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RESEARCH PAPER

Molecular characterization of mirid bugs infesting cotton

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Abstract: Mirid bug is an important emerging insect pest in the various crops and predominant in cotton. Both nymphs and adults pierce their stylet and suck the sap from immature bolls and watery saliva is secreted by the bug, when they pierce into the bolls which leads to cell necrosis and in-turn leads to dropping of cotton squares and ultimately reduce the yield. Conventional taxonomic studies were already well established but there is a lack of knowledge in the area of molecular characterization. The present investigation on the molecular characterization of mirid bugs was done during 2016-17 in Department of Biotechnology, University of Agricultural Sciences, Dharwad. The collection of mirid bug species from different crops at various locations confirmed that, the existence of brown, green and red colour morphs of (*Creontiades bisrratense* Distant) in Karnataka infesting on cotton and (*Creontiades pallidus* Rambur) on sunflower ecosystem in Latur, Maharashtra and which is a pest of cotton in Australia. Through available matching sequences of mitochondrial cytochrome oxidase DNA fragments resulted in two cluster phylogeny and all color morphs were matched each other.

Key words: *Creontiades,* Cytochrome oxidase, Mirid bug, Mitochondrial

Introduction

Cotton, the white gold and principal fiber crop of the world, plays an important role in Indian agriculture, industrial development and contribution to the national economy. Out of 50 species in genus *Gossypium*, only four are cultivated worldwide 77 countries and in India (Zhang *et al.,*2008). India is leading cotton grower (122.38 lakh hectare (Anonymous, 2019). After broad scale adoption of Bt cotton, sucking insect pests incidence was increased, among such emerging insect pest, the cotton mirid bugs are major in cotton growing countries. *Creontiades dilutes* (Stal) and *C. pacificus* (Stal) emerged as important sucking pest in Bollgard-II in Australia (Khan *et al.,* 2007). Similarly, mirid bug *Creontiades biserentese* (Distant) has been reported as new pest in Karnataka infecting cotton (Patil *et al.,* 2006). In short notice, this has occupied a key pest status in Karnataka, Tamil Nadu and Maharastra (Udikeri *et al.,* 2011). Mirid bug possess piercing and sucking type of mouth parts. Mirid bugs watery saliva contains the digestive enzymes, which are thought to be involved in both penetrating the surface of plant tissue and the pre-oral digestion of cells. The salivary gland and midgut may be related to extra-oral digestion and defense, leading to tissue necrosis and abscission shedding damage in plant (Taylor *et al.,* 1990).

The complex life cycles, significant polymorphism, immature taxonomy and absence of trained manpower make the identification of these pests difficult (Rebijith *et al.,* 2012). On the basis of the pattern of nucleotide arrangement in short standardized DNA sequence, 658 bp fragment of the mitochondrial cytochrome oxidase (COI 1), in taxonomy, this is used as a method to achieve rapid species descriptions in the context of the current biodiversity crisis (Hebert *et al.,* 2003). Molecular characterization of insects is carried out by DNA sequences from a short segment (mtDNA) of the whole genome, with the intention of advocating a broad range of environmental and conservation (Jalali *et al.,* 2015). Hence the present investigation on molecular characterization of mirids was carried out, which helps in strengthening the existing knowledge of managing the mirid bugs.

Material and methods

Mirid bugs specimens (both nymphs and adults) were collected from different host crops (cotton, sunflower, pigeon pea and sorghum) at UAS Dharwad, Karnataka, Agriculture College, Lathur, Maharashtra, ANGRAU, ICRISAT and Directorate of Oilseeds Hydrabad, Telagana and were stored in 70 per cent alcohol at -20°C for further analysis. All the species collected were submitted to the conventional taxonomic studies and were identified by Dr. C. A. Viraktamath, Department of Entomology, GKVK Bengaluru, Karnataka (Table 1 and Plate 1).

Mirid bug adults were taken in a micro centrifuge tube, 180µl of buffer animal tissue lyses (ATL) was added and crushed using mortor and pestle. Later, 20 µl proteinase k was added, mixed by vortexing and incubated at 56 C until completely lysed. After overnight incubation, 200 µl buffer ATL was added and vortexed immediately. And samples are incubated at 56 C for 10 minutes, 200 µl ethanol (96-100%) was added and mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini spin column, placed in a 2 ml collection tube and centrifuged at ≥ 6000 ×g (8000 rpm) for 1 minute. The flow through and collection tube were discarded. The spin column was placed in a new two ml collection tube, 500 µl buffer AW1 was added and centrifuged at ≥ 6000 ×g. The flow through and collection tube were discarded. The spin column was placed in a new 2 ml collection tube and 500 µl buffer AW2 was added and centrifuged for 3 minutes at 14000 rpm. The flow through and collection tube were

