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Research Article

CYTOCHROME B GENE AS A PHYLOGENETIC MARKER IN ESTIMATING MOLECULAR PHYLOGENY OF STINK BUGS (HEMIPTERA: PENTATOMIDAE) IN INDIA

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ABSTRACT

In the present study, a partial sequence of 440 bp of Cyt b gene were subjected to phylogenetic analysis to understand the intrageneric, interspecific and intraspecific variations in nine species of stink bugs of family Pentatomidae. The final aligned data pertained to 18 Cyt b sequences of 440 bp representing 10 species and 5 genera. Sequence of Aeschyntelus notatus, belonging to family Rhopalidae, was included in the analysis as the out-group taxon. The intraspecific divergence ranged from 0.2 to 7.2% whilst interspecific divergence ranged from 3.8 to 10.3% and intergeneric distance ranged from 12.1 to 20.2%The pairwise genetic distances were calculated and phylograms were constructed using Maximum Likelihood, Maximum Parsimony, and Neighbour-Joining methods. The database analysis showed hierarchical increase in percentage divergence across different taxonomic levels and revealed a clear pattern of increased nucleotide diversity from conspecific to congeneric to intra subfamily level using Cyt b gene fragment.

Keywords: Stink bugs, Cyt b, Phylogenetic relationships, MEGA, Neighbour-joining method

INTRODUCTION

Pentatomids are generally known as "Stink Bugs", because of the emission of disagreeable pungent odour from a pair of scent glands that open in the region of the metapleura. They produce large quantities of strong smelling and irritating defensive chemicals, which are released when they are disturbed or molested. The high reproductive rates and an exceptional ability to transmit diseases make them worst agricultural pests (Song et al., 2009). The present study was undertaken based on 11 Cyt b sequences of 440 bp representing seven species of four genera belonging to two subfamilies (Pentatominae and Podopinae) were finally taken for further analysis (Table 1). The corresponding amino acid sequence for each of the Cyt b gene fragment was also obtained. There were no stop codon or frame shifts detected indicating that sequences were not pseudogenes (NUMTs) and all sequences corresponded to a functional mitochondrial gene. Sequences were then submitted to Genbank (NCBI) database. 7 Corresponding sequences of five species deposited by other workers were procured directly from Genbank (NCBI) (Table 2). The final aligned data pertained to 18 Cyt b sequences of 440 bp representing 10 species and 5 genera of family Pentatomidae. Sequence of Aeschyntelus notatus, belonging to family Rhopalidae, was included in the analysis as the out-group taxon and added to the alignments in order to compare the findings. Nucleotide sequences were aligned using the multiple alignment program MUSCLE (Multiple Sequence Comparison by Log-Expectation) incorporated in MEGA 6.06.

Table 1: Details of species analysed for Cyt b in the present study.

S. No	Taxa	Specimen code	Collection state	Collection month/ year	Accession number
1	Graphosoma lineatum (Linnaeus, 1758)	HP25 HP14	Uttarakhand Himachal Pradesh	Area	Area
2	Halyomorpha picus (Fabricius, 1794)	HP22 HP16	Uttarakhand Himachal Pradesh	Oct, 2015 May, 2014	MG994913 MG821869
3	Nezara antennata Scott, 1874	P18	Punjab	June, 2013	MG994914
4	Nezara viridula (Linnaeus, 1758)	P9 P10 HP19	Punjab Punjab Himachal Pradesh	March, 2013 April, 2014 June, 2014	MG821868 MG821867 MG821866
5	Plautia crossota (Dallas, 1851)	U8	Uttarakhand	Oct, 2015	MG994917
6	Plautia stali Scott, 1874	HP17	Uttarakhand	Oct, 2015	MG994915
7	Plautia viridicollis (Westwood, 1837)	HP26	Himachal Pradesh	Aug, 2015	MG994916

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Table 2: List of taxa whose Cyt b sequences were downloaded from Genbank for alignment.

