RESEARCH ARTICLE

MITOCHONDRIAL CYTOCHROME C OXIDASE I (*MTCOI*) GENE-BASED MOLECULAR DIAGNOSTIC KIT FOR *PYRILLA PERPUSILLA* WALKER (HEMIPTERA: LOPHOPIDAE)

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Abstract

A DNA barcode was developed for sugarcane leaf hopper *Pyrilla perpusilla* Walker (Hemiptera: Lophopidae). The DNA barcode developed in this study is 658 bp in size and its amino acid sequence does not have any stop codon in it. The uninterrupted open reading frame indicated the flawlessness of the barcode generated in this study. The DNA barcode developed by us is the first report and it would certainly serve as an ideal molecular diagnostic kit for *P. perpusilla*. The extent of identity between the barcode sequences of *P. perpusilla* and other important hemipteran pests of sugarcane varied widely between 61.33 and 72.04%.The wide variation that exists between the barcode sequences of *P. perpusilla* and other cane-infesting hemipterans clearly indicates that the barcode developed in this study certainly identifies the species without any ambiguity.

Key words : Sugarcane, *Pyrilla perpusilla*, DNA barcode, molecular diagnostic kit, *mtCOI*

Introduction

Sugarcane is an important cash crop of India. Cane farming provides livelihood to more than seven million farmers in the country (Srivastava et al. 2013). India, though occupies the second place in area and production of sugarcane in the world (Department of Agriculture and Cooperation 2015), the productivity in the country is much lower than that is being realized in many developed countries like U.S.A. and Australia. Besides, there has been wide disparity in the productivity between the tropical and subtropical cane belts of the country. Although wide variation in the edaphoclimatic factors prevailing in the two different cane ecosystems has largely been accepted to be the major cause for the wide gap in cane yield, yield loss due to comparatively higher incidence of insect pests in the subtropical belt cannot be ignored. *Pyrilla perpusilla* Walker (Hemiptera: Lophopidae), though pan-India in occurrence, often reaches epidemic form only in the subtropical sugarcane production systems of the country. The pest alone is sometimes responsible for 31.6% and 2-3% reduction in cane yield and sugar recovery, respectively (Directorate of Sugarcane Development 2015). Unlike in other annual crop ecosystems, the control of sugarcane pests is by and large achieved by inundative releases of biocontrol agents, particularly parasitoids specific to the target pests. In contrast, insecticides, which are mostly broad-spectrum in their activity, may bring down pest populations significantly irrespective of

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the species complex in the population. Therefore, it is needless to mention that correct identification of the target pest is a prerequisite for the success of bio-intensive integrated pest management programme in sugarcane. Unambiguous identification of pest species has been a great challenge for insect taxonomists across the world. Taxonomy of crop pests, including those of sugarcane, has been under constant revision by taxonomists, possiblydue to the phenotypic plasticity of key morphological traits as one of the reasons. Besides, the conventional taxonomy cannot identify cryptic species which are morphologically similar in key taxonomic traits such as male and/or female genitalia. The science of biological identification in India suffers from a lack of sufficient number of taxonomists. Moreover, established insect taxonomists generally specialize in a narrow range of orders of class Insecta as insects outnumber other members of the kingdom Animalia. Under these circumstances, we are in need of an alternative or complementary tool for correct identification of insect species. DNA barcoding approach has emerged as a simple, reliable and handy tool to identify the insect species. It also has the potential to identify the cryptic species, if any, in the population. Though the science of DNA barcoding has evolved more than a decade back and a large volume of literature has been generated across the world, it is still in its infancy in India. Although a few entomologists in the country could succeed in their efforts in generating the full length DNA barcodes, efforts are still underway to obtain the ideal DNA barcode of 658 bp in size. Development of DNA barcodes for insects in sugarcane ecosystem is yet to be addressed adequately and, hence, as a part of a larger study, an attempt was made to develop ideal DNA barcode for the sugarcane hopper *P. perpusilla*.