Table 1. Mirid bug collections and their conventional taxonomy

Sl. No.	Place	Crops infested	Species, Order: Family
1	College of Agriculture, Dharwad.	Cotton Sunflower Sorghum	Creontiades biseratense (Distant), Hemiptera: Miridae Taylorilygus apicalis (Fieber), Hemiptera: Miridae Creontiades pallidus (Rambur), Hemiptera: Miridae
2	College of Agriculture, Hyderabad	Cotton	Nymphs, Hemiptera: Miridae (No confirmation of species)
3	Director of Oilseed Research, Hyderabad	Sunflower	Creontiades pallidus (Rambur), Hemiptera: Miridae Taylorilygus apicalis (Fieber), Hemiptera: Miridae Campylomma livida Reuter, Hemiptera: Miridae
4	ICRISAT ,Hyderabad	Sorghum Pigeon pea	Calocoris sp., Hemiptera: Miridae Clavigralla gibbosa Spinola, Hemiptera: Coreidae
5	College of Agriculture, Latur,	Cotton Sorghum	Nymphs, Hemiptera: Miridae (No confirmation of species) Creontiades pallidus (Rambur), Hemiptera: Miridae

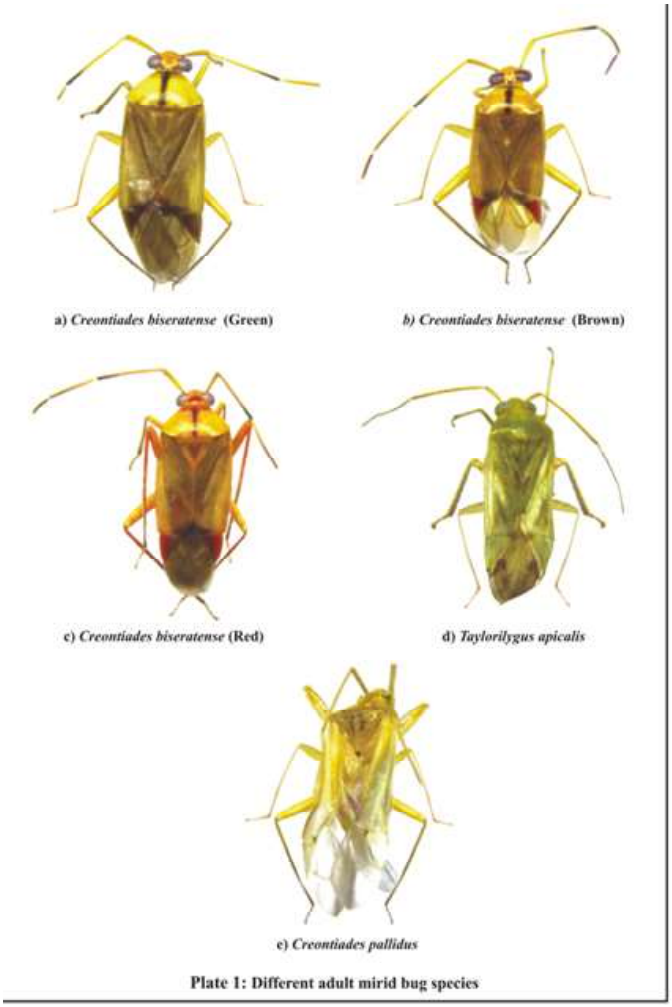


Plate 1: Different adult mirid bug species

discarded. The spin column was transferred to a new 1.5 ml or 2 ml micro centrifuge tube. The DNA was eluted by adding 200 µl buffer AE to the center of the spin column membrane and incubated for 1 minute at room temperature (15-25 C) and centrifuged for 1 minute at ≥ 6000 ×g.

Gel casting plate and comb was washed with water and wiped with 70 per cent alcohol. The two open ends of the gel casting plate were sealed with cellotape and placed on perfectly horizontal levelled platform with comb inside the gel casting plate. Agarose gel (0.8%) was prepared by adding agarose powder to 1X TAE buffer (prepared from 50X TAE buffer), it was boiled until the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was added to the agarose prior to pouring in the gel plate. After solidification of the agarose, the comb and cello tape were removed carefully and the casted gel was placed in the electrophoresis unit with wells facing towards the cathode and submerged with 1X TAE buffer to a depth of about 1 cm.

