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DNA BARCODING OF MAJOR PESTS OF THE PIGEON PEA *Cajanus cajan* (L) Millsp USING CO1 GENE

PUSHPARAJ KARTHIKA* AND NATRAJ KRISHNAVENI

Department of Zoology, Avinashilingam Institute for Home Science and higher education for women, Coimbatore-641043.

ABSTRACT

Six major pests *Cajanus cajan* (L) Millsp were collected from the agricultural fields of Karamadai, Tamilnadu. DNA was sequenced and identified using CO1 gene. The sequence content, nucleotide information and substantial saturation were studied and utilized for phylogenetic studies. Homology of sequence comparisons was done and the sequence of four species matched exactly with the respective species that existed in the database. However, the nucleotide sequences of two species, *Eurybrachys tomentosa* and *Gametis versicolor* were reported for the first time in our study. A strong AT bias was observed among the sequences and the ratio of the substitutions(R) was high in the first and third codon positions. The threshold value was 1.97%, inter and intra nucleotide divergences of the six species were also calculated and no overlap existed among the species. Distinct barcoding gap was noted. High nucleotide diversity P(i) and maximum intraspecific variation was found among the individuals of *N. viridula*.

KEYWORDS: **DNA barcoding, pests, nucleotide divergence, phylogenetic analysis.**

PUSHPARAJ KARTHIKA
Department of Zoology, Avinashilingam Institute for Home Science and higher education for women,Coimbatore-641043.

INTRODUCTION

After the advent of intensive agriculture there has been drastic change in the scenario of pests in relevance to the field of plant protection. Very often even minor pests and diseases emerge as a major drawback in monocrop cultivation[1]. In India there are about 200 major varieties of pests of economic importance, in such a back drop the importance of documentation of agriculturally important insects (pests) need to be precisely carried out to meet out the loss produced by pests in an essentially agrarian country like India. High incidence of pests are found in the vegetable and field crops, therefore usage of pesticides is maximum in them. Their indiscriminate usage not only creates environmental problems but causes complete destruction of non-target organisms, subsequently the targeted pests become resistant toward the insecticides[2].Hence for any biological problem, the potential, accurate and rapid identification of the causative organism is mandatory to find a wholesome solution. In this case, the present study was framed upon the identification of major pests in the field crop *Cajanuscajan*(Pigeon pea), based on the standard region of CO1 gene. The integration of traditional and modern molecular techniques was utilized to identify the pests. Since the classical use of morphological traits for species identification has several problems like phenotypic plasticity, polymorphic forms, immature stages (larvae, egg and pupa), existence of cryptic species and the insufficiency of morphological keys. Thus, a high level of expertise is required to correctly identify species, hence to counteract this problem the best solution could be the application of DNA barcoding. In addition, it has been shown to be valuable for insect surveillance and quarantine purposes, and displays potential as a tool for unified and coordinated global pest species detection [3].Rapidly evolving genes and nucleotide regions are considered useful for comparisons of closely related taxa[4]. Studies utilizing a 658 base pair region of the mitochondrial gene cytochrome c oxidase subunit I (COI) have demonstrated the ability of that marker to confidently link field collected organisms with a reference sequence of a previously identified species[5]. DNA barcoding is already well established in many organisms such as mosquitoes[6], cicadas[7], genus *Cricotopus* of non-biting midges[8] mayflies[3], springtails[9] and butterflies[10]. A critical step in a DNA barcoding approach also relies on the criteria and assumptions used to group barcode sequences in defined entities as they share some characteristics (e.g. sequence similarity, geographical provenance of the specimen)[11]. Consequently, the ideal DNA barcoding analysis mirrors the distributions of intra- and inter-specific variability separated by a distance called DNA barcoding gap[12,13].Phylogenetic analysis exploits maximum information content from the nucleotide sequences as the specimen identification using suitable gene region in phylogenetic analysis would work provide that the species of the unidentified specimen is represented in the database[14].The NJ method has been promoted as the analysis tool of choice for the construction of barcoding databases, due to its advantage of speed and its performance when the sequence divergences are low[3].Therefore based on the above information we have identified the six major pests of pigeon pea using the CO1 sequences and checked their utility of sequence information in phylogenetic studies based on the homology of the sequences, substitution rates and genetic divergence among the selected insect pests.