S. No	Taxa	Family	Accession no.	Country
1	Graphosoma rubrolineatum	Pentatomidae	KX267740.1	China
2	Halyomorpha halys	Pentatomidae	FJ685650.1	Korea
3	Nezara viridula	Pentatomidae	AB020514.1 AY839170.1 FJ418864.1	Japan Slovenia China
4	Nezara antennata	Pentatomidae	1 FJ418867.1	China
5	Aeschyntelus notatus	Rhopalidae	EU427333.1	China

MATERIALS AND METHODS

Taxon sampling

The first step subsumed the collection of stink bugs from different regions of North India (Himachal Pradesh, Punjab and Uttarakhand), followed by sorting, pinning, labelling and identification of the collected specimens. The collection of stink bugs involved both day and night collection. The localities visited were selected to include different range of altitudinal variations, from plains to hilly areas. DNA was either extracted immediately or specimens were preserved in 95% ethanol and held at -20°C until use. Briefly, insects were homogenized individually in homogenising and lysing buffer (100 mm Tris-HCl, pH 7.5, 50 mm EDTA, 0.5% SDS, and 0.2 M sucrose), proteins were precipitated by 8 M potassium acetate, and finally DNA was purified by repeated phenol-chloroform extractions (Kambhampati et al., 1991). Precipitated DNA pellets were re-suspended in 50 µl of distilled water with 0.5 µl of 10 mg/ml of RNase A.

PCR and sequencing

Partial fragments of Cyt b gene sequences were amplified using standard PCR conditions and specific primers (Muraji et al., 2000) (Table 3, 4). The amplification of the Cyt b sequences has been done in molecular laboratory of the Department of Zoology and Environmental Science and Sophisticated Instruments Centre, Punjabi University, Patiala. The integrity of amplified products was checked under UV light on 2% agarose gels. The amplified products were run for 45 minutes at 80 V along with 5 µl of DNA ladder (Thermo Scientific) and were then visualized on UV transilluminator. DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. The most commonly used method of sequencing DNA, the dideoxy method or chain termination method, was developed by (Sanger et al., 1977). The amplified were got sequenced directly from Agrigenome, Cochin (India).

Table 3: PCR program for Cyt b gene.

S. No	Steps involved	Number of cycles	Temperature (°C)	Time per cycle
1.	Initial denaturation	1	98	5 minutes
2.	Denaturation	40	98	1 minute
3.	Annealing		50	30 seconds
4.	Extension		72	2 minutes
5.	Final/last Extension	1	72	7 minutes

Table 4: Primer set for Cyt b gene.

Oligo name	Sequence ((5'-3')	Length (bp)	References
Cyt b	TAGGATATGTTTTACC- -TTGAGGACA TCCTCCTAATTT- -ATTAGGAATTG	25 24	Muraji et al., 2000

Nucleotide sequence alignments and phylogenetic analysis

Raw sequence chromatograms were obtained in both forward and reverse direction. These nucleotide sequences were proofread and assembled into contigs using Codon Code Aligner 3.7.1 (Codon Code Corp). The edited sequences were compared with related sequences from the nucleotide database of the National Centre for Biotechnology Information (NCBI), using Basic Local Alignment Search Tool (BLAST) algorithm to make sure that correct target sequences were amplified. The final sequences were then shortlisted by looking at the query cover, scores and e-values of the matching sequences (Altschul et al., 1997). The sequences of the species selected for this study as out group taxa are listed in (Table 2). The trees were constructed using MEGA 6.0 software, these were then analysed and interpreted for deriving the phylogenetic relationships with in the family. These trees were finally considered for intraspecific, interspecific and intrageneric divergence studies (Tamura et al., 2013).

