UTTAR PRADESH JOURNAL OF ZOOLOGY

40(3): 114-119, 2019
ISSN: 0256-971X (P)

FIRST RECORD OF POLYMORPHISM IN *Nezara viridula* (L.), THE SOUTHERN GREEN STINK BUG (HETEROPTERA: PENTATOMIDAE), FROM KERALA, INDIA AND ITS CONFIRMATION USING MITOCHONDRIAL GENE SEQUENCES

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AUTHORS' CONTRIBUTIONS
This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Received: 02 August 2019
Accepted: 16 October 2019
Published: 22 October 2019

Original Research Article

ABSTRACT

Nezara viridula (L.), the southern green stink bug is a pest of different economically important crops all over the world. It damages the crops by sucking the plant sap. Both adults and nymphs cause serious damage to the crops. *N. viridula* shows polymorphism and about 12 different colourmorphs has been reported throughout the world. The present study is the first report of three colourmorphs from Kerala, India. This study also proves the easiness of identification of specimens by means of DNA barcoding. Mitochondrial CO1 gene has been used as a molecular tool for identifying organisms. The colourmorphism produce confusion for identification of the species. Molecular taxonomy is an easiest way to find the identity of a species. This study aims to confirm the identity of the *N. viridula* by molecular taxonomy and phylogenetic analysis using mitochondrial CO1 sequences. Accurate identification of species is required for pest management strategies. Phylogenetic analysis of the *N. viridula* species helps in correct identification and to know about the variations among the species. This study proves the significance of molecular taxonomy in easy and speedy identification of pest organisms, thus provide way for correct control measures.

Keywords: Polymorphism; *N. viridula*; Kerala; phylogeny.

1. INTRODUCTION

Family Pentatomidae is considered as one of the largest families in the suborder Heteroptera that constitutes the true bugs. Stink bugs of family Pentatomidae are divided into eight subfamilies [1]. Among them, subfamily Pentatominae is the largest and comprises the phytophagous stink bugs. Most of the members of this group are pests of agricultural crops of various families, especially Fabaceae.

Nezara viridula, commonly known as southern green stink bug is a notorious pest. It has cosmopolitan distribution and distributed in regions of South America, New Zealand, North America, tropical and subtropical regions of Africa, America, Europe and Asia [2]. *N. viridula* is a common pest in India attacking wide variety of agricultural crops including almost 30 dicotyledon families and some monocots [3]. It is a pest of economically important plants like, cabbage, cotton, rice, sugarcane, soyabean, wheat,

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olives, mango, citrus, macadamia nuts and so on [2,4]. It has been reported in India from Jammu and Kashmir, Himachal Pradesh, Assam, Karnataka, Madhyapradesh, Maharashtra, and West Bengal [2]. Adult stages are more harmful to the crops. They cause damage to almost all parts of plants by piercing and sucking of plant sap using their mandibular and maxillary stylets.

Intraspecific variations like colour polymorphisms can be seen greatly in many insect groups. Among the insects, aphids, the members of order Hemiptera shows colour polymorphism in common [5]. Among the true bugs, *Nezara viridula* is a great example for groups that shows colour polymorphism. Pioneer studies on colour polymorphism in southern green stink bugs were done by Yukawa and Kiritani in 1965 [6]. Nine forms of colourmorphs including the basic forms like G, O, F and R were reported by them. The basic polymorphic forms G, O, F and R were described on the basis of the colour pattern seen on the dorsal body surface [5,7]. Colour polymorphism in this pentatomid bug has been reported only from some parts of the world like Vietnam, Hawaii, Brazil [7,8-10]. From India, three studies has been made on the polymorphic character of *N. viridula* [5,6,11]. The very recent report is from Chandgad Tehsil, Maharashtra that reports three forms from this region [7].

As *N. viridula* is a serious pest causing damage to many economically important crops, the correct identification of the same is required for doing the accurate pest control measures. The accuracy in identification of a species is the base of all aspects of entomology [5]. Inappropriate identification among the polymorphic species may lead to mistakes and thus it may be considered as new species [6]. Proper and faultless identification can only lead to exact method of pest management. Only those have expertise can identify the different forms without flaws. Classical taxonomy has some limitation in identifying insects during the larval or nymphal stages. DNA barcoding is a taxonomic method that provides speedy and error-free identification using short molecular marker regions [12]. The major advantage is that identification can be done with any stage of the insect life and even with a damaged part of the specimen. Mitochondrial gene regions like cytochrome c oxidase 1 (CO1) and other markers are used now for identifying and discovering new species [13].

