BIOSYSTEMATICS OF CHOSEN REDUVIINE ASSASSIN BUGS (INSECTA: HEMIPTERA: REDUVIIDAE)

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DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This thesis entitled "**BIOSYSTEMATICS OF CHOSEN REDUVIINE ASSASSIN BUGS (INSECTA: HEMIPTERA: REDUVIIDAE**" submitted by **Mr. E. Arockia Lenin** for the award of Degree of Doctor of Philosophy in Zoology of Manonmaniam Sundaranar University is a record of bonafide research work done by him and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University or Institution.

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DECLARATION

I hereby declare that the thesis entitled "**BIOSYSTEMATICS OF CHOSEN REDUVIINE ASSASSIN BUGS (INSECTA: HEMIPTERA: REDUVIIDAE**" submitted by me for the Degree of Doctor of Philosophy in Zoology is the result of my original and independent research work carried out under the guidance of Dr. Dunston P. Ambrose, D.Sc., Director, Entomology Research Unit, St. Xavier's College (Autonomous), Palayamkottai- 627 002 and it has not been submitted for the award of any degree, diploma, associateship, fellowship of any University or Institution.

(E. AROCKIA LENIN)

Place: Palayamkottai

Date: .06.2014

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"Delight yourself in the Lord and He will give you the desires of your heart"

- Psalms 37: 4

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CONTENTS

INTRODUCTION

The thesis entitled "**Biosystematics of chosen Reduviine Assassin Bugs (Insecta: Hemiptera: Reduviidae)"** is the outcome of part of my continued research on the biosystematics of Reduviinae. It covers important taxonomic characters of four genera of Reduviinae, viz., *Acanthaspis, Edocla, Empyrocois* and *Velitra,* i.e., the findings on intrageneric and intergeneric and interspecific markers as multidisciplinary tools in the biosystematics of Reduviidae with morphometrical and molecular tools to realize a comprehensive multidisciplinary biosystematics approach. Though diverse aspects of multidisciplinary biosystematics have been suggested I confine myself to morphometry, electrophoretic body protein profile, mitochondrial Cytochrome C Oxidase subunit I gene sequences and restriction fragment length polymorphism for my Ph.D thesis.

Taxonomy is the scientific discipline of describing, delimiting and naming organisms, both living and fossil, and systematics is the process of organising taxonomic information about organisms into a logical classification that provides the framework for all comparative studies. The systematics and taxonomy are referred to collectively as biosystematics.

Latreille (1807) is credited with the first time usage of the family name Reduviidae to indicate a group above the genus level although Fabricius (1775) first described the species *Reduvius personatus* under Reduviidae. As more species and genera were described, the reduviids were raised in taxonomic status and subdivided into subfamilies and tribes. Much reduviid taxonomy was done in the nineteenth century without type specimens and often without adequate descriptions or illustrations to provide distinguishing characteristics. For example, Latreille's (1807) description of 'Reduvide' mentions that these are large, black bugs, longer than broad, and allied to bed bugs. Many heteropterans in many different families fit into this description.

Amyot and Serville (1843) first classified the 'Reduvides' (modern subfamily Reduviinae) as a tribe in the Cimicidae. They considered the "Ectrichodides," Emesides," "Harpactorides," "Peiratides," "Phymatides," "Salyavatides," "Sphaeridopides" and Stenopides" to be groups of equal rank. Stål (1874) placed all these groups in the family Reduviidae. Champion (1899), Davis (1969) and Miller (1971) presented comprehensive summaries of Reduviidae classification.

Most twentieth century taxonomic revisions of Reduviidae have been made to characterize and distinguish all the subfamilies of the Reduviidae. The family Reduviidae has been divided into about 30 subfamilies, but following studies by Davis (1957, 1961, 1966, 1969), the number has been reduced to 23 subfamilies and 33 tribes recognized by Putshkov and Putshkov (1985) and Putshkov (1987) or 25 subfamilies recognized by Maldonado (1990). The Reduviidae has been conceived as a unit by many authors, excluding Elasmodeminae and Phymatinae (Wygodzinsky, 1944; China and Miller, 1959; Maldonado, 1990) as separate families. Arguments for inclusion of the Elasmodeminae within the Reduviidae were made convincingly by Davis (1957) and for Phymatinae by Carayon *et al*., (1958), the latter's arguments accepted by Davis (1961) were rejected by Froeschner and Kormilev (1989). Should these two subfamilies be regarded as distinct families, then at least the Holoptilinae, which also belong to the Phymatinae- Elasmodeminae clade (Carayon *et al*., 1958), would also have to be elevated to family rank in order to maintain a monophyletic Reduviidae. Such a splitting might also affect the rank of some other reduviid taxa, because according to Davis (1961) the Phimophorinae, Mendanocorinae and Centrocneminae also belong to the "phymatine complex." The Emesinae have often been treated as a distinct family, but no modern specialist has accepted this treatment (Wygodzinsky, 1966).

The knowledge of biosystematics of any group of organisms is the foundation of all meaningful further research (Narendren, 2001). One must know not only what an organism is

but also what its relatives and the phylogenetic relationships are; such knowledge broadens and deepens the biological information and thereby makes it more useful (Schaefer, 1988; Ambrose, 2000). The biosystematics helps one to determine the organism and to trace its phylogenetic relationship. Such knowledge has great relevance in the field of biodiversity, agriculture, medicine, forestry etc. (Ambrose, 2004a, b).

The multidimensional concept of species occupies a predominant place in life sciences. Increasing problems in biocontrol programmes have led to the involvement of multidisciplinary approaches towards a better assessment of the concerned species. The present day awareness regarding the need for such an integrated approach to biosystematics studies has bloomed more acute.

Biosystematics is not only confined with traditional customary tool of morphology since it has its own limitations. Hence, it is further supplemented by various observations in interpreting the relationships. For instance, biometrical, genetical, biochemical, immunological and karyological information are being increasingly used as additional parameters to justify the phylogenetic relationships. Therefore, biosystematics can now be called a truely interdisciplinary approach with basic and applied potential. The study of insect biosystematics has become increasingly significant in explaining problems which go beyond the classical systematic approach and which are of great importance in agriculture, medicine and forestry. There is an increasing tendency for the occurrence of biotype in species and that has created serious problems in applied entomology. To trace their phylogeny and fix their taxonomic position biosystematic approach is employed in classificatory schemes.

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 (Froeschner and Kormilev, 1989; Maldonado, 1990; Cassis and Gross, 1995). After Maldonado (1990) 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 (Ambrose, 1999, 2006).

Distant (1902, 1910) in his Fauna of British India, described 342 species of reduviids belonging to 106 genera and 13 subfamilies including the Nabidae and treating the Ectinoderinae under the Ectrichodiinae, the Physoderinae under the Peiratinae and the Centrocneminae, the Reduviidae and Triatominae together as the Acanthaspidinae. Ambrose (2006) prepared a checklist of assassin bugs including 14 subfamilies with 144 genera and 464 species from Indian faunal limits.

The family Reduviidae is a family of predaceous cimicomorphan bugs called the assassin bugs with cosmopolitan distribution (Maldonado, 1990). Reduviids are abundant, occur worldwide and are voracious predators. Hence, they are referred to as "assassin bugs". They may not be useful as predators of specific pests as they are polyphagous, but they are highly useful in managing diverse group of pests in any agroecosystem and integrated pest management programme (IPM) (Ambrose, 1999, 2000, 2003; Ishikawa *et al*., 2007).They kill more prey than they need to satiate themselves by their behaviour of indiscriminate killing (Ambrose, 1996a, b). Hence, assassin bugs deserve to be conserved and augmented for their utilization in biological control programmes. Moreover, they occur in every ecosystem, even in the most adverse micro-habitats (Ambrose, 1987a, b, 1988, 1991, 1995, 1999; Ables, 1978; Schaefer and Ahmad, 1987; James, 1994; Ambrose *et al*., 2006, 2007). Despite the high potential of this group as biological control agents in agriculture and ecological information is available only for a less number of species from a rather restricted area in the world. Such a situation is partially due to the difficulty in the identification of the species because of their small size and scarcity of clear morphological differences especially among closely related species. Therefore, it is necessary to develop new criteria for species identification and for specifying their position in taxonomic grouping and molecular phylogeny.

Among 25 subfamilies of assassin bugs (Maldonado, 1990), the Reduviinae is one of the most heterogeneous including 141 genera and more than 1070 species occurring in all biogeographic regions (Melo, 2007).

Although preliminary attempts were made on the exploration of multidisciplinary tool in the biosystematics of Reduviidae (Louis and Kumar, 1973; Ambrose, 1980, 1987a, b, Goel, 1984; Livingstone and Ambrose, 1984; Ambrose, 1999; Ambrose, 2004a, b; Singh, 2012) the possible utilization of the same has not been made. This prompted me to explore the advantages of various parameters in the biosystematic of Reduviidae. In the present thesis, multidisciplinary tools in the biosystematics of six species of Reduvinae viz., *Acanthaspis pedestris* Stål, *Acanthaspis quinquespinosa* (Fabricius), *Acanthaspis siva* Distant*, Empyrocoris annulata* (Distant)*, Edocla slateri* Distant and *Velitra sinensis* (Walker) were analysed.

The findings on multifaceted biosystematics of Reduviinae is presented in my Ph. D thesis entitled "Biosystematics of a chosen Reduviine Assassin Bugs (Insecta: Hemiptera: Reduviidae)" under the following four Chapters: 1) Taxonomy 2) Electrophoretic body protein profile 3) Mitochondrial cytochrome c oxidase subunit I gene sequences and 4) Restriction fragment length polymorphism of six reduviine species under four genera viz., *Acanthaspis pedestris* Stål, *Acanthaspis quinquespinosa* (Fabricius), *Acanthaspis siva* Distant, *Empyrocoris annulata* (Distant), *Edocla slateri* Distant and *Velitra sinensis* (Walker).

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REVIEW OF LITERATURE

Introduction

Reduviidae is a diverse group of predatory insects with currently close to 7000 species and subspecies in 913 genera and 25 subfamilies described worldwide (Froeschner and Kormilev, 1989; Maldonado, 1990; Cassis and Gross, 1995). Reduviids are abundant, occur worldwide and are voracious predators. Hence, they are referred to as "assassin bugs". After Maldonado's (1990) 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 (Ambrose, 1999, 2004b).

Distant (1902, 1910) in his Fauna of British India, described 342 species of reduviids belonging to 106 genera and 13 subfamilies including the Nabidae and treating the Ectinoderinae under the Ectrichodiinae, the Physoderinae under the Peiratinae and the Centrocneminae, the Reduviinae and Triatominae together as the Acanthaspidinae. Ambrose (2006) published a checklist of assassin bugs of 14 subfamilies with 144 genera and 464 species from Indian faunal limits.

1. Taxonomy

The taxonomy of Indian reduviids has been studied since Distant's (1902, 1910). The Fauna of British India volumes. He recorded 342 species of reduviids belonging to 106 genera and 13 subfamilies from Indian faunal limits. After Distant, contributions to taxonomy of Indian reduviids came from Paiva (1918, 1919) who described one new genus of Ectrichodiinae viz., *Paralibavius* and six new species: two under Harpactorinae viz., *Harpactor* (=*Rhynocoris*) *varians* and *Sycanus dubis*; three under Ectrichodiinae viz., *Ectrychotes relatus*, *Paralibavis singularis* and *Scadra castanea* and one under Emesinae viz., *Bagauda cavernicola*. Then, China (1924) described a new species of Emesinae under

the genus *Myiophanes* viz., *M. kempi*. Later, Samuel and Joseph (1953) described two species of Harpactorinae under the genus *Rhinocoris* (= *Rhynocoris*) viz., *R. lapidicola* and *R. nysiiphagus*. Thereafter, Muraleedharan (1976) described two species of Harpactorinae under *Henricohahnia* viz., *H. viroopa* and *H. milleri*. Maldonado (1990) in his World Reduviidae Catalogue listed 420 Indian species belonging to 146 genera and 14 subfamilies. After Maldonado's (1990) 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 (Ambrose, 1999, 2004a). Thereafter, Ambrose (2006) in his checklist of Indian assassin bugs added 58valid species under 9 genera and 9 subfamilies that were either not catalogued by Maldonado or described after the publication of his catalogue. He also included the details on the presence or absence of tibial pad or fossula spongiosa in both fore- and midlegs or only in the foreleg, diversity of curvature of rostrum, alary polymorphic characteristic features, morphology of egg and the oviposition behaviour. Thereafter, twenty four new species were described by Entomology Research Unit, St. Xavier's College, Palayamkottai (to be published). They include:

Ectrichodiinae:

1. *Synectrychotes calimerei* (Livingstone and Murugan, 1987) 2. *Ectrychotes bharathii* (Murugan and Livingstone, 1989) 3. *Physorhyncus* (=*Haematorrhophus*) *javadiensis* (Hegde, 1989) 4. *Echinocoris coromandelensis* (Livingstone and Ravichandran, 1992) 5. *Labidocoris tuberculatus* (Ambrose and Vennison, 1993) 6. *Hemihaematorrhophus planidorsatus* 7. *Haematorrhophus fovealis* and 8. *H. ruguloscutellaris* (Murugan and Livingstone, 1995).

Emesinae:

9. *Ploiaria nude* and 10. *Emesopsis bimedia* (Ravichandran and Livingstone, 1989).

Harpactorinae:

11. *Rhynocoris kumarii* (Ambrose and Livingstone, 1986) 12. *Irantha pepparii* 13. *Polididus brevispina* 14. *Sphedanolestes nigrocephala* 15. *S. bicolourous* 16.*Coranus carinata* 17. *C. ruthii* 18. *C. ambroseii* 19. *Euagoras erythrocephala* and 20. *Platerus bhavanii* (Livingstone and Ravichandran, 1988a, 1989b, c, 1990a, 1991) 21. *Rhaphidosoma madukaraiensis* (Ravichandran and Livingstone, 1994) 22. *Harpactor* (= *Rhynocoris*) *shevroyensis* (Hegde, 1989) 23. *Coranus soosaii* (Ambrose and Vennison, 1989) 24. *Neovillanovanus macrotrichiatus* (Ambrose and Vennison, 1991) 25. *Neonagusta bituberculata* (Ambrose and Kumaraswami, 1992) and 26. *Coranus nodulosus* (Ambrose and Sahayaraj, 1993) and 27 to 37. Krishnan (2009) described 11 new species of the genus, *Endochus* in his Ph.D thesis (to be published).

Peiratinae:

38. *Sirthenea bharati* (Sucheta and Chopra, 1988) and 39. *S. nigripes* (Murugan and Livingstone, 1990b) 40. *Pirates* (= *Peirates*) *unipunctatus* and 41. *Ectomocoris tuberculatum* (Livingstone and Murugan, 1988b) and 42. *E. xavierei* (Vennison and Ambrose, 1990a).

Reduviinae:

43. *Acanthaspis philomanmariae* and 44. *A. livingstonei* (Vennison and Ambrose, 1988) 45.*Acanthaspis nigripes* 46. *A. minutum* and 47. *A. siruvanii* (Livingstone and Murugan, 1988a) 48. *Acanthaspis maculata* 49. *A. nigra* and 50. *A. hisarensis* (Sucheta and Chopra, 1989) 51.*Velitra neelai* 52. *Edocla maculates* 53. *E. heberii* 54. *E. punctatum* 55. *Neoacanthaspis maculates* 56. *Acanthaspis alagiriensis* and 57. *A. carinata* (Murugan and Livingstone, 1987, 1990a, 1991, 1994) 58. *Mesacanthaspis kovaiensis* (Livingstone and Murugan, 1993)59. *Acanthaspis nigricans* (Ambrose, 1994) and 60 to 72. Rajan (2012) described 13 new species of the genus, *Acanthaspis* in his Ph.D thesis (to be published).

Salyavatinae:

73. *Nudiscutella frontispina* (Murugan, 1988).

Stenopodainae:

74. *Oncocephalus anniei* (Ambrose and Vennison, 1988) 75. *Hemisastrapada gandhi-gramensis* 76. *Bardesanes sericinotatus* 77. *Sastrapada elongate* 78. *Oncocephalus chamundcus* 79. *O. yasphali* and 80. *O. bipunctatus* (Livingstone and Ravichandran, 1988b, 1989a, 1990b).