MATERIALS AND METHODS

Sample collection and morphological examination

Six major pests were collected from *Cajanuscajan* (Pigeon pea) were collected from fields in Karamadai (11.16° N, 76.58° E) in Coimbatore, India by using aspirator and hand picking method. The collection comprised of insects belonging to adult and larval stages which were identified by ZSI (Zoological Survey of India) with prescribed taxonomical keys. Finally samples were preserved in 75 to 95% ethanol (based on the nature of insects the percentage of ethanol should be maintained for avoiding the decolouration) for further analysis.

Molecular analysis, PCR and Sequencing

Genomic DNA was isolated from a portion of the tissues from the leg or whole insect was used depending on their size. The DNA was isolated by DNeasy kit (Germany) according to manufacturer's protocol. 1% Agarose Gel Electrophoresis (GENEI, Bangalore) was performed to detect the genomic DNA using Gel documentation (Medic care, India)**.** DNA amplification of MT-COI gene was carried out in Eppendorf Thermo Cycler by using the forward (LCO1490: 5'-GGTCAACAAATCATAAAGATATTG-3') and reverse (HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers[16]**.** Amplification was performed in a total volume of 50µl containing 4µl of DNA template, 20 pM of each primers, 400µM of dNTP and 0.4µl of Taq DNA polymerase (Qiagen). Thermo cycler conditions were as follows: 5 min at 95°C for pre-running, then 35 cycles of 60 s at 95°C for denaturation, 60s at 49-52°C for annealing, and 90 s at 72°C for extension followed by 5 min at 72°C for a final extension. The final product was stored at -20°C for further usage. The amplified product was resolved with 2% AGE. Sequencing was done by using ABI 3500 XL Genetic Analyzer with manufacturer's protocol of Chromos Biotech, Pvt. Ltd., Bangalore, India. The sequences were trimmed and edited using clustalW and BioEdit v7.2.5[17] and authenticated in GenBank. Multiple sequence alignment was done with T-COFFEE and represented in MAS (Multiple Align Show).

Data analysis

Fourteen species (haplotypes) were downloaded from GenBank and used for comparisons. The nucleotide composition and AT bias and the nucleotide diversity P(i) among the species was estimated by Jukes Cantor method using DnaSp v 5.1. The ratio (R) of the of transitions (TS) and transversions (TV) at the first, second and third codon positions were calculated and subsequently plotted against the uncorrected p-distance for all the three codons was performed using MEGA v.6.2[18]. The test for substantial saturation of the sequences was checked by using [19, 20](DAMBE).The

intra and inter nucleotide divergence was calculated using the Kimura-2-parameter and the NJ –clustering was used to estimate the overlap and conspecific nucleotide divergence among the haplotypes by using MEGA.v.6.

Phylogenetic analysis

The optimum substitution models were determined by the best fit model test for the selection of model to be applied for sequences. The Tamura 3-paramerter model was selected from 24 different nucleotide substitution models for the original sequence of the selected species in the present study based on the Akaike Information Crierion (AIC) and lowest BIC values. The robustness of the clades of the tree was estimated using bootstrap analysis of 1000 replications with the elimination of all the codons containing gaps and missing data. The ML tree was constructed with the out group *M. lamarrei .The* Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 2.5055)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches).The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing contains gaps and missing data were eliminated. There were a total of 167 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Statistical analysis are integrated in the above analysis itself.

