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Identification of Invasive Pest, *Plautia corossota* (Green stink bug) Using DNA Barcoding

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ABSTRACT

Plautia corossota, is a Heteropteran insect belonging to family Pentatomidae. A polyphagous insect, commonly known as the green stink bug, or shield bug, considered as a pest, is frequently found in and around local vegetables market of Chennai. To learn about its biology and to precisely identify the exact species, a specimen was collected from local terrain of Chennai. This insect is cosmopolitan is distribution, and is a serious pest known to infest almost all of the vegetable, fruit and commercial crops. No specific insecticide or effective pest management strategies are in place till date, probably due to obscure identification of pest species. This insect is polymorphous, and its identification simply based upon morphological features, may result in ambiguous, conclusions, and so a more scientific method, may be employed to ascertain its identity. Precise identification, of the insect pest is the key for developing pest management strategies, and so DNA barcoding was used for specific identification. The mitochondrial CoI was amplified using corresponding forward and reverse primers, and the query sequence was submitted to Genbank. (ID MF125272). Its relationship with other species of Pentatomidae, was ascertained, by constructing a Phylogenetic tree using the software MEGA, 5.2. Bootstarp values, using 500 replicates, were found to be 99% match with species such as *Nezaraviridula voucher*, *Plautia crossota isolate U4c, Plautia crossota voucher ROPI*etc; of other green stink bugs. Tajima neutrality test D value was found to be 1.3, suggesting that the selection of this stink bug (*Plautia corossota*) was balanced.

KEYWORDS: *Plautia corossota*, Green stink bug, DNA barcoding, Pentatomidae, CoI , Genbank

INTRODUCTION

Insects belonging to Heteroptera, are serious pests which are known to feed upon the soft tissues, of the plants, causing huge damage to majority of agricultural and commercial crops. Green stink bug or shield bug, belongs to this order, reported to be a nuisance pest, and difficult to control. This is a polymorphous insect (More *et. al.*, 2017, Vivan and Panizza, 2002, Esquivel *et. al.*, 2015) known to be found in various colors and also polyphagous (More *et. al.*, 2017) known to feed upon many of agricultural crops such as tomatoes(Lye *et. al* 1988, Zalom *et. al.*,1997, McPherson and McPherson 2000, Nault and Speese 2002) soya, (Underhill 1934, McPherson and McPherson 2000) and commercial crops such as Pistachio (Barman *et. al.*, 2017) causing huge damage to the produce. It is cosmopolitan in distribution, found almost all over the globe including India. Indian states, of

Maharashtra and Tamil Nadu have reported the menace of stink bug. (Jadhav and Hedge, 2018)Quite a number of different species of green stink bugs, are reported from India, (Salini, 2011, Chandra *et al.*, 2014).Most of these insects resemble each other by color, but all of them may not belong to same species. Apart from this, detection of the stink bug invasion becomes, challenging as a number of other green insects, such as little green bugs (*Lygocoris publinus*, leaf hopper (*Cicadellidae*), thistle tortoise beetle, (*Cassida rubiginosa)* aphids and thirps which infest the agricultural crops (García *et. al.*, 2018) are look likes of the stink bug and thus, leading to obscurity in identification of the pest. Besides, damaging the crops, stink bugs are known to emerge from their hiding places, such as buildings and houses, to feed (Kiritani *et. al.*, 1966; Todd and Herzog 1980) and aggregate in groups, produce obnoxious odor from their stink glands, thus causing distress to the local inhabitants. An interesting fact about this insect is that in addition to being recognized as a pest, it is also consumed, by certain sections of populations, in certain parts of India, as the best and cheapest source of protein (Rooparao, 2018). Barring this exception, green stink bugs, are known to be invasive pests, which need to be checked. These were originally pests of cotton since1900 (Morrill, 1910) but now, known to infest almost all the important agricultural crops. This rise in the pest population in recent times, and their versatility in infecting and adapting to new host tissues, may be due to the absence of a targeted insecticide or due to absence of efficient pest management practices, competent enough to control the menace. In lieu of these observations, it may be said that this invasive species, can be controlled either by developing a specific pesticide or by adopting effective pest management approaches which are obviously feasible, only by accurate identification of the insect. Therefore the present study is intended to factually identify the specific species, of green stink bug using mitochondrial CoI by the method of DNA barcoding.

MATERIAL AND METHOD

Green stink bug, *Plautia corossota*, was collected from the local vegetable market form Chennai. It was placed in a container and transferred to laboratory. The green stink bug was killed and later processed for DNA extraction and isolation.