Tribelocephalinae:

81. *Tribelocephala uppasii* (Livingstone and Ravichandran, 1988c)

Triatominae:

82. *Linshcosteus karupus* (Galvao *et al.,* 2002).

In addition to the new species described, the following species from Indian faunal limits were redescribed:

Harpactorinae:

Alcmena spinifex (Thunberg) (Das, 1996; Das *et al.,* 2008a); *Coranus siva* Kirkaldy (= *C. obscurus* Kirby) (Kumar, 1993) and *C. vitellinus* Distant (Ambrose, 1980); *Endochus albomaculatus* Stål and *E. merula* Distant (Krishnan, 2009; Krishnan and Ambrose, 2010), *E. atricapillus* Distant, *E. atrispinus* Stål, *E. carbonarius* Breddin, *E. inornatus* Stål and *E. nigricornis* Stål (Krishnan, 2009), *E. migratorius* Distant (Ambrose *et al.,* 2007a; Gunaseelan, 2005) and *E. umbrinus* Distant (Sahayaraj, 1991); *Euagoras plagiatus* (Burmeister) (Vennison, 1988); *Irantha armipes* (Stål) (Das, 1996; Das and Ambrose, 2008b); *Lophocephala guerini* Laporte (Ambrose and Livingstone, 1979); *Polididus armatissimus* Stål, (Vennison, 1988); *Rhynocoris fuscipes* (Fabricius) and *R. marginatus* (Fabricius) (Ambrose, 1980) and *R. longifrons* (Stål) (Ambrose *et al.,* 2003; Kumar, 1993); *Scipinia horrida* (Stål) (Das, 1996); *Sphedanolestes himalyensis* Distant (Das, 1996; Das *et*

al., 2008b), *S. minusculus* Bergroth (Ambrose *et al.,* 2006; Kumar,1993), *S. pubinotum* Reuter (= *S. aterrimus* Distant) (Ambrose, 1980; Kumaraswami, 1991; Kumaraswami and Ambrose, 1993) and *S. signatus* Distant (Vennison, 1988; Vennison and Ambrose, 1990b); *Sycanus pyrrhomelas* Walker (Paniadima, 1987), *S. reclinatus* Dohrn (Vennison,1988) and *S. versicolor* Dohrn (Kumaraswami, 1991) and *Vesbius sanguinosus* Stål (Das, 1996; Das and Ambrose, 2008a).

Peiratinae:

Catamiarus brevipennis (Serville) (Kasinathan, 1983); *Ectomocoris tibialis* Distant (Ambrose, 1980; Ambrose and Livingstone, 1989a) and *E. Vishnu* Distant (Vennison, 1988).

Reduviinae:

Acanthaspis biguttula Stål, *A. bistillata* Stål, *A. inscripta* Distant, *A. livingstonei* Vennison and Ambrose and *A. pedestris* Stål (Livingstone and Ambrose, 1978; Ambrose, 1980; Rajan, 2012), *A. philomanmariae* Vennison and Ambrose, *A. quinquespinosa* (Fabricius), *A. rugulosa* Stål, *A. sexguttata* (Fabricius) and *A. siva* Distant (Ambrose, 1980; Ambrose and Livingstone, 1987b; Rajan, 2012) and *A. subrufa* Distant (Rajan, 2012); *Alloeocranum quadrisignatum* (Reuter) (Sahayaraj, 1991; Sahayaraj and Ambrose, 1992); *Edocla slateri* Distant (Vennison, 1988); *Empyrocoris* (=*Edocla*) *annulata* (Distant) (Kumaraswami, 1991) and *Velitra sinnensis* Walker (Vennison, 1988).

Salyavatinae:

Paralisarda malabarica Miller (Ambrose and Ravichandran, 2007) and *Petalocheirus brachialis* Stål (Sahayaraj, 1991).

Stenopodainae:

Oncocephalus annulipes Stål (Vennison, 1988).

2. Multidisciplinary Biosystematics

Multidisciplinary facets of biosystematics such as ecology, morphology, behaviour, biology and cytology of family Reduviidae have been studied. Ambrose (1999) in his monograph on Assassin bugs extensively reviewed the multidisciplinary facets of biosystematics of Reduviidae such as eggs (Kershaw, 1909; Readio, 1926; Miller, 1953, 1971; Southwood, 1956; Cobben, 1968; Salkeld, 1972; Hinton, 1981); pretarsal appendages (Reuter, 1910; Tullgren, 1918); salivary glands (Baptist, 1941); digestive system (Miyamoto, 1961; Bahadur, 1963a,b; Goodchild, 1963, 1966); dorsal and lateral abdominal scent glands, venation, genitalia, eggs, antennae, ocelli, tarsi, tibial setae (Usinger, 1943; Villiers 1948b); reproductive system (Carayon, 1950, 1969); prosternal groove (China and Usinger, 1949); wing venation (Davis, 1961); abdominal characteristics, such as fusion of pregenital segments, development of connexival, the dorsal position of first spiracle, position and modification of eighth tergum, phallus, genital characters (Davis, 1961); male and female genitalia and salivary glands (Scudder, 1959; Kumar, 1962).

Ambrose (2006), in his checklist of 464 Indian species of assassin bugs under 144 genera and 14 subfamilies, analysed taxonomical status of each species with diagnostic characteristic features such as presence or absence of tibial pad or fossula spongiosa in both fore- and midtibiae or only in foretibia; pterygopolymorphism; morphology of eggs and oviposition. The subfamily Harpactorinae was reported as the most abundant group with 146 species and 41 genera followed by Reduviinae and the Ectrichodiinae.

Thereafter, Ambrose *et al.* (2007c) reported in their annotated checklist of Indian Peiratinae, 39 species under 9 genera with their taxonomical status, Indian and worldwide distribution and their diagnostic ecological and morphological characters such as microhabitats and habitats, the curvature of rostrum, presence or absence of tibial pads and nature of wings. The morphological characters are correlated to the ecological characteristics

and behavioural and biological functions. The genus, *Ectomocoris* Mayr is the most abundant group with 21 species followed by the genus, *Peirates* Serville with five species.

2.1. Morphology

Usinger (1943) and Villiers (1948) discussed a number of morphological features namely antennae, ocelli, venation, dorsal and lateral abdominal scent glands, tibial setae, tarsi, genitalia, eggs and their utility in the biosystematics of Reduviidae.

Carayon *et al.* (1958) reported the taxonomic utility of morphological characters in the higher classification of Reduviidae.

Ambrose (1987a, b, 1999) examined the morphological characters of one hundred and sixty five species, morphometry and biological parameters of twenty five species and behavioural parameters of forty species of reduviids and discussed their taxonomic utility and traced the phylogeny.

Murugan (1988) traced the evolutionary origin of reduviids from tropical rainforest ecosystem to drought prone scrub jungles and semiarid zone ecosystems and reported that Salyavatinae as the parental stock from which Reduviinae and Triatominae have evolved in one direction and Ectrichodiinae and Peiratinae in another direction.

Ravichandran (1988) studied the taxonomic characteristics of 95 species of non-tibiaroliate reduviids belonging to 38 genera and 6 subfamilies viz., Emesinae, Harpactorinae, Holoptilinae, Saicinae, Stenopodainae and Tribelocephalinae.

Rukmani (1992) studied morphometrical, biological, growth pattern and rate, linear regression co- efficients of postembryonic developmental characteristics and behavioural tools together and demarcated and distinguished three subfamilies viz., Harpactorinae, Reduviinae and Peiratinae. She included thirty six morphometric indices comparing different parts of twenty seven species of reduviids belonging to fourteen genera and concluded that these indices are useful at subfamily level.

Ambrose and Ambrose (1996a) reported that utility of morphological, morphometrical, growth, ecological, biological, behavioural, biochemical and cytogenetic characteristics in the insect biosystematics in general with special emphasis on assassin bugs.

Khokar (2000) traced the cladistic relationship between twenty five species of reduviids based on morphological characteristics such as head capsule, ocelli, labium, metacoxal comb, tarsi, cells of hemelytra, abdominal scent glands and the male and female genitalia. He further suggested that the subfamily Harpactorinae is the most plesiomorphic and Stenopodainae appears to have evolved from ancestor similar to Harpactorinae, i.e., symplesiomorphic, Reduviinae, Ectrichodiinae and Tribelocephalinae have several apomorphic, i.e., specialized derived characters while Peiratinae appears to be highly autapomorphic.

George *et al.* (2005) studied UPGMA cluster analysis of thirty three morphological and biological characteristics of thirty species of reduviids belonging to seventeen genera and three subfamilies and found intra- and intergeneric and interfamilial affinities. The phylogenetic relationship between the three subfamilies viz., Harpactorinae, Peiratinae and Reduviinae suggest that both Peiratinae and Reduviinae might have evolved from a common ancestor. The reduviines are closer to harpactorines and peiratines are broadly closer to harpactorines and narrowly closer to reduviines.

Weirauch (2008) analysed a cladistics of higher- level taxa of Reduviidae of twenty one subfamily level taxa and twenty eight tribes of Reduviidae based on one sixty two morphological characters and seventy five in-group and out-group species. She observed a sister group relationship of Hammacerinae with the remaining Reduviidae, the monophyly of the Phymatinae complex, the relative basal position of Harpactorinae within Reduviidae as well as a novel hypothesis on the relationships within this group, and the sister group relationship of Ectrichodiinae + Tribelocephalinae and their placement in a clade that also

contains Emesinae, Saicinae and Visayanocorinae. The analysis further supports a clade formed by paraphyletic Salyavatinae + Sphaeridopinae, renders Vesciinae non- monophyletic and demonstrates the polyphyly of Reduviinae. Pseudocetherinae is shown to group with some Reduviinae. Tritominae is supported as a monophyletic group and are nested among additional Reduviinae and Stenopodainae.

Ambrose and Ambrose (2009b) analysed 36 morphometric indices belonging to 27 species, 14 genera and 3 subfamilies viz., Harpactorinae, Peiratinae and Reduviinae by direct observation as well as unweighted pair group method using arithmetic mean (UPGMA) incorporating Euclidean distances, Nei and Li's genetic distance coefficient and Jaccard's coefficient to understand diagnostic intra- as well as intersubfamilial and generic affinities. Their analysis further revealed the position of genera in the existing divisions (tribes) of subfamilies and their phylogenetic relationship. Further, it clearly suggested two diversified lines of evolution of Peiratinae and Reduviinae from Harpactorinae than the earlier suggested straight line evolution of these two subfamilies from Harpactorinae.

Singh *et al.* (2011) analysed linear regression coefficient values of twenty one morphological characteristics of four *Rhynocoris* species viz., *R. fuscipes* (Fabricius), *R. kumarii* Ambrose and Livingstone, *R. longifrons* (Stål) and *R. marginatus* (Fabricius), twenty one such computed values were analysed at two levels, i.e., interspecific and intraspecific to find out the biosystematics utility and values are subjected to cluster analysis. The analysis of linear regression coefficient values (r) of postembryonic developmental morphometry of four *Rhynocoris* species revealed three *Rhynocoris* generic markers as confirmed by the dendrogram with 100 % similarity and interspecific and intraspecific affinity as confirmed by dendrogram node of grouped characters with up to 98.25% similarity.

2.1.1. External morphology:

2.1.1.1. Head and Cephalic appendages

Wygodzinsky and Lohdi (1989) and Zrzavý (1990) studied the antennal trichobothria as a morphological tool. Weirauch (2003) studied sensillar structures of the antennal pedicel in Reduviidae and Pachynomidae. The cave organ, a chemoreceptive structure, previously reported only for haematophagous Triatominae, is described here also for representatives of Peiratinae, Reduviinae and Stenopodainae. She discussed the systematic implication of the occurrence of this sensillar structure. Further, four sclerites located in the membrane between pedicel and preflagelloid are described and used as landmarks for the recognition of individual trichobothria in Reduviidae and Pachynomidae. She also systematically studied and discussed characters of the trichobothrial socket. Homology of the distal most trichobothrium of Reduviidae with the single trichobothrium in Pachynomidae is proposed. This hypothesis is based on the structure of the cuticle surrounding the trichobothria and on the trichobothrial position relative to the four sclerites of the pedicello - flagellar articulation.

Wygodzinsky (1966) illustrated and described enlarged setae or spines on the second labial segment in the subfamilies Emesinae, Saicinae and Vesciinae.

Murugan (1988) studied the mandibular and maxillary stylets and their taxonomic significance in seventy three species of reduviids under twenty eight genera and five subfamilies viz., Acanthaspidinae (Reduviinae and Tritominae together), Ectrichodiinae, Peiratinae, Salyavatinae and Triatominae.

Ravichandran (1988) made a similar study in ninety five species of non- tibiaroliate reduviids belonging to thirty eight genera and six subfamilies viz., Emesinae, Harpactorinae, Holoptilinae, Saicinae, Stenopodainae and Tribelocephalinae. He found direct relationship between the evolution of carnivory and the length, shape and segmentation of labium. He systematically analysed the mandibular and maxillary stylets of non- tibiaroliate reduviids

and found monomorphic mandibular stylets in Emesinae, Harpactorinae, Saicinae, Stenopodainae and Tribelocephalinae (except in Holoptilinae) and dimorphic maxillary stylets in Emesinae, Harpactorinae, Holoptilinae, Saicinae, Stenopodainae and Tribelocephalinae where the right maxillary stylet was found uniformly with one or two rows of barbs and the left stylet was completely smooth without any barbs. Right maxillary stylet was found uniformly with one or two rows of barbs. He traced carnivory in Reduviidae from a proreduviid ancestor which had straight labium with highly serrated stylets and trace of tibiarolium as found in Holoptilinae. According to him tibiarolium might have evolved from a Harpactoroid ancestor.

Cai *et al.* (1994) studied the morphology, postembryonic development and evolution of rostral stridulation in Reduviidae with special reference to their taxonomic importance.

Murugan *et al.* (1996) and later Livingstone *et al.* (1998) studied the functional organization of labium especially the mandibular stylets of seventy seven species of Reduviidae representing forty five genera and eleven subfamilies and analysed the phenotypic diversity. They reported isomorphous mandibular stylets in Reduviidae except in the subfamily Holoptilinae and the spatulated mandibular stylets of the myriophagus Ectrichodiinae that aid in sawing apart the septa between the segments of their prey. The terminal mandibular denticles were attributed to the nature of prey, feeding strategy and nutritional ecology of reduviids and on this basis the evolutionary origin of Reduviidae was proposed.

2.1.1.2. Thorax and Thoracic appendages

China and Usinger (1949) reported the presence of prosternal groove as the morphological characteristics of reduviids. Goel (1984) formulated a taxonomic key and demarcated four subfamilies viz., Peiratinae, Reduviinae, Stenopodainae and Tribelocephalinae based on the observations on thoracic skeleton.

Goel (1984) later reported that pyriform and fairly rotatory meso- and metathoracic coxae. He further reported the presence of equal sized supercoxal lobes and elongated prothoracic coxae, which support the fossula spongiosa to provide considerably a strong hold and forceful suction during feeding and this peculiar mode of feeding rendered effective by small opening of coxal sockets than the size of prothoracic coxae and absence of metapleural stink gland are the characteristics of reduviids.

Weirauch (2006) studied the biosystematics significance of metathoracic glands and associated evaporatory structure in Reduvioidea with observation on the mode of function of the metacoxal comb.

Reuter (1910) and Tullgren (1918) considered the taxonomical significance of pretarsal appendages in Heteroptera. Pretarsal structures have proven to be of systematic value in Heteroptera at the higher group level (Wheeler *et al.,* 1993). Goel (1969) reported the taxonomic value of setae of claws to demarcate Reduviidae from other heteropterous families. Reduction of tarsal segments and the substitution of fossula spongiosa in pro- and metathoracic legs for arolia and pseudoarolia (Goel and Schaefer, 1970) and unquitractor plate (Dashman, 1953) are taxonomically significant morphological characters. Data presented by Cobben (1978) imply that pretarsal structure among Reduviidae is rather uniform in contrast to the structural diversity observed in some other groups of Heteroptera. However, Wygodzinsky (1966) showed that there is a considerable amount of diversity in the pretarsi of Emesinae, on which he based part of his phylogenetic scheme of this group.

