Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(5): 538-543

Received: 13-07-2019
Accepted: 15-08-2019

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Phylogenetic analysis of mitochondrial cytochrome C oxidase subunit I gene sequences of four species of *Rhynocoris* Kolenati, four *Rhynocoris kumarii* Ambrose and Livingstone and the morphs of *Rhynocoris marginatus* (Fabricius) (Insecta: Hemiptera: Reduviidae: Harpactorinae)

V Jeba Singh and Dunston P Ambrose

Abstract
The present study is on the phylogenetic analysis of mitochondrial COI gene of four species of *Rhynocoris* Kolenati, viz., *Rhynocoris fuscipes* (Fabricius), *Rhynocoris kumarii* Ambrose and Livingstone, *Rhynocoris longifrons* (Stål) and *Rhynocoris marginatus* (Fabricius), four ecotypes of *R. kumarii* viz., Kalthuruthy (RK-KAZ), Maruthuvazhmalai (RK-MAR), Muppandal (RK-MUP), Theni (RK-THE) and three morphs of *R. marginatus* viz. Niger (RM-NIG), Nigrosanguineous (RM-NSAN), and Niger banded morphs of *R. marginatus* (RM-NB). The nucleotide sequences of *Rhynocoris* species, ecotypes and morphs revealed intrageneric diagnostic characteristics as well as interspecific genetic variations among the four *Rhynocoris* and interecotypic and intermorphic peculiarities. The understating of such characteristics and variation in the phylogenetic reconstruction of family Reduviidae is discussed.

Keywords: *Rhynocoris*, assassin bugs, biocontrol agents, molecular biosystematics, speciation, ecotypism, polymorphism

Introduction
Reduviidae is a diverse group of mostly predatory insects with currently close to 7000 species and subspecies in 913 genera and 25 subfamilies described worldwide [1]. Reduviids are abundant, occur worldwide and are voracious predators. Hence, they are referred to as "assassin bugs". After Maldonado's [1] world checklist on assassin bugs many Indian species have been described and redescribed and considerable changes have been incorporated at species, generic, tribe and subfamily levels [2, 3]. Ambrose [4] prepared the checklist 14 subfamilies with 144 genera and 464 species from Indian faunal limits.
Mitochondrial genes are utilized to study the phylogeny [5] and phylogeography [6] and the revision of insect phylogenetics [7, 8] particularly, Reduviidae [9, 10]. Up to date, complete sequences of mitochondrial genes of assassin bugs viz., *Brontostoma colossus* from subfamily Ectrichodiinae, *Oncocephalus breviscutum* from subfamily Stenopodainae, *Peirates arcuatus* and *Sirthenea flavipes* from subfamily Peiratinae, *Triatoma dimidiata* (Latreille) from subfamily Triatominae, *Valentia hoffmanni* Stål from subfamily Salyavatinae, *Agriosphodrus dohrni* (Signoret) a representative of Harpactorinae were sequenced [8].
The most abundant harpactorine assassin bugs, *Rhynocoris* in Oriental region are the promising biological control agents [2, 11]. Since they occur as diverse morphs and ecotypes accurate identification of these biocontrol potential reduviids is imperative for their utilization in Insect Pest Management programmes.
Ambrose *et al.* [12] analysed the phylogenetics of intrageneric and intraspecific variations of fifteen species of *Rhynocoris* and four ecotypes of *R. kumarii* and three morphs of *R. marginatus* of Indian and Non Indian origin and the role of geographical isolation on speciation using three genes viz., 16S, Cyt b and COI genes. Their analysis of 16S gene revealed the affinity between Indian species *R. fuscipes* and *R. segmentarius* of South Africa. The Cyt b revealed affinity between the non-Indian *R. fuscipes* with Indian *R. fuscipes* and the four Indian species of *Rhynocoris* having two affinity clusters between viz., *R. kumarii* and *R.*

longifrons and *R. marginatus* and *R. fuscipes*. The COI gene analysis revealed the affinity between the Indian *R. marginatus* and two non- Indian species of *R. ventralis* and *R. ornatus*. The COI-like gene analysis revealed the affinity of Indian *R. fuscipes* with the ecotypes of *R. kumarii*-KAZ and THE with the same line of lineage. Interestingly, the MAR ecotype of *R. kumarii* showed affinity with the morph of *R. marginatus*-Unknown. Moreover, the Niger morph of *R. marginatus* showed affinity with *R. marginatus*-Nigrosanguineous.