A piece of parafilm was placed on the solid surface and 7 µl of genomic DNA was pipetted out on to a parafilm. To the DNA samples, 3 µl loading dye (bromophenol blue) was added and mixed thoroughly by pippeting up and down several times gently. The contents were loaded into wells carefully with the help of micropipette. DNA ladder (100 bp) was loaded as standard marker. Cathode and anode were connected to a power pack and gel was run at the constant voltage (70 V). The gel was run for 60 min until the tracking dye reached half of the gel and bands were visualized and documented in the gel documentation system.

PCR amplification and confirmation

A fragment of the mitochondrial cytochrome oxidase I (COI) gene was amplified with the primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) described as conserved primers for invertebrate DNA. The PCR reaction volume (20 µl) contained 13.7 µl water, 2.0 µl dNTP's (2 mM), 2.0 µl Taq buffer (10X), primers (10 pmoles per µl) of 0.5 µl each, 1.0 µl DNA template and Taq polymerase (5 U/µl) of 0.3 µl. PCR amplification conditions for COI gene in mirid bugs is given in Table 2.

About 5 µl of amplified products from each tube along with 2 µl loading dye were separated on 1 per cent agarose gel along with 100 bp DNA ladder and gel image was visualized and documented in the gel documentation system. The excised PCR fragments were subjected for QIAGEN Min Elute® PCR purification kit to elute the PCR product from agarose gel. The purification of PCR product was done as described in the user's manual. The purified PCR product was quantified by using the nanodrop. The fragment of the cytochrome oxidase I (COI) gene of about 658 bp amplicon was sequenced by using both forward (LCO-1490) and reverse (HCO-2198) primers by commercial sequencing centre, Applied bio system Pvt. Ltd., Bangalore

Table 3. Nucleotide composition COI gene fragment of four isolates of mirid bug contigs.

	T(U)	C	A	G	Total in bp	COI 1 gene in bp
Creontiades biseratense (Green)	27.9	18.4	32.5	21.1	1027	723 bp
Creontiades biseratense (Brown)	0.9	18.4	27.8	23.9	1236	750 bp
Creontiades biseratense (Red)	28.5	21.1	30.4	20	1073	740 bp
Creontiades pallidus	31.7	20.1	28.4	19.9	1051	629 bp
Average	29.4	19.5	29.4	21.6	908.6	-

The PCR products were sent for sequencing at Applied bio system Pvt. Ltd. Bangalore and the resulting sequences were cleaned up by using Bio edit software to remove vector backbone and contigs developed. Further, the contigs were subjected for the homology search analysis at BLASTn search tool (NCBI) to confirm the resulted sequences of COI. Later, the multiple sequence alignment and phylogenetic tree was developed at MEGA x software with bootstrap value 1000 and other default parameters.

Three factorial CRD and student (paired) T-test were applied suitably using three factorial CRD T-test and OP stat software's during the statistical analysis of the results and interpretation.