Materials and methods

DNA isolation and PCR

Insects collected from the research fields at ICAR-Sugarcane Breeding Institute, Coimbatore, were used for identification through conventional taxonomy and for developing DNA barcodes. DNA was isolated from single insect as described by Ramasubramanian et al. (2015b). The insect was taken in a sterile 1.5 mL microcentrifuge tube containing 700 µL of warm CTAB buffer (Tris 100 mM, EDTA 20 mM, NaCl 1.4 M, CTAB 2%, β-mercaptoethanol 0.2%). The insect tissues were homogenized and kept in water bath at 65°C for 1.5 h. The contents were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected and equal volume of chloroform: isoamyl alcohol (24:1) mixture was added. The microcentrifuge tube was shaken vigorously and the contents were again centrifuged at 12,000 rpm for 10 min at 4°C. The top layer was collected carefully and equal volume of ice-cold alcohol was added to the recovered layer. The contents were kept in deep freezer (-20°C) overnight to precipitate the DNA. The DNA pellet was recovered after centrifugation at 12,000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol, dried carefully and then dissolved in sterile Milli-Q water. One µl of RNaseA (10 mg/mL) was added to the isolated DNA and kept at 37°C for 30 min. The enzyme was inactivated by keeping the contents at 65°C for 10 min. The resultant RNA-free DNA was used immediately or 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). In addition, the quality of the DNA was also checked by running the DNA in 0.8% agarose (Sigma-Aldrich India Pvt. Ltd., Bengaluru) gel. PCR was performed in S-1000 PCR Touch Cycler (BioRad, USA) in 20 µl reaction volume. The primer pair LCO1490 and

HCO2198 (Folmer et al.1994) was used to amplify the target fragment of the mitochondrial cytochrome c oxidase-I *(mtCOI)* gene. The PCR mixture consisted of 25-30 ng of template DNA, 0.2 µM each of forward and reverse primers (Sigma-Aldrich India Private Ltd., Bengaluru), 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 PCR programme used to amplify the target fragment consisted of one cycle of 4 min at 94°C; 35 cycles of 30 sec at 94°C, 45 sec at 47°C and 45 sec at 72°C and a final cycle of 20 min at 72°C. After PCR amplification, the samples were subjected to electrophoresis in 1.5% agarose gel.

Cloning and characterization

Cloning and characterization were performed as detailed by Ramasubramanian et al. (2015b). The PCR products were purified using GenElute Gel Extraction Kit (Sigma-Aldrich India Private Ltd., Bengaluru) as per the manufacturer's instructions. The final concentration of the purified PCR products was checked in the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific Inc., USA). The quality of purified PCR products was also checked by resolving them in 1.5% agarose gel. The purified PCR products were cloned into the plasmid vector pTZ57R/T using InsTAclone PCR cloning kit as per the manufacturer's instructions (Thermo Scientific Inc., USA). This is followed by transformation of *Escherichia coli* (strain DH5α) competent cells by heat-shock method. The recombinant clones were confirmed by colony PCR using the barcode primers LCO1490 and HCO2198 as detailed above 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 manufacturer's instructions. The recombinant plasmid containing target fragment was digested with *Eco*R1*/Hin*dIII. Digestion was done in 20 µL reaction with 4 µL of recombinant plasmid, 2 µL of NE buffer, 2 µL each of *Eco*R1 and *Hind*III, 1 µL of 1x BSA and 8 µL of nuclease free water. The release of insert (~700bp in size) confirmed the success of transformation. Sequencing of purified plasmids was done through outsourcing (Chromas Biotech India Pvt. Ltd., Bengaluru). Sequencing was done in both forward and reverse directions to find out mismatch, if any, in the target sequence. Homology search was made in the 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 of *P. perpusilla* was finally submitted in the GenBank of NCBI.

Results and discussion

DNA of good quality and yield was isolated from the sugarcane leaf hopper by adopting the protocol standardized earlier (Ramasubramanian et al. (2015b). Quantity of the DNA obtained from individual insects varied between 970 and 1470 ng/µL. Quality of the DNA as determined by 260/280 ratio was in the range of 1.76-1.82 indicating its suitability for PCR. The target fragment of approximately 700 bp was amplified from the genomic DNA of *P. perpusilla*, purified, cloned and sequenced as detailed earlier. The barcode sequence of *P. perpusilla* was observed to be 658 bp in size (Fig.1) and the amino acid sequence without any stop codon in it (Fig. 2). The uninterrupted open reading frame indicated the flawlessness of the sequence cloned in this study. Besides, the sequencing was done in both the directions to eliminate the chances of sequencing

