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Development of DNA Barcoding Signatures for Pink Bollworm in Cotton Ecosystem Based on the Mitochondrial *COI* Gene

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ABSTRACT

Background: Species identification is a highly specialized, time-consuming and rely significantly on the diagnostic features that are present mostly in the adult life stages which constrains the recognition, as many specimens lack these characters.

Methods: In this study, we used molecular strategy to develop markers for the identification of the pink bollworm in the cotton ecosystem. This research was conducted at the Department of Agricultural Entomology and Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu State, during 2021 and 2022. DNA sequences of the *cytochrome c oxidase subunit I* gene were used to successfully develop DNA barcoding signatures for pink bollworm.

Result: Using the MUSCLE alignment method in MEGA X software and by the use of phylogenetic tree, the sequences were segregated into Clade 1 (had a sequence length of 264 bp) and Clade 2 (had a sequence length of 164 bp) and for each clade DNA barcoding signatures were developed. With such molecular identity would be simple, rapid, precise and more reliable to identify *Pectinophora gossypiella,* as it is difficult to accurately identify this bollworm during its early life stages.

Key words: Cotton, DNA barcoding signatures, InDel regions, Mitochondrial *COI* gene, Pink bollworm.

INTRODUCTION

DNA isolated from any life phase of an organism *viz*., egg, larva, pupa, adult or tissue fragments will generate a similar identification, while the conventional keys often depend mainly on the adult features. Social insects with varied morphologies have been misidentified as separate species in several situations. Sexual dimorphism has also long been recognized as a source of complications to taxonomists. Conversely, DNA sequencing can be used to eliminate such misunderstandings (Zhai *et al*., 2017). Until DNA sequencing revealed that males and females shared the same *cytochrome c oxidase subunit I* (*COI*) sequences, an inventory of the butterfly *Saliana severus* recorded each sex as a distinct species. This led to the identification of a highly sexual dimorphic species (Janzen *et al*., 2005). An important advantage of a DNA sequence-based approach is it relies mostly on the use of algorithms enabling DNA sequences such as comparisons tools, DNA-sequence repositories *viz*., BLAST (Basic Local Alignment Search Tool), GenBank or BOLD (Barcode of Life Data Systems), *etc* (Altschul *et al*., 1990; Ratnasingham and Hebert 2007). Taxonomic accuracy, low cost, ease of application in varied contexts (including by non-specialists), portability, repetitive and instantaneous access to information and functionality across a broad phylogenetic and taxonomic spectral range of organisms, including many species new to science, are all advantages of using DNA sequence for species identification, assessment and taxonomic description (Jalali *et al*., 2015). Using this (Onah *et al*., 2017) successfully identified *Bactrocera dorsalis* and *Ceratitis anonae* (Graham) infesting citrus in South-Eastern Nigeria using PCR-RFLP based on the COI gene. These methods have been used to find economically significant insect pest species.

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Pectinophora gossypiella (Lepidoptera: Gelechiidae) a member of the bollworm complex, is one of the significant pest in cotton (Dhurua and Gujar 2011). The characteristics that distinguish the pink bollworm larva and pupa from those of other Lepidoptera attacking cotton or related malvaceous plants, were the presence of the adfrontal setae are widely separated and seta on the segment 2 is at the apex of the front; the mandible has four teeth with the last one smaller than the others; a crescent-shaped marking is often present on the prothoracic shield; the abdominal prolegs have crochets in a uniordinal penellipse; the anal crochets are in a single uninterrupted band and seta on the dorsal side of

segment 9, not hair-like (Gilligan and Passoa 2014). Unlike some other gelechiids lack an anal comb, while the lateral group of setae in segment 9 is usually bisetose (Stehr 1987). Busck (1917) and Heinrich (1921) discovered a third microscopic seta on the lateral side of the larva. *Dicymolomia julianalis* Walker and *Cracidosema plebeiana* Zeller also closely resembled the pink bollworm in their habits and the larval stages (Heinrich 1921). The diagnostic methods based on DNA and PCR-based markers to distinguish between species can be an alternate method for the identification of insects. The detection of the pest and monitoring of its status is an important principle of integrated pest management (Barzman *et al*., 2015; Yones *et al*., 2019).