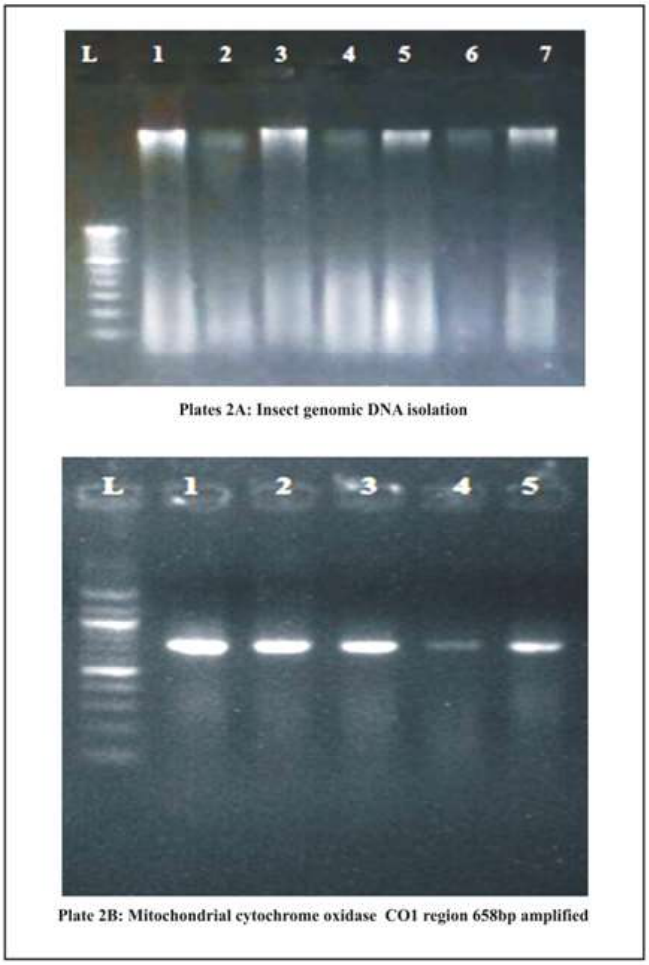


Plate 2A: L = Lader, (genomic DNA)*1 = Creontiades biseratense (Green), 2 = Creontiades biseratense (Brown), 3 = Creontiades biseratense (Red), 4=Taylorigus apicalis, 5= Creontiades pallidus, 6= Campylomma livida, 7= Clavigralla gibbosa.*

Plate 2B: L = Lader, (amplified 658bp of CO1 region)

1 = Creontiades biseratense (Green), 2 = Creontiades biseratense (Brown), 3 = Creontiades biseratense (Red), 4=Taylorigus apicalis, 5= Creontiades pallidus.

Results and discussion

DNA based molecular diversity study is a technique for species identification and biodiversity analysis. These DNA molecular markers are more powerful tool than biochemical and morphological studies as they are least affected by environment and stably heritable (Folmer *et al.,* 1994). The total genomic DNA of the all four isolates (*C. biseratense* Green, *C. biseratense*-Brown, *C. biseratense* -Red and *C. palidus*) (Plate 1) were isolated by Qiagen Blood and tissue DNA extraction as per the instructions. The obtained DNA integrity was confirmed on 0.8 per cent agarose gel electrophoresis (Plate 2). The concentration of whole genomic DNA was 250-300 ng/µl.

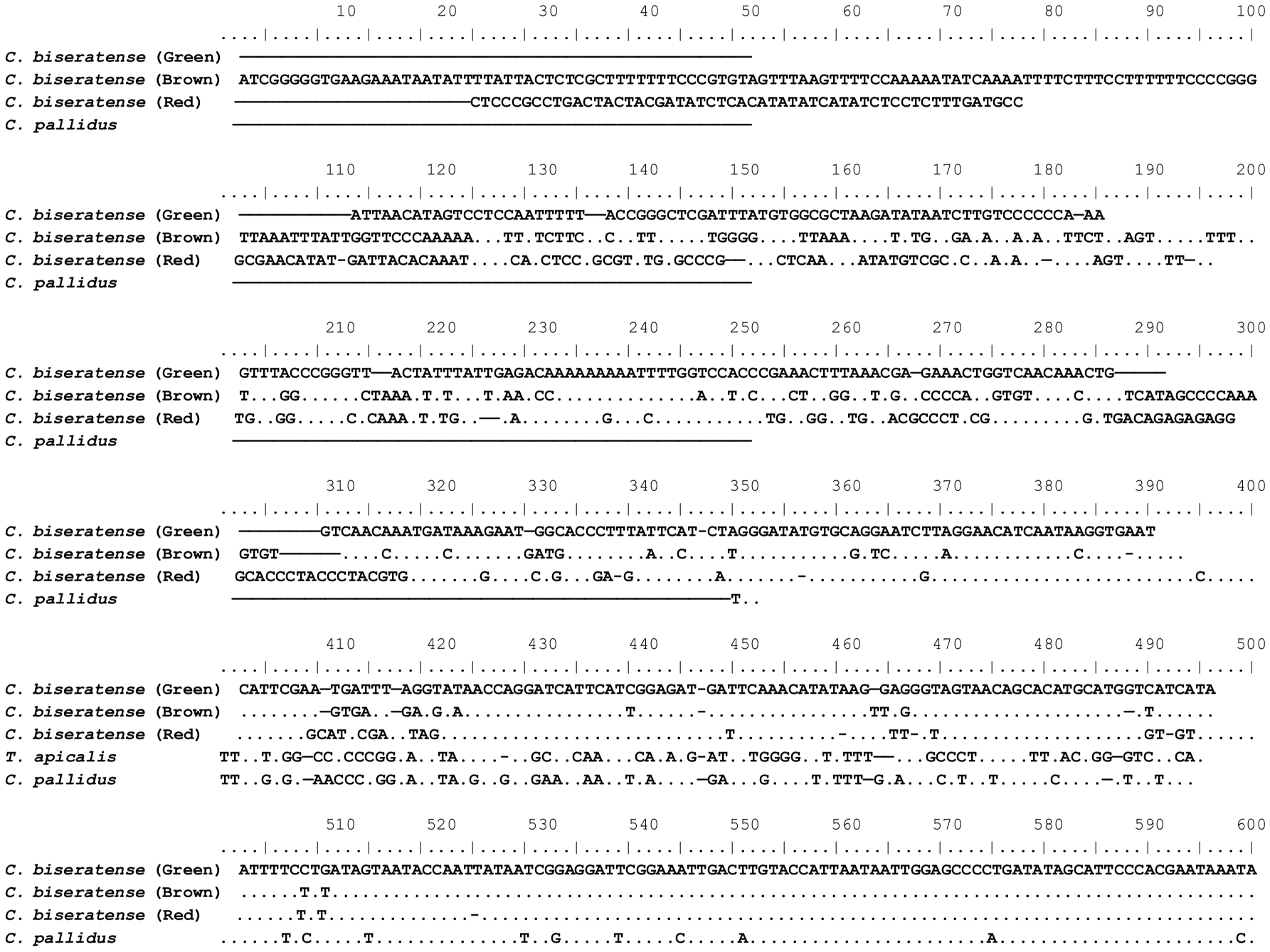
In insects, the molecular characterization is carried out by profiling the DNA sequences from a short segment of Mitochondrial DNA (mtDNA) *viz.,* cytochrome c oxidase (COI) gene. Mitochondrial genome is maternally inherited; any change in the mitochondrial DNA is transmitted to the entire progeny. Evolutionary changes in the conserved regions of mitochondrial DNA spread rapidly within populations (Jalali *et al.,* 2015). PCR amplification of mitochondrial cytochrome oxidase I (COI) gene resolved on one per cent agarose gel along with 100 bp of a standard DNA marker. A very sharp and specific amplicon of expected size (658-700 bp) was observed in all samples (Plate 2). The visualized PCR product contained no double bands on agarose gel, thus indicating that sequences obtained were mitochondrial DNA and not nuclear pseudo genes. The 658-700 bp amplicon of all four test insect population