RESULTS

In the present study we generated DNA barcode sequences of the six major pests affecting the economically important agricultural crop *Cajanus cajan* (Pigeon pea). The selected species belonged to three different orders and each of the samples was represented by three to four individuals. The pests were collected based on the extent of damage caused by them to the crop and it comprised of both adult and larval (immature specimens).Adults were morphologically distinguishable and identified with standard taxonomical keys but identification of the larval stages were certainly difficult as a non-taxonomical expertise. The DNA isolated from the insect tissues produces clear bands devoid of the protein contaminations, appreciable good quality of sequence range of 627-695 bp were obtained. The similarity search for the obtained sequences was done by BLAST in NCBI, most of the sequences matched perfectly with the respective species that already existed in the database. Whereas, *Eurybrachys tomentosa* and *Gametis versicolor* were the first time record therefore it did not match exactly. The BLAST parameters and the similarity results for the six nucleotide sequences are presented in Table 1.All the sequences were edited, aligned and authenticated in GenBank with the following accession numbers KJ5559402, KJ559403, KJ559405-08.

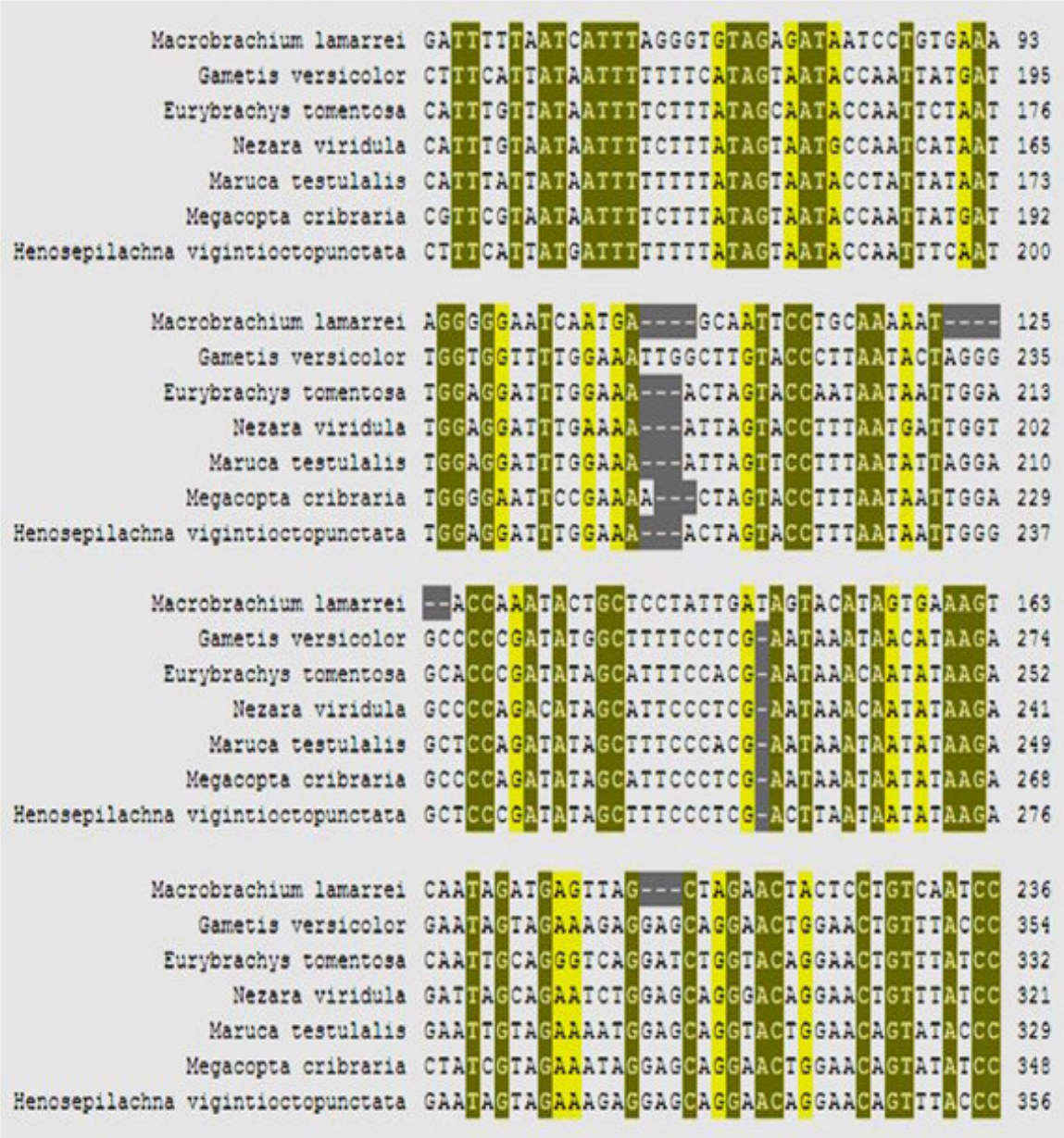
Table.1
Summary *of identification based on each nucleotide sequences of fifteen species of insects using BLASTN search from GenBank*

