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DNA BARCODING OF MAJOR INSECT PESTS AND THEIR NATURAL ENEMIES FROM CUCURBITACEOUS CROPS IN NORTHEAST INDIA

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ABSTRACT

Correct identification of insect pest is a prerequisite for any control measures, and DNA barcoding facilitates this. In this study, assigning of 28 specimens (insect pests and natural enemies) to known species using DNA barcode by sequencing partial cytochrome oxidase I (COI) gene of mitochondrial DNA has been accomplished. Quick identification of a non-indigenous species *Bactrocera cilifera* (Diptera: Tephritidae) in Meghalaya has been enabled and taxonomic ambiguity of *Henosepillachna pusillanima* (Coleoptera: Coccinellidae) resolved. Molecular identity of *Malcus* sp., *Paridea* sp. and *Coridius* sp. has been established with NCBI GenBank registrations.

Key words: Cucurbits, insect pests, natural enemies, species identification, COI gene, sequencing, non-indigenous, ambiguity, DNA barcodes, molecular identity.

Cucurbits are widely cultivated in India, and northeast India is known for its good quality of produce. Insect pests' infestation and yield loss from 30 to 100% to cucurbits is known from different parts of the world (Dhillon et al., 2005). Besides insect pests, several natural enemies also harbour cucurbit ecosystems. Some of these provide biological control against insect pests and keeping them below economic injury level (Chambers and Adams, 1986) and help the farmers (Gul et al., 2017). With millions of insect species and their various lifestages, correct identification becomes a challenge for taxonomy (Zhang, 2011). The accurate taxonomic identification is an essential step before implementing any control measures. On the other hand, misidentifications could lead to ineffective management (Rivera and Currie, 2009), and there is a dire need to accelerate species discovery with new initiatives which the advancement of technology has to offer (Godfray, 2002; Hebert et al., 2003; La Salle et al., 2009).

The north eastern (NE) region of India comprises of eight states namely Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Tripura and Sikkim, is one of the biodiversity hot spots of India. Its uniqueness lies in its sharing international borders with China, Bhutan, Myanmar, Bangladesh and Nepal (Gogoi et al., 2009), making transboundary insect migration inevitable (Behere et al., 2007). Most of these borders are porous and the quarantine setup is almost poorly maintained. Due to the remoteness, the resources are not properly explored and as a result, little information is available on insect diversity (pests and natural enemies), especially in cucurbits ecosystem. With the advances in science, it is now possible to facilitate the identification of new or invasive species very quickly using various molecular techniques (Behere et al., 2008). Amongst the molecular techniques, DNA barcoding is gaining attention for identification of taxonomically difficult species concisely. It is a taxonomic method that uses mitochondrial COI gene which is a short genetic marker in an organism's DNA in order to identify a particular species (Hebert et al., 2003). Comprehensive molecular information on insect pests and natural enemies of cucurbit crops is very limited as India has generated a total of only 4.6% barcodes of known species with its contrast to an approximate of 59,000 described insect species. On the other hand the corresponding global scenario is about 16% of described species, therefore a lot of emphasis is required to catch up with the world scenario (Jalali et al., 2015). Considering these, the present study analyses the insect pests and their natural enemies in cucurbits through species specific DNA barcodes using mtCOI gene.

MATERIALS AND METHODS

This study was carried out during 2017-2018 in the insect molecular biology laboratory of ICAR (Indian

Council of Agricultural Research) research complex for Northeastern Hills (NEH) Region, Meghalaya. Experimental farms of ICAR research complex and College of Post Graduate Studies (CPGS), Umiam, Meghalaya (25°41'N,91°55'E) supported the field work. Insects were collected from the major cucurbitaceous crops viz., pumpkin (*Cucurbita maxima*), cucumber (*Cucumis sativus*), bottle gourd (*Lagenaria siceraria*), spine gourd (*Momordica dioica*) and chow-chow (*Sechium edule*). The samples were collected by various methods (hand picking, net sweeping, aspirator) and stored in clean glass vials. The parasitoids were either collected directly or with rearing parasitized insect pests The collected specimens were either dry preserved in boxes or wet preserved in 70% ethanol in vials after labelling, the latter were preserved at -20°C. Voucher specimens have been deposited at the Insect Museum of Entomology Section of Crop Protection Division, ICAR Research Complex for North Eastern Hill (NEH) Region, Umiam, Meghalaya.