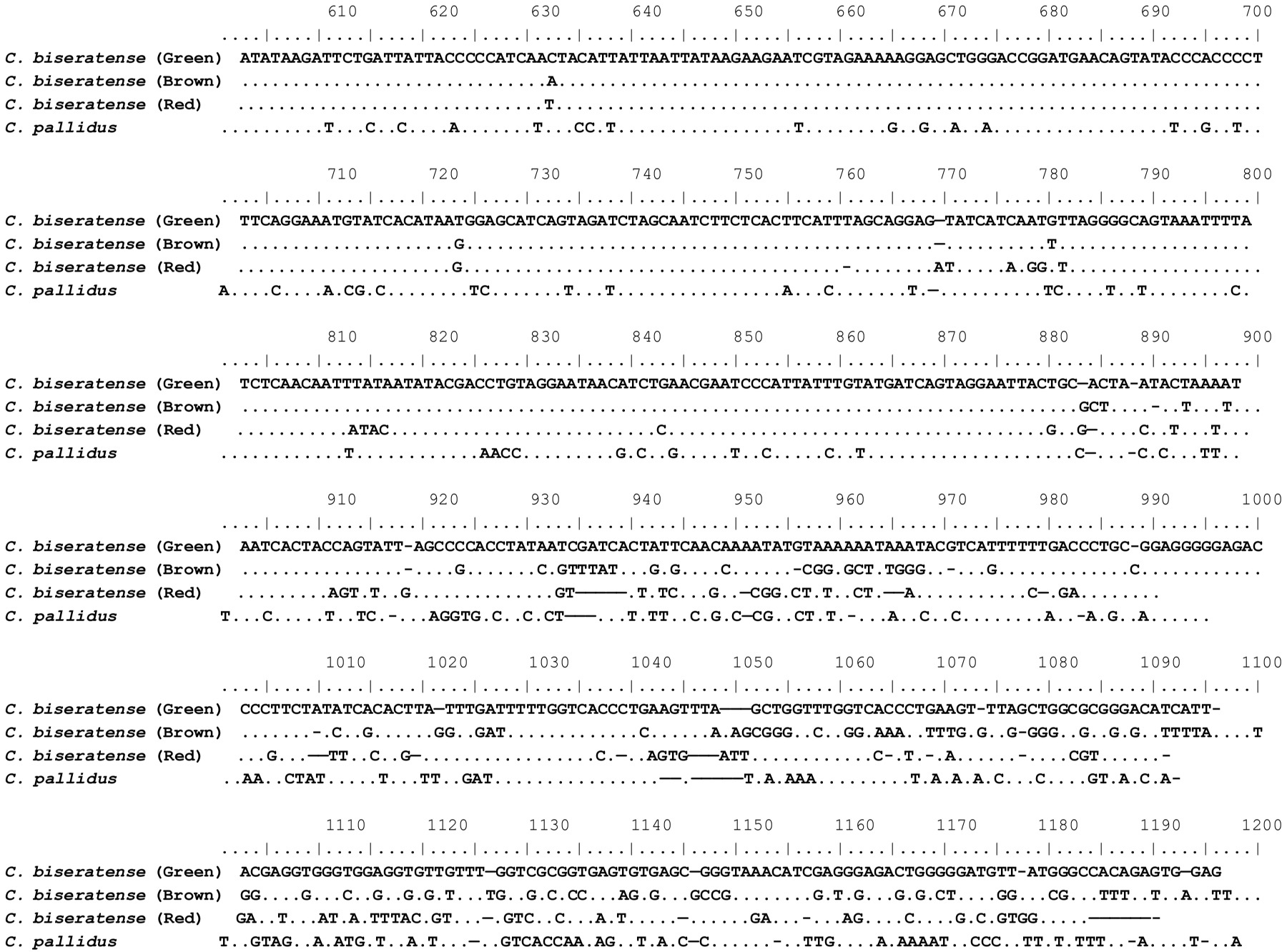
Table 4. Distant matrix of mirid bug species