RESULTS AND DISCUSSION

Multiple Sequence Alignment: 11 sequences of 440 bp representing seven species of four genera belonging to two subfamilies (Pentatominae and Podopinae) were finally taken for analysis (Table 1). 7 corresponding sequences of five species deposited by other workers were procured directly from Genbank (Table 2). The final aligned data pertained to 18 Cyt b sequences of 440 bp representing 10 species and 5 genera. Sequence of Aeschyntelus notatus, belonging to family Rhopalidae, was included in the analysis as the outgroup taxon and added to the alignments in order to compare the findings.

Nucleotide Alignment: Of the 440 sequence characters sampled across all the Pentatomid species, 258 (58.63%) were found to be conserved and 182 (41.37%) were variable sites. 46 (10.45%) variable characters were found to be phylogenetically uninformative while 136 (30.90%) variable characters were found to be phylogenetically (parsimony) informative (Table 5).

Table 5: Characterstics of Cyt b gene sequences in all the species of family Pentatomidae included in the study.

Type of character	Total
Characters	440

Constant Characters	258
Variable sites	182
Parsimony Informative sites	136
Parsimony Uninformative sites	46

Nucleotide Composition: As observed in the mtDNA of many other insects (Simon et al., 1994), a high A+T content was observed in family Pentatomidae. The average A+T content was found to be 70.9% for Cyt b gene (Table 6, 7). When nucleotide compositions were compared among positions of codons in the Cyt b gene, the highest average A+T content was seen in the third codon position, at which 81.5% of all nucleotides were either A or T followed by 73.0% at second and 58.5% at the first codon position (Table 7). Muraji et al. (2000) also found similar results showing 84.9% high A+T content at the third codon position followed by 67.27% at the second position and 60% at the first codon position. The high A+T content (84.3%) at the third codon position following 74.0% at the second and 60.3% at the first codon position of partial Cyt b gene region variation of the predatory bug Pilophorus typicus in Japan was studied by (Ito et al, 2010).

Table 6: Base composition of different species of present study using Cyt b sequences.

S.No	Species name	T	C	A	G
1	Aeschyntelus notatus (EU427333.1)	36.5	18.1	34.5	10.9
2	Graphosoma lineatum* (MG994918)	37.3	17.6	34.0	11.1
3	Graphosoma lineatum* (MG994919)	36.4	18.2	34.5	10.9
4	Graphosoma rubrolineatum (KX267740.1)	35.8	19.1	34.7	10.5
5	Halyomorpha halys (FJ685650.1)	37.9	17.0	33.4	11.7
6	Halyomorpha picus* (MG821869)	37.3	18.0	32.1	12.6
7	Halyomorpha picus * (MG994913)	37.4	17.9	32.1	12.6
8	Nezara antennata (FJ418867.1)	38.7	17.3	32.8	11.2
9	Nezara antennata* (MG994914)	40.6	17.3	30.9	11.2
10	Nezara viridula (AB020514.1)	38.6	16.1	34.5	10.8
11	Nezara viridula (AY839170.1)	38.7	15.8	34.8	10.7
12	Nezara viridula (FJ418864.1)	38.4	16.1	34.5	10.9
13	Nezara viridula* (MG821866)	36.7	19.0	32.2	12.2
14	Nezara viridula* (MG821867)	36.4	18.9	32.6	12.1
15	Nezara viridula * (MG821868)	36.8	18.9	32.1	12.1
16	Plautia crossota* (MG994917)	39.9	16.1	31.5	12.5
17	Plautia stali* (MG994915)	40.7	15.9	32.1	11.2
18	Plautia viridicollis* (MG994916)	39.3	17.7	31.4	11.5
	Average	38.0	17.6	32.9	11.5

*Indicates specimens of the present study

Table 7: Average nucleotide composition at the first, second and third coding positions of the Cyt b gene sequences.