Baskar *et al*. [13 a, b, c] reported genetic diversity based on the mitochondrial genes COI and Cyt-b genes among the ecotypes of four Indian *Rhynocoris* species, *R. fuscipes*, *R. kumarii*, *R. longifrons* and *R. marginatus* and correlated the affinity with the ecological diversity of semiarid, scrub jungle, and tropical rainforest habitats. Their neighbour-joining distance method analysis of the COI gene revealed that the *R. marginatus* was distinctly separated from the other three *Rhynocoris* species which grouped together.

Baskar *et al.* [14] further reported the phylogenetic analysis of intrageneric and intraspecific variations based on COI gene sequences of four species of *Rhynocoris* viz., *R. fuscipes*, *R. kumarii*, *R. longifrons* and *R. marginatus*. Their analysis revealed the highest affinity between *R. kumarii* and *R. longifrons* and less affinity between *R. marginatus* and *R. fuscipes* as reported by Ambrose *et al*. [12]. The highest nucleotide base substitution in the third codon and the lowest in the second codon and base composition showed extreme bias being AT from 62% to 70% and GC from 30% to 37.9%.

Lenin [15] analysed the phylogenetics of intergeneric and intraspecific variations based on COI gene sequences of six species of Reduviinae viz., *Acanthaspis pedestris* (Stål), *A. quinquespinosa* Amyot and Serville, *A. siva* Amyot and Serville, *Edocla slateri* Stål, *Empyrocoris annulata* Miller and *Velitra sinensis* Stål. The three phylogenetic trees he constructed viz., maximum likelihood, maximum parsimony and neighbor-joining revealed the affinity between *A. pedestris* and *A. quinquespinosa* and *E. annulata* and *E. slateri* whereas *V. sinensis* remained as a separate clade.

Manimuthu *et al.* [16] analysed phylogenetics of intrageneric and intraspecific variations based on 28S rRNA gene in five *Ectomocoris* species viz., *Ectomocoris atrox* (Stål), *E. ornatus* (Stål), *Ectomocoris* sp.$_1$, *Ectomocoris* sp.$_2$ and *Ectomocoris* sp.$_3$ and COI gene of four species of *Ectomocoris* viz., *Ectomocoris cordiger* Stål, *E. quadriguttatus* (Fabricius), *E. tibialis* Distant and *Ectomocoris* sp. and a lone species of *Catamiarus*, *C. brevepennis* (Serville). The affinity found among the 28S rRNA of four Australian species of *Ectomocoris* viz., *E. ornatus*, *Ectomocoris* sp.$_1$, sp.$_2$ and sp.$_3$ and one Indian *E. atrox* supported monophyly despite geographical isolation. Their 28S rRNA analysis further revealed the intrageneric affinity between *E. atrox, E. ornatus* and *Ectomocoris* sp. The Cyt c gene of three *Ectomocoris* species from Asia, one species of *Ectomocoris* sp. from Australia and Indian *C. brevipennis* revealed closer affinity. The Asiatic *E. quadriguttatus* exhibited close affinity with the other two Aisatic species viz., *E. cordiger* and *E. tibialis* but interestingly distantly related to them than to *C. brevipennis*. Thus, their study revealed intrageneric as well as intergeneric affinity.

Ambrose *et al*. [17] analysed the phylogenetics of intrageneric and intraspecific variations of thirteen species of *Coranus* Curtis and two ecotypes of *Coranus callosus* Stål from four countries and three continents and the role of geographical isolation on speciation by using four genes viz., 16S, Cyt b, COI and 28S rRNA. The 16S rRNA analysis revealed monophyly. *Coranus* sp.$_2$ of Australia instead of clustering with *C. callosus* of Australia clustered with *Coranus* sp.$_3$ of Nigeria and *Coranus* sp.$_1$ of Brunei. These species also exhibit affinity despite their geographical isolation as observed by the gene of Cyt c Manimuthu *et al.* [16].