The reduviids have been categorized into two major groups viz., reduviids with tibial pads and without tibial pads. The shape, size and development of tibiae and tibial pads vary in different species certain subfamilies could be easily differentiated from the other subfamilies based on their structural diversity (Livingstone and Ambrose, 1984; Ambrose, 1999; Weirauch, 2007; Ambrose and Ambrose, 2009a). Ravichandran (1988) reported that

shifting of basitarsus slightly dorsal as a prerequisite for the development of tibiarolium and such shifting has followed a distinct course in different subfamilies and traced the phylogenetic relationship of Harpactorinae with Tribelocephalinae, Saicinae with Stenopodainae and Emesinae with Holoptilinae.

Weirauch (2005) systematically analyzed pretarsal structures in Reduviidae of 22 higher-level taxa and several outgroup representatives using scanning electron microscopy (SEM) and light microscopy. Structures of the distal tarsomer, which appear to be functionally correlated with the pretarsus, are documented for the first time in Heteroptera. She also discussed pretarsal structures, including the unguitractor plate and the tarsal marginal setae among reduviid groups in a phylogenetic context.

According to Davis (1961) the reduviid venation is distinct from that of other Cimicomorpha and is best characterized by a narrow postorbital sector containing sub-parallel PCU and 1st A veins. The venation is useful in characterizing three major group of subfamilies Reduviidae viz., Phymatinae complex, Peiratinae complex and Harpactorinae complex. He also correlated the nature of venation and their prey capturing methods in Phymatinae and Peiratinae complex of Reduviidae.

Goel (1966, 1970) reported the taxonomic values of wing coupling apparatus and wing venation to demarcated Reduviidae from heteropterous families. He (1970) also studied marginal radius and cubitus and absence of arbital extension from the hind wing.

2.1.1.3. Abdomen and Abdominal appendages

Davis (1957) studied the morphology of abdomen of Reduviidae and discussed its bearing on phylogeny. He (1966) also studied abdominal characteristics such as fusion of pregenital segments, development of connexiva, the dorsal position of first spiracle, position and modification of eighth tergum, phallus, genital characters and male and female genitalia.

Pruthi (1925) described the male genitalia of reduviids representing ten subfamilies and their taxonomic significance. Carayon (1944, 1950) studied the male reproductive system of reduviids representing nine subfamilies and demonstrated the unique family characteristic of male system. Giacchi (1983) proved that the morphology and chaetotaxy of the female genital segments and genitalia have characters of taxonomic value useful to separate genera and species. He also analysed the ovipositor's structure in Stenopodainae and other Reduviidae, reaching to the conclusion that the so called "styloid" or "third valvula" are the gonocoxae of the ninth segment. Lent and Jurberg (1984) analyzed and compared eight structures of male external genitalia of tribe of Bolboderini.

Murugan (1988) examined the male genitalia and analysed the functional relationship between the development of pygopore spine with the armature of endotheca, phallotheca, dorsal phallic sclerite and spermatophore pouch.

Male genitalia studies of Ravichandran (1988) indicated the diverse nature of pygophore in Harpactorinae. Maximum development is seen in Saicinae and could be compared to that of Acanthaspidinae. Parameres differ among families as well as among the members of Emesinae. Ravichandran *et al.* (1998) studied the structural and functional significance of male intromittent organ of Harpactorinae, Stenopodainae, Emesinae, Saicinae and Holoptilinae and discussed their utility in the biosystematics of Reduviidae.

Gutiérrez (1999) studied the taxonomic value of genitalia of 8 Apiomerini genera. He also studied a comparative analysis among the genital structures allowed to identify the different genera and subgenera. Yang (2003) described and compared external male genitalia of five reduviids. Moulet (2005) described the female genitalia of some *Sphedanolestes* species.

Sucheta and Khokhar (2004) studied male genitalic characters and traced the phylogenetic relationships amongst different subfamilies of Reduviidae. The subfamilies

Stenopodainae and Harpactorinae share some plesiomorphic characters; Holoptilinae appears to be distinct due to whip- like process of each strut and reduced endosoma while Tribelocephalinae and Peiratinae indicate apomorphic characters especially possession of either the median and lateral symmetrical pygophoral lobe or median asymmetrical pygophoral lobe. Both claspers are similar, simple, somewhat sickle- shaped, curved near apex in all the subfamilies except Peiratinae and some Reduviinae where left and right claspers are dissimilar, broad and asymmetrical. Peiratinae are autapomorphic because of asymmetry of clasper, pygophore and phallus as well as symmetrical, sclerotized eighth sternum with median process. Ectrichodiinae, Reduviinae, Salyavatinae and Triatominae occupy intermediate position because of some plesiomorphic and apomorphic characters of claspers, pygophore and phallus.

Forero and Weirauch (2012) analysed the comparative genitalic morphology in the New World resin bugs Apiomerini genera, the male and female ectodermal genitalic structures are documented for all but one of the 12 extant genera of Apiomerini, including 12 species of the speciose genus *Apiomerus*. Descriptions and digital micrographs are provided for the pygophore, parameres, and phallus of the male, and for the tergite 8, syntergite 9/10, gonocoxae, gonapophyses, gonoplac and bursa copulatrix of the female.

2.1.2. Internal organs:

2.1.2.1. Digestive system and Salivary glands

Salivary glands of reduviids were studied by Baptist (1941), Kumar (1962), Haridass and Ananthakrishnan (1981a) and Agnes (1990). Kumar (1962) in addition to the characteristics of the salivary glands described the male and female genitalia of representatives of six subfamilies of Reduviidae and commented on their possible taxonomic value at subfamily level. Haridass and Ananthakrishnan (1981a) studied the functional morphology of salivary system of sixteen reduviids belonging to seven subfamilies.

According to them the salivary system of most reduviids studied typically conforms to the general heteropteran plan of principal and accessory glands with their ducts, their vesicular gland being similar to those found in other Cimicomorpha families. But the structure and number of the principal glands are found to be specific to different subfamilies.

Digestive system of reduviids was studied by Miyamoto (1961), Bahadur (1963a, b), Goodchild (1963, 1966), Haridass and Ananthakrishnan (1981b), Santha (1986), Sivaraj (1986), Udayakumar (1986) and Vellingirinathan (1986). Louis and Kumar (1973) studied the digestive and reproductive organs of Reduviidae and correlated their findings with phylogeny of reduviids.

Different feeding strategies of reduviids were studied by Cobben (1978), Santha (1986), Udayakumar (1986), Haridass *et al.* (1987) and Ambrose (1999).

2.1.2.2. Reproductive organs and Accessory glands

Haridass and Ananthakrishnan (1981b) studied the functional morphology of pylorus and rectal glands of Reduviidae belonging to seven subfamilies and observed the differences among the subfamilies.

Ambrose (1980) described the external morphology of female reproductive system with special emphasis on pseudospermatheca in nine reduviids. The reproductive systems of a harpactorine, a reduviine and two peiratine reduviids are reported in detail (Agnes, 1980; Lalithamani, 1983; Felicia, 1984;Itty, 1984; Swaminathan, 1984; Dhanapal, 1988; Samuel, 1991). Haridass (1987) described and studied the female reproductive system and activities of seven reduviids.

Haridass (1987) described the sub- rectal glands as lateral expansions of the bursa storing eggs before oviposotion. The shape and size vary in different species. George (1988a) studied the bursal glands of Reduviidae and classified into three types. She found it vermiform in Harpactorinae, Stenopodainae, Salyavatinae and Acanthaspidinae, ampulliform

in Ectrichodiinae, Emesinae and Salyavatinae and characteristically distal ampulliform in Acanthaspidinae and Peiratinae.

George (1988b) also studied the sub- rectal glands of Reduviidae and distinguished them as a characteristic feature of Harpactorinae. She correlated the presence of these glands with their habits of gluing their eggs in compact masses to the substratum by using large quantum of spumaline secreted by them.

Ambrose and Vennison (1990) reported the diversity of spematophore capsule of reduviids belonging to four subfamilies viz., Acanthaspidinae, Ectrichodiinae, Harpactorinae and Peiratinae.

2.1.2.3. Egg

The morphology of eggs of reduviids belonging to various subfamilies were studied by Kershaw (1909), Readio (1926), Bose (1951), Miller (1956, 1971), Southwood (1956), Odhiambo (1959), Cobben (1968), Salkeld (1972), Ambrose and Livingstone (1979, 1989b), Ambrose (1980, 1987a, b, 1999), Hinton (1981), Haridass (1985b, 1986a, b, 1988), Haridass *et al.* (1987), George (1988c), Vennison and Ambrose (1990c), Das and Ambrose (2008b) and Ambrose and Ambrose (2009a).

2.2. Ecotypic diversity

Ambrose (1999) reported that the reduviid predators of a particular species collected from different habitats exhibit substantially different colours, shapes and sizes from each other and yet members of one microhabitat could successfully breed with members of another microhabitat. Such apparent dissimilarity seen among individuals of the same species collected from different habitats, is referred to as ecotypic diversity. This could be useful to the interpretation of species concept and this specialization was considered by Mayr (1963, 1969) as an ecological race or habitat race, otherwise referred to as ecotype.

Ecotypism among reduviids was reported among the member of Ectrichodiinae such as *Neohaematorrhophus therasii* Ambrose and Livingstone (Sahayaraj, 1991; Ambrose, 1999) and Harpactorinae viz., *Euagoras plagiatus* (Burmeister) (Das, 1996; Ambrose *et al.,* 2008a), *Rhynocoris longifrons* (Stål) (Ambrose *et al.,* 2007), *Rhynocoris marginatus* (Fabricius) with three morphs viz., Niger, Sanguineous and Nigrosanguineous (Ambrose, 1980, 1987c; 1999), *Sphedanolestes minusculus* Bergroth (Das, 1996; Ambrose *et al.,* 2006) and *Sphedanolestes pubinotum* Reuter (Ambrose, 1986; Das, 1996; Ambrose, 1999) and Peiratinae viz., *Ectomocoris tibialis* Distant (Sahayaraj, 1991; Ambrose, 1999) and Reduviinae viz., *Acanthaspis pedestris* Stål (Ambrose, 1980; Ambrose and Livingstone, 1987, 1988a; George *et al.,* 2007) and *Acanthaspis siva* Distant (Ambrose, 1980).

2.3. Polymorphism

Polymorphism is the variability within a population (Mayr, 1963, 1969). Ford (1937) reported polymorphism as "the occurrence together in the same habitat at the same time of two or more distinct forms of the same species in such a proportion that the rarest of them cannot be maintained by recurrent mutation".

The differential development of wing in between the sexes or both sexes of a species and by different external morphological features indicated that the intra- and interspecific polymorphism in reduviids (Readio, 1927).

Reduviids exhibit morphs by their differential development of wings as well as by their differential external morphological features associated to thorax, leg or connexivum (Ambrose, 1999; Distant, 1902, 1910; Louis, 1974; Wallace, 1953; Wygodzinsky, 1979; Ambrose and Livingstone, 1988b, 1990; Vennison, 1988).

The following reduviids exhibit intraspecifc polymorphism: members of Ectrichodiinae viz., *Neohaematorrhophus therasii* Ambrose and Livingstone (Sahayaraj, 1991; Ambrose, 1999) and Harpactorinae viz., *Rhynocoris marginatus* (Fabricius) (Ambrose,

1980; Ambrose and Livingstone, 1988b) and Peiratinae viz., *Ectomocoris tibialis* Distant (Sahayaraj, 1991; Ambrose, 1999) and Reduviinae viz., *Acanthaspis siva* Distant (Ambrose, 1980; Ambrose and Livingstone, 1990).

Ambrose and Maran (2000) studied the polymorphic diversity in salivary and haemolymph proteins and digestive physiology of *R. marginatus* based on endopeptidase and exopeptidase activities.

George (2000) studied the polymorphic adaptations in reproductive strategies of *R. marginatus* on *Earias vittella* (Fabricius) with reference to fecundity, longevity rate of population multiplication and cohort generation time.

George and Ambrose (2001) studied the adaptive polymorphic resistance of three morphs of *R. marginatus* viz., niger, sanguineous and nigrosanguineous to the toxic effects of the 50 % lethal concentration (LC_{50}) of methylparathion quinalphos and endosulfan.

Ambrose *et al.* (2008b) studied the relative toxicity of two organophosphorous insecticides viz., monocrotophos and methylparathion and polymorphic resistance by detoxification through carboxyl esterase activity .

The conspicuous alary polymorphism reported by Campos *et al.* (2011) in *Mepraia spinolai* (Porter) and *Mepraia gajardoi* Frías from natural populations, unique in the triatominae subfamily revealed that the brachypterous wings of both species are smaller than macropterous wings of *M. spinolai.* This analysis revealed that based on the wings of *Mepraia* species two separate distinct groups are consistent with the two described species.

2.4. Biology

Ambrose (1987b, 1999) demarcated the three subfamilies of Reduviidae viz., Acanthaspidinae, Harpactorinae and Peiratinae based on their biological characteristic such as time and duration of eclosin and ecdysis, incubation period, oviposition, hatchability, stadial period, adult longevity, sex ratio and number of generation(s) per year. Later,

Ambrose and Ambrose (1996a, b) further demarcated and distinguished three subfamilies viz., Harpactorinae, Peiratinae and Reduviinae based on biological characteristic.

Rukmani (1992) studied the postembryonic developmental growth indices using growth rate of twenty five different parts of twenty four species belonging to thirteen genera suggests that they are highly useful in the demarcation of species, genera and to a certain extent the subfamilies and to trace their affinity.

Ambrose and Ambrose (2003) also revealed that linear regression coefficient values (r) of postembryonic development morphometry are an effective tool in the biosystematics of reduviids. The (r) values were found useful to identify and understand the affinity of reduviids at subfamily, generic and species level.

Ambrose and Ambrose (2004) further studied the utility of growth indices in the biosystematics of 30 species of reduviids in three subfamilies viz., Harpactorinae, Peiratinae and Reduviinae.

Das *et al.* (2008a, b) studied the biology and behaviour of *Alcmena spinifex* Thunberg and *Sphedanolestes himalayensis* Distant and observed that harpactorine characteristic the pin and jab mode of predation and the precopulatory female cannibalism over male were not present.

Ambrose *et al.* (2009) studied the biology and behaviour of *Sphedanolestes variabilis* Distant and also its positive functional response to the prey density. Laboratory breeding observations indicated it as a multivoltine species.

2.5. Behaviour

Miller (1971) differentiated the subfamilies of Reduviidae based on their prey or host. Blood feeding was reported as a characteristic of haematophagous reduviid subfamily Triatominae (Lent and Wygodzinsky, 1979; Haridass and Ananthakrishnan, 1980a). Haridass

and Ananthakrishnan (1980a) discussed the predatory models of entomophagous reduviids and the predatory behaviour of haematophagous reduviids.

The subfamilies of Reduviidae were further classified according to their prey capturing methods (Wygodzinsky, 1966; Miller, 1971). Chase and pounce method of prey capturing was observed in the subfamilies like Peiratinae and Ectrichodiinae (Miller, 1953, 1971; Haridass and Ananthakrishnan, 1980a, b; Livingstone and Ambrose, 1984; Ambrose, 1987a, b; Haridass, 1985). Wait and grab method of feeding was observed in the members of the subfamilies such as Reduviidae, Salyavatinae and Stenopodainae (Odhiambo, 1958a; Miller, 1971; Livingstone and Ambrose, 1978; Haridass, 1985; Ambrose *et al.,* 1986). Pin and jab method of prey capturing was reported as the characteristic of the reduviid subfamilies viz., Harpactorinae and Rhaphidosominae (Edwards, 1962, 1966; Miller, 1971; Parker, 1969, 1971, 1972; Haridass, 1985; Ambrose, 1999; Das *et al.,* 2008a, b).

Based on their prey preference and their mode of predations in reduviids, Ambrose and Ambrose (2009a) categorized them into six types viz., sticky-trap, raptorial, wait and grab, pin and jab, chase and pounce and blood feeding types.