S.No	Average Nucleotide composition in %					
	A	T	G	C	A+T	G+C
1st codon	24.5	34	20	21.5	58.5	41.5
2nd codon	33.0	40	7.2	19.8	73.0	27.0
3rd codon	41.3	40.2	7.3	11.2	81.5	18.5
Overall	32.9	38.0	11.5	17.6	70.9	29.1

Estimates of evolutionary divergence over sequence pairs within groups: The genetic divergence among the selected species of family Pentatomidae was estimated using partial Cyt b gene sequences. The intraspecific divergence ranged from 0.2 to 7.2% with an average of 3.75 ± 3.0% whilst interspecific divergence ranged from 3.8 to 10.3% with an average of 7.49 ± 1.7% and intergeneric distance ranged from 12.1 to 20.2% with an average of 16.37 ± 2.2%. The percentage divergence values revealed a clear pattern of increased nucleotide diversity from conspecific to congeneric to intra subfamily level using Cyt b gene fragment. Intergeneric divergence was quite high in the present study for all the genera and no ambiguity was observed as it was above 10% for all the species. All the species exhibited discriminative values of intra and interspecific (Tables 8, 9) and intergeneric divergence (Table 10). The percent divergence values revealed a clear pattern of increased nucleotide diversity from conspecific to congeneric to intra subfamily level.

Table 8: Intraspecific divergence of different species on the basis of Cyt b gene sequences.

S. No.	Sequence I	Accession No.	Sequence II	Accession No.	Divergence (%)
1	Graphosoma lineatum*	MG994918	Graphosoma lineatum*	MG994919	1.9
2	Halyomorpha picus*	MG994913	Halyomorpha picus*	MG821869	0.5
3	Nezara antennata*	MG994914	Nezara antennata	FJ418867.1	2.8
4	Nezara viridula*	MG821866	Nezara viridula*	MG821868	0.5
5	Nezara viridula*	MG821866	Nezara viridula*	MG821867	0.2
6	Nezara viridula*	MG821866	Nezara viridula	AB020514.1	6.4
7	Nezara viridula*	MG821866	Nezara viridula	AY839170.1	6.7
8	Nezara viridula*	MG821866	Nezara viridula	FJ418864.1	7.2
9	Nezara viridula*	MG821867	Nezara viridula	AB020514.1	6.1

10	Nezara viridula*	MG821867	Nezara viridula	AY839170.1	6.4
11	Nezara viridula*	MG821867	Nezara viridula	FJ418864.1	7.0
12	Nezara viridula*	MG821868	Nezara viridula*	MG821867	0.2
13	Nezara viridula*	MG821868	Nezara viridula	AB020514.1	6.4
14	Nezara viridula*	MG821868	Nezara viridula	AY839170.1	6.7
15	Nezara viridula*	MG821868	Nezara viridula	FJ418864.1	7.2
16	Nezara viridula	AB020514.1	Nezara viridula	AY839170.1	0.2
17	Nezara viridula	AB020514.1	Nezara viridula	FJ418864.1	0.7
18	Nezara viridula	AY839170.1	Nezara viridula	FJ418864.1	0.5

*Indicates specimens of the present study

Table 9: Interspecific divergence of different species on the basis of Cyt b gene sequences.