Genomic DNA isolation

Reagents required

Lysis Buffer (pH 7.5)

- 50 mM Tris
- 100 mMNaCl
- 10 mM EDTA
- 1 % SDS
- 0.2 to 0.4 mg/ml Proteinase K

Phenol: Chloroform: Isoamylalcohol (25:24:1) Potassium Chloride, Ice Cold Isopropanol70% Ethanol TE Buffer (1X)

- Tris-HCl : 10mM
- EDTA : 1mM

Procedure

50- 70 mg of Fresh / Frozen sample were placed on 2- 3 mm thick Aluminum foil and the tissue was smashed between two sheets of foil. The squashed tissue was transferred to 2ml tube.1 ml of lysis buffer was added to the tube and vortexed and incubated at 55° C for 1 to 2 hour. The clear lysate was transferred to another fresh tube, to which 100µl KClis added and mixed by inversion and incubated on ice for 5 mins. The content was centrifuged for 10 to 15 mins at maximum speed and transferred the supernatant to another tube. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added the tubes were centrifuged for 10 minutes at 10000rpm.The Upper aqueous phase was transferred to a new centrifuge tube. Equal volume of absolute ice-cold isopropanol was added and & incubated for 5 to 10 min at room temperature. The tubes were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% (v/v) ethanol. The pellet was air-

dried at room temperature and then dissolved in 50μl TE buffer. The DNA samples were stored at -20°C until further use.

Agarose gel electrophoresis

Principle
For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because DNA molecules and their fragments are considerably larger than proteins; therefore larger size agarose gels are required. Under an electric field, any given fragment of DNA should move towards the anode with the same mobility. This is due to the charge per unit length owing to the phosphate groups. Separation on agarose gel is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficulty moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size. Gel concentrations must be chosen based on the molecules to be separated. For mitochondrial DNA – 0.8% and amplified samples at 1.5% was used

Reagents
TAE buffer (stock solution 50X- 1000ml)
- Tris base – 242g
- Acetic acid glacial – 57.1ml
- EDTA 0.5M

Working TAE Buffer concentration 1X
Gel loading Dye
- Bromo-phenol blue
- Xylene cyanol
- Sucrose

Ethidium bromide 20mg/ml
Agarose 0.8%

Procedure: Agarose was weighed and transferred to a conical flask.25 ml of 1X TAE was added and Agarose was melted to a clear solution by heating. It was allowed to cool until it reached bearable temperature. 5 μl of ethidium bromide stock solution was added. A gel casting tray was placed in a leveling table and the melted agarose was poured. After the gel solidified, the comb was taken out carefully. The casted gel was placed in an electrophoresis tank and 1X TAE buffer was added until the gel was completely submerged.DNA sample was mixed with the gel loading buffer and loaded into the well. The samples were then electrophoresed at 50V until the gel loading dye reached 2/3rd of the gel. This gel was then viewed under UV Trans-illuminator

Qualitative and Quantitative Determination of DNA by Spectrophotomeric Method

Principle
A solution of nucleic acids strongly absorbs UV with an absorbance maximum of 260nm and proteins at 280nm which is linearly related with the concentration of DNA and RNA and the amount of contamination I n the solution in the solution. The intense absorption is primarily due to the presence of aromatic rings in the purines and pyrimidines. The concentration of nucleic acid in a solution can be calculated if one knows the value of A_{260} of the solution. A solution of double-stranded DNA at a concentration of 50ug/ml in a 1cm quartz cuvette will give A_{260} reading of 1.A solution of single-stranded DNA/RNA that has A_{260} of 1 in a cuvette with a 1cm path length has a concentration of 40ug/ml. Proteins are usually the major contaminants in nucleic acids extract and these have absorption maximum at 280nm. The ratio of absorbance at 260 and 280nm hence provides a clear idea about the extent of contamination in the preparation. A ratio between 1.7 and 1.9 is indicative of fairly pure DNA preparation. But values less than 1.8 signify the presence of proteins as impurities. The

values greater than 1.8 signify the presence of organic solvent in the DNA preparations. A ratio of 1.8 determines the pure DNA preparation.

Procedure: The spectrophotometer and the UV lamp were switched on. The wavelength was set at 260nm and 280nm. The instrument is set at zero absorbance with T.E buffer as blank. 5 or 7ul of the sample is taken in a quartz cuvette and made up to 3ml with TE buffer Absorbance of the solution with the sample was read. The concentration of DNA in the sample was calculated using the given formula:

Concentration of ds DNA

A_{260} * 50µg * dilution factor

Purity of the DNA

$A_{260:}$ A_{280}ratio = A260/ A280

= 1.8: pure DNA

= 1.7 – 1.9; fairly pure DNA (acceptable ratio for PCR)

= less than 1.8; presence of proteins.