Livingstone and Ambrose (1984) reported the nature of egg deposition in different subfamilies of Reduviidae. Ambrose (1987a, b, 1999) and Ambrose and Ambrose (2009a) categorized the oviposition of reduviids belonging to Oriental subfamilies into five distinct groups: 1. eggs laid in clusters and cemented to each other and to the substratum such as tree trunks, leaves, boulders etc., e.g. Harpactorinae 2. eggs laid in clusters glued basally and to the substratum but not glued to each other longitudinally e.g. Ectinoderinae and Ectrichodiinae 3. eggs individually cemented to the substratum e.g. Holoptilinae 4. eggs buried deep inside the soil e.g. Peiratinae, Physoderinae and Stenopodainae and 5. eggs loosely strewn around eratically without any pattern just below or above the surface of soil e.g. Centrocneminae, Reduviinae, Salyavatinae and Triatominae.

Camouflaging was reported as a characteristic of reduviid subfamilies like Reduviinae, Stenopodainae and Salyavatinae (Odhiambo, 1958b; Miller, 1953a, 1971; Ambrose, 1980; Livingstone and Ambrose, 1984). Ambrose (1987b, 1999) distinguished Acanthaspidinae from Peiratinae and Harpactorinae by the presence of nymphal camouflaging only in the Acanthaspidinae.

Ambrose (1987b, 1999) and Ambrose and Ambrose (2009a) utilized the chronological sequential events and the finer details of predation such as arousal, approach, capturing, paralyzing and sucking and other aspect of predation such as number of sucking sites, nymphal congregational feeding and nymphal cannibalism in the biosystematics of three subfamilies of Reduviidae. Ambrose also differentiated Harpactorinae from Acanthaspidinae (Reduviinae and Triatominae) and Peiratinae by the presence of death feigning, spitting behaviour and head nodding behaviour.

Ambrose (1987a, 1999) and Ambrose and Ambrose (1996c) demarcated three subfamilies of Acanthaspidinae (Reduviinae and Triatominae), Harpactorinae and Peiratinae of Reduviidae based on their premating period, mating acts, precopulatory and postcopulatory cannibalism, duration of copulation, time taken for the ejection of spermatophore capsule, frequency of mating and the interval between two successive matings.

Rukmani (1992) extensively analysed the parameters of predatory, mating ovipositional, parental care, nymphal camouflaging, death feigning and spitting behaviour of large number of reduviids and found their usefulness to demarcate the subfamilies and to identify their affinity.

Ambrose and Ambrose (2001) analysed behavioural aspects viz., predation and offensive and defensive behaviours such as nympal camouflaging, death feigning and rolling, extension of rostrum and spitting, head nodding and stridulation, stinging and emitting odour

belonging to the members of three subfamilies viz., Harpactorinae, Reduviinae and Peiratinae.

2.6.Cytology

Reduviidae have holocentric or holokinetic chromosomes in which the centromere is distributed along the entire length of the chromosome and the highest number of multiple chromosomes in Hetroptera (Schuh and Slater, 1995). Jande (1959a,b) studied chromosome number and sex mechanism of Indian Heteroptera.

Manna and Mallick (1980) reported that reduviids possess relatively high diploid number and having mostly multiple X chromosomes but the single Y with an exception in *Ectrychotes abbreviatus* that has XO males with prereductional meiosis.

Cytotaxonomy based on meiotic chromosome number and constitution in mainly males of 41 species of Heteroptera including 12 of species of reduviids under 7 subfamilies were studied by Manna and Mallick (1981).

Dey and Wangdi (1988) analysed chromosome number and sex chromosome system in forty four species of Heteroptera.

The main source of chromosome pholymorphism in Triatominae is the variation in amount, behaviour, and position of highly repetitive DNA regions, identified as hetrochromatin by C-banding. The variation of this C-hetrochromatin leads to the identification of cryptic species such as *Triatoma sordida* (Stål) and *Triatoma garciabesi* Carcavallo *et al*. (Panzera *et al.,* 1997; Jurberg *et al.,* 1998).

Poggio *et al.* (2007) studied karyotype evolution of Reduviidae and explained the reduction in autosomal number through fusions, and the increase in the number of sex chromosomes through fragmentations. The presence of multiple sex chromosomes system is much more frequent in Harpactorinae and Stenopodainae than in the other subfamilies.

Kaur *et al.* (2012) studied the internal male reproductive organs and course of meiosis in a predatory species, *Repipta taurus* (Fabricius). Kaur and Kaur (2013) analysed chromosomes and their meiotic behavior in twelve species of subfamily Harpactorinae from North India. All the species show twelve pairs of autosomes and X multiplicity with 9 species having X1X2X3Y and 3 species having X1X2Y.

2.7. Haemocytes:

The role of haemocytes and types were observed in reduviids by Wigglesworth (1933,1937,1955,1956a,b,c, 1965, 1973,1975), Harington (1961), Jones and Liu (1961), Coles (1963), Jones (1964, 1965a,b, 1967a,b), Barrett and Friend (1966), Lai-Fook (1970) and Gringorten and Friend (1979) in *Rhodnius prolixus* Stål and Agosin *et al.* (1965) in *Triatoma infestans* Klug; Ambrose and George (1994) and George and Ambrose (1998) studied the total and differential haemocytes count and their morphometry in the life stages and adult of *C. brevipennis* and *A. pedestris* respectively. Ambrose and George (1996) studied the total and differential haemocytes count in three morphs of *R. marginatus.* Ambrose *et al.* (1999) estimated the total and differential haemocyte counts among the life stages of *R. marginatus.*

Ambrose and George (1994,1996), George and Amrose (1998, 2000a,b) and Ambrose *et al.* (1999) stated that granular haemocytes and plasmatocytes are the most abundant types in predatory assassin bugs.

Ambrose and George (1994), George and Amrbrose (1998) stated that haemocytes number increased during development and maximum number was attained in the adults in *C. brevipennis* and *A. pedestris.*

3. Molecular Markers

Protein Profile

Description: The Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) is one of the most powerful experimental techniques used for the separation of proteins, and most proteins are separated according to their molecular size by this technique (Okada *et al.,* 2011).

Though electrophorectic analysis was carried out to understand the functions and biochemical components of the salivary glands of reduviids such as *Platymerus rhadamanthus* Gerst (Edwards, 1962), *Acanthaspis pedestris* Stål (Morrision, 1989) and three *Rhynocoris* Hahn species (Kumaraswamy, 1991) electrophoresis as a biosystematic tool was not fully exploited except the work of Brodie and Ryckman (1967) in kissing bugs, Salkeld (1969) in twenty three insect species representing seven orders, Adams and Ryckman (1969) in *Triatoma rubida* Uhler, Soares *et al.* (1998) in two *Rhodnius* species, Barbosa *et al.* (1999) in *Panstrangylus megistus* Burmiester, *Pineda et al.* (2008) in *Triatoma dimidiate* Latreille. They used either salivary or egg protein profile as a biosystematic tool.

Baskar *et al.* (2010) analysed the protein profile of fat body in different life stages of *Rhynocoris marginatus* (Fabricius). Also the protein profiles pattern and their importance was studied in *Trypanasoma cruzi* Chagas and *Trypanasoma rangeli* Tegera by Mejia *et al.* (2004).The salivary protein profile of three *Rhynocoris* species viz., *Rhynocoris fuscipes* Fabricius, *R. kumarii* Ambrose and Livingstone and *R. marginatus* Fabricius were studied and compared to find out the paralytic potential of three prey species viz., tobacco cutworm, *Spodoptera litura* F., blister beetle, *Myoslabris pustulata* Thunberg and red cotton bug, *Dysdercus cingulatus* F. (Maran *et al.,* 2011).

Though protein profile was used as a marker for other insects, such work is very limited for reduviids.

Merits

The gel electrophoresis can provide information about the molecular weights and charges of proteins, the subunit structures of proteins, and the purity of particular protein preparation. It is relatively simple to use and it is highly reproducible. The most common use of gel electrophoresis is the qualitative analysis of complex mixtures of proteins (Monk, 1987, Schagger, 2003).

Demerits

This method does have limitations. For example, identification of a band on a protein gel is not considered as a positive proof of identity. A great many different polypeptides have very similar molecular masses. One band may mask the presence of more than one polypeptide. Incomplete denaturation, unusual amino acid sequences, and/or presence of non-protein residues can affect mobility, resulting in considerable error estimating molecular mass. Highly basic protein, which contains an abundance of positively charged aminoacids migrate more slowly in SDS-PAGE due to a reduced charge-to-mass ratio, resulting in a higher apparent Molecular Weight. Proteins with high proline content or with other usual amiono acid sequences show a decreased electrophoretic mobility as a result of links and structural rigidity caused by the primary sequence (Bollag *et al.,* 2002).

Application: Analysis of whole-cell protein by SDS-PAGE contributed to a notable progress in the taxonomy (Giammarinaro *et al.,* 2005).

These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate genetic marker will depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and know-how, time constraints and financial limitations.

Allozymes

Description: The allozymes are allelic variants of enzymes encoded by structural genes. Allelic variations can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt, e.g. nitro-blue tetrazolium. Usually two, or sometimes even more loci can be distinguished for an enzyme and these are termed isoloci. Therefore, allozyme variation is often also referred to as isozyme variation (Kephart, 1990; May, 1992).

Merits: The zymograms (the banding pattern of isozymes) can be readily interpreted in terms of loci and alleles, or they may require segregation analysis of progeny of known parental crosses for interpretation. Sometimes, however, zymograms present complex banding profiles arising from polyploidy or duplicated genes and the formation of intergenic heterodimers, which may complicate interpretation (Tanksley and Orton, 1983).

The strength of allozymes is its simplicity. Because allozyme analysis does not require DNA extraction or the availability of sequence information, primers or probes, they are quick and easy to use. Some species, however, can require considerable optimization of techniques for certain enzymes. Simple analytical procedures allow some allozymes to be applied at relatively low costs, depending on the enzyme staining reagents used. Allozymes are co-dominant markers that have high reproducibility (Hamrick and Godt, 1997).

Demerits: The main weakness of allozymes is their relatively low abundance and low level of polymorphism. Moreover, proteins with identical electrophoretic mobility (co-migration) may not be homologous for distantly related germplasm. In addition, their selective neutrality may be in question (Berry and Kreitman, 1993; Hudson *et al.,* 1994; Krieger and Ross, 2002). Lastly, often allozymes are considered as molecular markers since they represent enzyme variants, and enzymes are molecules. However, allozymes are in fact phenotypic markers, and as such they may be affected by environmental conditions. For example, the banding

profile obtained for a particular allozyme marker may change depending on the type of tissue used for the analysis. This is because a gene that is being expressed in one tissue might not be expressed in other tissues. On the contrary, molecular markers, because they are based on differences in the DNA sequence, are not environmentally influenced, which means that the same banding profiles can be expected at all times for the same genotype.

Applications: The allozymes have been applied in many population genetics studies, including measurements of outcrossing rates (Erskine and Muehlenbauer, 1991). Allozymes are particularly useful at the level of conspecific populations and closely related species (Flores *et al.,* 2001; Noireau *et al.,* 2002; Monteiro *et al.,* 2002). They have been used, often in concert with other markers, diversity studies (Lamboy *et al.,* 1994; Ronning and Schnell, 1994), to study interspecific relationships (Garvin and Weeden, 1994), for fingerprinting purposes (Maass and Ocampo, 1995), allelic frequencies in germplasm collections over serial increase cycles in germplasm banks (Reedy *et al.,* 1995) and to identify parents in hybrids (Parani *et al.,* 1997) and the mode of genetic inheritance (Warnke *et al.,* 1998). Dujardin *et al*. (1998) studied the allozyme frequencies and their epidemiological features among Andean (localities in Bolivia) *Triatoma infestans* Klug.

Restriction Fragment Length Polymorphism (RFLP)

Description: The DNA sequence variation affecting the absence or presence of recognition sites of restriction enzymes, and insertions and deletions within two adjacent restriction sites, form the basis of length polymorphisms (Botstein *et al.,* 1980; Burr *et al.,* 1983; Helentjaris *et al.,* 1985; Evola *et al.,* 1986). The Restriction Fragment Length Polymorphism are bands that correspond to DNA fragments, usually within the range of 2–10 kb, that have resulted from the digestion of genomic DNA with restriction enzymes. The DNA fragments are separated by agarose gel electrophoresis and are detected by subsequent Southern blot hybridization to a labeled DNA probe (Burke *et al.,* 1991). Labelling of the probe may be

performed with a radioactive isotope or with alternative non-radioactive stains, such as digoxigenin or fluorescein (Karp *et al.,* 1997).

Merits: The RFLPs are generally found to be moderately polymorphic. In addition to their high genomic abundance and their random distribution, RFLPs have the advantages of showing co-dominant alleles and having high reproducibility (Neale and Williams, 1991).

Demerits: The main drawbacks of RFLPs are the requirement of laborious and technically demanding methodological procedures, and high expense (Castiglione *et al.,* 1993). Moreover, large quantities of purified, high molecular weight DNA are required for each DNA digestion. Larger quantities are needed for species with larger genomes, and for the greater number of times needed to probe each blot. The lack of collaboration among research teams for distribution of discovered RFLP probes makes RFLP not amenable to automation (Karp *et al.,* 1997).

Applications: The RFLPs can be applied in diversity and phylogenetic studies ranging from individuals within populations or species, to closely related species. They have been widely used in gene mapping studies because of their high genomic abundance due to the ample availability of different restriction enzymes and random distribution throughout the genome (Neale and Williams, 1991). They also have been used to investigate relationships of closely related taxa (Miller and Tanksley, 1990; Lanner *et al.,* 1997), for diversity studies (Debreuil *et al.,* 1996), for fingerprinting tools (Fang *et al.,* 1997) and for studies of hybridization and introgression. For instance, population structure and genetic variability of *Rhodnius prolixus* Stål from various geographic regions of Columbia were studied using PCR/RFLP technique (Lopez *et al*., 2007).

Minisatellites

Description: The minisatellite analysis involves digestion of genomic DNA with restriction endonucleases, but minisatellites are a conceptually very different class of marker. They

consist of chromosomal regions containing tandem repeat units of a 10–50 base motif, flanked by conserved DNA restriction sites. A minisatellite profile consisting of many bands, usually within a 4–20 kb size range, is generated by using common multilocus probes that are able to hybridize to minisatellite sequences in different species. Locus specific probes can be developed by molecular cloning of DNA restriction fragments, subsequent screening with a multilocus minisatellite probe and isolation of specific fragments. Variation in the number of repeat units, due to unequal crossing over or gene conversion, is considered to be the main cause of length polymorphisms. Due to the high mutation rate of minisatellites, the level of polymorphism is substantial, generally resulting in unique multilocus profiles for different individuals within a population. Minisatellite loci are also often referred to as Variable Number of Tandem Repeats (VNTR) loci (Jeffreys *et al.,* 1985a, b).

Merits: The main advantages of minisatellites are their high level of polymorphism and high reproducibility (Karp *et al.,* 1997).

Demerits: The disadvantages of minisatellites are similar to RFLPs due to the high similarity in methodological procedures. If multilocus probes are used, highly informative profiles are generally observed due to the generation of many informative bands per reaction. In that case, band profiles cannot be interpreted in terms of loci and alleles and similar sized fragments may be non-homologous. In addition, the random distribution of minisatellites across the genome has been questioned (Schlötterer, 2004).

Applications: The term DNA fingerprinting was introduced for minisatellites, though DNA fingerprinting is now used in a more general way to refer to a DNA-based assay to uniquely identify individuals. Minisatellites are particularly useful in studies involving genetic identity, parentage, clonal growth and structure, and for population-level studies (Wolff *et al.,* 1994). Minisatellites are of reduced value for taxonomic studies because of hypervariability. So far, no minisatellite analysis has been reported in reduviids.

