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Arensungla Pongen
School of Agricultural Sciences and Rural Development (SASRD), Nagaland University, Medziphema, Nagaland, India

GT Behere
ICAR-Central Citrus Research Institute (CCRI), Amravati Road, Nagpur, Maharashtra, India

Huirem Diana Devi
School of Agricultural Sciences and Rural Development (SASRD), Nagaland University, Medziphema, Nagaland, India

Rumki Heloise CH Sangma
ICAR- Research Complex for NEH Region, Umiam, Meghalaya, India

M Alemla AO
School of Agricultural Sciences and Rural Development (SASRD), Nagaland University, Medziphema, Nagaland, India

Pankaj Neog
School of Agricultural Sciences and Rural Development (SASRD), Nagaland University, Medziphema, Nagaland, India

Corresponding Author:
Arensungla Pongen
School of Agricultural Sciences and Rural Development (SASRD), Nagaland University, Medziphema, Nagaland, India

The use of DNA barcoding for identification of major insect pests and their natural enemies of tomato in Nagaland, India

Arensungla Pongen, GT Behere, Huirem Diana Devi, Rumki Heloise CH Sangma, M Alemla AO and Pankaj Neog

Abstract
In today's scenario of diversity crisis, the initiative of DNA barcoding has appeared to be a useful tool to achieve quick and accurate species identification of existing species and also aid in discovery of unknown species. In the present study 6 major insect species (insect pests & natural enemies) were collected and identified *viz*., *Aphis spiraecola, Chrysodeixis eriosoma, Helicoverpa armigera, Glyptapanteles* sp., *Coccinella septempunctata* and *Coccinella transversalis*. DNA barcodes were successfully developed by sequencing partial Cytochrome oxidase I (COI) gene of mitochondrial DNA. The molecular identities of the insect species were established through BLAST NCBI. All the analyzed sequences have been deposited to International Gene Bank (NCBI) with accession numbers ON460288, ON460289, ON461368, ON461370, ON489304 and ON496461. The present study has resulted in the quick identification of *A. craccivora, C. eriosoma* and *Glyptapanteles* sp. in tomato ecosystem which has not been reported previously from Nagaland, India. The comprehensive molecular database developed in this study could be used as diagnostic guide at both morphological and molecular level.

Keywords: Vegetable, northeast India, pests, natural enemies, species identification, COI gene, DNA barcodes

Introduction
Tomato (*Solanum lycopersicum* L.) belongs to the family Solanaceae and is the second most important vegetable crop next to potato (*Solanum tubersum* L.). India is one of the largest producers of tomatoes in the world, second only to China. It is one of the most important commercial vegetable grown mainly due to its health-promoting compounds which are easily integrated as a nutritious part of a balanced diet (Martí *et al*., 2016) [1]. In addition to consuming the fresh fruits, consumers use tomatoes in processed products such as soups, juices, and sauces (Li *et al*., 2018) [2]. The day by day growth in the awareness of vegetable's profound nutritive value has persuaded the consumers to include more vegetables in their daily diet. In the present day context, insect pests are major constraint because all parts of the plant offer food, shelter and reproduction site for insects. Insects can cause unthrifty growth or death of the tomato plant and damage to fruits in the form of scarring tissue destruction and aberration in shape or colour. With millions of species and their different life stages, correct identification becomes a challenge for taxonomy (Zhang, 2011) [3]. Pest management tools depend on proper identification of arthropod species, which are usually classified relying on morphological keys. However due to the remoteness, the resources are not properly explored and as a result, taxonomic identification is sometimes very difficult due to the lack of expertise, especially in respect to taxonomically difficult species and entire process is time consuming. The advances in science, has made it possible to complete the identification of new or invasive taxonomically difficult species very quickly and reliably using various molecular techniques (Behere *et al*., 2008) [4]. Besides other molecular techniques, DNA barcoding is getting more attention in identification of taxonomically difficult species, it is a method that uses a short genetic marker in an organisms DNA as to identify it as belonging to a particular species. (Hebert *et al*., 2004) [5]. Comprehensive molecular information is very limited, especially on insect pests and natural enemies of tomato ecosystem in Nagaland. Considering all these facts, this research aims to establish the correct identity of individual insect pests and natural enemies by developing the species specific DNA barcodes by using COI gene of mitochondrial DNA.

Materials and Methods

The research study was carried out during the year 2019-2020 and 2020-2021 in the insect molecular biology laboratory of ICAR (Indian Council of Agricultural Research) research complex for Northeastern Hills (NEH) Region, Meghalaya and experimental farms of School of Agricultural Sciences and Rural Development, Medziphema, Nagaland supported the field work. The insect species were collected by various methods (hand picking, net sweeping, aspirator) and stored in clean glass vials. For the parasitoids, parasitized insect pests were collected from the field and later, on emergence these parasitoids were collected and subsequently used for identification. The insect specimens were either dry preserved in insect box with labelled information or by preserving it in 70% ethanol in vials with proper labelling and preserved at -20 °C in laboratory for further molecular investigations. Voucher specimens were maintained at Insect Museum of Entomology Section of Crop Protection Division, ICAR Research Complex for North Eastern Hill (NEH) Region, Umiam, Meghalaya.