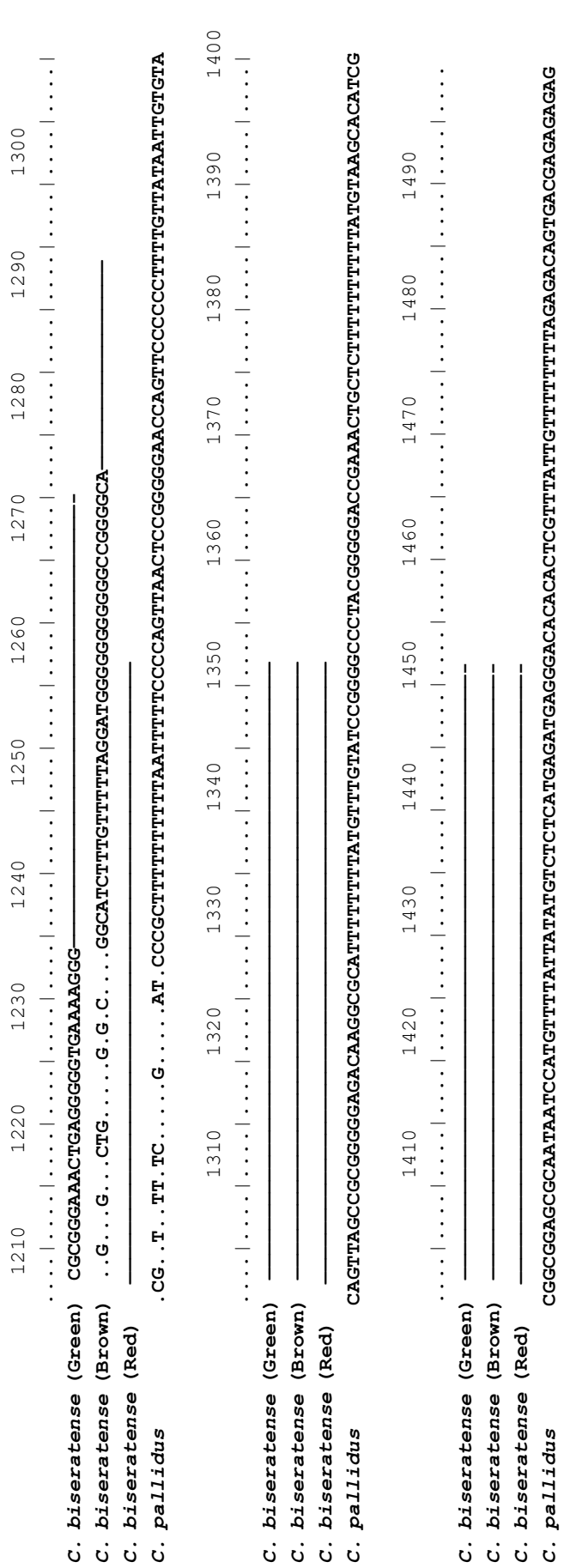
Mirid bug isolates	1	2	3	4	5
Creontiades biseratense (Green)	ID				
Creontiades biseratense (Brown)	24.529	ID			
Creontiades biseratense (Red)	23.423	34.170	ID		
Creontiades pallidus	44.558	48.173	46.598	90.953	ID