S.no	Name, Ac.no and bp size of queried organism	Name .Ac.no and location of matched organism	BLAST parameters*					Author details
			Q	S	E	G	I	
1.	*Gametis versicolor* KJ559408	*Protaetia aurichalcea* KM033437, India	99	686	0	23	85	Rangeshwaran *et al*., 2014
2.	*Eurybrachys tomentosa* KJ559407	*Geisha distinctissima* JN087438, Japan	100	640	1e-179	18	85	Lee, 2011
3.	*Nezara viridula* KJ559406	*Nezara viridula* KJ866507, India	100	1077	0	15	97	Rakshit*et al*., 2014
4.	*Maruca testulalis* KJ559405	*Maruca testulalis* KJ623250. China	100	1109	0	15	98	Yuan *et al*., 2014
5.	*Megacopta cribraria* KJ559403	*Plataspidae sp* KC153670, USA	100	1083	0	21	95	Jenkins *et al*., 2013
6.	*Henosepilachna vigintioctopunctata* KJ559402	*Henosepilachna vigintioctopunctata* AB002180, Japan	99	1066	0	13	94	Kobayashi *et al*., 2008

BLAST parameters Q- query coverage; S-maximum score; E- E-value; G- no. of gaps; I- percentage of identity

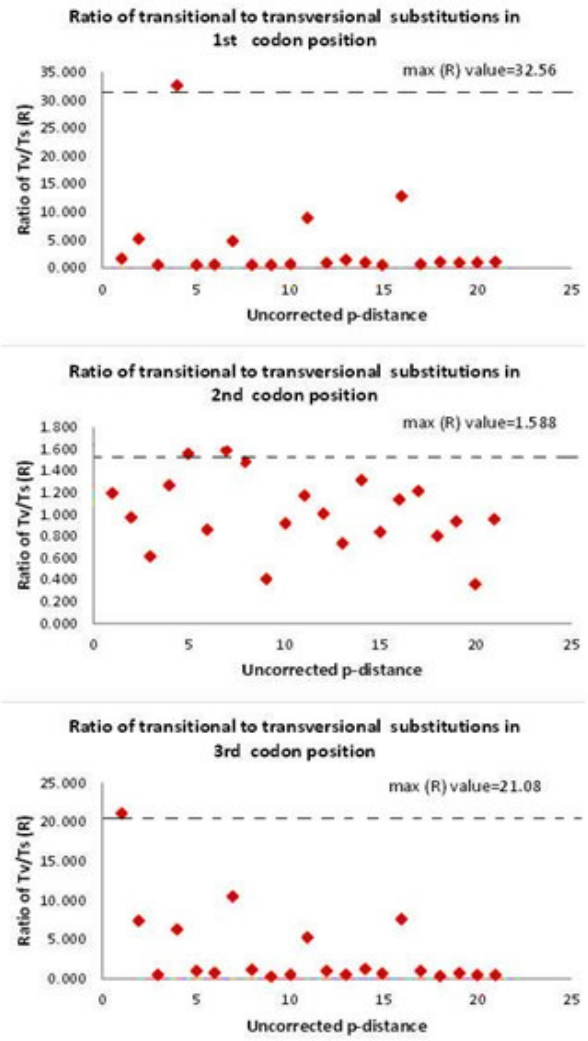
The multiple sequence alignment was performed in which the similar and identical residues were highlighted and represented using multiple align show (Fig. 1).Comparative analysis of the individual nucleotide composition of the standard CO1 fragment exhibited strong AT bias (66.95%) whereas the GC content was 33.04%.The individual base composition varied among the species, the mean A=30.80%,C=17.80%,G=15.24% and T=34.65%.The nucleotide diversity P(i) of the three species along with their haplotypes were calculated in which minimum diversity was observed in the haplotypes of *Maruca testulalis* (0.0296) and maximum in *Nezara viridula* (0.1125) whereas *Henosepilachna vigintioctopunctata*was 0.0555.