>*Pyrillaperpusilla*

CACTCTTTACTTTATCTTTGGAATATGAGCAGGAATTATAGGAACTGCCTTAAGTATAATTATTCGAATAG
AACTTATTCAACCAGGTAACCTTATTAAAAATGACCAAATTTATAATACTGTGGTTACATCACATGCATTC
ATTATAATTTTCTTTATAGCTATACCAACTATAATTGGAGGATTTGGAAACTGATTAGTCCCTTTAATAAT
TGGAGCACCAGACATAGCCTTCCCCCGACTTAATAATATAAGATTTTGGCTCCTACCAATATCTATTTCCC
TTCTTATTTCGAGTTCAATTACAGGTTCGGGTACAGGAACAGGATGAACTGTTTATCCACCTCTGTCCAGT
CAACCAGCTCACTCTGGTCCATCAGTAGATTTAACAATTTTTTCTTTACATATTGCAGGAATTAGATCTAT
CCTTGGAGCAATTAACTTCATCTCAACTATCCTAAATATACGAGTTAAAGGAATAACTTTAGAAAAAACTC
CCCTATTCTGTTGATCTGTATTAATTACAGCTATTCTTCTTCTTTTATCTCTTCCTGTACTAGCAGGTGCA
ATTACAATATTAATTATAGATCGAAACTTTAATACTTCATTTTTTGACCCTTCAGGAGGAGGTGACCCGAT
TCTTTACCAACATTTATTT

Fig.1 DNA barcode of *Pyrilla perpusilla*

>*Pyrilla perpusilla*

TLYFIFGMWAGIMGTALSMIIRMELIQPGNLIKNDQIYNTVVTSHAFIMIFFMAMPTMIGGFGNWLVPLMI
GAPDMAFPRLNNMSFWLLPMSISLLISSSITGSGTGTGWTVYPPLSSQPAHSGPSVDLTIFSLHIAGISSI
LGAINFISTILNMRVKGMTLEKTPLFCWSVLITAILLLLSLPVLAGAITMLIMDRNFNTSFFDPSGGGDPI
LYQHLF

Fig. 2 Uninterrupted ORF of *COI* gene fragment from *Pyrilla perpusilla*

error. DNA sequence of 658 bp invariably located in the 5' region of the mitochondrial cytochrome c oxidase I (*mtCOI*) gene of insects has been designated as DNA barcode for the insect concerned (Hajibabaei et al. 2005; Floyd et al. 2009). It has been proved across the orders of the class Insecta that this mitochondrial DNA sequence is unique for a particular species and is sufficient enough to delineate the closely related species. The DNA barcode cloned by us for the sugarcane leaf hopper *P. perpusilla* is the first report as there is no such barcode for this species in the public domain. The barcode sequence of *P. perpusilla* was submitted in the GenBank of NCBI and unique accession number (KJ013412) obtained.

Full-length barcode sequences from important hemipteran pests of sugarcane, viz. *Aleurolobus barodensis, Neomaskellia bergii* (Hemiptera: Aleyrodidae)*, Melanaphis sacchari, Tetraneura javensis* (Hemiptera: Aphididae) and *Melanaspis glomerata* (Hemiptera: Diaspididae) were cloned successfully earlier by Ramasubramanian and Ramaraju (2014); Ramasubramanian et al. (2015a); Ramasubramanian et al. (2015b) and Ramasubramanian et al. (2015c). Although ideal DNA barcode should be 658 bp in size, the DNA barcodes of *A. barodensis* and *M. glomerata* are of 649 bp in size (Ramasubramanian et al. 2015a; Ramasubramanian et al. 2015b). The loss of 3-9 bases (1-3 amino acids) is quite common among the barcode sequences of insects. These deletions were reported as real deletions in the functional proteins of insects (COI enzyme). Moreover, it has been speculated that the standard barcode length of diaspidids may be 649 instead of 658bp in size as reported by Ramasubramanian et al. (2015a). The

Table 1. Extent of identity among the barcode sequences of hemipterans infesting sugarcane

Hemipteran	*Pyrilla*	*Melanaphis*	*Aleurolobus*[1]	*Neomaskellia*[1]	*Tetraneura*	*Melanaspis*[2]
Pyrilla	100.00	72.04	70.42	66.57	71.43	61.33
Melanaphis	-	100.00	71.19	68.39	72.49	61.02
Aleurolobus	-	-	100.00	72.27	73.04	65.33
Neomaskellia	-	-	-	100.00	70.82	64.41
Tetraneura	-	-	-	-	100.00	69.34
Melanaspis	-	-	-	-	-	100.00