DNA extraction was done from 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) [6]. For COI gene based barcoding, the primer used was LCO/HCO (Hebert *et al*., 2004) [5]. PCR amplifications were carried out in the thermal cycler (Eppendorf, India) to test the amplifications of all the samples with standard DNA barcoding primers. The reaction mixture contain 2µl of g DNA (~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. This premix master mix has composition of 5µl of 2mM dNTPs, 1.5 µlof 50 mM MgCl2, 0.25µl of 5U *Taq*DNA polymerase and 5µl of 10X PCR buffer. PCR profile consist of initial denaturation at 94 °C for 2 minutes, followed by 5 cycles of denaturation at 94 °C for 30 seconds, annealing at 45°C for 40 seconds and extension for 1 minute at 72 °C, again followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 51 °C for 40 seconds and extension for 1 minute at 72°C. A final extension was allowed for 10 minutes 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. 1.5% Agarose Gel Electrophoresis was performed to detect the genomic DNA using Gel documentation (Care stream Gel Logic 212 Pro). The amplified products were sent to commercial sequencing company, M/s Eurofins Genomics India Pvt. Ltd, Bangalore, India. The DNA sequences were analyzed using the Molecular Biology software, Staden Package (Staden, 2000) [7] 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 each species identified in this study was deposited to NCBI and accession numbers of all the submitted sequences were also obtained. All sequences were uploaded to Gen Bank and Barcode of Life Data (http://www.boldsystems.org). The DNA barcode images for all nucleotide sequences submitted to NCBI were developed using web based software http://www.cib.res.in/ibin/create-barcode.phpavailable at Insect Barcode Informatica (IBIn), ICAR-NBAIR, Bengaluru, India.

Results and Discussion

From the present study, a total of six insect species were collected and identified *viz*., *Aphis spiraecola, Chrysodeixis eriosoma, Helicoverpa armigera*, *Glyptapanteles* sp., *Coccinella septempunctata* and *Coccinella transversalis*. DNA was successfully extracted from multiple specimens of collected insect species and to avoid cross contamination during PCR amplification, a negative control (a sample without DNA) was kept for all the batches. By employing standard DNA barcoding primer (LCO/HCO) multiple specimens of all six species were successfully amplified. The barcoding primers were designed to amplify partial COI gene which targeted 709bp PCR fragment. The targeted 709bp PCR fragment was successfully amplified for all the collected specimens. Irrespective of the insect order, no variation at band size was detected. Amplified bands were detected by gel electrophoresis on 1.5% agarose gel. For accuracy purpose, sequencing was carried out bi-directionally (from both the ends 5' and 3') for all the samples. Sequencing analysis was carried out utilizing the pregap and gap program within the software staden 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 621bp - 675bp (Table 1). The final analysed sequences were submitted to Gen Bank maintained by NCBI, with accession number (Table 1).

Table 1: List of identified species along with nucleotide length, protein length and NCBI accession number

Common Name	Scientific name	Nucleotide sequence length (bp)	Protein Sequence	NCBI Accession number
Green garden looper	*Chrysodeixis eriosoma*	658	219	ON460288
Transverse ladybird	*Coccinella transversalis*	658	219	ON460289
Seven spot ladybird	*Coccinella septempunctata*	621	207	ON461368
Green citrus aphid	*Aphis spiraecola*	637	212	ON461370
Parasitic wasps	*Glyptapanteles* sp.	658	219	ON489304
Tomato fruit borer	*Helicoverpa armigera*	675	225	ON496461

Establishment of correct identity of target insect pest is a prerequisite for undertaking any control measures in integrated management because, misidentifications could lead to ineffective control and may potentially increase the impact caused by a particular pest species (Rivera and Currie, 2009) [8]. Morphological data are usually time consuming and with the dwindling number of taxonomists and other identification experts (Jinbo *et al*., 2011) [9], cytochrome oxidase I (COI) based technique has provided an alternative practical method of species identification of insects and can be used for the identification of all developmental stages of insects, their food webs and biotypes which may not be possible with morphology-based taxonomy (Jalali *et al*., 2015) [10]. This holds true as the insect pest *A. craccivora* and *C. eriosoma* identified in this research through COI based barcoding were performed with the nymphal and larval stage of the insect,

respectively. Extraction and PCR amplification of DNA and subsequent sequencing presented no challenge. With the advancement of science, it is now possible to carry out the DNA work very quickly and reliably with minimum technical skill.

DNA barcoding on insect pests of agricultural importance has lead to identifying cryptic and potentially new species (Burns *et al*., 2008) [11]. In the present study, the insect species *A. craccivora*, *C. eriosoma* and *Glyptapanteles* sp. observed in tomato ecosystem has not been reported previously from Nagaland, India and this demonstrates the efficiency of DNA barcoding as a tool to correctly identify or detect overlooked species. Likewise many new insect pest species have been reported from India as well as from northeast India and has facilitated in establishing the correct identity of insect pest species. South American tomato pinworm (*Tuta absoluta*) has successfully invaded into India and was reported for the first time in 2014 in Maharashtra and the pest was detected and identified using DNA barcoding in Meghalaya in 2017 (Sankarganesh *et al*., 2017) [12]. Simlarly, invasive tomato leaf miner *Liriomyza sativae* was also detected and identified by DNA barcoding from North East India in 2017 (Firake *et al*., 2017) [13]. Comprehensive molecular information on insect species is still very limited in India as it has generated a total of only 3,694 barcodes of known species with its contrast to an approximate of 59,000 described insect species. On the other hand the corresponding global scenario global scenario is about 1, 63,617 barcodes of described species, therefore a lot of emphasis is required to catch up with the world scenario (IBIn, 2022) [14]. Over the last decade this technique has proven to be an authentic and efficient tool achieving species level resolution in 95% to 97% of cases (Hebert *et al*., 2004) [5]. The comprehensive data on DNA barcodes generated in this study would certainly help as a diagnostic guide for identification and designing of better management strategies for the management of insect pests of tomato.

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