Figure 1
Multiple Sequence Analysis represented using Multiple Align Show (MAS)



Identical residues of amino acids are given in dark yellow colour and that of similar residues are highlighted in light yellow. Gaps and missing data are shaded in grey. The ratio of transition and transversion substitutions (R) for all the three codon positions were calculated and plotted against the uncorrected p-distance and represented as scattergram (Fig. 2).Among them the first codon position possessed the maximum R value (32.56) whereas the second and third codon positions had 1.588 and 21.08 respectively. Substitution saturation of the sequences was checked on the fully resolved sites where, the Iss (0.3040) was found to be less than Iss.c (0.7717) (Table 2) therefore the sequences were devoid of substantial saturation and certainly suitable for phylogenetic studies.

Figure 2
Ratio of Substitution rates among the codons



Scatterplots showing the ratio of substitutions (TS-Transitions and TV-Transversions) in y-axis against uncorrected p-distance in x-axis for each codon position.

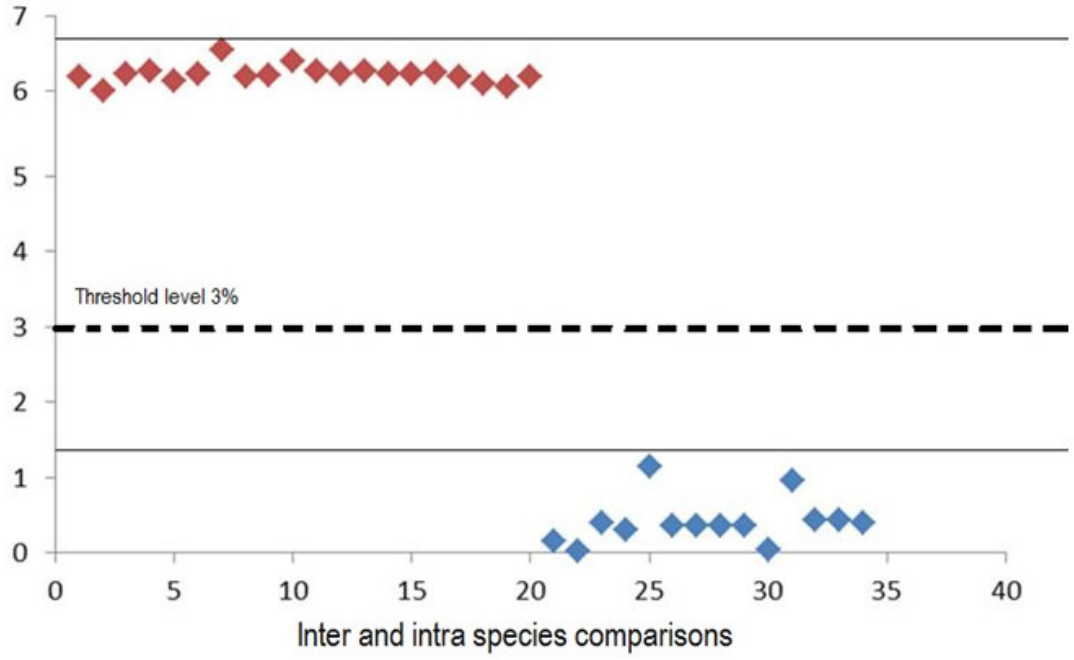
Table.2
Results of the nucleotide substitution saturation test (DAMBE) on the CO1 mt DNA dataset of 6 sequences