The present study is based on the collection made by the author during field trips. The objective of the present study is to report the first record of three colourmorphs of the southern green stink bug, *Nezara viridula* from Kerala, India and to confirm its identity by means of mitochondrial cytochrome oxidase 1 gene sequences using phylogenetic analysis.

2. MATERIALS AND METHODS

2.1 Collection, Identification and Preservation

The *Nezara viridula* specimens were collected from grasslands and paddy fields from different parts of Kerala. Collections were done by sweeping net method and hand picking. The primary morphological identification was done by classical taxonomic keys under the assistance of by Dr. S. Salini, Scientist, Division of Insect Systematics, National Bureau of Agricultural Insect Resources, Bengaluru, India. The identified specimens were photographed and stored at -20°C in Molecular Biology Laboratory, Department of Zoology, University of Calicut, Kerala as vouchers.

2.2 DNA Extraction and Phylogenetic Analysis

DNA of the bugs was isolated from legs using commercially available DNA extraction kit following the instructions. The isolated DNA was confirmed using 1% Agarose gel and then was PCR amplified using forward primer 5'-CGAATTGAGTTAGGTCAACCCG - 3' and reverse primer 5'- GGATCTCCTCCTCCTGAAGGATC - 3'. The thermo cycler conditions were modified as follows; 1 initial cycle of 5 minute at 95°C followed by 30 cycles of 95°C for 10 seconds, 50°C for 1minute, 72°C for 45 seconds followed by a final step of 72°C for 3 minutes. The PCR product was confirmed using 2% Agarose gel electrophoresis and were sequenced with both primers in automated sequencer ABI 3730XL by Sangers method. The sequences were analysed for gaps, consistency, non-sense codons etc. The aligned sequences were used for species identification and confirmation using NCBI BLAST tool. Mitochondrial COI sequence data after confirming the identification was submitted in NCBI GenBank and accession numbers were obtained (Table 1). Phylogenetic analyses were done by MEGA6 software [14]. The most similar sequences from GenBank were retrieved (Table 2) and sequences of three colourmorphs generated from this study were compared and aligned using the ClustalW program. Divergence between the species were analyzed by Kimura 2 parameter [15] model of MEGA6 software. The phylogenetic tree was constructed using the Neighbour joining (NJ) method [16] with help of K2P distance method using MEGA6. The bootstrap values were set to 1000 replicates [17].

Table 1. Three colourmorphs of *Nezara viridula* (Linnaeus) collected from Kerala, India with the NCBI-GenBank accessesion numbers

Sl. no.	Taxa name	Colour Type	Place of collection	Length of segment (bp)	GenBank Accession No:
1.	*Nezara viridula var. smaragdula* (Fabr.)	G Type	Chemmappilly, Thrissur, Kerala	478	KX503046
2.	*Nezara viridula var. torquata* (Fabr.)	O Type	Chemmappilly, Thrissur, Kerala	483	KX50347
3.	*Nezara viridula* L.	R Type	Athirapilly, Thrisur, Kerala	470	KX503045

Table 2. CO1 gene sequences of *N. viridula* retrieved from GenBank for analyses

Sl.No.	Place	Accession No.	Authors
1	India	KX351397	Kuotsuk,K. et al.
2	India	KX467340	Rakshit, O. et al.
3	India	KX467339	Rakshit, O. et al.
4	India	KR028341	Reetha, B. et al.
5	India	KR028340	Reetha, B. et al.
6	India	KR028339	Reetha, B. et al.
7	India	KJ866507	Rakshit, O. et al.
8	India	GQ306225	Tembe, S. et al.
9	India	HQ236460	Tembe,S. et al.
10	India	KY859196	Srinivas,N. et al.
11	India	KX587504	Priya, B. et al.
12	China	KC155924	Zhang, X. et al.
13	Korea	GQ292245	Jung, S. et al.
14	Canada	KU601566	Dhani, M.K. et al.
15	Canada	KU601565	Dhani, M.K. et al.
16	Canada	KU601564	Dhani, M.K. et al.
17	USA	KJ642018	Brown, V.A. et al.

3. RESULTS AND DISCUSSION

With the classical taxonomic keys, the specimens were identified as three colourmophs of Nezara viridula, the southern green stink bug. These are the first report of Colour polymorphism from Kerala, India. The three colour morphs are described below.