= greater than 1.8; presence of organic solvent

Polymerase Chain Reaction (PCR)

Procedure: 100ng of DNA is used for molecular based detection of insect sample. The PCR reaction is performed for 20µl. PCR reaction was performed for COI gene. The PCR tubes were placed in thermo cycler and the reaction was carried inside the thermo cycler.

Primer Details:

COI-5′ F: GGTCAACAAATCATAAAGATATTGG

COI-5′ R: TAAACTTCAGGGTGACCAAAAAATCA

Reaction Set up For CoI Gene Amplification

Components	Stock Concentration	Final Concentration	Volume for 20 µl Setup
Milli Q water			10.3µl
dNTP mix	2mM	0.2mM	2 µl
Taq buffer	10X	1X	2 µl
Forward Primer	3µM	0.3µM	2 µl
Reverse Primer	3µM	0.3µM	2 µl
DNA Template	100ng/µl		1.5 µl
Taq DNA polymerase	5U/µl	1U	0.2 µl

PCR Reaction Conditions

Initial denaturation	:	94◦C – 3min
Denaturation	:	94◦C – 45Sec
Annealing	:	48◦C – 1min
Extension	:	72◦C – 1min 20Sec
Final extension	:	72◦C – 7min
Hold	:	4◦C

Total number of cycles 35

RESULTS

PCR, successfully amplified the desired mitochondrial sequence and the sequence was analyzed using Agilent bioanalyzer. Electrophoregram, of CoI gene, shows, an amplified mt DNA, as seen as a significant, peak in the middle, at 1000-1050. Electropherogram shows, Y axis, representing the intensity of the signal and X axis represents the time expressed in scan numbers.

When the query sequenced was matched with BLAST search, it showed a 99% match with other similar species, of *Plautia corossota*. Phylogenetic tree was constructed, using MEGA 5.2. as illustrated below. Tajima neutrality test, "D" value was found to be 1.3, suggesting that there is a balancing selection of *Plautia corossota*.