Genomic DNA (gDNA) was extracted from two specimens of each species (a single leg or antennae in case of large insect and whole insect in case of small insects) using modified phenol: chloroform protocol (Behere et al., 2007). These were tested for presence of *Wolbachia* infection using *Wolbachia* genes specific primer viz., Wol16SF/Wol16SR (O'Neill et al., 1992) and WSP81F/WSP96R (Zhou et al., 1988); PCR protocol was followed according to the composition and profile described by Murthy et al. (2011). The detection of *Wolbachia* was done prior to DNA barcoding as the presence of *Wolbachia* DNA in total genomic extracts made from insects is unlikely to compromise the accuracy of the DNA barcode library (Jalali et al., 2015). For mtCOI gene-based barcoding, PCR amplification was carried out in the thermal cycler (Eppendorf, India) to test the amplification of all the sample using a partial 709 bp cytochrome oxidase I (COI) gene base marker LCO/HCO (Folmer et al., 1994) and LepF1/LepR1 (Hebert et al., 2004). The reaction mixture contained 2µl of gDNA (~40-50 ng), 0.5µl each of forward and reverse primers, 5µl of ready to use EmeraldAmp® MAX PCR Master Mix (2x) (Takara) and 2µl of molecular biology grade water. The standard PCR profile consisted of one cycle of 2 min at 94°C, 5 cycles of 30 s at 94°C, 40 s annealing at 45°C, 1 min extension at 72°C, followed by 35 cycles of 94°C for 30 s, 51°C for 40 s and 72°C for 1 min. A final extension was allowed for 10 min at 72°C and samples were allowed to hold at 10°C in PCR machine after completion of all the cycles and then stored in -20°C for further use. Gel electrophoresis was performed using 1.5 % agarose to detect the genomic DNA using gel documentation (Care stream Gel Logic 212 Pro). The amplified products were got sequenced by M/s Eurofins Genomics India Pvt. Ltd, Bangalore, India. Sequencing was performed for all the samples from both the ends (5' and 3'). The DNA sequences were analyzed using the Molecular Biology software, Staden Package (Staden, 2000) under pregap and gap mode. Thereafter, Basic Local Alignment Search Tool (BLAST) search in online portal of National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) was conducted for identity and homology of all the analyzed sequences. The representative sequence of partial COI gene of species identified was deposited with NCBI and accession numbers obtained. All sequences were uploaded to GenBank and Barcode of Life Data (http://www.boldsystems.org). The DNA barcode images of the sequences submitted were developed using web based software http://www.cib.res.in/ibin/create-barcode.pzhpavailable at Insect Barcode Informatica (IBIn), ICAR-NBAIR, Bengaluru, India.

RESULTS AND DISCUSSION

A total of 31 insect species were observed in the study, classified under six orders viz., Coleoptera (12), Hemiptera (7), Diptera (4), Lepidoptera (3), Hymenoptera (4) and Araneae (1) (Table 1). These results corroborate with those on arthropods associated with cucurbits reported from other regions (Gameel, 2013; Vinutha et al*.,* 2017). The collected insect pests were preliminary identified based on known taxonomic keys and in cases of ambiguities, the insect specimens were sent to ICAR-Indian Agricultural Research Institute (IARI), New Delhi; University of Agricultural Sciences (UAS), Bengaluru; ICAR- National Research Centre for Banana, Tiruchirappalli, Tamil Nadu. The analyses of bacterial endosymbiont *Wolbachia* confirmed the fact that in the reproductive tissues of arthropods, as many as 25 to 70% of all insect species are potential hosts (Werren and Windsor, 2000). Three species viz., *Diadegma* sp., *Diachasmimorpha* sp. and *Hyposoter* sp. resulted positive and thus were not further used (Table 1). Multiple specimens were subjected to this step and those specimens which resulted positive were discarded.

The DNA barcode was successfully developed for 28 species by sequencing partial mtCO1, and sequencing analysis was carried out utilizing the pregap and gap program within the software staden