MATERIALS AND METHODS

Pink bollworm samples were collected from the major cotton-growing tracts of Tamil Nadu *viz*., Coimbatore, Srivilliputhur, Veppathanttai and Salem were used since they include the irrigated, rainfed and rice fallow cotton cultivation belts which were later compared with the Indian and world population using the selected NCBI sequences. The collected larva were stored in Eppendorf tubes with 70% ethanol @ -20°C before DNA extraction.

The genomic DNA was extracted from pink bollworm larvae using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle 1991) with necessary modifications. The laboratory works where conducted at the Department of Agricultural Entomology and Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu State, during 2021 and 2022. Homogenization of larvae with 600ìl of CTAB buffer (CTAB buffer: 1 mM Tris HCl; 0.5 mM EDTA; 5 mM NaCl; 2% beta-mercaptoethanol, 2% CTAB and pH-8.0) and made up to 1ml with CTAB buffer. The mixture was incubated for 30-60 min at 65°C. Then treated with 500ìl phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged at 12000rpm for 10 min. The supernatant was transferred to a fresh Eppendorf tube containing 300ìl chloroform-isoamyl alcohol (24:1) and the mixture was centrifuged at 12000 rpm for 10 min. Again the supernatant was transferred to the fresh Eppendorf tube containing 250 to 500 µl of isopropanol, for precipitation. The pellets were recovered by centrifugation @ 12000 rpm for 10 min and washed with 70% ethanol and re-suspended in 30-50 µl DEPC treated water. The quality and quantity of DNA were assessed using a NanoDrop spectrophotometer (Thermos Scientific, USA) and 0.8% agarose gel.

The mitochondrial genes are inherited maternally and they show a sufficient degree of diversity among the species. Hence, the primers (Table 1) which amplify the mitochondrial genes *viz*., *cytochrome c oxidase subunit I* was employed to generate PCR products. PCR analyses were performed in 25-µl total reaction volume using 1 µl of each forward (LCO1490) and reverse (HCO2198) primer. Polymerase Chain Reaction (PCR) was carried out in a thermal cycler with the following cycles: 94°C for 4 minutes as initial denaturation followed by 35 cycles of 94°C for 30 seconds, 48°C for 45 seconds, 72°C for 45 seconds and 72°C for 20 minutes as a final extension.

The amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10ìg/ml) and visualized in a gel documentation system (UVP) (Asokan *et al*., 2011). The amplicons were commercially sequenced bidirectional using the SANGER sequencing method (@ Biokart India Pvt Ltd, Bangalore). The sequences were characterized using bioinformatics tools such as BLAST to check their homology. The sequences including all of the geographic populations were submitted to GenBank repositories for accession numbers and BOLD software to generate the DNA barcodes (Jiang *et al*., 2014).

The differences in the *COI* region of all the sequences used in the current study (Table 2 and 3) were determined by CLUSTAL multiple sequence alignment, aligned by MUSCLE (Multiple Sequence Comparison by Log-Expectation) using MEGA X software (Fig 1). After alignment, they were further analysed by constructing a phylogenetic (Neighbour-joining) tree using MEGA X software to find the genetic diversity among the pink bollworm population (Kumar *et al*., 2018; Zheng *et al*., 2019).

The DNA barcoding signatures were designed to the developed DNA sequence based on the variations in the *COI* gene sequence between the pink bollworm populations. The amplification of fragments that were very specific to the species under investigation where used as a marker for that species as same approach as species specific markers mentioned by Rebijith *et al*., (2012). The PCR amplified fragments resulting from bio-markers were used for further quality analysis using OligoAnalyzer (© Integrated DNA Technologies, Inc.) software.