Polymerase Chain Reaction (PCR)-Sequencing

Description: The PCR sequencing involves determination of the nucleotide sequence within a DNA fragment amplified by the PCR, using primers specific for a particular genomic site. The method that has been most commonly used to determine nucleotide sequences is based on the termination of in vitro DNA replication. The procedure is initiated by annealing a primer to the amplified DNA fragment, followed by dividing the mixture into four subsamples. Subsequently, DNA is replicated in vitro by adding the four deoxynucleotides (adenine, cytosine, guanine, thymidine; dA, dC, dG and dT), a single dideoxynucleotide (ddA, ddC, ddG or ddT) and the enzyme DNA polymerase to each reaction. Sequence extension occurs as long as deoxynucleotides are incorporated in the newly synthesized DNA strand. However, when a dideoxynucleotide is incorporated, DNA replication is terminated. Because each reaction contains many DNA molecules and incorporation of dideoxynucleotides occurs at random, each of the four subsamples contains fragments of varying length terminated at any occurrence of the particular dideoxy base used in the subsample. Finally, the fragments in each of the four subsamples are separated by gel electrophoresis (Sanger *et al.,* 1977; Landegren *et al.,* 1988). The Polymerase Chain Reaction (PCR)-sequencing was a major breakthrough for molecular markers in that for the first time, any genomic region could be amplified and analyzed in many individuals without the requirement for cloning and isolating large amounts of ultra-pure genomic DNA (Schlötterer, 2004). PCR sequencing of four *Rhynocoris* species viz., *Rhynocoris kumarii* Ambrose and Livingstone, *R. longifrons* Stål *, R. marginatus* Fabricius and *R. fuscipes* Fabricius based on cytochrome c oxidase I gene which can be used to develop molecular markers important for examining molecular genetic variation or gene diversity and understanding deep phylogenetic relationships within the *Rhynocoris* species. Singh (2012) also analysed the same four *Rhynocoris* species as resulted in the possibility of establishing certain interspecific and

intraspecific markers for the purpose of identification. He also analysed the mitochondrial COI gene of four ecotypes of *R. kumarii* and three morphs of *R. marginatus* which could be used to develop molecular markers important for examining intraspecific molecular genetic variations at ecotypic and morphic levels.

Merits: Due to the amplification of fragments by PCR only low quantities of template DNA (the "target" DNA used for the initial reaction) are required, e.g. 10–100 ng per reaction. Moreover, most of the technical procedures are amenable to automation (Mullis and Faloona, 1987; Saiki, *et al.,* 1988; Erlich *et al.,* 1991). Because all possible sequence differences within the amplified fragment can be resolved between individuals, PCR sequencing provides the ultimate measurement of genetic variation. Universal primer pairs to target specific sequences in a wide range of species are available for the chloroplast, mitochondrial gene. Since it high reproducibility is greater the chance of detecting truly homologous differences in the known sequences (Beckmann, 1988).

Demerits: The PCR-sequencing restricts genome coverage and together with the fact that different genes may evolve at different rates, the extent to which the estimated gene diversity reflects overall genetic diversity is yet to be determined (Karp *et al.,* 1997).Disadvantages include low genome coverage and low levels of variation below the species level. In the event that primers for a genomic region of interest are unavailable, high development costs are involved. If sequences are visualized by polyacrylamide gel electrophoresis and autoradiography, analytical procedures are laborious and technically demanding. Fluorescent detection systems and reliable analytical software to score base pairs using automated sequencers are now widely applied. This requires considerable investments for equipment or substantial costs in the case of outsourcing. Because sequencing is costly and time-consuming, most studies have focused on only one or a few loci (Rokas *et al.,* 2003).

Applications: In general, insufficient nucleotide variation is detected below the species level, and PCR sequencing is most useful to address questions of interspecific and intergeneric relationships (Sanger *et al.,* 1977). Low-copy nuclear markers generally circumvent problems of uniparental inheritance frequently found in plastid markers (Corriveau and Coleman, 1988) that limits their utility and reliability in phylogenetic studies (Bailey *et al.,* 2003). In addition to biparental inheritance, low-copy nuclear markers exhibit higher rates of evolution (particularly in intron regions) than cpDNA and nrDNA markers (Small *et al.,* 2004) making them useful for closely related species. Yet another advantage is that low-copy sequences generally evolve independently of paralogous sequences and tend to be stable in position and copy number. Until recently, chloroplast DNA and nuclear ribosomal DNA have provided the major datasets for phylogenetic inference because of the ease of obtaining data due to high copy number. Recently, single- to low-copy nuclear DNA markers have been developed as powerful new tools for phylogenetic analyses (Mort and Crawford, 2004; Small *et al.,* 2004). The PCR sequencing was reported to be helpful to identify the blood meals of *Triatoma* bugs which act as vectors of Chagas' disease (Dawn *et al*., 2013).

Random Amplified Polymorphic DNA (RAPD)

Description: The Random Amplified Polymorphic DNAs are DNA fragments amplified by the PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primers, and are usually able to amplify fragments from 1–10 genomic sites simultaneously. Amplified fragments, usually within the 0.5–5 kb size range, are separated by agarose gel electrophoresis, and polymorphisms are detected, after ethidium bromide staining, as the presence or absence of bands of particular sizes. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites (Williams *et al.,* 1990; Hadrys *et al.,* 1992).

Merits: The main advantage of RAPDs is that they are quick and easy to assay. Because PCR is involved, only low quantities of template DNA are required, usually 5–50 ng per reaction (Carlson *et al.,* 1991; Hu and Quiros, 1991). Since random primers are commercially available, no sequence data for primer construction are needed. Moreover, RAPDs have a very high genomic abundance and are randomly distributed throughout the genome.

Demerits: The main drawback of RAPDs is their low reproducibility (Klein-Lankhorst *et al.,* 1991; Black, 1993; Schierwater and Ender, 1993) and hence highly standardized experimental procedures are needed because of their sensitivity to the reaction conditions. The RAPD analyses generally require purified, high molecular weight DNA (Ayad *et al.,* 1995), and precautions are needed to avoid contamination of DNA samples because short random primers used are able to amplify DNA fragments in a variety of organisms. Altogether, the inherent problems of reproducibility make RAPDs unsuitable markers for transference or comparison of results among research teams working in a similar species and subject. As for most other multilocus techniques, RAPD markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous (Karp *et al.,* 1997).

Applications: The RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers (Hadrys *et al.,* 1992). Variants of the RAPD technique include Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) which uses longer arbitrary primers than RAPDs (Welsh and McClelland, 1990, 1991) and DNA Amplification Fingerprinting (DAF) that uses shorter, 5–8 bp primers to generate a larger number of fragments (Caetano-Anolles *et al.,* 1991). Multiple Arbitrary Amplicon Profiling (MAAP) is the collective term for techniques using single arbitrary primers (Caetano-Anolles *et al.,* 1992; Caetano-Anolles, 1996). The RAPDs have been used for many purposes, ranging from studies at the individual level (e.g. genetic identity), studies involving closely related species

(Jaramillo *et al.,* 2001; Pacheco *et al.,* 2003). Taxonomic studies of triatomine bugs have become easier with Random Amplified Polymorphic DNA analysis (Garcia *et al*., 1998). The genetic diversity of assassin bugs, *Rhynocoris longifrons* (Stål) (Baskar *et al.,* 2011) and *R. marginatus* (Fabricius) (Baskar *et al.,* 2013) was studied based on RAPD analysis.

Microsatellites

Description: Microsatellites are repeat motifs and shorter nucleotide sequences (1–6 base pairs). If nucleotide sequences in the flanking regions of the microsatellites are known, specific primers (generally 20–25 bp) can be designed to amplify the microsatellites by PCR (Tautz, 1989). Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library, screening the library with a synthetically labelled oligonucleotide repeat and sequencing the positive clones. Alternatively, microsatellites may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed. In addition, primers may be used that have already been designed for closely related species. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the number of repeat units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis (Levinson and Gutman, 1987; Moxon *et al.,* 1999; Schlotterer, 2000).

Merits: The strengths of microsatellites include the co-dominance of alleles, their high genomic abundance in eukaryotes and their random distribution throughout the genome, with preferential association in low-copy regions (Morgante *et al.,* 2002). Because the technique is PCR-based, only low quantities of template DNA (10–100 ng per reaction) are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high and analyses do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap (Ghislain *et al.,* 2004).This

decreases significantly the analytical costs. Furthermore, the screening of microsatellite variations can be automated, if the use of automatic sequencers is an option.

Demerits: One of the main drawbacks of microsatellites is that high development costs are involved if adequate primer sequences for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although microsatellites are in principle co-dominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring (Callen *et al.,* 1993). The potential presence of null alleles increases with the use of microsatellite primers generated from germplasm unrelated to the species used to generate the microsatellite primers (poor "crossspecies amplification"). Null alleles may result in a biased estimate of the allelic and genotypic frequencies and an underestimation of heterozygosity (Amos and Rubinsztein, 1996). A very common observation in microsatellite analysis is the appearance of stutter bands that are artifacts in the technique that occur by DNA slippage during PCR amplification. These can complicate the interpretation of the band profiles because size determination of the fragments is more difficult and heterozygotes may be confused with homozygotes. However, the interpretation may be clarified by including appropriate reference genotypes of known band sizes in the experiment (Hearne *et al.,* 1992; Jarne and Lagoda, 1996). Furthermore, the underlying mutation model of microsatellites is still under debate. Homoplasy may occur at microsatellite loci due to different forward and backward mutations, which may cause underestimation of genetic divergence (Goodman, 1998).

Applications: In general, microsatellites show a high level of polymorphism. As a consequence, they are very informative markers that can be used for population genetics studies (Bruford and Wayne, 1993). Conversely, their high mutation rate makes them unsuitable for studies involving higher taxonomic levels. Microsatellites are also considered

ideal markers in gene mapping studies (Hearne *et al.,* 1992; Morgante and Olivieri, 1993; Queller *et al.,* 1993; Jarne and Lagoda, 1996) and microsatellite data are used to estimation of genetic distances of populations and individuals. (Sodhi *et al.,* 2005; Tapio *et al.,* 2005). Microsatellite markers were found to be useful in assessing the dispersal of *Triatoma dimidiate* Latreille in the Yucatan Peninsula of Mexico (Dumonteil *et al*., 2007). Bertha *et al*. (2013) has established the phylogeny of certain triatomine bugs of Northern America based on microsatellite sequence analysis.

Inter Simple Sequence Repeats (ISSR)

Description: The Inter Simple Sequence Repeats are DNA fragments of about 100–3000bp located between adjacent, oppositely oriented microsatellite regions. The ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16–18 bp). About 10–60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. Techniques related to ISSR analysis are Single Primer Amplification Reaction (SPAR) that uses a single primer containing only the core motif of a microsatellite (Staub *et al.,* 1996) and Directed Amplification of Minisatellite region DNA (DAMD) that uses a single primer containing only the core motif of a minisatellite (Heath *et al.,* 1993; Somers and Demmon, 2002).

Merits: The main advantage of ISSRs is that no sequence data for primer construction are needed. Because the analytical procedures include PCR, only low quantities of template DNA are required (5–50 ng per reaction). Furthermore, ISSRs are randomly distributed throughout the genome (Zietkiewicz *et al.,* 1994).

Demerits: Because ISSR is a multilocus technique, disadvantages include the possible non-homology of similar sized fragments. Moreover, ISSRs, like RAPDs, can have reproducibility problems (Zietkiewicz *et al.,* 1994).

Applications: Because of the multilocus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Zietkiewicz *et al.,* 1994; Godwin *et al.,* 1997).

Single-Strand Conformation Polymorphism (SSCP)

Description: The Single-Strand Conformation Polymorphisms are DNA fragments of about 200–800bp amplified by PCR using specific primers of 20–25bp. Gel electrophoresis of single-strand DNA is used to detect nucleotide sequence variation among the amplified fragments. The method is based on the fact that the electrophoretic mobility of single-strand DNA depends on the secondary structure (conformation) of the molecule, which is changed significantly with mutation. Thus, SSCP provides a method to detect nucleotide variation among DNA samples without having to perform sequence reactions. In SSCP, the amplified DNA is first denatured, and then subject to non-denaturing gel electrophoresis. Related techniques to SSCP are Denaturing Gradient Gel Electrophoresis (DGGE) that uses double stranded DNA which is converted to single stranded DNA in an increasingly denaturing physical environment during gel electrophoresis (Riedel *et al.,* 1990) and Thermal Gradient Gel Electrophoresis (TGGE) which uses temperature gradients to denature double stranded DNA during electrophoresis (Glavac and Dean, 1993; Cotton, 1997).

Merits: The advantages of SSCP are the co-dominance of alleles and the low quantities of template DNA required (10–100ng per reaction) due to the fact that the technique is PCR-based (Peterson *et al.,* 1995; Slabaugh *et al.,* 1997).

Demerits: The drawbacks include the need for sequence data to design PCR primers and the necessity of highly standardized electrophoretic conditions in order to obtain reproducible results. Furthermore, some mutations may remain undetected, and hence absence of mutation cannot be proven (Hayashi and Yandell, 1993).

Applications: The SSCPs have been used to detect mutations in genes using gene sequence information for primer construction (Hayashi, 1992). The mitochondrial sequence variation has been studied among triatomine species using PCR-based SSCP (Stothard *et al*., 1998). So far no such work has been reported in reduviids

Cleaved Amplified Polymorphic Sequences (CAPS)

Description: The Cleaved Amplified Polymorphic Sequences are DNA fragments amplified by PCR using specific 20–25 bp primers, followed by digestion of the PCR products with a restriction enzyme. Subsequently, length polymorphisms resulting from variation in the occurrence of restriction sites are identified by gel electrophoresis of the digested products (Karp *et al.,* 1997; De Vicente *et al.,* 2004). The CAPS have also been referred to as PCR-Restriction Fragment Length Polymorphism (PCR-RFLP).

Merits: The advantages of CAPS include the involvement of PCR requiring only low quantities of template DNA (50-100ng per reaction), the co-dominance of alleles and the high reproducibility (Dellaporta *et al.,* 1983). Compared to RFLPs, CAPS analysis does not include the laborious and technically demanding steps of Southern blot hybridization and radioactive detection procedures (Karp *et al.,* 1997).

Demerits: In comparison with RFLP analysis, CAPS polymorphisms are more difficult to find because of the limited size of the amplified fragments (300–1800bp). Furthermore, sequence data are needed to design the PCR primers (Tragoonrung *et al.,* 1992; Ghareyazie *et al,* 1995).

Applications: The CAPS markers have been applied predominantly in gene mapping studies (Akopyanz *et al.,* 1992; Konieczny and Ausubel, 1993). CAPS markers have not been so far attempted in reduviids.

Sequence Characterized Amplified Regions (SCAR)

Description: The Sequence Characterized Amplified Regions are DNA fragments amplified by the PCR using specific 15–30bp primers, designed from nucleotide sequences established from cloned RAPD fragments linked to a trait of interest (Bodenes *et al.,* 1997). By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs. Obtaining a co-dominant marker may be an additional advantage of converting RAPDs into SCARs, although SCARs may exhibit dominance when one or both primers partially overlap the site of sequence variation. Length polymorphisms are detected by gel electrophoresis (Paran and Michelmore, 1993; De Vicente *et al.,* 2004).

Merits: The main advantage of SCARs is that they are quick and easy to use. In addition, SCARs have a high reproducibility and are locus-specific (Karp *et al.,* 1997). Due to the use of PCR, only low quantities of template DNA are required (10–100ng per reaction).

Demerits: Disadvantages include the need for sequence data to design the PCR primers (Karp *et al.,* 1997).

Applications: The SCARs are locus specific and have been applied in gene mapping studies and marker assisted selection (Paran and Michelmore, 1993). SCARs markers have not been used so far for reduviids.