3.1 Colourmorphs of *N. viridula*

The colour polymorphisms of *N. viridula* is because of the genetic mechanism and studies suggest that it is controlled by two sets of genes on two independent loci [9,18]. In this study, three colourmorphs which belong to the four basic colourmorphs in *N. viridula* has been reported (Fig. 1).

Nezara viridula var. *smaragdula (Fabr.)* – G type (Fig. 1a): This is the common green form. Although it is green, a narrow yellow margin is seen in entire body. Ventral surface is pale green.

Nezara viridula var. *torquata (Fabr)* – O type (Fig. 1b): Similar to G type except having broad yellow fasciae on head region excluding base and yellow colour in anterior margin of pronotum.

Nezara viridula L. – R type (Fig. 1c): Green spots are present on yellow dorsal body. The arrangement of spots are as follows - two small spots on the base of ocelli, three transverse spots on pronotum, a large spot on mid basal region of scutellum, Single spots on each basal angle, one spot near to the caudal apex and spot on hemelytra.

In *N. viridula* 12 colourmorphs has been reported and the combination of the morphs varies in different regions. Highest polymorphic diversity is seen in Japan [9]. The basic green type, *N. viridula* var. *smaragdula* or G type is seen all around world as it is best adapted to colonize different environments [9,7].

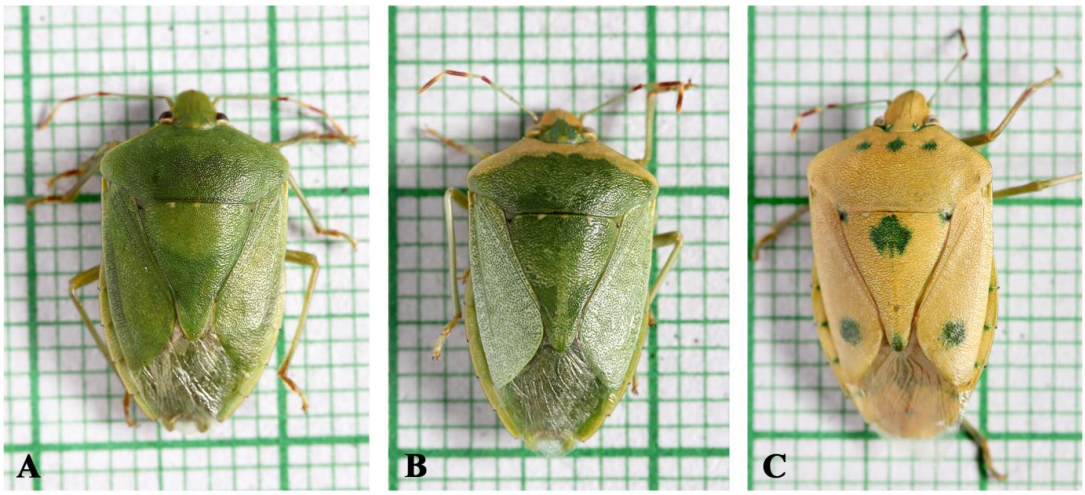

Fig. 1. The colourmorphs of *Nezara viridula* (L.) from Kerala, India. A) *Nezara viridula* var. *smaragdula*, Type G. B) *Nezara viridula* var. *torquata*, Type O. C) *Nezara viridula* (L.), Type R

3.2 Identity Confirmation of Colourmorphs by Phylogenetic Analysis

The three sequences of present study showed more than 98% similarity with other *N. viridula* sequences in the BLAST analysis. It confirms its taxonomic identification. For further confirmation and studies, along with the three sequences of present study, 17 other CO1 sequences of *N. viridula* were retrieved from NCBI GenBank. The final aligned data after incorporating the GenBank sequences had 20 CO1 sequences of 443 bp length and they were analysed.