([1]Ramasubramanian et al. 2015b; [2]Ramasubramanian et al. 2015a)

barcode sequences of hemipterans infesting sugarcane were aligned together and analysed for the extent of identity between the sequences (Table 1).The barcode sequences of hemipteran pests showed wide variation among the species: the barcode of scale insect *M. glomerata* shares only 61.02-69.34% identity with other hemipterans; the barcode sequence of *P. perpusilla* showed 61.33-72.04% identity with other hemipteran pests of sugarcane. The wide variation that exists between the barcode sequences of *P. perpusilla* and other cane-infesting hemipterans clearly indicated that the barcode developed in this study certainly identifies the species without any ambiguity. There may hardly be any region in the insect genome that is 100% perfect in delineating species with sufficiently high level of barcoding gap while maintaining very low level of intra-specific sequence variation. However, there are reports wherein DNA barcoding with *COI* gene fragment was found highly successful in the correct identification of closely associated species in Hemiptera (suborders Homoptera and Heteroptera). After detailed investigation with true bugs (Hemiptera:Heteroptera), Park et al. (2011) and Jung et al. (2011) concluded that the *COI* sequences were found efficient enough to delineate the species belonging to Heteroptera. Rebijith et al. (2012 a, b) have cloned and characterized the *COI* gene sequences of important hemipteran pests in horticultural ecosystem. They could differentiate the closely related species of Hemiptera by employing the DNA barcoding approach. The sequence divergence of 9.0% between the *COI* gene fragments of *Aphis gossypii* and *Myzus persicae* (Hemiptera: Aphididae), 7.6% between *Helopeltis antonii* and *Helopeltis bradyi* (Hemiptera: Miridae) and 7-13% among the barcode sequences of *H. antonii, H. bradyi, Helopeltis theivora* and *Pachypeltis maesarum* observed for the populations collected from the horticultural ecosystems of Indian subcontinent showed the potential of *COI* gene fragment in delineating the species. Asokan et al. (2012) also observed more than 10% variation between the sequences of closely related species of tea mosquito bugs, viz. *H. antonii* and *H. theivora* (Hemiptera: Miridae). Ramasubramanian et al. (2015b) have cloned and characterized the 'Folmer region' of *COI* gene from sugarcane aleyrodids *Aleurolobus barodensis* and *Neomaskellia bergii*. The *COI* sequences of these two species showed only 73.53% identity between them. In the present study, the extent of identity between the *COI* sequences of *P. perpusilla* and other hemipteran pests of sugarcane was in the range of 61.33-72.04% and thus, more than 25%

variation does exist between the sequences. The inter-specific sequence variation of more than 25% would undoubtedly delineate the species without any ambiguity.

Besides being a powerful tool, the conventional alpha taxonomy also suffers from several inherent limitations. In conventional taxonomy, the phenotypic variation that arises in the key taxonomic traits over a period of time may lead to ambiguous identification of the species. The key-based taxonomyby and large relies on the morphological traits of adult insects and generally does not give any weightage to the immature stages of the insects. Besides, it often fails to identify the cryptic species (morphologically similar, but reproductively isolated) in the population. The skipper butterfly *Astraptes fulgerator,* originally described in 1775, had long been considered a single species for more than two centuries. The dissection of genitalia from many individuals of both sexes did not show any variations and failed to reveal the existence of cryptic species in *A. fulgerator* (Hebert et al. 2004). The limitations associated with the conventional taxonomy are often adequately addressed by the DNA barcoding approach. The DNA barcode may be developed irrespective of the sex and stage of the insect and it,therefore, helps in identifying the pests before they become adults. This will certainly help us to initiate appropriate management strategies in time. The barcode generated for the first time through this study would serve as an ideal DNA barcode for *P. perpusilla,* with which the barcodes to be developed for different geographical populations will be compared to fish out the cryptic species, if any, in the small scale sugarcane production systems of the country. In a nutshell, the DNA barcode developed for the sugarcane leaf hoper is reliable and can be employed for unambiguous identification of the species even by insect molecular biologists with little expertise in insect taxonomy. This is the first DNA barcode for sugarcane leaf hopper *P. perpusilla* which would certainly serve as the ideal molecular diagnostic kit for the species.

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