Amplified Fragment Length Polymorphism (AFLP)

Description: The Amplified Fragment Length Polymorphism are DNA fragments (80–500 bp) obtained from digestion with restriction enzymes, followed by ligation of oligonucleotide adapters to the digestion products and selective amplification by the PCR. The AFLPs, therefore involve both RFLP and PCR (Karp *et al.,* 1997; Matthes *et al.,* 1998). The PCR primers consist of a core sequence, and a restriction enzyme specific sequence and 1–5 selective nucleotides (the higher the number of selective nucleotides, the lower the number of bands obtained per profile). The AFLP banding profiles are the result of variations in the

restriction sites or in the intervening region. The AFLP technique simultaneously generates fragments from many genomic sites (usually 50–100 fragments per reaction) that are separated by polyacrylamide gel electrophoresis and that are generally scored as dominant markers (Karp *et al.,* 1997). Selective Fragment Length Amplification (SFLA) and Selective Restriction Fragment Amplification (SRFA) are synonyms sometimes used to refer to AFLPs (Zabeau and Vos. 1993, 2006). A variation of the AFLP technique is known as Selectively Amplified Microsatellite Polymorphic Locus (SAMPL). This technology amplifies microsatellite loci by using a single AFLP primer in combination with a primer complementary to compound microsatellite sequences, which do not require prior cloning and characterization (Morgante and Vogel, 1994; Witsenboer *et al.,* 1997).

Merits: The strengths of AFLPs lie in their high genomic abundance, considerable reproducibility, the generation of many informative bands per reaction, their wide range of applications, and the fact that no sequence data for primer construction are required. The AFLPs may not be totally randomly distributed around the genome as clustering in certain genomic regions, such as centromers (Young *et al.,* 1999; Saal and Wricke, 2002). They can be analyzed on automatic sequencers, but software problems concerning the scoring of AFLPs are encountered on some systems.

Demerits: The disadvantages include the need for purified (Zabeau and Vos, 1993), high molecular weight DNA, the dominance of alleles, and the possible non-homology of co-migrating fragments belonging to different loci. In addition, due to the high number and different intensity of bands per primer combination, there is the need to adopt certain strict but subjectively determined criteria for acceptance of bands in the analysis. Special attention should be paid to the fact that AFLP bands are not always independent. For example, in case of an insertion between two restriction sites the amplified DNA fragment results in increased band size. This will be interpreted as the loss of a small band and at the same time as the gain

of a larger band. This is important for the analysis of genetic relatedness, because it would enhance the weight of non-independent bands compared to the other bands (Blears *et al.,* 1998; Caterino *et al.,* 2000).

Applications: Because of the highly informative fingerprinting profiles generally obtained, AFLPs can be applied in studies involving genetic identity, parentage and identification of clones (VanToai *et al.,* 1997) and phylogenetic studies of closely related species. Their high genomic abundance and generally random distribution throughout the genome make AFLPs a widely valued technology for gene mapping studies (Vos *et al.,* 1995). SAMPL (Selectively Amplified Microsatellite Polymorphic Loci) is considered more applicable to intraspecific than to interspecific studies due to frequent null alleles. Identification of six *Trypanosoma cruzi* Chagas lineages, causative organism of Chagas' disease transmitted by triatomine bugs was done using sequence-characterised amplified region markers by Brisse *et al*. (2000).

Expressed Sequence TAGS (ESTs)

The expressed sequence tags are short DNA molecules (300-500bp) reverse-transcribed from a cellular mRNA population (MacIntosh *et al.,* 2001). They are generated by large scale single-pass sequencing of randomly picked cDNA clones and have proven to be efficient and rapid means to identify novel genes (Adams *et al.,* 1991). The ESTs thus, represent informative source of expressed genes and provide a sequence resource that can be exploited for large-scale gene discovery (Whitefield *et al.,* 2002). They are useful for the development of cDNA microarrays that allow analysis of differentially expressed genes to be determined in a systematic way, in addition to their great value in genome mapping and physical mapping in animal genomics. They can be mapped to physical maps by hybridization and integration of physical and genetic linkage maps would in turn anchor the ESTs to the linkage maps. No such work available for ESTs markers.

Single Nucleotide Polymorphisms (SNPs)

The single nucleotide polymorphisms are excellent markers for association mapping of genes controlling complex traits and provide the highest map resolution (Bhattramakki *et al.,* 2002; Botstein and Risch, 2003). The SNPs are DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genome sequence is altered. It reveals hidden polymorphism not detected with other markers and methods. For example, a SNP might change the DNA sequence A**A**GGCTAA to A**T**GGCTAA. The SNP can occur in both coding (gene) and non coding regions of the genome. They are inherited as co-dominant markers. They have properties and a density in the human genome that makes them attractive as marker for identification of genes in as yet uncharacterized parts of the genome that may have some relation to specific disease. The DNA sequencing has been most accurate and most used approach for SNP discovery and genotyping.

Merits

The DNA provides many advantages that make it especially attractive in studies of diversity (Hillis and Moritz, 1990; Avise, 1994) and relationships (Crawford, 1990). These advantages include: 1) Freedom from environmental and pleiotropic effects. Molecular markers do not exhibit phenotypic plasticity, while morphological and biochemical markers can vary in different environments. The DNA characters have a much better chance of providing homologous traits. Most morphological or biochemical markers, in contrast, are under polygenic control, and subject to epistatic control and environmental modification (plasticity) and 2) DNA characters can be more easily scored as discrete states of alleles or DNA base pairs, while some morphological, biochemical and field evaluation data must be scored as continuously variable characters that are less amenable to robust analytical methods. On the contrary, morphological, ecological and other "traditional" data will continue to provide practical and often critical information needed to characterize genetic

resources. Molecular markers differ in many qualities and must therefore be carefully chosen and analyzed differently with their differences in mind (Brown and Kresovich, 1996; Spooner *et al.,* 2005).

Demerits

The DNA barcoding also has its own limitations like genetic introgression and pseudogenes (Buhay, 2009). The use of mitochondrial genes character has been controversial (Seberg *et al.,* 2003; Tautz *et al.,* 2003) and recent debates have been particularly heated (Meyer and Paulay, 2005; Will *et al.,* 2005) in the light of increasing popularity of projects such as the phylogeny of the insect life. Pavan *et al.* (2013) reported that the differentiation among the members of *Rhodnius* species was made possible using single nucleotide polymorphism studies.

Cytochrome c Oxidase Subunit I Gene

Description: Short species specific sequences known as DNA barcodes have been used as tools in biodiversity and ecological research. A certain fragment of the mitochondrial gene COI (Cytochrome Oxidase I), coding for a subunit of the enzyme cytochrome oxidase is one such DNA barcode which is being widely used for the identification of animal species. The DNA barcode, COI gains much importance since it varies a lot between species and very little between the individuals of a given species, hence making the identification easier.

The study of mtDNA sequence has become the method of choice in recent years for a wide range of molecular genetics, phylogeny, taxonomic, population and evolutionary study of insects. Different optimal criteria and dataset compilation techniques have been applied to find the best method of analyzing the complex COI gene data (Cameron *et al.,* 2004; Castro and Dowton, 2005; Kim *et al.,* 2005).The current view, as advanced by Cameron *et al.* (2007) is that the COI gene data recover the most phylogenetic information when all available genes are analyzed as nucleotide sequences and the results from different optimality criteria are

then compared for the sensitivity of resulting phylogenetic hypotheses to the underlying model assumptions of the tree building methods. Many aspects of the structure and evolution of mtDNA have made it a valuable evolutionary tool and these include its ease of isolation, high copy number and lack of recombination, conservation of sequence and structure across insects and the range of mutational rates in different regions of the molecule (Moritz *et al.,* 1987; Harrison, 1989; Simon, 1991; Wolstenholme, 1992). Baskar (2010) analysed the molecular genetic variation and phylogeny of four *Rhynocoris* species viz., *Rhynocoris kumarii* Ambrose and Livingstone, *R. longifrons* Stål *, R. marginatus* Fabricius and *R. fuscipes* Fabricius based on cytochrome c oxidase I gene which can be used to develop molecular markers important for examining molecular genetic variation or gene diversity and understanding deep phylogenetic relationships the utility across the available heteropteran mitochondrial genomes to facilitate informed gene choice for molecular studies within the *Rhynocoris* species. Singh (2012) analysed the mitochondrial COI gene of four ecotypes of *R. kumarii* and three morphs of *R. marginatus* which could be used to develop molecular markers important for examining intraspecific molecular genetic variations at ecotypic and morphic levels.

Merits: The mitochondrial COI gene is the terminal catalyst in the mitochondrial respiratory chain, relatively well studied at the biochemical level and its size and structure appears to be conserved across all organisms investigated (Saraste, 1990). Mutational studies have been used to map the reaction centers of this COI gene (Gennis, 1992) and these provide a background which enables interpretation of sequence differences in terms of gene function. It is involved in both electron transport and the associated translocation of protons across the membrane and it has been shown to contain a range of different types of functional domain including ligand sites, components of the proton channel and interspersing hydrophilic loops (Saraste, 1990; Gennis, 1992). It is potentially well suited to establishing a temporal

framework for comparative studies of at least tertiary-originating lineages such as insects (Dobler and Farrell, 1999). The mitochondrial COI gene has proved useful in resolving relationships among closely related species and genera of insects and other arthropods (Harrison and Crespi, 1999). Moreover, several studies have also shown that mitochondrial COI gene exhibits sufficient rate constancy to provide the means for inference of dates of divergence (Sandoval *et al.,* 1998; Pellmyr and Leebens-Mack, 1999; Sequeira *et al.,* 2000).

Demerits: The Mitochondrial COI genes are clonally inherited, non-recombining, making recombination, paralogy and heterozygosity. However, the nuclear copies of mitochondrial genes may exist and creating problems for the analysis of mitochondrial gene sequences (Sunnucks and Hales, 1996; Zhang and Hewitt, 1996). The mitochondrial genes are generally evolved at higher rates than nuclear protein-coding genes. In insects, mitochondrial genes are estimated to evolve 2 to 9 times faster than nuclear protein-coding genes (DeSalle *et al.,* 1987; Monteiro and Pierce, 2001).

Since all the mitochondrial genes are linked on the same chromosome, one could argue that they do not provide an independent estimate of phylogeny in the same way as the unlinked single-copy (Harrison, 1989). Furthermore, the higher rate of substitutions can be disadvantageous when one is trying to resolve divergences of more than 5-10 million years. Most importantly for phylogenetic analysis, mitochondrial genes have attributed to lead high levels of homoplasy when analyzed by standard phylogenetic methods, such as an extreme A/T bias in third positions (Frati *et al.,* 1997).

Applications: The mitochondrial COI gene has been frequently used for inferring phylogenetic relationships at different taxonomic levels in many animal groups (Avise, 2000). It has been used to resolve the relationships not only at the generic level or below (Joy and Conn, 2001; Morris *et al.,* 2002; Bull *et al.,* 2003), but also at higher taxonomic ranks (Sequeira and Farrell, 2001; Carlini *et al.,* 2001; Kjer *et al.,* 2002). Different methods of

analyzing mitochondrial genomes have been proposed and applied, spanning from selecting a few key genes from the entire mitochondrial genome data (Nardi *et al.,* 2003) to the inclusion of all available genes excluding the control region (Castro and Dowton, 2007), and from analyzing amino acid sequences (Nardi *et al.,* 2001) or purine / pyrimidine recoding of nucleotide sequences (Saitoh *et al.,* 2006) to the traditional approach of analyzing native nucleotide sequences (Cameron *et al.,* 2007). In terms of the ability to resolve deeper level relationships in insects, the mitochondrial COI gene data were able to unambiguously resolve the relationships among major arthropod lineages (Cameron *et al.,* 2004).

The effectiveness of using DNA barcodes for the purpose of identification has been proved in case of certain forest insect pest species. A total of 103 mitochondrial COI barcode sequences were made available for 77 forest insect pests belonging to Coleoptera, Hemiptera and Lepidoptera. All 77 species studied, had unique COI sequences that brings about low intraspecific genetic divergence and high interspecific genetic divergence. A very clear demarcation has been observed between the species of the same genera of the forest insect pests. This way of using DNA barcodes as tools for identification helps to overcome difficulty in identification due to polymorphism and environmental induced variation which are commonly observed (Lee *et al.,* 2006).

Restriction Fragment Length Polymorphism (RFLP)

Description: The RFLP probes are maintained as clones in suitable bacterial vectors that conveniently allow the isolation of the DNA fragments they hold. Probes from related species may be used (heterologous probes). The DNA sequence variation affecting the absence or presence of recognition sites of restriction enzymes, and insertions and deletions within two adjacent restriction sites, form the basis of length polymorphisms (Botstein *et al.,* 1980; Burr *et al.,* 1983; Helentjaris *et al.,* 1985; Evola, *et al.,* 1986). The locus specific RFLP probes consist of a homologous sequence of a specific chromosomal region. Probes are generated

through the construction of genomic or complementary DNA (cDNA) libraries and therefore may be composed of a specific sequence of unknown identity (genomic DNA) or part of the sequence of a functional gene (exons only, cDNA). The RFLP probes are maintained as clones in suitable bacterial vectors that conveniently allow the isolation of the DNA fragments they hold and RFLP probes from related species may be used (heterologous probes). The DNA sequence variation affecting the absence or presence of recognition sites of restriction enzymes, and insertions and deletions within two adjacent restriction sites, form the basis of length polymorphisms (Botstein *et al.,* 1980; Burr *et al.,* 1983; Helentjaris *et al.,* 1985; Evola *et al.,* 1986). The RFLPs are generally found to be moderately polymorphic. In addition to their high genomic abundance and their random distribution, RFLPs have the advantages of showing co-dominant alleles and having high reproducibility (Neale and Williams, 1991).

The Restriction Fragment Length Polymorphism are bands that correspond to DNA fragments, usually within the range of 2–10 kb, that have resulted from the digestion of genomic DNA with restriction enzymes. DNA fragments are separated by agarose gel electrophoresis and are detected by subsequent Southern blot hybridization to a labelled DNA probe (Burke *et al.,* 1991). A simple and rapid technique for identification of methanogenic species which play important roles in the biodegradation of organic matter was developed. The 16SrDNA fragments were amplified and digested with *Hae* III or *Hha* I which yielded species-specific RFLP profiles (Hiraishi *et al.,* 1995). Labelling of the probe may be performed with a radioactive isotope or with alternative non-radioactive stains, such as digoxigenin or fluorescein (Karp *et al.,* 1997).

Labelling of the probe may be performed with a radioactive isotope or with alternative non-radioactive stains, such as digoxigenin or fluorescein (Karp *et al.,* 1997). Taxonomy based on geographical origin or conidial morphology was rechecked using PCR-

RFLP technique for 26 isolates of the genus *Entomophthora*. The Internal Transcribed Spacer (ITS) II and the first part of the Large Serum Values (LSV) rDNA were amplified and cut using eight different restriction endonucleases and analysed by Unweighted Pair Group Method with Arithmatic Mean(UPGMA). Host-range widening was also observed based on the clustering of the species studied (Jensen and Eilenberg, 2000).

The diagnosis and identification of *Leishmania* in clinical specimens, infected reservoirs and vectors, were made easier by the development of PCR-RFLP. This method was compared with PCR-hybridization method which gave a highly concordant value. The conserved region of the minicircle of *Leishmania* kDNA was amplified and digested using *Hae* III (Angela *et al.,* 2004).

Differentiation of *Anopheles culicifacies* Giles species A,B,C,D,E was done by PCR-RFLP method. The ITS2 region, 5.8S and 28S rDNA sequences and mitochondrial cytochrome oxidase II were amplified and digested with different restriction endonucleses *Alu* I, *Rsa* I and *Dde* I (Goswami *et al.,* 2005).

The PCR-RFLP was employed for species identification of beef, buffalo meat, mutton and chevon. The identification of origin of meat was made feasible by the use of PCR-RFLP of the mitochondrial 12S rRNA gene. The PCR amplification resulted an amplicon size of 456-bp fragment in each of these species. The amplicons were digested with Alu I, Hha I, Apo I and BspTI restriction enzymes. Genetic structure of three forms of *Anopheles stephensi* Liston was studied using PCR-RFLP method. One of the three forms of this species is considered to be an efficient vector of malaria (Oshaghi *et al.,* 2006).

Identification of coagulase-negative staphylococci (CNS) causing ovine infections was made feasible by PCR-RFLP technique. The staphylococcal 16SrRNA and gap genes were amplified and subjected to restriction fragment length polymorphism analysis using endonucleases Alu I, Rsa I, Pst I and Alu I. The PCR-RFLP patterns of CNS were obtained,

suggesting the reliable way of identifying CNS causing mastitis in sheep and goats (Onni *et al.,* 2010).