S. No	Species I	Accession No.	Species II	Accession No.	Divergence (%)
1	Graphosoma lineatum*	MG994919	Graphosoma rubrolineatum	KX267740.1	4.6
2	Graphosoma lineatum*	MG994918	Graphosoma rubrolineatum	KX267740.1	3.8
3	Halyomorpha halys	FJ685650.1	Halyomorpha picus*	MG821869	10.3
4	Halyomorpha halys	FJ685650.1	Halyomorpha picus*	MG994913	9.8
5	Nezara viridula*	MG821866	Nezara antennata*	MG994914	7.2
6	Nezara viridula*	MG821866	Nezara antennata	FJ418867.1	6.7
7	Nezara viridula*	MG821867	Nezara antennata*	MG994914	7.5
8	Nezara viridula*	MG821868	Nezara antennata*	MG994914	7.2
9	Nezara viridula*	MG821867	Nezara antennata	FJ418867.1	6.4
10	Nezara viridula*	MG821868	Nezara antennata	FJ418867.1	6.1
11	Nezara viridula	AB020514.1	Nezara antennata*	MG994914	9.2
12	Nezara viridula	AB020514.1	Nezara antennata	FJ418867.1	6.7
13	Nezara viridula	AY839170.1	Nezara antennata*	MG994914	9.5
14	Nezara viridula	AY839170.1	Nezara antennata	FJ418867.1	7.0
15	Nezara viridula	FJ418864.1	Nezara antennata*	MG994914	9.8
16	Nezara viridula	FJ418864.1	Nezara antennata	FJ418867.1	7.2
17	Plautia stali*	MG994915	Plautia crossota*	MG994917	5.9
18	Plautia stali*	MG994915	Plautia viridicollis*	MG994916	8.6
19	Plautia viridicollis*	MG994916	Plautia crossota*	MG994917	8.9

*Indicates specimens of the present study

Table 10: Divergence among species belonging to different genera on the basis of Cyt b gene sequences.

S. No	Genus I	Accession No.	Genus II	Accession No.	Divergence (%)
1	Halyomorpha halys	FJ685650.1	Plautia stali*	MG994915	12.8
2	Halyomorpha halys	FJ685650.1	Plautia crossota*	MG994917	14.4
3	Halyomorpha halys	FJ685650.1	Plautia viridicollis*	MG994916	13.4
4	Halyomorpha halys	FJ685650.1	Nezara antennata	FJ418867.1	13.7
5	Halyomorpha halys	FJ685650.1	Nezara antennata*	MG994914	15.0
6	Halyomorpha halys	FJ685650.1	Nezara viridula*	MG821866	16.7
7	Halyomorpha halys	FJ685650.1	Nezara viridula*	MG821867	17.0
8	Halyomorpha halys	FJ685650.1	Nezara viridula*	MG821868	17.4
9	Halyomorpha halys	FJ685650.1	Nezara viridula	AB020514.1	15.3
10	Halyomorpha halys	FJ685650.1	Nezara viridula	AY839170.1	15.7
11	Halyomorpha halys	FJ685650.1	Nezara viridula	FJ418864.1	16.0
12	Halyomorpha halys	FJ685650.1	Graphosoma lineatum*	MG994919	16.3
13	Halyomorpha halys	FJ685650.1	Graphosoma lineatum*	MG994918	17.0
14	Halyomorpha halys	FJ685650.1	Graphosoma rubrolineatum	KX267740.1	17.7
15	Halyomorpha picus*	MG994913	Plautia stali*	MG994915	17.7
16	Halyomorpha picus*	MG994913	Plautia crossota*	MG994917	19.1
17	Halyomorpha picus*	MG994913	Plautia viridicollis*	MG994916	19.5
18	Halyomorpha picus*	MG994913	Nezara antennata	FJ418867.1	17.0
19	Halyomorpha picus*	MG994913	Nezara antennata*	MG994914	15.7
20	Halyomorpha picus*	MG994913	Nezara viridula*	MG821866	18.4
21	Halyomorpha picus*	MG994913	Nezara viridula*	MG821867	18.8
22	Halyomorpha picus*	MG994913	Nezara viridula*	MG821868	18.4
23	Halyomorpha picus*	MG994913	Nezara viridula	AB020514.1	18.0
24	Halyomorpha picus*	MG994913	Nezara viridula	AY839170.1	18.4
25	Halyomorpha picus*	MG994913	Nezara viridula	FJ418864.1	18.0