Mean H	0.4518
Standard Error	0.0211
Hmax	1.4862
Iss	0.3040
Iss.c	0.7717
T	22.1365
DF	620
Prob(two tailed)	0.02
95% lower limit	0.2625
95% upper limit	0.3455

The nucleotide substitution test was calculated based on the index of saturation (Iss), which was compared against the critical value computed for symmetrical (Iss.cSym) tree topology and the analysis was performed on fully resolved sites. T: Tvalue, DF: degrees of freedom, P: probability that *Iss* are significantly lower than the critical value(*Iss.cSym*).Two-tailed tests was performed with 10000 replications. Inter and intra species nucleotide divergence was calculated for the six species and their available haplotypes. The average inter nucleotide divergence was 0.623, maximum --divergence was observed in *Nezara viridula* (AB021151) 0.55;minimum divergence in *Eurybrachys tomentosa* (KJ559407) 0.601.The haplotype sequences were available for the three species *Henosepilachna vigintioctopunctata*, *Maruca testulalis* and *Nezara viridula* therefore the intra nucleotide divergence was calculated based on the above data set. In such case, the average intra nucleotide divergence was 0.191, with maximum divergence for *M. testulalis* (KJ559398) 0.048 and minimum divergence for *N. viridula* (KJ86607) 0.031 and *H. vigintioctopunctata* (KJ559395) 00403.

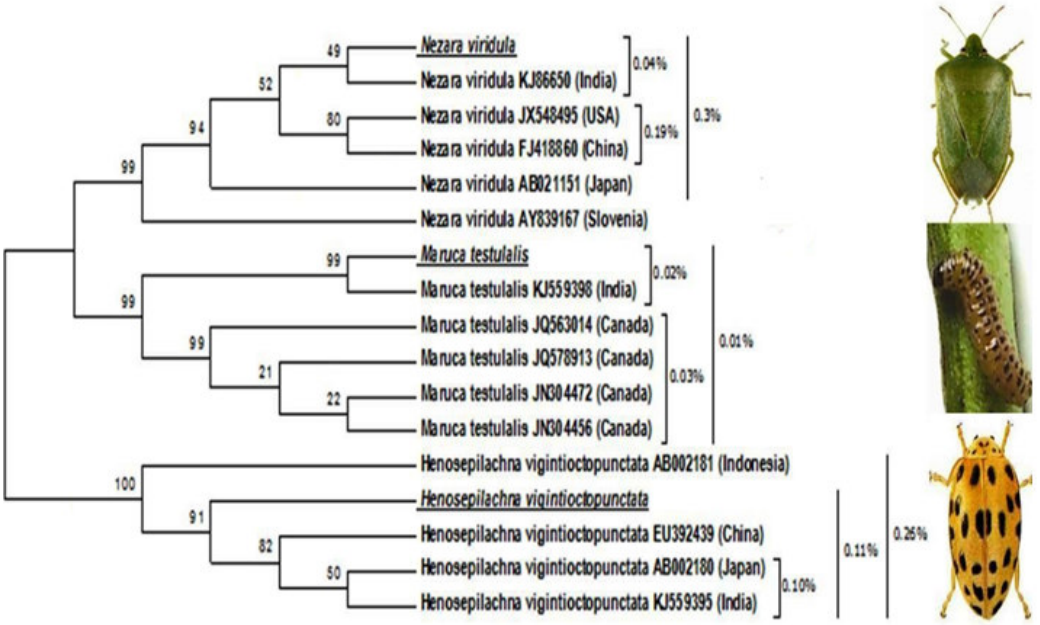
The 10X rule postulated by Hebert *et al*[10] was utilized in the present study. According to it, majority of the samples were completely resolved and it certainly discriminated to the species level. The threshold value obtained among the interspecies was 6.23% (0.632x 10) which is > 3% and that of intraspecies was 1.97% (0.197x 10) which is <3%.Therfore the species of the species of the present study clearly demonstrated the distinct interspecies and intraspecies variations (Fig. 3).The NJ tree of the haplotypes constructed formed monophyletic clades (Fig. 4) with an average nucleotide divergence of 0.3% among the species of *N.viridula*, 0.01% in *M. testulalis* and 0.11% in *H. vigintioctopunctata*. The haplotypes comprised of individuals from the Indian as well as from other geographical locations but they maintained a constant and notable difference among the conspecifics and wide variations as a whole in the interspecies comparisons.