RESULTS AND DISCUSSION

The accession numbers and DNA barcodes were generated (Table 2) and using them as reference templates, two pairs of DNA barcoding signatures were successfully designed (Forward primer 1-5′GAA AAT GGA GCA GGA ACC G 3′, reverse primer 1-5′CCTGT TTT AGC AGG AGC TA 3′, forward primer 2-5′ GAG CTG TAT TTG CAA TTT TAG GAG G 3′, reverse primer 2-5′ GAT TAC CCC GAT GCT TAT 3′). (Table 4). The selected sequences from table 2 and 3 were arranged in CLUSTAL multiple sequence alignment, aligned by MUSCLE using MEGA X software, showed that the sequences were divided between Clade 1 and Clade 2 (Fig 1). A portion where most variation followed by the conservation

Table 1: Details of the primers used in the current study.

Primer name	Sequence (5′-3′)	Melting temperature	Reference
LCO1490	GCTCAACAAATCATAAGATATTGG	61.2°C	Folmer *et al*., 1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	66.9°C	Folmer *et al*., 1994

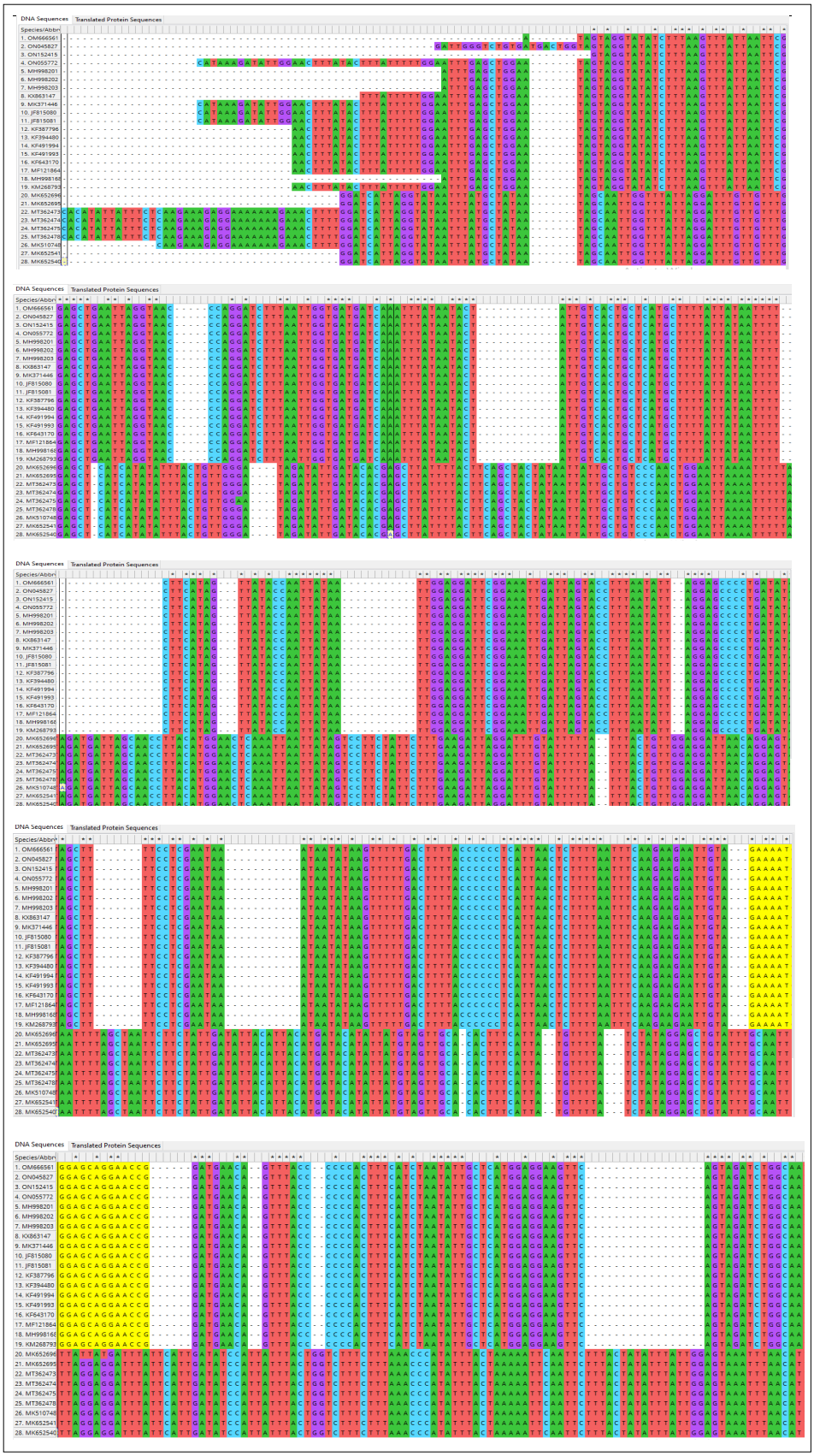

Fig 1: Continue...

Fig 1: Continue...

The InDels that were present in conserved regions were marked as gaps (-). The conserved positions are marked by asterisks (*). In examining these MSAs, only those sequence gaps (-) or InDels were considered, which were flanked on both sides by a number of (*) in the neighbouring 30-40 aa.

Fig 1: CLUSTAL multiple sequence alignment of mt *COI* gene sequences, aligned by MUSCLE (3.8) using MEGA X software.

Table 2: mt COI gene sequences developed in the current study.

Species	Collection site	Latitude and longitude	Accession numbers	BIN (Barcode index number)	Size
P. gossypiella	Coimbatore, India	11.0167N, 76.9350E	OM666561	BOLD:AAH4802	638 bp
P. gossypiella	Srivilliputhur, India	9.5045N, 77.6440E	ON055772	BOLD:AEO7403	624 bp
P. gossypiella	Veppanthattai, India	11.3516N, 78.8043E	ON045827	BOLD:AAH4802	551 bp
P. gossypiella	Salem, India	11.6560N, 78.4690E	ON152415	BOLD:AAH4802	624 bp