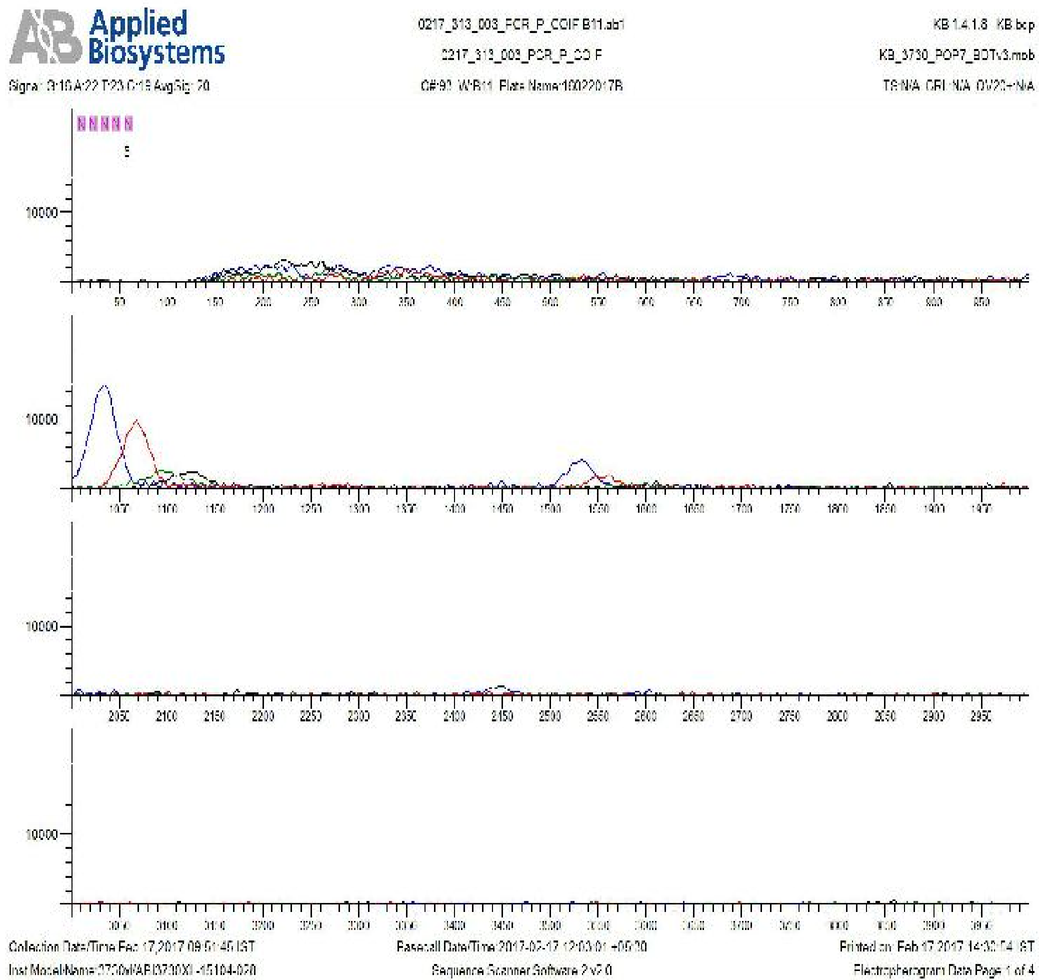

Fig: 1: Sequencing data output, of mt DNAPCR product using amplified mt DNA

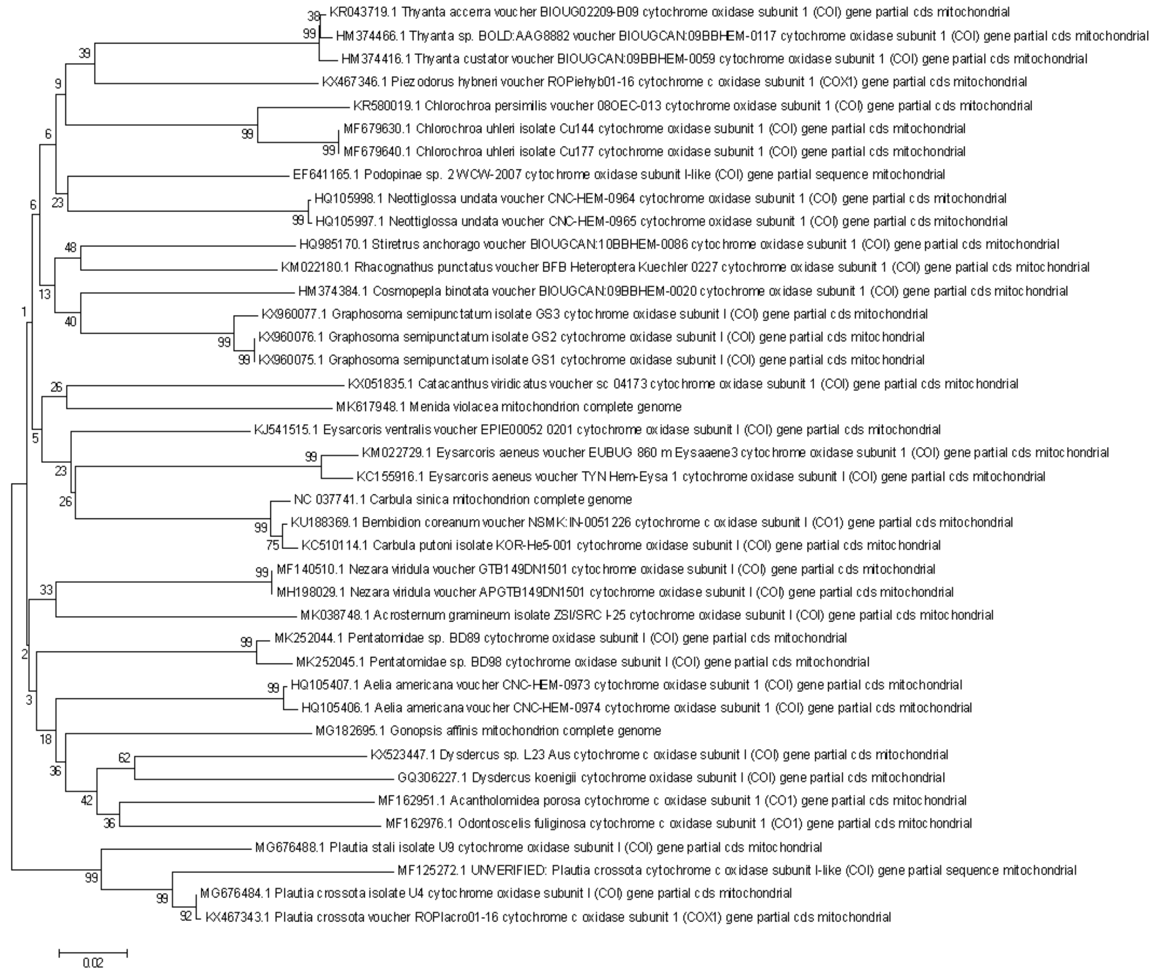

Figure 2: Phylogenetic tree

Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.94721893 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 554 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.2.