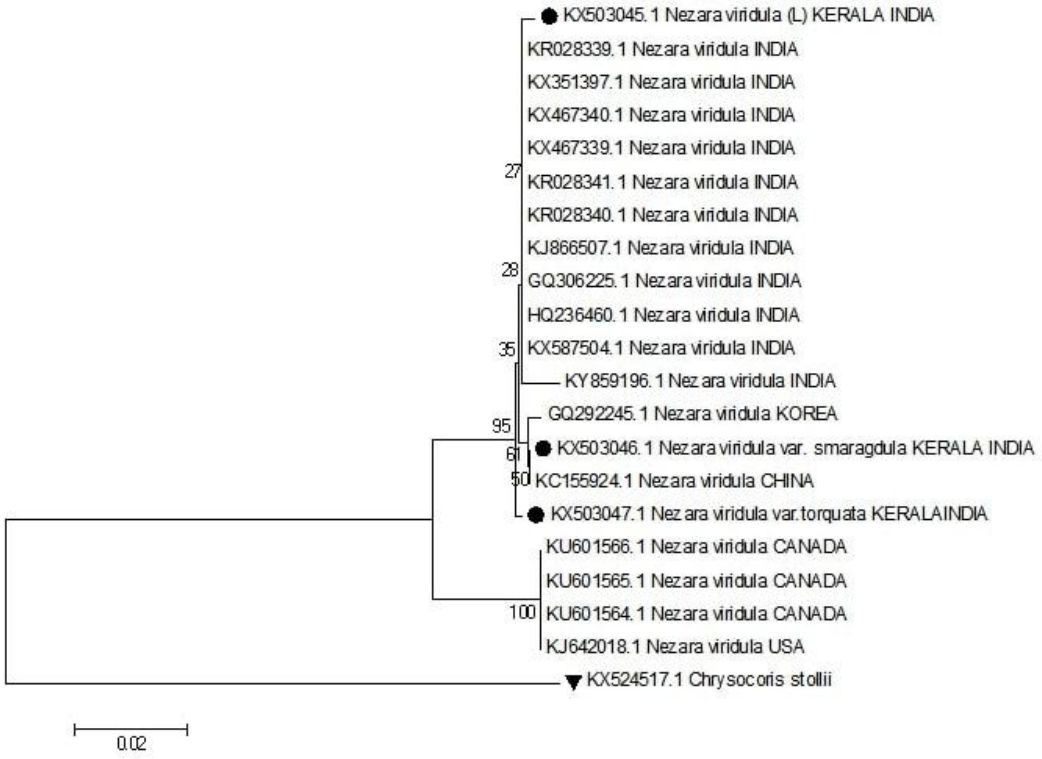

Fig. 2. Phylogenetic tree of *Nezara viridula* based on CO1 (K2P mode) using Neighbour-Joining method. Numbers indicate the percentage of 1000 bootstrap replicates

The intraspecific divergence was calculated using K2P method. As all the sequences are of same species *N. viridula*, the intraspecific variation was very low as below 2% and this is according to many previous studies [19]. The very low intraspecific divergence confirms that the species in this study belongs to *Nezara viridula* inspite of its different morphological appearance. Phylogenetic tree using MEGA6 software was also constructed for all the 20 sequences. Sequence of *Chrysocoris stolli* of Scutellaridae family retrieved from GenBank is taken as outgroup and bootstrap repeats has been set as 1000 for the checking the reliability of phylogenetic tree (Fig. 2). From the tree it is evident that all *N. viridula* species originated from the same ancestor confirming its identification. The interesting fact is that, inspite of the same origin, sequences has divided into two branches, one with Neartic species (Canada and USA) and other with Oriental species (India, China, Korea). This reveals the changes in sequences due to the geographical separation. As *Nezara viridula* is a widely spread species throughout the world, this variations in the genetic makeup in distant populations causes intraspecific divergence [19]. With its different phenotypic appearance, many of the colourmorphs of *Nezara viridula,* are greatly adapted to unfavourable environmental conditions. The different colour polymorphism can be attributed as the reason for it being a successful pest of wide variety of crops all over the world. This shows DNA barcoding will assist in rapid identification of such pests and thus proper pest management strategies can be taken [9,19].

4. CONCLUSION

Nezara viridula is a serious pest of many economically important crops worldwide. It is present in different colour polymorphic appearance which makes dubiety in identification. This study reports three colourmorphs of *N. viridula* species from Kerala for the first time. Using the mitochondrial CO1 gene sequences of the species, present work confirms that DNA barcoding is very advantageous in identification. This method can be used to identify the pests of economically important crops very easily and accurately. This study also reveals the intraspecific variation among *N. viridula* species of Neartic and Oriental regions of the world.

ACKNOWLEDGEMENT

Authors would like to thank Dr. S. Salini Scientist, Division of Insect Systematics, National Bureau of Agricultural Insect Resources, Bengaluru, India for the assistance in identification of specimens. Thanks to UGC- Maulana Azad National Fellowship scheme for the funding assistance. All facilities for the work were provided by Molecular Biology Labouratory, Department of Zoology, University of Calicut, Kerala. We also appreciate help from our friends in specimen collection and for constant support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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