26	Halyomorpha picus*	MG994913	Graphosoma lineatum*	MG994919	19.1
27	Halyomorpha picus*	MG994913	Graphosoma lineatum*	MG994918	18.8
28	Halyomorpha picus*	MG994913	Graphosoma rubrolineatum	KX267740.1	20.2
29	Halyomorpha picus*	MG821869	Plautia stali*	MG994915	17.7
30	Halyomorpha picus*	MG821869	Plautia crossota*	MG994917	19.1
31	Halyomorpha picus*	MG821869	Plautia viridicollis*	MG994916	18.8
32	Halyomorpha picus*	MG821869	Nezara antennata	FJ418867.1	17.7
33	Halyomorpha picus*	MG821869	Nezara antennata*	MG994914	16.3
34	Halyomorpha picus*	MG821869	Nezara viridula*	MG821866	19.1
35	Halyomorpha picus*	MG821869	Nezara viridula*	MG821867	19.5
36	Halyomorpha picus*	MG821869	Nezara viridula*	MG821868	19.1
37	Halyomorpha picus*	MG821869	Nezara viridula	AB020514.1	18.8
38	Halyomorpha picus*	MG821869	Nezara viridula	AY839170.1	19.1
39	Halyomorpha picus*	MG821869	Nezara viridula	FJ418864.1	18.8
40	Halyomorpha picus*	MG821869	Graphosoma lineatum*	MG994919	19.1
41	Halyomorpha picus*	MG821869	Graphosoma lineatum*	MG994918	18.8
42	Halyomorpha picus*	MG821869	Graphosoma rubrolineatum	KX267740.1	19.5
43	Nezara antennata	FJ418867.1	Graphosoma lineatum*	MG994919	14.7
44	Nezara antennata	FJ418867.1	Graphosoma lineatum*	MG994918	14.4
45	Nezara antennata	FJ418867.1	Graphosoma rubrolineatum	KX267740.1	17.4
46	Nezara antennata*	MG994914	Graphosoma lineatum*	MG994919	16.3
47	Nezara antennata*	MG994914	Graphosoma lineatum*	MG994918	16.0
48	Nezara antennata*	MG994914	Graphosoma rubrolineatum	KX267740.1	19.1
49	Nezara viridula	AB020514.1	Graphosoma lineatum*	MG994919	16.0
50	Nezara viridula	AB020514.1	Graphosoma lineatum*	MG994918	16.0
51	Nezara viridula	AB020514.1	Graphosoma rubrolineatum	KX267740.1	18.8
52	Nezara viridula	AY839170.1	Graphosoma lineatum*	MG994919	16.3
53	Nezara viridula	AY839170.1	Graphosoma lineatum*	MG994918	16.3
54	Nezara viridula	AY839170.1	Graphosoma rubrolineatum	KX267740.1	18.4
55	Nezara viridula	FJ418864.1	Graphosoma lineatum*	MG994919	16.3
56	Nezara viridula	FJ418864.1	Graphosoma lineatum*	MG994918	16.3
57	Nezara viridula	FJ418864.1	Graphosoma rubrolineatum	KX267740.1	18.4
58	Nezara viridula*	MG821866	Graphosoma lineatum*	MG994919	18.4
59	Nezara viridula*	MG821866	Graphosoma lineatum*	MG994918	17.0
60	Nezara viridula*	MG821866	Graphosoma rubrolineatum	KX267740.1	19.5
61	Nezara viridula*	MG821867	Graphosoma lineatum	MG994919	18.8
62	Nezara viridula*	MG821867	Graphosoma lineatum*	MG994918	17.4
63	Nezara viridula*	MG821867	Graphosoma rubrolineatum	KX267740.1	19.8
64	Nezara viridula*	MG821868	Graphosoma lineatum*	MG994919	18.4
65	Nezara viridula*	MG821868	Graphosoma lineatum*	MG994918	17.0
66	Nezara viridula*	MG821868	Graphosoma rubrolineatum	KX267740.1	19.5
67	Plautia crossota*	MG994917	Nezara antennata	FJ418867.1	14.0
68	Plautia crossota*	MG994917	Nezara antennata*	MG994914	13.7
69	Plautia crossota*	MG994917	Nezara viridula*	MG821866	14.7
70	Plautia crossota*	MG994917	Nezara viridula*	MG821867	15.0
71	Plautia crossota*	MG994917	Nezara viridula*	MG821868	14.7
72	Plautia crossota*	MG994917	Nezara viridula	AB020514.1	15.0
73	Plautia crossota*	MG994917	Nezara viridula	AY839170.1	15.3
74	Plautia crossota*	MG994917	Nezara viridula	FJ418864.1	15.7
75	Plautia crossota*	MG994917	Graphosoma lineatum*	MG994919	12.8
76	Plautia crossota*	MG994917	Graphosoma lineatum*	MG994918	13.4
77	Plautia crossota*	MG994917	Graphosoma rubrolineatum	KX267740.1	15.0
78	Plautia stali*	MG994915	Nezara antennata	FJ418867.1	13.4
79	Plautia stali*	MG994915	Nezara antennata*	MG994914	13.1
80	Plautia stali*	MG994915	Nezara viridula*	MG821866	12.1
81	Plautia stali*	MG994915	Nezara viridula*	MG821867	12.5
82	Plautia stali*	MG994915	Nezara viridula*	MG821868	12.1
83	Plautia stali*	MG994915	Nezara viridula	AB020514.1	12.1