Figure 3
Dot plot analysis of the nucleotide divergence among the species.



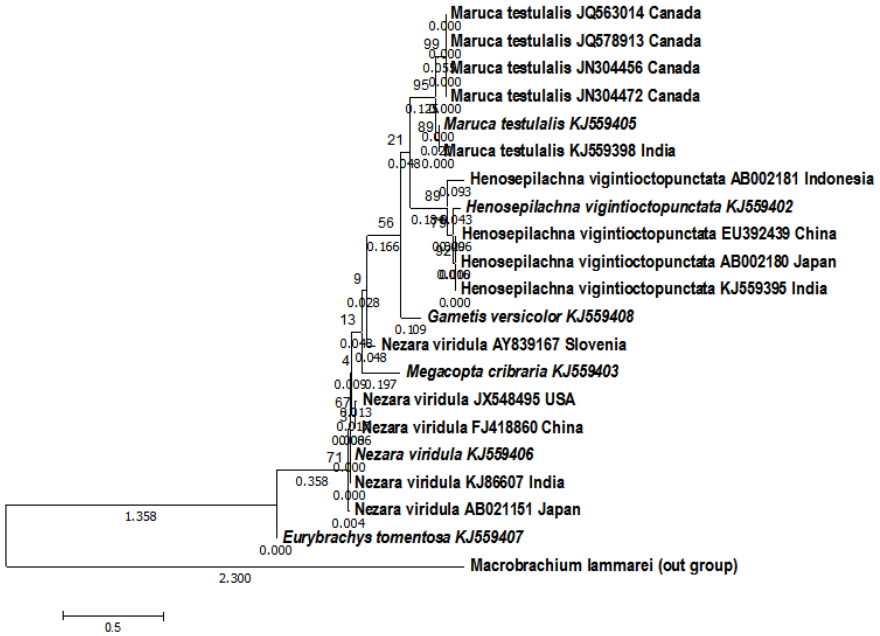
The genetic divergence of each species comparisons are plotted, where the inter nucleotide divergence are represented in blue squares and intra nucleotide divergence in red squares. The maximum values for the inter and intra specific divergence is represented by a straight line .The threshold values 3% suggested by Hebert et al represented by dotted line

Figure 4
NJ clustering analysis showing the intraspecies divergence among the selected haplotypes species in the study



Three different species are represented here with distinct intraspecific divergences .Each species formed a separate cluster as monophyletic clade .No overlap of the species were found and the lowest divergence was found within the conspecifics of Indian origin, whereas discrete divergence was noted in the species from a distant geographical location. Underlined name of the species are original records of the present study. The Tamura 3-prameter was selected as the best fit model and ML tree was constructed with the out group as *M. lamerri* of log likelihood (1288.84) (Fig.5).The phylogenetic tree was monophyletic associated with discrete branch lengths. Typical clustering of the conspecifics were formed and the two species *G. versicolor* and *E. tomentosa* emerged as separate clades illustrating the wide genetic differences among the other species in the data set.