region were chosen. The sequence denoted by (*) are the conserved regions and they were chosen (Fig 1). The primers were designed from both the clades based on the InDel regions in the both clades and which were considered as the DNA barcoding signatures for the pink bollworm clades. Conserved Signature InDels in gene/proteins sequences provide an important category of molecular markers for understanding microbial phylogeny and systematics. The InDels which provide useful molecular markers are generally of defined size and they are flanked on both sides by conserved regions to ensure that they constitute reliable molecular characteristics. The InDels which were not present in conserved regions are not investigated (Gupta 2014). Clade 1 sequences had 38bp inserted codons and 41 bp deleted codons in the selected portion, whereas Clade 2 sequences had 41 bp inserted

Table 3: mt COI gene sequences from GenBank used in the current study.

Species	Collection site	Accession numbers	Size
P. gossypiella	New Delhi, India	MH998201	651 bp
P. gossypiella	New Delhi, India	MH998202	651 bp
P. gossypiella	New Delhi, India	MH998203	651 bp
P. gossypiella	Pakistan	KX863147	570 pb
P. gossypiella	Pakistan	MK371446	687 bp
P. gossypiella	USA	JF815080	688 bp
P. gossypiella	USA	JF815081	688 bp
P. gossypiella	Australia	KF387796	658 bp
P. gossypiella	Australia	KF394480	658 bp
P. gossypiella	USA	KF491994	642 bp
P. gossypiella	USA	KF491993	658 bp
P. gossypiella	Kenya	KF643170	658 bp
P. gossypiella	Kenya	MF121864	658 bp
P. gossypiella	Junagarh, India	MH998168	651 bp
P. gossypiella	Bangalore, India	KM268793	658 bp
P. gossypiella	Parbhani, India	MK652696	686 bp
P. gossypiella	Parbhani, India	MK652695	686 bp
P. gossypiella	Bangalore, India	MT362473	753 bp
P. gossypiella	Guntur, India	MT362474	753 bp
P. gossypiella	Raichur, India	MT362475	753 bp
P. gossypiella	Warangal, India	MT362478	753 bp
P. gossypiella	Akola, India	MK510748	711 bp
P. gossypiella	Akola, India	MK652541	686 bp
P. gossypiella	Akola, India	MK652540	686 bp