DISCUSSION

In the present study, the identity of the green stink bug which was collected from the local terrain of Chennai was successfully deciphered.DNA barcoding, using mitochondrial CoI of the query insect, revealed that, it was *Plautia corossota*, a species of green stink insect, and the sequence was submitted to public data base Genbank (ID MF125272). BLAST search of the query sequence, revealed that this sequence, shares similarities, with other species, of greenstink insects, such as *Plautia corossotastali*, *Plautia corossota isolate* and *Nezera virdula*, a species, which was reported in certain parts of India (More *et. al., 2017).* To learn, about its affiliation with other species, its evolution and its diversity, a phylogenetic tree was constructed using the software, MEGA 5.2. Phylogenetic tree was constructed

using neighbor joining method, with bootstrap test using 500 replicates. The query sequence showed 99% similarity with species such as *Eysarcoris aeneus*, (brown stink bug), *Nezera virdula*, (green stink bug) *Pentatomidae* (green stink bug), *Alia Americana* (brown stink bug) and *Bembidion* coreamum (useful ground beetles) illustrating, that *Plautia corossota*, was closely associated with these species genetically, but may differ, in morphology and physiology.

The phylogenetic tree, reveals, that there is a vast diversity in the green stink bug species, and majority of them are designated as pests, which are spread out all over the globe. Among all of these species, the population of *Plautia*, seems to have increased in great numbers and also escalated its scope of infestation to almost all crops, greatly impacting the crop yield and quality. The enormous rise in the pest population in recent times may be attributed to broad diversity, greater adaptability to newer environments or due to non availability of effective pest control measures. Broad diverse evolution of stink bugs, may be because of the polyphagous nature, where in it feedsupon, variety of plants as the insect requires, a series of plants with overlapping periods, of seed and fruit to complete its life cycle (Underhill, 1934) and thus becoming capable of infesting large variety of agricultural and commercial crops. Success of polyphagy, may be attributed to inter domain symbiosis with gut bacteria which seems to have helped the bug, in feeding, adapting, and surviving the most harshest of environments, thereby enabling it to colonize underutilized niches of almost all ecosystems. (Kalia *et. al.*, 2014)

Presently no specific insecticide, isin place to control *plautia*, but certain pest control measures such as pheromone traps, light traps, broad spectrum pesticides, used to control other pests, such as boll weevil *Anthonomus grandis grandis Boheman*, and the bollworm, *Helicoverpazea* (Boddie) (Greene *et. al.*, 1997)are being used, which seem to have largely become ineffective. In recent times, there are instances, where certain special signaling substances, known as semiochemicals, are being used, as a part of integrated pest management IPM (Sharma *et. al.*, 2019). One such example of a semiochemical, is the aggregation pheromone reported by Tsutsumi, *et. al.*, 2003, which is being used to control this insect. In addition to chemical methods, biological control methods have also been reported. A species of samurai wasp, *Trissolocus japonicus*, is known to parasitize the eggs of pentatomidae, particularly the species of brown marmorated stink bug, *Halyomorpha halys* and also rarely found to parsitize the eggs of *plautia corossota*, (Zhang *et. al*, 2017). Additionally Mermithidnematodes have also been reported to attack stink bug adults and nymphs (Fuxa *et. al.*, 2000, Riberiro *et. al.* and Castiglioli 2008) thereby aiding in controlling its population.

However no concrete effective pesticide targeting *plautia* has so far been developed or reported. This may be because, of improper identification of the pest species, or may be the routinely used insecticide, and commonly practiced pest management measures seem to have become, unproductive. These studies, clearly illustrate that, *Plautia corossota*, is a very hardy, highly adaptable and extremely diverse insect pest, and needs a customized insecticide, or customized pest management strategy. As stated earlier, for successful development of a pesticide, or to develop, efficient pest management practices, exactness in identification of the species, is crucial, and in this regard, DNA barcoding is the best scientific method, in place. This study, successfully identified the green stink bug to be as *Plautia corossota*, and this data, might be useful in developing customized solutions for this menace. In summary, it may be suggested that a best tailor made, solution, to control this pest, is probably to develop an insecticide, targeting the gut bacteria, of *plautia*, rather than targeting the pest itself, which may prove to be effective.

In conclusion, it may be said, that for development of effective insecticide, or for IPM approaches to be fruitful, accurate identification of the pest, by DNA barcoding, is more reliable, rather than solely banking upon morphological data to ascertain the species.

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