The above reported analyses revealed the importance of mitochondrial genes in phylogenetics. Hence, the present study was undertaken to understand the utilization of cytochrome c oxidase subunit I gene sequence as a molecular marker to reveal the interspecific, interecotypic and intermorphic genetic variations among four species of *Rhynocoris* Kolenati, four ecotypes of *R. kumarii* Ambrose and Livingstone and three morphs of *R. marginatus* (Fabricius).

Materials and methods

DNA isolation

The legs of adult reduviid predators of four species of *Rhynocoris* Kolenati, viz., R. *fuscipes* (Fabricius), *R. kumarii* Ambrose and Livingstone, *R. longifrons* (Stål) and *R. marginatus* (Fabricius) and four ecotypes of *R. kumarii* viz., Kalthuruthy (RK-KAZ), Maruthuvazhmalai (RK-MAR), Muppandal (RK-MUP), Theni (RK-THE) and three morphs of *R. marginatus* viz. Niger (RM-NIG), Nigrosanguineous (RM-NSAN), and Niger banded (RM-NB) from the type specimens deposited at Entomology Research Unit, St. Xavier's College (Autonomous), Palayamkottai, India were selected as the tissue of choice for the source of genomic DNA. The genomic DNA was isolated by Sambrook *et al*., method [18].

PCR amplification

PCR was carried out to amplify the partial mitochondrial COI gene using COI gene forward primer CI-J-1751 'GGA TCA CCT GAT ATA GCA TTC CC' and reverse primer CI-N-2191- 'CCC GGT AAA ATT AAA ATA TAA ACT TC' [5]. The primer combination yielded a fragment size of ~500 bp, in all the *Rhynocoris* species.

Gene sequencing

The partial nucleotide sequence of interspecies, interecotypes and intermorphs were submitted to NCBI GenBank database and accession numbers are given below: four *Rhynocoris* species of *R. fuscipes*- GU967411, *R. kumarii*- HQ846916, *R. longifrons*- HQ245922, *R. marginatus*- HM768319; four ecotypes of *R. kumarii*- KAZ- HM768317, *R. kumarii*- MAR-HQ846916, *R. kumarii*- MUP- HQ846917, *R. kumarii*- THE-HM768318; three morphs of *R. marginatus*- NIG- JN634062, *R. marginatus*- NSAN- JN634063, *R. marginatus*- NB-HM768319.

Phylogenetic analysis

The phylogenetic analysis of four species of *Rhynocoris* and four ecotypes of *R. kumarii* was conducted with three different methods namely, maximum likelihood, maximum parsimony and neighbour-joining distance method and three morphs of *R. marginatus* was performed using two methods such as maximum likelihood and neighbour-joining distance method [19].

Results

Basic sequence statistics

The nucleotide composition of four species of *Rhynocoris* reveals that highest AT rich for *R. marginatus* (66.9%) and lowest was observed for *R. fuscipes* (61.3%) and four ecotypes of *R. kumarii* the highest AT rich for *R. kumarii*-MAR (65.5%) and lowest was observed for *R. kumarii*-MUP (60.4%) and three morphs of *R. marginatus* the highest AT rich for *R. marginatus*-NB (66.6%) and lowest was observed for *R. marginatus*-NIG (59.2%). The CG rich composition in four species of *Rhynocoris* reveals that highest CG rich for *R. fuscipes* (38.8%) and lowest was observed for *R. marginatus* (33.1%) and four ecotypes of *R. kumarii* the highest CG rich for MUP (39.6%) and lowest was observed for MAR (34.5%) and three morphs of *R. marginatus* the highest NIG (40.8%) and lowest was observed for NB (33.4%). The absence of methionine in *R. fuscipes*, *R. longifrons* and *R. marginatus* and only traces in *R. kumarii* could be considered for the generic character of *Rhynocoris*.

The codon frequency of four species of *Rhynocoris* reveals that highest codon frequency for TTA (2.58) and the lowest for ATT (2.08). Among the four ecotypes of *R. kumarii*, the highest codon was observed for TTA (2.83) but the lowest for TCA (2.17), instead of ATT observed in *Rhynocoris*. Among the three morphs of *R. marginatus*, the highest codons observed were GCT and AGG (2.61) and the lowest for GTT (2.32), again a deviation from the *Rhynocoris* character.