codons and 38bp deleted codons (Table 4). To confirm this, a phylogenetic analysis was performed and a phylogenetic tree was constructed. Clade 1 and Clade 2 were identified on the phylogenetic tree (Fig 2). The Neighbour-Joining method was used to infer the evolutionary history (Saitou and Nei 1987). The optimal tree was shown (next to the branches). The evolutionary distances were processed utilizing the Tamura 3-parameter technique (Tamura 1992) and the number of base substitutions per site was used as units. This analysis involved 28 nucleotide sequences. The given pink bollworm sequences were separated into two clades, Clade 1 and Clade 2, with sub-clades forming clusters according to evolutionary analysis. Clades 1 and 2 were identified based on sequence comparisons and phylogenetic analysis, which revealed significant genetic variability (King *et al*., 2002; Chu *et al*., 2008; Chu *et al*., 2014). Accordance to the current study, multiple alignment of nucleotide sequences analysis conducted by Latina *et al*., (2022) have also created a conserved 16-bp insertion-deletion (InDel) site common to all *Aspidiotus rigidus* specimens discovered, from which the *A. rigidus* unique oligonucleotide (RIG1) primer targeting marker was identified.

The following guidelines were applied to the developed DNA barcoding signatures (Nadipineni 2021):

1. At the initial site of attachment, there should be a strong bond. At the 5' prime end, we must ensure that G/C is present.
2. The ideal GC content is in between 40% and 60%.