84	Plautia stali*	MG994915	Nezara viridula	AY839170.1	12.5
85	Plautia stali*	MG994915	Nezara viridula	FJ418864.1	12.8
86	Plautia stali*	MG994915	Graphosoma lineatum*	MG994919	15.0
87	Plautia stali*	MG994915	Graphosoma lineatum*	MG994918	15.3
88	Plautia stali*	MG994915	Graphosoma rubrolineatum	KX267740.1	16.0
89	Plautia viridicollis*	MG994916	Nezara antennata	FJ418867.1	15.0
90	Plautia viridicollis*	MG994916	Nezara antennata*	MG994914	15.7
91	Plautia viridicollis*	MG994916	Nezara viridula*	MG821866	15.7
92	Plautia viridicollis*	MG994916	Nezara viridula*	MG821867	15.3
93	Plautia viridicollis*	MG994916	Nezara viridula*	MG821868	15.0
94	Plautia viridicollis*	MG994916	Nezara viridula	AB020514.1	14.4
95	Plautia viridicollis*	MG994916	Nezara viridula	AY839170.1	14.7
96	Plautia viridicollis*	MG994916	Nezara viridula	FJ418864.1	15.0
97	Plautia viridicollis*	MG994916	Graphosoma lineatum*	MG994919	13.7
98	Plautia viridicollis*	MG994916	Graphosoma lineatum*	MG994918	13.1
99	Plautia viridicollis*	MG994916	Graphosoma rubrolineatum	KX267740.1	14.4

*Indicates specimens of the present study

Phylogenetic Analysis 18 sequences of 440 bp representing 9 species of four genera belonging to two subfamilies (Pentatominae and Podopinae) were finally taken along with 1 species of family Rhopalidae (taken as an out group) using partial Cyt b gene fragment for phylogenetic analysis in family Pentatomidae with a bootstrap value of 1000 replications. The NJ, ME and ML trees for the 440 bp region of Cyt b gene of mtDNA isolated from the nine species of Pentatomidae and their world sequences are shown in Figures 1-3. It is clear from the data that all the samples of the nine species are well separated from the other species and grouped together with their world sequences with high bootstrap values. In all three trees Graphosoma lineatum and Graphosoma rubrolineatum belonging to subfamily Podopinae acted as a sister taxon of genus Nezara and Plautia of subfamily Pentatominae with 81% BT in NJ, 82% in ME tree. Aeschyntelus notatus (out group) was most basal followed by Halyomorpha picus belonging to subfamily Pentatominae in all the trees. (Figures 1, 2 and 3) are clearly showed that all the nine species are well separated with high bootstrap support. Species Plautia stali, P. crossota and P. viridicollis have no Gen Bank sequence for comparison and they are new for the Gen Bank database from India. All the present sequences belonging to Nezara viridula were clustered together showing 99% bootstrap value in NJ and ME tree. The three species of genus Plautia whose world sequences were not available grouped together within their own samples and separated from other species with 82% bootstrap value in NJ and ME trees. However, in ML tree species of subfamily Pentatominae appeared as a sister clade to genus Graphosoma of subfamily Podopinae with 78% bootstrap value.