The Transition/Transversion ratios of first three codons of four species of *Rhynocoris* reveals that the highest ratio was observed for the first codon (0.51) and the lowest for second codon (0.43). However, among the four ecotypes of *R. kumarii*, the highest ratio was observed for the first codon (0.83) and the lowest for third codon (0.50). Among the three morphs of *R. marginatus*, the highest ratio was observed for the second codon (0.86) and the lowest for the first codon (0.59) instead of the first codon. The nucleotide base composition analysis in species of *Rhynocoris*, ecotypes of *R. kumarii* and morphs of *R. marginatus* reveals common features i.e., the highest percentage of AT and the lowest percentage of CG and the most frequently used codon is TTA.

Genetic distance estimation

The genetic distance estimation of four species of *Rhynocoris* reveals the longest evolutionary distance between *R. fuscipes* and *R. longifrons* (0.543 ± 0.067) and the shortest between *R. fuscipes* and *R. kumarii* (0.408 ± 0.049). Among the four ecotypes of *R. kumarii*, the longest evolutionary distance was observed between KAZ and MAR and MAR and THE (0.995 ± 0.341) and the shortest between MAR and MUP (0.532 ± 0.195). Among the three morphs of *R. marginatus*, the longest evolutionary distance was observed between the NSAN and the NB (1.262 ± 0.396) and the shortest between the NIG and the NB (0.884 ± 0.261). The longer genetic distance observed between *R. fuscipes* and *R. longifrons* suggests lesser affinity and the shorter distance between *R. fuscipes* and *R. kumarii* suggests greater affinity.

Test of neutral evolution

Codon-based Z-test for four species of *Rhynocoris* reveals the lowest neutral evolution in *R. kumarii* and *R. marginatus* (0.000 ± 4.300) and the highest in *R. fuscipes* and *R. longifrons* (0.050 ±1.981). Among the four ecotypes of *R. kumarii*, the highest neutral evolution was observed in KAZ and MAR and MAR and THE (0.009 ± 2.659). Among the three morphs of *R. marginatus*, the highest neutral evolution was observed in NIG and NSAN (0.225 ± 1.218) ($P < 0.05$). The higher rate of neutral evolution between *R. fuscipes* and *R. longifrons* suggests lesser affinity and the lower rate of neutral evolution between *R. kumarii* and *R. marginatus* suggests greater affinity.

Homogeneity of substitution patterns

(i) Disparity index test

The disparity index tested for four species of *Rhynocoris* reveals the highest homogeneity i.e., greater affinity between *R. fuscipes* and *R. marginatus* (0.004 ± 2.468) and the lowest between *R. fuscipes* and *R. longifrons* (1.000 ± 0.000) i.e., lesser affinity. Among the four ecotypes of *R. kumarii*, the highest homogeneity was observed between MAR and MUP (0.010 ± 0.741) and the lowest between KAZ and THE, KAZ and MUP and MUP and THE (1.000 ± 0.000) suggesting their varied affinity. Among the three morphs of *R. marginatus*, the highest homogeneity was observed between NIG and NB (0.008 ± 1.738) suggesting higher affinity and the lowest between NIG and NSAN (0.369 ± 0.029) suggesting lower affinity.

(ii) Composition distance test

The Composition distance between four species of *Rhynocoris* reveals the longest distance between *R. fuscipes* and *R. marginatus* (3.210) suggesting lower affinity and the shortest distance between *R. fuscipes* and *R. longifrons* (0.329) indicating greater affinity. Among the four ecotypes of *R. kumarii*, the longest distance was observed between MAR and MUP (1.041), i.e., lower affinity and the shortest between KAZ and THE (0.000), i.e., greater affinity. Among the three morphs of *R. marginatus*, the longest distance was observed between NIG and NB (2.297), i.e., lower affinity and the shortest between NIG and NSAN (0.259), i.e., greater affinity.