The mitochondrial cytochrome oxidase I gene is also used for identification of meat species. In a study carried out by (Haider *et al.,* 2011), the amplified COI gene was digested using 7 different enzymes, out of which Hpa II was suggested to be the enzyme needed to generate species specific restriction profile for each of the species studied. This makes the routine identification cheaper and faster.

Merits: The RFLPs are generally found to be moderately polymorphic. In addition to their high genomic abundance and their random distribution, RFLPs have the advantages of showing co-dominant alleles and having high reproducibility (Neale and Williams, 1991).

Demerits: The main drawbacks of RFLPs are the requirement of laborious and technically demanding methodological procedures, and high expense (Castiglione *et al.,* 1993). Moreover, large quantities (1–10 μg) of purified, high molecular weight DNA are required for each DNA digestion. Larger quantities are needed for species with larger genomes, and for the greater number of times needed to probe each blot. The identified RFLPs are not amenable to automation due to no collaboration among researchers in distributing of the probes (Karp *et al.,* 1997).

Applications: The RFLPs can be applied in diversity and phylogenetic studies ranging from individuals within populations or species, to closely related species. They have been widely used in gene mapping studies because of their high genomic abundance due to the ample availability of different restriction enzymes and random distribution throughout the genome (Neale and Williams, 1991). They also have been used for diversity studies (Debreuil *et al.,* 1996), to investigate relationships of closely related taxa (Lanner *et al.,* 1997), as fingerprinting tools (Fang *et al.,* 1997) and for studies of hybridization and introgression.

The mitochondrial rDNA sequences of following the reduviids are currently available in the NCBI- GenBank:

Apiomerinae: *Agriocoris flavipes* (Fabricius), *Apiomerus apicalis* Burmeister, *A. binotatus* Champion, *A. claripennis* Lima *et al., A. colombianus* Lima *et al., A. crassipes* (Fabricius), *A. elatus* Stål, *A. emarginatus* Stål, *A. flaviventris* Schaeffer, *A. geniculatus* Erichson, *A. hirtipes* (Fabricius), *A. lanipes* (Fabricius), *A. lituratus* Stål, *A. lobulatus* Breddin, *A. longispinis* Lima *et al., A. luctuosus* Lima *et al., A. nigricollis* Stål, *A. nitidicollis* Stål, *A. ochropterus* Stål, *A. peruvianus* Lima *et al., A. pictipes* Schaeffer, *A. pilipes* (Fabricius), *A. rubrocinctus* Lima *et al., A. spissipes* (Say), *A. subpiceus* Stål, *A. venosus* Stål, *A. vexillarius* Champion, *Calliclopius nigripes* (Linnaeus), *Heniartes flavicans* (Fabricius), *H. Putumayo* Wygodzinsky, *Manicocoris rufipes* (Fabricius), *Micrauchenus lineola* (Fabricius), *Ponerobia bipustulata* (Fabricius) and *Sphodrolestes vittaticollis* Stål.

Cetherinae: *Cethera musiva* (Germar), *Eupheno histrionicus* (Stål) and *E. pallens* (Laporte).

Ectrichodiinae: *Ectrichodia lucida* (Lepeletier & Serville), *Ectrychotes andreae* (Thunberg), *Haematoloecha nigrorufa* (Stål), *Rhiginia cruciata* (Say);

Emesinae: *Emesaya brevipennis* (Say), *E. incisa* Mc Atee & Malloch, *Ploiaria hirticornis* (Banks) and *Stenolemoides arizonensis* (Banks).

Hammacerinae: *Microtomus cinctipes* (Stål).

Harpactorinae: *Agriosphodrus dohrni* (Stål), *Ambastus villosus* Stål, *Arilus cristatus* (Linnaeus), *Biasticus flavus* (Distant), *Castolus subinermis* (Stål), *Coranus callosus* Stål, *C. dilatatus* (Matsumura), *C. emodicus* Kiritschenko, *C. fuscipennis* Reuter, *C. hammarstroemi* Reuter, *C. lativentris* Jakovlev, *C. marginatus* Hsiao, *C. sichuensis* Hsiao & Ren, *C. spiniscutis* Reuter, *C. subapterus* (De Geer), *Cydnocoris geniculatus* Hsiao, *C. russatus* Stål, *Endochus albomaculatus* Stål, *Epidaus annulipes* (Reuter), *E. nebulo* (Stål), *E. tuberosus* Yang, *E. xuliangae* Stål, *Euagoras plagiatus* (Burmeister), *Fitchia spinosula*Stål,

Isyndus obscurus (Dallas), *I. reticulatus* Stål, *Panthous bimaculatus* Distant, *Pselliopus cinctus* (Fabricius), *P. spinicollis* (Champion), *P. zebra* (Stål), *Pyrrhosphodrus amazonus* Stål, *Rhaphidosoma decorsei* Jeannel, *Rhynocoris fuscipes* (Fabricius), *R. iracundus* (Poda), *R. kumarii* Ambrose & Livingstone, *R. longifrons* (Stål), *R. marginatus* (Fabricius), *R. marginellus* (Fabricius), *R. monticola* (Oshanin), *R. segmentarius* (Germar), *R. ventralis* (Say), *Ricolla quadrispinosa*(Linnaeus), *Rihirbus trochantericus* Stål, *Sinea diadema* (Fabricius), *Sphedanolestes gularis* Hsiao, *S. impressicollis* (Stål), *S. trichrous* Stål, *Sycanus croceus* Hsiao, *S. croceovittatus* Dohrn, *S. croceus* Hsiao, *Vesbius purpureus* (Thunberg), *Zelus longipes* (Linnaeus),*Z. luridus* Stål, *Z. renardii* kolenati and *Z. tetracanthus* Stål.

Holoptilinae: *Ptilocnemus femoralis* Horváth.

Reduviinae: *Acanthaspis pedestris* Stål, *Acanthaspis quinquespinosa* (Fabricius), *Acanthaspis siva* Distant, *Empyrocoris annulata* (Distant), *Edocla slateri* Distant and *Velitra sinensis* (Walker).

Peiratinae: *Catamiarus brevipennis* Serville, *Ectomocoris tibialis* Distant, *Ectomocoris* cordiger Stål and *Ectomocoris quadriguttatus* Fabricius.

CHAPTER I

MORPHOMETRY

Introduction

Morphometric methods are powerful tools used in the context of sound biological knowledge. They are most frequently used to achieve objectives that are not obtainable at presently by any other means. They allow us to summarise morphological data numerically and graphically, to express and test hypothetical relationship exactly, and with multivariate techniques to examine relationships in many dimensions in a manner not otherwise possible (Daly, 1985).

Morphometrics, i.e., the quantitative description and interpretation of shape and shape variation in biology is a fundamental area of research. Technique of descriptions and comparison of shapes of structures are needed in any systematic study that is based on the morphology of organisms. Measurements of morphological diversity are of interest in ecological and genetic studies. Ways of dealing with shape change are also important for developmental studies and for practical application in the medical sciences (Rohlf, 1990).

Though it is not a coherent discipline, it is practised in partial isolation in various fields of science including Entomology (Daly, 1985). Insects are ideal subjects for morphometric analysis. Although often used for exploration purposes, the most rewarding applications have been where appropriate background knowledge of an insect's life history, genetic ecology etc., already exists. With an increasing number of techniques available for measurement and analysis, entomologists can use morphometry as a splendid research tool (Daly, 1985).

Morphometry has been interrelated to biochemical, genetic, cytological and ecogeographical information in systematics. For example, morphometry of social bees and wasps (Daly, 1973), asymmetrical relationship between the prey size and the possibility of

the prey capture (Wilson, 1975), positive correlation between the mandible length of the workers of seed eating ants, mandible size and the prey size of the coccinellid beetles (Pearson and Mury, 1979), morphometry and geographic variations in the gall forming aphids (Sokal *et al.,* 1980), length of the wing pad of the fourth instar nymph of delphacid rice pest with the wing length of brachypterous and macropterous adults to understand their population dynamics (Cook and Perfect, 1982), correlation between the wing length of African armyworm to geographical areas of East Africa (Aidley and Lubega, 1979), size differences in the monogeographic races of a coastal dune beetle within the distance up to 500 m from the highest tide (Doyen and Slobodchikoff, 1984), forewings size of *Bombus* and closely related cuckoo bee *Psithyrus* and their generic relationship (Plowright and Stephen, 1973), correlation between the morphometry and the dispersal and the distribution of bush fly population in South Australia (Greenham and Hughes, 1971), morphometric asymmetry and developmental instability of extreme phenotypes in honey bees biosystematics (Bruckner, 1976), analysis of races of honey bee population from Europe to Africa (Gadbin *et al.,* 1979), measurements of the wings of species of various genera, families of calyptrate Diptera (Brown, 1979a, b) and the measurements of male genital organ of *Psammolestes tertius* Lent & Jurberg species (Soares *et al*., 2001). Within the morphological context, morphometry (Rohlf and Marcus, 1993; Adams *et al*., 2004) appears as an important taxonomic tool for species discrimination and species variations (Dujaridin *et al*., 1998; Calle *et al*., 2002; Jaramillo *et al*., 2002; Monroy *et al*., 2003; Yurtas *et al*., 2005; Feliciangeli *et al*., 2007). Delgado and Rubio-Palis (1993) examined the morphometric variation of *Anopheles* (*Nyssorhynchus*) *nuneztovari* Gabaldon and detected variability within Venenzuelan populations. Size variation is more influenced by environmental factors, whereas shape variation has a stronger genetic component (Klingenberg *et al*., 2004; Dujardin and Slice,

2007). The morphometric analysis (discriminant analysis) of *Anopheles pseudopunctipennis* Theobald (Juri *et al*., 2011) did not reveal a pattern between Bolivian and Argentinean populations based on geographical distances.

Rukmani (1992) expanded the work of Ambrose (1987b) of which she calculated the linear regression coefficients (r) of the relative postembryonic development of 27 combinations of parts of 24 species belonging to 13 genera. According to her, these values can be used to identify a particular genus as well as to trace the affinity between different genera of a particular subfamily and to diagnose family Reduviidae from other heteropterous families but it might not be useful to demarcate the subfamilies of Reduviidae. Morphological and biological characteristics of ecotypes and morphs of several species of assassin bugs etc., were studied and correlated (Ambrose, 1999).

Ambrose and Ambrose (2003) studied the linear regression coefficient values (r) of postembryonic developmental morphometry as an effective tool in biosystematics of reduviids. The (r) values were found useful to identify and understand the affinity of reduviids at subfamily, generic and species levels.

Monroy *et al*. (2003) examined the *Triatoma nitida* Usinger intraspecific variations using morphometry as a tool to compare three populations from different localities, in order to understand its population structure. They also reported that the morphometric analysis of 47 *T. nitida* males from three localities that showed quantitative differences between the populations. Lehmann *et al*. (2005) analysed the morphometry of *Triatoma dimidiata* (Latreille) populations and reported that although wing fluctuating asymmetry was present in all populations, only head characters were necessary to distinguish population level differences. Kwadjo *et al*. (2008) studied the morphometrical changes of eggs of *Rhynocoris*

albopilosus Signoret during their development. Wignall and Taylor (2008) studied the morphometric analysis in an araneophagic assassin bug, *Stenolemus bituberus* Stål.

Ambrose and Ambrose (2009b) analysed of 36 morphometric indices belonging to 27 species, 14 genera and 3 subfamilies viz., Harpactorinae, Peiratinae and Reduviinae by direct observation as well as unweighted pair group method using arithmetic mean (UPGMA) incorporating Euclidean Distances, Nei and Li's Genetic Distance Coefficient and Jaccard's Coefficient which reveals diagnostic intra-as well as intersubfamilial and generic affinities. Moreover, it gives an insight into the placement of genera in the existing divisions (tribes) of subfamilies and their phylogenetic relationship. Further, it clearly suggests two diversified lines of evolution of Peiratinae and Reduviinae from Harpactorinae than the earlier suggested straight line evolution of these two subfamilies from Harpactorinae (Ambrose, 1999). Villacis *et al*. (2010) studied the variation between the two geographical populations of *Rhodnius ecuadoriensis* Lent and León with wing size and shape. The morphometry of the antennae of *Panstrongylus megistus* Burmeister, *Rhodnius neglectus* Lent, *R. prolixus* Stål and *Triatoma vitticeps* Stål was also studied (Rosa *et al*., 2010).

Singh (2012) studied generic identity and interspecific differentiation of postembryonic developmental morphometry of four *Rhynocoris* species. The analysis of these species by linear regression coefficient values (r) of postembryonic developmental morphometric suggests its utility as a biosystematics tool at generic and species levels and also the morphometric results confirmed by the dendrogram showed in 100 % similarity among the four *Rhynocoris* species.

In the present thesis, an attempt was made to understand whether the linear regression coefficient (r) values of size of various parts of life stages of six species belonging to subfamily Reduvinae viz., *Acanthaspis pedestris* Stål*, A. quinquespinosa* (Fabricius)*, A. siva*

Distant*, Empyrocoris annulata* (Distant)*, Edocla slateri* Distant and *Velitra sinensis* (Walker) could be used as a tool in their demarcation either at generic or species level. Since reduviids are natural enemies of several insect pests, present study helps their accurate identification for their effective utilization in the Integrated Pest Management.

Materials and Methods

The measurement of one body part (a) was compared to that of another (b) from the first nymphal instar to the adult. Thus, six pairs of (I, II, III, IV, V nymphal instars and adult) (a) and (b) values were computed together and the regression coefficient (r) was calculated for a particular part. Equal number of males and females represented the adult measurement. Twenty one such sets of values were computed and the regression coefficient values were analysed to find out biosystematic significance, if any. Affinity was calculated by identifying exact values or relatively closer values (Rukmani, 1992). The analysed sets of values include: 1. Head length/ Head width (HL/HW), 2. Head length/ Prothoracic length (HL/PTL), 3. Head length/ Abdominal length (HL/AL), 4. Head length/ Diameter of eye (HL/DE), 5. Head width/ Width between eyes (HW/WBE), 6. Head width/ Prothoracic width (HW/PTW), 7. Entire Antennal length/ Head length (EAL/HL), 8. Entire Antennal length/ Prothorax length (EAL/PTL), 9. Entire Antennal length/Foretibial length (EAL/FTL), 10. Entire Antennal length/ Abdominal length (EAL/AL), 11. Rostral length/ Head length (RL/HL), 12. Rostral length/ Prothoracic length (RL/ PTL), 13. Rostral length/ Foretibial length (RL/ FTL), 14. Rostral length/ Abdominal length (RL/ AL), 15. Prothoracic length/ Prothoracic width (PTL/ PTW), 16. Prothoracic length/ Foretibial length (PTL/ FTL), 17. Prothoracic length/ Midtibial length (PTL/ MTL), 18. Prothoracic length/ Hind tibial length (PTL/ HTL), 19. Prothoracic length/ Abdominal length (PTL/ AL), 20. Prothoracic length/ Abdominal width (PTL/ AW) and 21. Abdominal length / Abdominal width (AL/AW).

Cluster analysis was made using the software, MultiVariate Statistical Package for Windows, ver. 3.1. A dendrogram was constructed with Unweighted Pair Group Method wing arithmetic mean (UPGMA) and the similarity matrix index was calculated by the per cent similarity coefficient (Kovach, 2007).

Results

1. Head Length/Head width (HL/HW)

The (r) values are the highest and exactly similar (0.99) among three species belonging to three genera *A. pedestris, E. slateri* and *E. slateri,* lower and unique in *E. annulata* (0.98) and the lowest and exactly similar in *A. quinquespinosa* and *A. siva* (0.97) (Table 1).

2. Head Length/Prothoracic length (HL/PTL)

Though the r values are the lowest and exactly similar in *A. quinquespinosa* and *A. siva* (0.92) it is higher and unique in the rest of the four species: *E. slateri,* (0.99), *A. pedestris* (0.98) *V. sinensis* (0.97) and *E. annulata,* (0.96).

3. Head length/ Abdominal length (HL/AL)

The (r) values are exactly similar in *E. slateri* and *V. sinensis* (0.96). It is the highest in *A. quinquespinosa* (0.99) and lower in *A. siva* (0.92) and the lowest in *A. pedestris* (0.90) and unique among themselves.