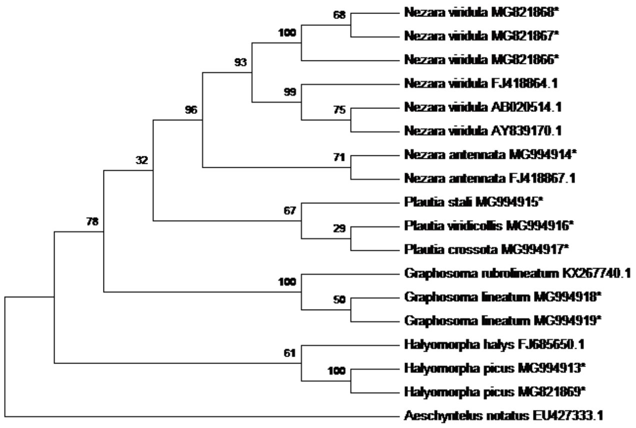

Figure 1: *Maximum Likelihood tree of family Pentatomidae constructed from Cyt b gene sequences. Numbers indicate the percentage of 1000 bootstrap replicate.*

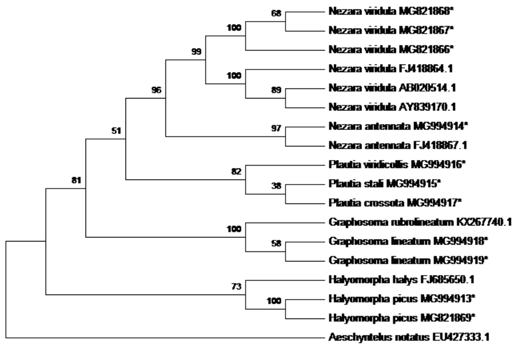

Figure 2: *Neighbour Joining tree of family Pentatomidae constructed from Cyt b gene sequences. Numbers indicate the percentage of 1000 bootstrap replicate.*

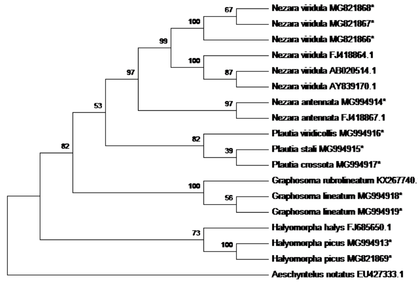

Figure 3: *Minimum Evolution tree of family Pentatomidae constructed from Cyt b gene sequences. Numbers indicate the percentage of 1000 bootstrap replicate.*

CONCLUSION

On the basis of above discussion it can be concluded that Cyt b gene fragment of mitochondrial DNA are useful in the identification and phylogenetic analysis of Indian stink bugs. It acts as wonderful markers for both phylogenetic analysis describing monophyly and polyphyly of the groups and at the same time it also throws some light on the apparent geographical population distribution pattern with the genetic variation in these gene fragments. In Heteroptera, the Cyt b gene has been used to infer the evolution of taxa, such as the infraorder (Pentatomomorpha) and superfamily (Pentatomoidea) (Muraji et al., 2000). No work in India has been done so far in family Pentatomidae using these gene fragments. The present study has generated molecular database for different species of family Pentatomidae on the basis of Cyt b gene region.

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