Phylogenetic analysis

The phylogenetic analysis in four species of *Rhynocoris* by maximum likelihood, maximum parsimony and neighbour-joining distance methods reveals two main clusters. The first cluster exhibits affinity between *R. fuscipes* and *R. longifrons* and second cluster exhibits affinity between *R. kumarii* and *R. marginatus*. (Fig. 1.1 to 1.3)

The phylogenetic analysis in four ecotypes of *R. kumarii* by maximum likelihood, maximum parsimony and neighbour-joining distance methods reveals two main clusters. The first cluster exhibits affinity with *R. kumarii*-KAZ, THE and MUP whereas *R. kumarii*-MAR forms a separate lineage. (Fig. 2.1 to 2.3)

The phylogenetic analysis in three morphs of *R. marginatus* by maximum likelihood and neighbour-joining distance methods reveals two main clusters. The first cluster exhibits affinity between *R. marginatus*-NIG and NSAN whereas *R. marginatus*-NB forms a separate lineage. (Fig. 3.1 and 3.2)

Intrageneric and Interspecific analysis in four species of *Rhynocoris*

The amino acid composition analysis in four species of *Rhynocoris* reveals that absences of methionine in all these species. The secondary structure of protein analysis revealed that highest coil structure for *R. longifrons* (50.32%) and the lowest for *R. marginatus* (17.85%). The highest strand was observed for *R. kumarii* (48.96%) and the lowest for *R. fuscipes* (15.89%). The highest helix was observed for *R. marginatus* (40%) and the lowest for *R. longifrons* (15.68%).

Intraspecific and Interecotypic analysis in four ecotypes of *R. kumarii*

The amino acid composition analysis in four ecotypes of *R. kumarii* reveals that absences of methionine in all the ecotypes. The secondary structure of protein analysis reveals the highest coil structure for *R. kumarii*-MAR (35.17%) and the lowest for *R. kumarii*-THE (14.86%). The highest strand was observed for *R. kumarii*-MAR (48.96%) the lowest for *R. kumarii*-KAZ and absence of strand structure in *R. kumarii*-THE. The highest helix was observed for *R. kumarii*-THE (17.07%) and the lowest for *R. kumarii*-KAZ (11.78%) and absence of helix structure in *R. kumarii*-MUP.

Intraspecific and Intermorphic analysis in three morphs of *R. marginatus*

The amino acid composition analysis in three morphs of *R. marginatus* reveals the absence of quantified amount of methionine in all the three morphs whereas traces of methionine in the morph NSAN alone. The secondary structure of protein analysis reveals that the highest coil structure for *R. marginatus*-NIG (18.63%) and lowest for *R. marginatus*-NSAN (15.80%). The highest strand was observed for *R. marginatus*-NB (42.14%) and the lowest for *R. marginatus*-NIG (0.55%). The highest helix was observed for *R. marginatus*-NB (40%) and the lowest for *R. marginatus*-NIG (12.73%).

Discussion

The highest percentage of A+T composition ranging from 61.3 to 66.9% observed in the four species of *Rhynocoris* corroborates with the findings for insects in general and Reduviidae in particular [20, 9]. In addition to this, the most frequently occurred codon TTA in all the four species of *Rhynocoris* could be also considered as a specific generic characters. The absence of methonine in *R. fuscipes*, *R. longifrons* and *R. marginatus* and traces in *R. kumarii* could be also considered as a supplementary generic character of *Rhynocoris*.

The highest percentage GC composition (38.7%) in *R. fuscipes*; the highest secondary strand structure of protein (48.96%) and the presence traces of methionine in *R. kumarii*; the highest coiled secondary structure of protein (50.32%) in *R. longifrons* and the highest AT composition (66.9%) and highest secondary helix structure of protein (40%) in *R. marginatus* could be considered for species specific characters.

The higher affinity observed between *R. kumarii* and *R. marginatus* (0.000 ± 4.300) than between *R. fuscipes* and *R. longifrons* (0.050 ± 1.981) based on intrageneric affinity analysis from the codon-based Z-test of neutrality between the sequences was further confirmed by phylogenetic tree reconstruction using maximum likelihood, maximum parsimony and neighbour-joining distance methods. These observations suggest that even the closely related species are genetically richly diversified [21].