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

Fig. 1. Multiple sequence alignment of four isolates of mirid bug




610 620 630 640 650 660 670 680 690 700
C. biseratense (Green) ATATAAGATTCTGATTATTACCCCCATCAACTACATTATTAATTATAAGAAGAATCGTAGAAAAAGGAGCTGGGACCGGATGAACAGTATACCCACCCCT
C. biseratense (Brown) .....A.....
C. biseratense (Red) .....T.....
C. pallidus .....T...C..C....A.....T...CC.T.....T.....G..G..A..A.....T..G..T..
710 720 730 740 750 760 770 780 790 800
C. biseratense (Green) TTCAGGAAATGTATCACATAATGGAGCATCAGTAGATCTAGCAATCTTCTCACTTCATTTAGCAGGAG—TATCATCAATGTTAGGGGCAGTAAATTTTA
C. biseratense (Brown) .....G.....—.....T.....
C. biseratense (Red) .....G.....-.....AT.....A.GG.T.....
C. pallidus A....C....A.CG.C.....TC.....T...T.....A...C.....T.—.....TC....T..T.....C.
810 820 830 840 850 860 870 880 890 900
C. biseratense (Green) TCTCAACAATTTATAATATACGACCTGTAGGAATAACATCTGAACGAATCCCATTATTTGTATGATCAGTAGGAATTACTGC—ACTA-ATACTAAAAT
C. biseratense (Brown) .....GCT....-..T...T...
C. biseratense (Red) .....ATAC.....C.....G..G—....C..T...T...
C. pallidus .....T.....AACC.....G.C..G.....T..C.....C..T.....C—...-C.C...TT..
910 920 930 940 950 960 970 980 990 1000
C. biseratense (Green) AATCACTACCAGTATT-AGCCCCACCTATAATCGATCACTATTCAACAAAATATGTAAAAAATAAATACGTCATTTTTTGACCCTGC-GGAGGGGGAGAC
C. biseratense (Brown) .....-....G.....C.GTTTAT...G.G....C.....-CGG.GCT.TGGG..-...G.....C.....
C. biseratense (Red) .....AGT.T..G.....GT———.T.TC...G..—CGG.CT.T..CT.——A.....C—.GA.....
C. pallidus T...C.....T..TC.-...AGGTG.C..C.CT——...T.TT..C.G.C—CG..CT.T.-...A..C..C.....A..-A.G..A.....
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
C. biseratense (Green) CCCTTCTATATCACACTTA—TTTGATTTTTGGTCACCCTGAAGTTTA——GCTGGTTTGGTCACCCTGAAGT-TTAGCTGGCGCGGGACATCATT-
C. biseratense (Brown) .....-.C..G.....GG..GAT.....C.....A.AGCGGG..C..GG.AAA..TTTG.G..G-GGG..G..G.G..TTTTA....T
C. biseratense (Red) ...G...——TT..C..G—.....C.—..AGTG——ATT.....C-.T.-.A.....-....CGT.....-
C. pallidus ..AA..CTAT.....T...TT..GAT.....—.———T.A.AAA.....T.A.A.A.C...C....GT.A.C.A-
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
C. biseratense (Green) ACGAGGTGGGTGGAGGTGTTGTTT—GGTCGCGGTGAGTGTGAGC—GGGTAAACATCGAGGGAGACTGGGGGATGTT-ATGGGCCACAGAGTG—GAG
C. biseratense (Brown) GG....G...C..G..G.G.T...TG..G.C.CC...AG.G...GCCG.....G.T.G...G.G.CT....GG...CG...TTT..T..A..TT...
C. biseratense (Red) GA..T...AT.A.TTTAC.GT...—.GTC..C...A.T.....—.....GA...-...AG....C....G.C.GTGG.....———-
C. pallidus T..GTAG..A.ATG.T..A.T...—..GTCACCAA.AG..T.A.C—C.....-..TTG....A.AAAAT..CCC..TT.T.TTT..-A....T-..A



was purified by gel extraction method (QIAGEN MinElute® PCR purification kit). The purified PCR products were sequenced. The gene sequence (after vector backbone cleanup) results from both the forward and reverse primer of a sequences were assembled to develop the contig (Bio Edit software) of COI gene of each collected insects. Nucleotide composition sequences of all the genes were analyzed and presented (Table 3 and 4).

