNA barcode). The protein sequence of the *COI* gene fragment (219 amino acids) does not have any stop codon (Fig. 4) and the uninterrupted open reading frame reaffirmed the impeccability of the barcode developed in this study. We could retrieve a *COI* sequence of *S. excerptalis* (KC306948) from the NCBI which was 611 bp in size. Although it has been proved by many researchers that the 658 bp *COI* gene fragment is efficient enough in delineating the insect species with sufficiently high and low inter- and intra-specific sequence divergence respectively, the loss of bases from the

Fig. 2. DNA barcode of *Scirpophaga excerptalis* (GenBank Accession No. KJ013411)

Fig. 3. Illustrative barcode of *Scirpophaga excerptalis*

(Sample I.D. SE_CBE_SBITR of BOLD SYSTEMS)

ideal barcode length of 658 bp may sometimes lead to ambiguous identification of insect species. Hence, it was aimed to clone 658 bp *COI* gene fragment of *S. excerptalis*, and we could succeed. The *COI* sequence of *S. excerptalis* cloned in this study was aligned with the *COI* sequences generated elsewhere for two other closely related species of the genus *Scirpophaga viz., S. nivella* and *S. imparellus.* Although *COI* sequences of *Scirpophaga incertulas*, *Scirpophaga innotata*,

Scirpophaga percna and *Scirpophaga praelata* are available in the NCBI, none of them was of 658 bp in size. Their *COI* sequences were hence, ignored in the multiple sequence analysis performed in the ClustalW2 of European Bioinformatics Institute of European Molecular Biology Laboratory (EMBL-EBI). There were significant levels of variations between the *COI* sequences of *Scirpophaga* spp. The *S. excerptalis COI* sequence shared only 86.78% and 87.39% identity with *S. nivella* and

Fig. 4. Uninterrupted ORF of translated *Scirpophaga excerptalis* DNA barcode

Table 1. Percent identity among the *COI* sequences of selected *Scirpophaga* spp.

Scirpophaga spp.	GenBank Accession No.	Percent identity created by Clustal2.1		
		S. excerptalis	*S. nivella*	*S. imparellus*
Scirpophaga excerptalis	KJ013411	100.00	86.78	87.39
Scirpophaga nivella	KF399959*	86.78	100.00	88.91
Scirpophaga imparellus	JN278989*	87.39	88.91	100.00

(*the nucleotide sequences were retrieved from NCBI at www.ncbi.nlm.nih.gov for multiple sequence alignment using ClustalW2)

S. imparellus, respectively (Table 1). Thus, the DNA barcode of *S. excerptalis* developed in this study could easily diagnose the sugarcane top borer from other closely related species of *Scirpophaga*.

The 658 bp fragment lies in the 5' region of mitochondrial *COI* gene has been designated as the standard DNA barcode for animal species including insects (Hebert *et al.,* 2003). This fragment has been shown to identify the species from across the orders of the class Insecta without any ambiguity. Hebert *et al.* (2003) were successful in identifying 196 of 200 lepidopteran species involved in their study by using the *COI* gene sequences, when the minimum threshold variation between two *COI* sequences to delineate a species has been fixed at 3%. Only four congeneric species of recent origin were shown to have 0.6-2.0% variation between their *COI* sequences. The *COI* gene sequence cloned in the study has been proved as a powerful molecular tool to correctly identify the top borer of sugarcane.

The *COI*-based DNA barcoding approach has been reported as a decisive tool for quick and reliable identification of insect species belonging to the orders Hymenoptera, Hemiptera, Diptera Lepidoptera and Coleoptera (Asokan *et al*., 2012; Rebijith *et al.,* 2012; Rebijith *et al.,* 2013; Ojha *et al*., 2014; Tembe *et al.*, 2014; Jalali *et al.,* 2015). The size of the *COI* fragments was found to vary between 500 and 744 bp in these studies. Although there had been several justifications for the deviation in size of *COI* gene fragments from 658 bp, after a thorough examination of methodologies adopted in the studies conducted by many of the researchers, we speculate that the direct sequencing of PCR products without performing cloning may have led to the loss of nucleotides in the 5' and 3' region of the target sequence.

Besides being a highly conserved region in the genome of insects and maternal in inheritance, the *COI* gene fragment carries the phylogenetic information with significantly higher level of interspecific sequence divergence while maintaining lower level of intraspecific sequence variations. Hence, the *COI* gene fragment is preferred over other regions/gene fragments of insect genome to serve as DNA barcode. The potential of *COI* sequence in delineating the species belonging to different orders of the class Insecta has been demonstrated by many researchers worldwide. Hence, this study was also aimed to amplify the *COI* gene fragment from *S. excerptalis* and the same was flawlessly achieved. By and large, the *COI*-based DNA barcoding approach has emerged as a handy, reliable, cost-effective and complementary tool to conventional alpha taxonomy. The full-length DNA barcode cloned in this study would certainly serve as a molecular diagnostic kit for unambiguous identification of sugarcane top borer, *S. excerptalis* by non - taxonomists.

Acknowledgement

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