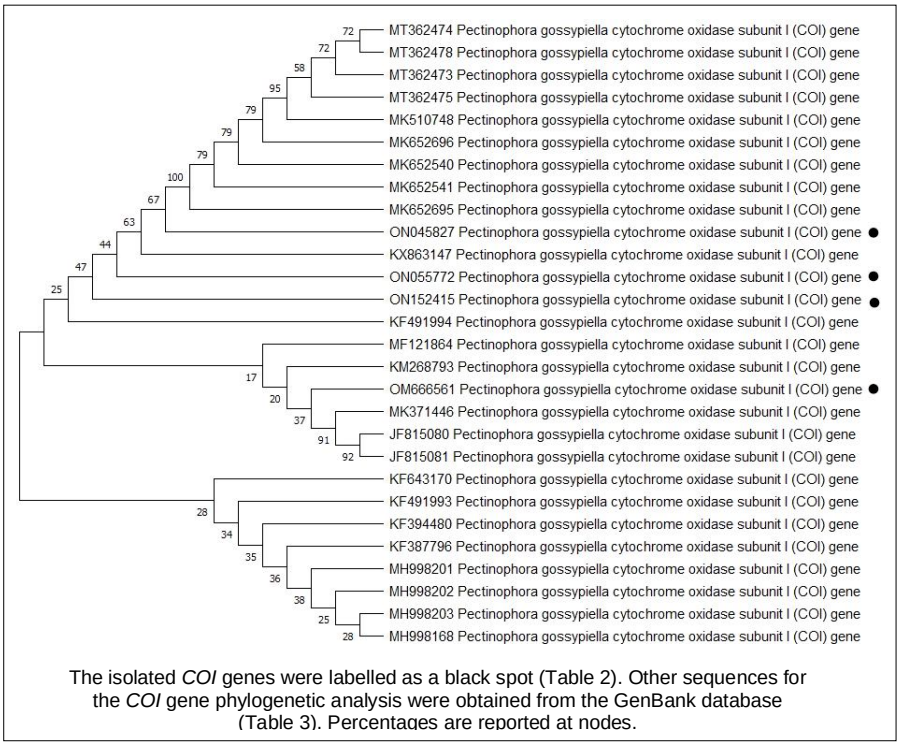

Fig 2: Phylogenetic tree of the given mt *COI* gene sequences of *Pectinophora gossypiella*. The GenBank accession number with scientific names were included.

3. The optimal length is 18 to 22 bp; less than 18 bp may result in unspecific bindings whereas greater than 22 bp forms secondary structures.

According to Jiang *et al*., (2014), primers quality is the most important element affecting specific amplification and sensitivity. The quality of the primers that were developed in the current study was checked for the quality with OligoAnalyzer (© Integrated DNA Technologies, Inc.) software and it fulfilled the rules that were needed to be followed in designing the molecular primers (Table 5).

Chua *et al*. (2010) effectively created two sets of species-specific markers based on the mtDNA *COI* gene that could differentiate *Bactrocera papayae* and *Bactrocera carambolae* under standard PCR settings. Asokan *et al.* (2011) have developed a method for identifying *Bactrocera dorsalis* and *Bactrocera zonata* using species-specific

Table 4: DNA barcoding signatures obtained for the given mt COI gene sequences based on the indel regions.

Clade 1

>OM666561.1 Pectinophora gossypiella isolate TNAUPBWCBE01 cytochrome c oxidase subunit I (COX1) gene, partial cds; Mitochondrial

ATAGTAGGTATATCTTTAAGTTTATTAATTCGAGCTGAATTAGGTAACCCAGGATCTTTAATTGGTGATGATCAAATTTATAATACTA
TTGTCACTGCTCATGCTTTTATTATAATTTTCTTCATAGTTATACCAATTATAATTGGAGGATTCGGAAATTGATTAGTACCT
TTAATATTAGGAGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGACTTTTACCCCCCTCATTAACTCTTTT
AATTTCAAGAAGAATT GTAGAAAATGGAGCAGGAACCGTTCATTGATGAACATTGTTTACCGGCCCCACTTTCATCTAATATTGCT
CATGGAGGAAGTTCTTTACTATATTTATTGGAGTAGATCTGGCAATTTTTTCTTTACATTTTTTAGGATTAGCAGGTATACTTCATCAAT
TTTAGGAGCAATTAACTTTATTACTACAATTATTAATATACGAATTAATGGTTTATCATTCGATCAAATACCATTATTTGTTTGAGCTGTAGG
AATTACAGCCTTATTATTACTTATATTATCATTACCTGTTTTAGCAGGAGCTATTACTATATTACTAACAGATCGAAATTTAAATA
CTTCATTTTTTGATCCAGCTGGTGGAGGAGATCCAATCCTATACC AACACTTATTTTGATTTTTTGGTCA

Clade 2

>MK510748.1 Pectinophora gossypiella isolate Akola_B3 cytochrome oxidase subunit 1 (COI) gene, partial cds; Mitochondrial