The isolated Mitochondrial DNA of four mirid isolates employed for sequences doesn't able to predict the exact diversity and similarity. The mirid isolates were collected from Dharwad and Lathur. Raw fastq files were pre processed to trim the unwanted sequence and sequence reduced to 723 bp (*C. biseratense* green), 750 bp (*C. biseratense*-Brown), 740 bp (*C. biseratense*-Red) and 692 bp (*C. palidus*). The processed sequence subjected to blast to check the homology in the NCBI database. The per cent identical information with other genus of the Miridae family is shown in Table 3. The sequence of three strains of *C. biseratense* shows the similarities to *C. palidus* and other genus of the Miridae family. Mitochondrial COI genes of *C. pallidus* is subjected to blast with sequence of *C. biseratense* (Green) showed 48 per cent query cover and present identity was 82 per cent, and confirms that the insect belongs to same genus. The obtained sequence will be helpful for the identification of the mentioned cotton mirid bugs in the further research purpose (Fig. 1). The contig sequences were subjected for BLASTn search at NCBI nucleotide databank and it was confirmed that the four species (*C. biseratense* (Green), *C. biseratense* (Brown), *C. biseratense* (Red) and *C. pallidus* are shown homology with mirid bug. All four sequences were used to develop multiple alignment and phylogenetic tree construction by use of MEGA x software. Initially, the mitochondrial COI genes were subjected to multiple sequence alignment on Clustal W to speculate the sequence conservations among mirid bug species from different geographical locations. From the multiple sequences alignment results the total conserved region variable region, insertions and deletions were observed.

Evolutionary analyses were conducted using MEGA-X software to know the evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method which shows the inter and intra species divergence. Even the percentage nucleotide distances were calculated in MEGA-X by applying bootstrap value 1000 replicates (Fig. 2).

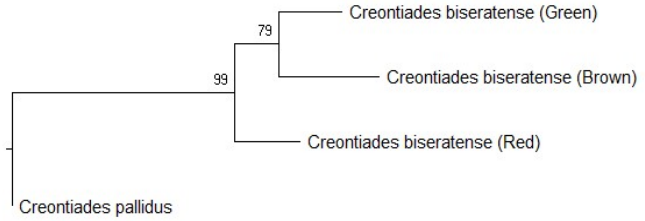


Fig.2. Phylogenic tree of all four mirid bugs. Evolutionary relation ships of taxa were inferred using the Neighbor-Joining method, MEGA X.

The phylogenetic tree developed had a clear indication of presence of two clusters. The one cluster consists of three isolates *viz., C. biseratense (*Green*), C. biseratense* (Brown), *C. biseratense* (Red) are same species with different color morphs it depicts the maximum similarity among the isolates. Second cluster where only one species *C. pallidus* belongs to a species but evolved very diversely. Within this 1st cluster we found maximum similarity between the species is only 52% indicating though these belongs to a species but evolved very diversely. Similar kind of observation were made by Rang *et al.* (2005) they found only 50 % maximum similarity between the *F. schultezei* species that are infecting to cotton plant.

The pairwise distant matrix was calculated with bootstrap 1000 replicates, the results are co-linear with phylogenetic tree where, distant between *C. biseratense* (Green) and *C. biseratense* (Brown) was 24.59%; *C. biseratense* (Green) and *C. biseratense* (Red) was 23.42% and *C. biseratense* (Green) v/s *C. pallidus* was 44.55 % indicating that green, brown and red are more closely related to each other than *C. pallidus* (Table 4).

The optimal tree with the sum of branch length is 1.67559187. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The CO1 sequences of the dataset were represented in a phylogenetic tree with distinctive clusters based on the nucleotide divergence among the species. Hence, the probability of substitution in all four genes during the course of evolution was calculated by Maximum Likelihood Estimate approach with Gamma Parameter of Tamura-Nei-93 (Tamura and Nie, 1993) model (+G). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G], parameter = 1.3789). Rates of different transitional substitutions are more predominant than transversional substitutions. The nucleotide frequencies are A = 29.43%, T/U = 29.43%, C = 19.52%, and G = 21.62% (Table 3). For estimating ML values, a tree topology was automatically computed. Therefore, in the present study CO I based DNA marker was deployed to study the diversity among mirid bug species collected from different regions of Karnataka, Telangana and Maharashtra. The study was limited to single specimen analyses and hence does not call for clearcut evidences on diversity or barcodes. However, with limited matching sequences efforts were made to describe molecular diversity in mirid bug species which could be considered as indicative only.

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