Figure 5
ML tree for the haplotypes along with the original sequences of the study



Phylogenetic tree based on the Tamura 3-parameter model with the haplotypes along with the selected species of the study (italics) in the monophyletic clade. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

DISCUSSION

India experiences an annual damage of 20-30% is caused by pests, diseases and weeds in various crops[1], in such a case rapid and timely identification of the crop pests is the actual need of the hour. However it is elapsed by several problems such as the existence of polymorphic forms, smaller size, insufficient discerning morphological characters and complex association within multiple hosts[21].Generally most of the pests include the bugs which cause damage by their mouth parts by sucking the plant parts and they also play an important role as vectors in transmitting viral infections (eg. Aphids). Secondly, the larvae of the lepidopteran insects occupy a major part in affecting the succulent parts (e.g *Leucinodes orbonalis*), while the beetles feed on the vegetative parts like flowers (flower chaffer beetles).Thus insect pest management approaches require a clear understanding on the pest species in relevance to their biology, ecology and population structure/genetics[22].Thus DNA barcoding has gained a crucial role the accurate and prompt identification of the species and play an important role in pest management strategy and the present study was the first attempt carried out to identify the pests of the economically important crop *Cajanus cajan*(Pigeon pea). Amplification using the universal primer for the 652bp CO1 region was effective for all the insect samples as they belonged to different insect orders. Strict analogy was observed in the identification of the species by their nucleotide sequences with their morphological keys. The nucleotide sequences of the six species showed a strict AT bias as it is a characteristic feature of arthropods[23]and this has been reported in the case of black flies[24], *Trialeurodes* sp. (Hemiptera) [25] and peach fruit fly *Bactrocera zonata*[26].The effectiveness of DNA barcoding using CO1 relies on the comparison of the intra and inter species nucleotide divergence. The level of nucleotide variation observed for the CO1 gene fragment was high in interspecies comparisons and low among intraspecies. Jung et al[27] reported the average intra and interspecific divergence to be 0.8 and 12.6% respectively in true bugs. In addition, Rebijith et al[22] demonstrated the discrete barcoding gap between the inter species and intra species of the Aphids inferred from the NJ tree. Thus, the 3%, threshold or cut off value demonstrated adequately to discriminate the species. The overlap among the individuals was minimum at the conspecific level whereas, no overlap was found among the congenerics. NJ clustering analysis were best suited for understanding the DNA barcode gap among the species comparisons and it was supported by several studies[28, 29]. The reliability of results from molecular phylogenetics of sequence data depends on how well the analysis deals with the problems such as the reliability of sequence alignment, variation of substitution rates substantially over sites[30] and whether some or all sequences in the data set have already lost phylogenetic information due to substitution saturation[31]. The substitution rate among the sequences is usually the deciding factor in reconstruction of a phylogeny such that the third codon position of the mitochondrial genes were used to infer phylogeny among the closely related organisms[32]. Moreover, in all the protein coding genes, the third codon position is the most variable and the second is considered to be most conserved[33]. The third codon position is often not excluded from the analysis, mainly for two reasons. First, excluding the third codon position would often leave us with a few substitutions to work on. Second, substitutions at the third codon position likely conform better to the neutral theory of molecular evolution than those at the other two codon positions. Consequently, the former may lead to better phylogenetic estimation than the latter, especially in estimating divergence time[34]. A low substitution rate usually ensures accurate phylogenetic inference, irrespective of the values of all other parameters[35]. The ratio (R) of transitions to the transversion substitutions of the third codon position (21.08) was found to be slightly lower than the first codon (32.56).Indeed, multiple substitutions at a site perhaps potentially obscure true phylogenetic signal since third codon position accumulates the majority of phylogenetic information. Test of substitution saturation of the sequences showed that the nucleotide sequences of the six samples were devoid of substantial saturation condition. Hence it was evident that the 3rd codon position retained the necessary phylogenetic data and thus the sequences were subsequently utilized for the DNA barcoding and phylogenetic analysis in the present study.

CONCLUSION

Since, Pigeon pea (Cajanus cajan [L] Millsp.) is an important grain legume of the semi-arid tropics[36] , the present study provides a limelight on the phylogenetic variations in the major pests occurring in the crop.

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