The presence of traces of strand (5.57%) structure in secondary structure of protein in the *R. kumarii* ecotype KAZ; the highest AT composition (65.5%) and the highest coil, strand and helix structure in secondary structure of protein (35.17%), (48.96%) and (15.86%) respectively in the ecotype MAR; the highest GC composition (39.5%) and the absence of helix structure in secondary structure of protein in the ecotype MUP; and the absence of strand structure in secondary structure of protein in the ecotype THE could be considered for ecotype specific characters. These observations suggest intraspecific diversity due to environmental influences and speciation [22]. This could be due to geographical isolation as observed by Mahendran *et al*. [23], Ambrose *et al*. [12], Manimuthu *et al*. [16] and Ambrose *et al*. [17]. However, we admit that it is premature to suggest the role of geographical isolation without knowing the molecular characteristics such as the number of segregating sites, nucleotide diversity and haplotype diversity and the geographical genetic structure. Moreover, the quantity of sampling is too small.

The highest affinity between ecotypes of *R. kumarii* KAZ and THE (0.000) and lowest between MAR and MUP (1.041) was also supported by composition distance. The phylogenetic trees reconstructed using maximum likelihood, maximum parsimony and neighbour-joining distance methods also reveals greater affinity between KAZ and THE and lesser affinity between MAR and MUP.

The highest GC composition (40.8%) and the highest coil (18.63%) and the traces of strand (0.55%) structures in secondary structure of protein in the *R. marginatus* morph NIG; and the presence traces of methionine in the morph NSAN; the highest AT composition (66.5%) and the highest percentage strand (42.14%) and helix (40%) structure in secondary structure of protein in the morph NB could be considered for morphs specific characters.

The highest affinity between morphs of *R. marginatus* NIG and NSAN (0.259) and the lowest between NIG and NB (2.297) were also supported by composition distance and neutrality. The phylogenetic trees reconstructed using maximum likelihood and neighbour-joining distance methods also reveals greater affinity between NIG and NSAN and lesser affinity NB. Moreover, the quantity of sampling is too small. The present results corroborates with the findings of Ambrose *et al*. [12] and further suggest the existence of genetic diversity, with low level of gene flow in four *Rhynocoris* species, four ecotypes of *R. kumarii* and three morphs of *R. marginatus* [12 and 17, 13 a, b, c and 14, 15 and 16]. Moreover, these observations further suggest the progression of speciation among the ecotypes of *R. kumarii* and the morphs of *R. marginatus*.

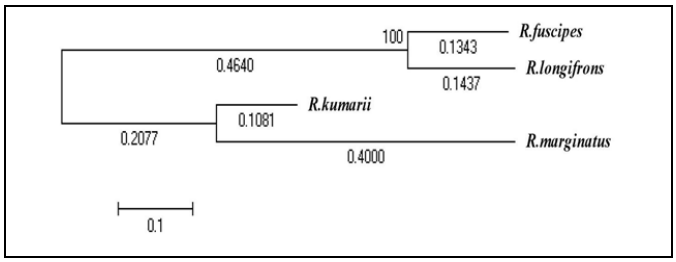

Fig 1.1: Phylogenetic relationship analysis of four *Rhynocoris* species by maximum likelihood method

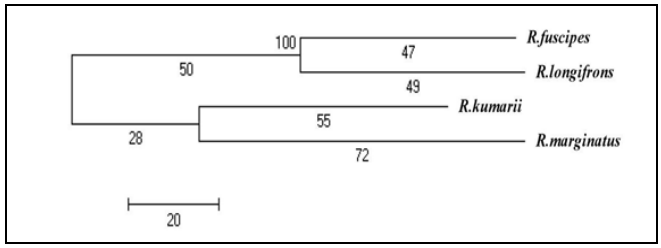

Fig 1.2: Phylogenetic relationship analysis of four *Rhynocoris* species by maximum parsimony method

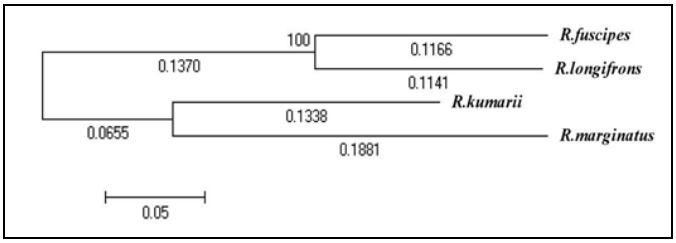

Fig 1.3: Phylogenetic relationship analysis of four *Rhynocoris* species by neighbour-joining distance method