Table 1. Details of species along with barcoding and NCBI accession numbers

Name of insect species	Order: Family	Insect status	Host	Wol16SF Wol16SR	WSP81F WSP96R	nt. length (bp) : Protein length	Accession number
Bactrocera cilifera	Diptera: Tephritidae	Pest	Spine gourd	-ve	-ve	663: 215	MH395849
Leptoglossus gonagra	Hemiptera: Coreidae	Pest	Spine gourd	-ve	-ve	669: 216	MH395857
Aulacophora lewisii	Coleoptera: Chrysomilidae	Pest	Gourds, pumkin, cucumber	-ve	-ve	673: 224	MH198035
Aulacophora foveicollis	Coleoptera: Chrysomilidae	Pest	Gourds, pumpkin, cucumber	-ve	-ve	536: 178	MH198036
Paridea sp.	Coleoptera: Chrysomilidae	Pest	Gourds, pumpkin, cucumber	-ve	-ve	639: 213	MH198026
Tiracola plagiata	Lepidoptera: Noctuidae	Pest	Chow chow	-ve	-ve	647: 210	MH395862
H. pusillanima (12 spots)	Coleoptera: Coccinellidae	Pest	Gourds, pumpkin, cucumber, chow-chow	-ve	-ve	650: 212	MH395853
H. pusillanima (14 spots)	Coleoptera: Coccinellidae	Pest	Gourds, pumpkin, cucumber, chow-chow	-ve	-ve	650: 216	MH395854
H. pusillanima (16 spots)	Coleoptera: Coccinellidae	Pest	Gourds, pumpkin, cucumber, chow-chow	-ve	-ve	596: 194	MH395855
Bactrocera cucurbitae	Diptera: Tephritidae	Pest	Bottle gourd, cucumber, pumpkin, chow-chow	-ve	-ve	666: 221	MH198034
Bactrocera tau	Diptera: Tephritidae	Pest	Bottle gourd, cucumber, pumpkin, chow-chow	-ve	-ve	591: 193	MH395850
Bactrocera carambolae	Diptera: Tephritidae	Pest	Bottle gourd, cucumber, chow-chow	-ve	-ve	640: 207	MH395848
Nezara viridula	Hemiptera: Pentatomidae	Pest	Cucumber, spine gourd	-ve	-ve	626: 208	MH198029
Mylabris sp.	Coleoptera: Meloidae	Pest	Pumpkin	-ve	-ve	637: 212	MH198030
Arthrotus flavocincta	Coleoptera: Chrysomellidae	Pest	Bottle gourd, pumpkin	-ve	-ve	642: 213	MH198037
Coridius sp.	Hemiptera: Pentatomidae	Pest	Cucumber, chow - chow	-ve	-ve	561: 180	MH395852
Oenopia sexareata	Coleoptera: Coccinellidae	Predator	Aphids	-ve	-ve	497: 165	MH198027
Apanteles sp	Hymenoptera: Braconidae	Parasitoid	Cucumber moth	-ve	-ve	670:218	MH395863
Oxyopes sp.	Araneae Oxyopidae	Predator	General predator	-ve	-ve	600: 197	MH395859
Bothrogonia tibetana	Hemiptera: Cicadellidae	Pest	Cucumber, pumpkin	-ve	-ve	669: 223	MH198033
Kolla paulula	Hemiptera: Cicadellidae	Pest	Cucumber	-ve	-ve	590: 194	MH395856
Spilarctia sp.	Lepidoptera: Eribidae	Pest	Pumpkin	-ve	-ve	667: 222	MH198025
Malcus sp.	Hemiptera: Malcidae	Pest	Cucumber	-ve	-ve	630: 204	MH395858
Micraspis sp.	Coleoptera: Coccinellidae	Predator	Aphids	-ve	-ve	589: 196	MH198031
Oenopia kirbyi	Coleoptera: Coccinellidae	Predator	Aphids	-ve	-ve	609: 203	MH198028
Coccinella septempunctata	Coleoptera: Coccinellidae	Predator	Aphids	-ve	-ve	523: 170	MH395851
Anadevidia peponis	Lepidoptera: Noctuidae	Pest	Bottle gourd	-ve	-ve	678: 220	MH395845
Aphis gossypii	Hemiptera: Aphididae	Pest	Cucumber, bottle gourd, pumpkin	-ve	-ve	669: 217	MH395846
Diachasmimorpha sp.	Hymenoptera: Braconidae	Parasitoid	Fruit flies	+ve	+ve	-	-
Diadegma sp.	Hymenoptera: Ichneumonidae	Parasitoid	Cucumber moth	+ve	+ve	-	-
Hyposoter sp.	Hymenoptera Ichneumonidae	Parasitoid	*Euproctis* sp.	+ve	+ve	-	-

package and the messy/ambiguous 5' and 3' end of the sequences were trimmed to obtain good quality sequence. The total length of the final sequence varied from species to species and it ranged between 497-678bp. The final analysed sequences were submitted to GenBank maintained by NCBI, with accession number (Table 1). DNA barcoding on insect pests of agricultural importance has led to identifying cryptic and potentially new species (Seifert et al., 2007; Vaglia et al., 2008; Burns et al., 2008). This fact is in line with the present finding of a non indigenous species *B. cilifera* in Meghalaya, which is a recently discovered fruit fly in India (Nair et al., 2017); also the taxonomical ambiguity in the identity of three species of the genus *Henosepilachna* with 6 spots, 7 spots and 8 spots on each elytron (Naz et al., 2008) was resolved. These results revealed that the barcoding detected no variation, and the sequences from these specimens were 100% identical to *H. pusillanima*. Over the last decade DNA barcoding has proven to be an authentic and efficient tool achieving species level resolution in 95 % to 97% of cases (Hebert et al., 2004; Ward et al., 2005). All the analysed sequences were subjected to BLAST and results with 99-100% homology to NCBI database were considered as similar species and molecular identity of the test species was confirmed.

However, for those species with blast result below 99%, the identity was established till genus level only (Table 1). The molecular identity of three species viz**.,** *Malcus* sp., *Paridea* sp. and *Coridius* sp. was established and the sequences have been deposited for the first time in the NCBI database. Hajibabaei et al. (2007), Carvalho et al. 2008 and Smith et al. (2008) corroborate with the potential of DNA barcoding results of the present study.

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AUTHOR CONTRIBUTION STATEMENT

GTB, DMF and AP conceived the research work plan. AP, GTB and BS conducted the experiment. DMF and TR contributed in identification. AP wrote the manuscript. All authors approved the manuscript.

CONFLICT OF INTEREST/ COMPETING INTERESTS

There is no conflict of interest/competing interests.

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