CAAGAAAGAGGAAAAAAAGAAACTTTTGGATCATTAGGTATAATTTAT GCTATAATAGCAATTGGTTTATTAGGATTTGTTGTTTGAGC
TCATCATATATTTACTGTTGGGATAGATATTGATACACGAGCTTATTTTACTTCAGCTACTATAATTATTG
CTGTCCCAACTGGAATTAAAATTTTTAGATGATTAGCAACCTTACATGGAACTCAAATTAATTATAGTCCTTCTATTCTTTGAAGATTAG
GATTTGTATTTTT ATTTACTGTTGGAGGATTAACAGGAGTAATTTTAGCTAATTCTTCTATTGATATTACATTACATGATACATATTATGT
AGTTGCACACTTTCATTATGTTTTATCTATAGGAGCTGTATTTGCAATTTTAGGAGGATTTATTCATTGATATCCATTATTTACTG
GTCTTTCTTTAAACCCATATTTACTAAAAATTCAATTCTTTACTATATTTATTGGAGTAAATTTAACATTTTTTCCTCAACA
CTTTTTAGGATTAGCAGGTATACCTCGACGATATTAGGAGCAATTAACCTGATTACCCCGATTATTAATATAC
GAATTAATGGCTTATCATCTCATGAAATATTGTTTCTTCTTTAG GTTCTTATATTTCCTTATTAGCAACTATATTAATATTAATTATTATCTGA
GAATCTATAATTAATCAACGAATTG TATTATTCCCATTAAATATCATCATCAATTGAATGATATCAAAA

AAAA - Primer designed
AAAA - Inserted
AAAA - Deleted

Sub-clades of Clade 1
ON055772 (Srivilliputtur) 624 bp
ON045827 (Vepanthattai) 551 bp
ON152415 (Salem) 624 bp
MH998201 (New Delhi) 651 bp
MH998202 (New Delhi) 651 bp
MH998203 (New Delhi) 651 bp
KX863147 (Pakistan) 570 bp
MK371446 (Pakistan) 687 bp
JF815080 (USA) 688 bp
JF815081 (USA) 688 bp
KF387796 (Australia) 658 bp
KF394480 (Australia) 658 bp
KF491994 (USA) 642 bp
KF491993 (USA) 658 bp
KF643170 (Kenya) 658 bp
MF121864 (Kenya) 658 bp
MH998168 (Junagarh) 651 bp
KM268793 (Bangalore) 658 bp

Sub-clades of Clade 2
MK652696 (Parbhani) 686 bp
MK652695 (Parbhani) 686 bp
MT362473 (Bangalore) 753 bp
MT362474 (Guntur) 753 bp
MT362475 (Raichur) 753 bp
MT362478 (Warangal) 753 bp
MK652541 (Akola) 686 bp
MK652540 (Akola) 686 bp

Table 5: Properties of the DNA barcoding signatures designed.

Primer 1 designed based on Clade 1		Primer 2 designed based on Clade 2	
Forward primer 1	5′-GAA AAT GGA GCA GGA ACC G-3′	Forward primer 2	5′- GAG CTG TAT TTG CAA TTT TAG GAG G-3′
Length	19	Length	25
GC content	52.6%	GC content	40%
Melting temperature	54°C	Melting temperature	54.4°C
Reverse primer 1	5′-CC TGT TTT AGC AGG AGC TA-3′	Reverse primer 2	5′-GAT TAC CCC GAT GCT TAT-3′
Length	19	Length	18
GC content	47.4%	GC content	44.4%
Melt Temp	51.7°C	Melt temp	48.7°C
Product size	223 bp	Product size	148 bp

markers based on DNA barcode sequences and this method could accurately identify the target species at all life stages. Jiang *et al*. (2014) employed DNA barcoding sequences to develop a species-specific genetic markers for *Bactrocera minax* and *Bactrocera tsuneonis*, two sibling species that are difficult to identify or distinguish based solely on morphological characteristics and had a significant quarantine importance in Asia. Accordingly, the DNA barcoding signatures in this study were generated using same approach for the given pink bollworm populations.

CONCLUSION

Thus the DNA barcoding signatures developed can be used for the molecular identification of *Pectinophora gossypiella,* as it is difficult to accurately identify this bollworm during its early life stages. DNA barcoding signatures reported in this study would also be useful in plant quarantine, pest surveillance and monitoring as well as in developing better management strategies to minimize the yield loss caused by pink bollworm.

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Conflict of interest: None.

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