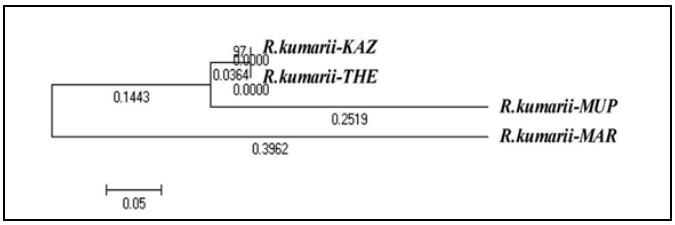

Fig 2.1: Phylogenetic relationship analysis of four ecotypes of R. kumarii by maximum likelihood method

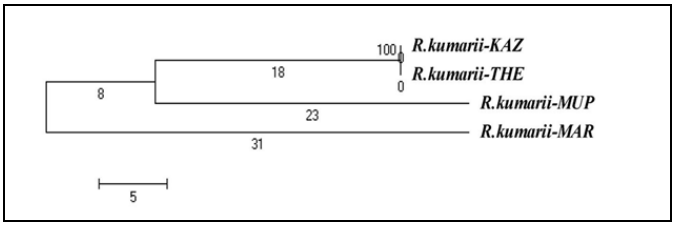

Fig 2.2: Phylogenetic relationship analysis of four ecotypes of R. kumarii by maximum parsimony method

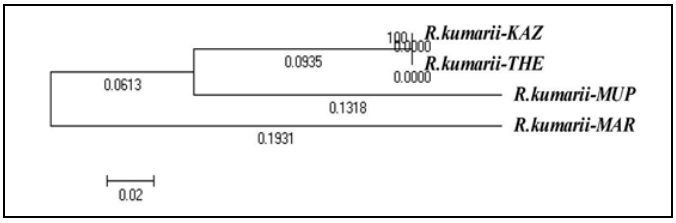

Fig 2.3: Phylogenetic relationship analysis of four ecotypes of *R. kumarii* by neighbour-joining distance method

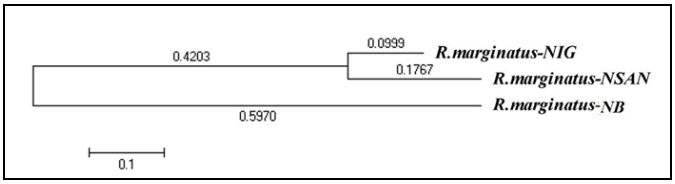

Fig 3.1: Phylogenetic relationship analysis of three morphs of *R. marginatus* by maximum likelihood method

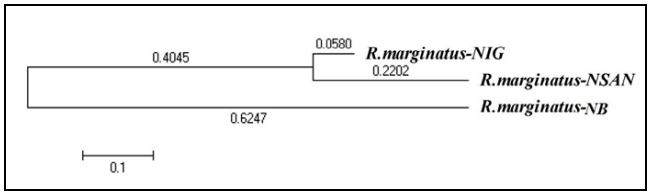

Fig 3.2: Phylogenetic relationship analysis of three morphs of *R. marginatus* by neighbour-joining distance method

Conclusion

The mitochondrial cytochrome c oxidase subunit I gene analysis using four *Rhynocoris* species, four ecotypes of *R. kumarii* and three morphs of *R. marginatus* resulted in the possibility of establishing certain interspecific and intraspecific markers for the purpose of identification. The study also revealed the intrageneric affinity within species and ecotypic and morphic affinity. These findings could be incorporated into the multidisciplinary biosystematics characteristics of *Rhynocoris* species. However, our sampling of only four species of the genus *Rhynocoris* which has more than 118 species emphasizes further studies with more species. Hence, further studies are warranted in this direction that could lead to meaningful revision, regrouping or replacement of species with new revelations through such molecular studies. The analysis further suggests the usefulness of cytochrome c oxidase subunit I gene in phylogenetics. The study could also be further extended analyzing other mitochondrial genes for a reasonable number of interspecific, interecotypic and intermorphic levels and the findings may contribute in evolutionary studies.

Acknowledgements

The authors are grateful to the authorities of St. Xavier's College (Autonomous), Palayamkottai, Tamil Nadu, India for facilities. One of us (DPA) thanks Council of Scientific and Industrial Research (21 (0865)/11/EMR-II, 2012-2013 Dated 28.12.2011), Govt. of India, New Delhi for financial assistance.

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