4. Head length/ Diameter of eye (HL/DE)

The (r) values are exactly similar (0.98) in three species belonging to two genera viz., *A. quinquespinosa, A. siva* and *E. slateri.* It is the highest in *A. pedestris* (0.99) and lower in *E. annulata* (0.96) and the lowest in *V. sinensis* (0.94) and unique among themselves.

5. Head width/ Width between eyes (HW/WBE)

The (r) values are unique among all the six species: *E. slateri* (0.99)>*A. quinquespinosa* (0.98)>*E. annulata* (0.97)>*A. pedestris* (0.93)>*A. siva* (0.89)>*V. sinensis* (0.84).

6. Head width/ Prothoracic width (HW/PTW)

The (r) values are exactly similar among four species belonging to three genera *A. pedestris*, *A. quinquespinosa, E. slateri* and *V. sinensis* (0.96) but it is higher and unique in *E. annulata* (0.98) and much lower and unique in *A. siva* (0.89).

7. Entire Antennal length/ Head length (EAL/HL)

The (r) values are again exactly similar in three species belonging to two genera viz., *A. pedestris, A. siva* and *V. sinensis* (0.99) and lower and similar in two species belonging to two genera viz., *E. annulata* and *E. slateri* (0.96) but it is intermediate and unique in *A. quinquespinosa* (0.97).

8. Entire Antennal length/ Prothorax length (EAL/PTL)

The (r) values are exactly similar in two species belonging to two genera viz., *V. sinensis* and *E. slateri* (0.98) but it is slightly higher and unique in *A. quinquespinosa* (0.99) and still lower and unique in the rest of the three species belong to two genera viz., *A. pedestris* (0.97), *E. annulata* (0.96) and *A. siva* (0.95).

9. Entire Antennal length/Fore tibial length (EAL/FTL)

The (r) values are exactly similar in two species of *Acanthaspis* viz., *A. quinquespinosa* and *A. siva* (0.98) but it is higher and unique in *V. sinensis* (0.99) and lower and unique in the rest of the three species belonging to three genera viz., *A. pedestris* (0.97), *E. annulata* (0.96) and *E. slateri* (0.95).

10. Entire Antennal length/ Abdominal length (EAL/AL)

The (r) values are exactly similar in three species belonging to two genera viz., *A. quinquespinosa, A. siva* and *E. annulata* (0.95) but it is higher and unique in the rest of the three species belonging to three genera *E. slateri* (0.98) and *V. sinensis* (0.96) and lesser in *A. pedestris* (0.94).

11. Rostral length/ Head length (RL/HL)

The (r) values are exactly similar in three species of *Acanthaspis* viz., *A. pedestris, A. quinquespinosa* and *A. siva* (0.99), slightly lower and unique in two species belong to two genera *E. annulata* and *V. sinensis* (0.98) and still it is slightly lower and unique in *E. slateri* (0.97).

12. Rostral length/ Prothoracic length (RL/ PTL)

The (r) values are unique in all the six species: *A. pedestris* (0.98)>*V. sinensis* (0.97)> *E. annulata* (0.96)>*A. quinquespinosa* (0.92)>*E. slateri* (0.90)>*A. siva* (0.89).

13. Rostral length/ Foretibial length (RL/ FTL)

The (r) values are exactly similar in three species belonging to two genera viz., *A. pedestris, A. quinquespinosa* and *V. sinensis* (0.99) and lower and unique in species belonging to three genera viz., *E. annulata* (0.97), *A. siva* (0.95) and *E. slateri* (0.92).

14. Rostral length/ Abdominal length (RL/ AL)

The (r) values are exactly similar in two species of *Acanthaspis* viz., *A. pedestris* and *A. quinquespinosa* (0.98) but it is lower and unique in the rest of the four species belonging to four genera viz., *E. slateri* (0.96), *V. sinensis* (0.95), *A. siva* (0.90) and *E. slateri* (0.89).

15. Prothoracic length/ Prothoracic width (PTL/ PTW)

The (r) values are exactly similar in three species belonging to three genera viz., *A. siva, E. annulata* and *E. slateri* (0.99) lower in two speies of *Acanthaspis* viz., *A. pedestris* and *A. quinquespinosa* (0.98) and still lower and unique in *V. sinensis* (0.88).

16. Prothoracic length/ Fore tibial length (PTL/ FTL)

The (r) values are exactly similar in two species belonging to two genera viz., *E. annulata* and *V. sinensis* (0.98) and lower and unique in *E. slateri* (0.97) and lowest and similar in three species of *Acanthaspis*: *A. pedestris, A. quinquespinosa* and *A. siva* (0.90).

17. Prothoracic length/ Midtibial length (PTL/ MTL)

The (r) values are exactly similar in two species belonging to two genera viz., *A. quinquespinosa* and *E. slateri* (0.99), lower and similar in two species belonging to two genera viz., *E. annulata* and *V. sinensis* (0.98) lower and unique in *A. pedestris* (0.94) and the lowest and unique in *A. siva* (0.86).

18. Prothoracic length/ Hind tibial length (PTL/ HTL)

The (r) value is the highest and unique in *E. slateri* (0.99) lower and exactly similar in two species belonging to two genera viz., *E. annulata* and *V. sinensis* (0.98) and still lower and similar in two species of *Acanthaspis* viz., *A. quinquespinosa* and *A. siva* (0.95) and the lowest and unique in *A. pedestris* (0.94).

19. Prothoracic length/ Abdominal length (PTL/ AL)

The (r) values are exactly similar in two species belonging to two genera: *A. pedestris* and *E. annulata* (0.93) and varies and unique in the remaining four species belonging to three genera viz., *A. siva* (0.99), *A. quinquespinosa* (0.97), *E. slateri* (0.92) and *V. sinensis* (0.90).

20. Prothoracic length/ Abdominal width (PTL/ AW)

The (r) values are the lowest and exactly similar (0.94) in three species of *Acanthaspis* viz., *A. pedestris, A. quinquespinosa* and *A. siva*, higher and unique among the rest of the three species belonging to three genenra: *E. slateri* (0.99), *E. annulata* (0.96) and *V. sinensis* (0.95).

21. Abdominal length / Abdominal width (AL/AW)

The (r) values are the lowest and exactly similar in three spceis of *Acanthaspis* viz., *A. quinquespinosa, A. siva* and *E. annulata* (0.96), lower in *A. pedestris* (0.97). It is higher and unique in two species belonging to two genera viz., *E. slateri* (0.98) and *V. sinensis* (0.99).

Exactly similar (r) values

1. *A. quinquespinosa* and *A. siva* have 11 similar (r) values, i.e., Head Length/Head width (HL/HW), Head Length/Prothoracic length (HL/PTL), Head length/ Diameter of eye (HL/DE), Entire Antennal length/Foretibial length (EAL/FTL), Entire Antennal length/ Abdominal length (EAL/AL), Rostral length/ Head length (RL/HL), Prothoracic length/ Fore tibial length (PTL/ FTL), Prothoracic length/ Hind tibial length (PTL/ HTL), Prothoracic length/ Abdominal width (PTL/ AW) and Abdominal length / Abdominal width (AL/AW).
2. *A. pedestris, A. quinquespinosa* and *A. siva* have three similar (r) values, i.e., Rostral length/ Head length (RL/HL), Prothoracic length/ Fore tibial length (PTL/ FTL), Prothoracic length/ Abdominal width (PTL/ AW). Hence, these values could be considered as generic markers.
3. *A. pedestris* and *A. quinquespinosa* have seven similar (r) values, i.e., Head width/ Prothoracic width (HW/PTW), Rostral length/ Head length (RL/HL), Rostral length/

Foretibial length (RL/ FTL), Rostral length/ Abdominal length (RL/ AL), Prothoracic length/ Prothoracic width (PTL/ PTW), Prothoracic length/ Fore tibial length (PTL/ FTL) and Prothoracic length/ Abdominal width (PTL/ AW).

4. *E. slateri* and *V. sinensis* have four similar (r) values, i.e, Head Length/Head width (HL/HW), Head length/ Abdominal length (HL/AL), Head width/ Prothoracic width (HW/PTW) and Entire Antennal length/ Prothorax length (EAL/PTL).
5. *A. pedestris* and *A. siva* have four similar (r) values, i.e., Entire Antennal length/ Head length (EAL/HL), Rostral length/ Head length (RL/HL), Prothoracic length/ Fore tibial length (PTL/ FTL) and Prothoracic length/ Abdominal width (PTL/ AW).
6. *A. pedestris* and *V. sinensis* have four similar (r) values, i.e., Head Length/Head width (HL/HW), Head width/ Prothoracic width (HW/PTW), Entire Antennal length/ Head length (EAL/HL) and Rostral length/ Foretibial length (RL/ FTL).
7. *E. annulata* and *V. sinensis* have four similar (r) values, i.e., Rostral length/ Head length (RL/HL), Prothoracic length/ Fore tibial length (PTL/ FTL), Prothoracic length/ Midtibial length (PTL/ MTL) and Prothoracic length/ Hind tibial length (PTL/ HTL).
8. *A. quinquespinosa* and *E. slateri* have three similar (r) values i.e., Head length/ Diameter of eye (HL/DE), Head width/ Prothoracic width (HW/PTW) and Prothoracic length/ Midtibial length (PTL/ MTL).
9. *A. siva* and *E. annulata* have three similar (r) values, i.e., Entire Antennal length/ Abdominal length (EAL/AL), Prothoracic length/ Prothoracic width (PTL/ PTW) and Abdominal length / Abdominal width (AL/AW)

10. *E. annulata* and *E. slateri* have three similar (r) values, i.e., Entire Antennal length/ Head length (EAL/HL), Entire Antennal length/ Prothorax length (EAL/PTL) and Prothoracic length/ Prothoracic width (PTL/ PTW)
11. *A. quinquespinosa* and *V. sinensis* have two similar (r) values, i.e., Head width/ Prothoracic width (HW/PTW) and Rostral length/ Foretibial length (RL/ FTL)
12. *A. pedestris* and *E. slateri* have two similar (r) values i.e., Head Length/Head width (HL/HW) and Head width/ Prothoracic width (HW/PTW).
13. *A. pedestris* and *E. annulata* have one similar (r) value, i.e., Prothoracic length/ Abdominal length (PTL/ AL).
14. *A. quinquespinosa* and *E. annulata* have one similar (r) value, i.e., Entire Antennal length/ Abdominal length (EAL/AL).
15. *A. siva* and *E. slateri* have one similar (r) value, i.e., Prothoracic length/ Prothoracic width (PTL/ PTW)
16. *A. siva* and *V. sinensis* have one similar (r) value, i.e., Entire Antennal length/ Head length (EAL/HL)

Closer (r) values

1. *A. pedestris* and *A. quinquespinosa* have four closer (r) values, i.e., Head length/ Diameter of eye (HL/DE), Entire Antennal length/Foretibial length (EAL/FTL), Prothoracic length/ Hind tibial length (PTL/ HTL) and Abdominal length / Abdominal width (AL/AW).
2. *A. pedestris* and *A. siva* have six closer (r) values, i.e., Head length/ Diameter of eye (HL/DE), Entire Antennal length/Foretibial length (EAL/FTL), Entire Antennal length/ Abdominal length (EAL/AL), Prothoracic length/ Prothoracic width (PTL/

PTW), Prothoracic length/ Hind tibial length (PTL/ HTL) and Abdominal length / Abdominal width (AL/AW).

3. *A. pedestris* and *E. annulata* have seven closer (r) values, i.e., Head Length/Head width (HL/HW), Entire Antennal length/ Prothorax length (EAL/PTL), Entire Antennal length/Foretibial length (EAL/FTL), Entire Antennal length/ Abdominal length (EAL/AL), Rostral length/ Head length (RL/HL), Prothoracic length/ Prothoracic width (PTL/ PTW) and Abdominal length / Abdominal width (AL/AW).
4. *E. annulata* and *E. slateri* have seven closer (r) values, i.e., Head Length/Head width (HL/HW), Entire Antennal length/Foretibial length (EAL/FTL), Rostral length/ Head length (RL/HL), Prothoracic length/ Fore tibial length (PTL/ FTL), Prothoracic length/ Midtibial length (PTL/ MTL), Prothoracic length/ Hind tibial length (PTL/ HTL) and Prothoracic length/ Abdominal length (PTL/ AL).
5. *A. quinquespinosa* and *E. annulata* have six closer (r) values, i.e., Head Length/Head width (HL/HW), Head width/ Width between eyes (HW/WBE), Entire Antennal length/ Head length (EAL/HL), Rostral length/ Head length (RL/HL), Prothoracic length/ Prothoracic width (PTL/ PTW) and Prothoracic length/ Midtibial length (PTL/ MTL).
6. *A. pedestris* and *E. slateri* have six closer (r) values, i.e., Head Length/Prothoracic length (HL/PTL), Head length/ Diameter of eye (HL/DE), Entire Antennal length/ Prothorax length (EAL/PTL), Prothoracic length/ Prothoracic width (PTL/ PTW), Prothoracic length/ Abdominal length (PTL/ AL) and Abdominal length / Abdominal width (AL/AW).
7. *A. quinquespinosa* and *V. sinensis* have six closer (r) values, i.e, Entire Antennal length/ Prothorax length (EAL/PTL), Entire Antennal length/Foretibial length

(EAL/FTL), Rostral length/ Head length (RL/HL), Prothoracic length/ Midtibial length (PTL/ MTL) and Prothoracic length/ Abdominal width (PTL/ AW).

8. *E. annulata* and *V. sinensis* have six closer (r) values, i.e., Head Length/Head width (HL/HW), Head Length/Prothoracic length (HL/PTL), Entire Antennal length/ Abdominal length (EAL/AL), Rostral length/ Prothoracic length (RL/ PTL), Rostral length/ Abdominal length (RL/ AL) and Prothoracic length/ Abdominal width (PTL/ AW).
9. *A. pedestris* and *V. sinensis* have five closer (r) values, i.e., Head Length/Prothoracic length (HL/PTL), Entire Antennal length/ Prothorax length (EAL/PTL), Rostral length/ Head length (RL/HL), Rostral length/ Prothoracic length (RL/ PTL) and Prothoracic length/ Abdominal width (PTL/ AW).
10. *A. quinquespinosa* and *E. slateri* have four closer (r) values, i.e., Head width/ Width between eyes (HW/WBE), Entire Antennal length/ Head length (EAL/HL), Entire Antennal length/ Prothorax length (EAL/PTL) and Prothoracic length/ Prothoracic width (PTL/ PTW).
11. *A. siva* and *V. sinensis* have four closer (r) values, i.e., Entire Antennal length/Foretibial length (EAL/FTL), Entire Antennal length/ Abdominal length (EAL/AL), Rostral length/ Head length (RL/HL) and Prothoracic length/ Abdominal width (PTL/ AW).
12. *E. slateri* and *V. sinensis* have four closer (r) values, i.e., Rostral length/ Head length (RL/HL), Prothoracic length/ Fore tibial length (PTL/ FTL), Prothoracic length/ Hind tibial length (PTL/ HTL) and Abdominal length / Abdominal width (AL/AW).

13. *A. siva* and *E. annulata* have three closer (r) values, i.e., Head Length/Head width (HL/HW), Entire Antennal length/ Prothorax length (EAL/PTL) and Rostral length/ Head length (RL/HL).
14. *A.siva* and E. slateri have two closer (r) values, i.e., Rostral length/ Prothoracic length (RL/ PTL) and Rostral length/ Abdominal length (RL/ AL).
15. *A. quinquespinosa* and *A. siva* have one closer (r) value, i.e., Prothoracic length/ Prothoracic width (PTL/ PTW).

Unique (r) values

1. Head Length/Head width (HL/HW)

It is unique only in *E. annulata* (0.98).

2. Head Length/Prothoracic length (HL/PTL)

It is unique in four species viz., *E. slateri,* (0.99), *A. pedestris* (0.98), *V. sinensis* (0.97) and *E. annulata,* (0.96) belonging to all the four genera.

3. Head length/ Abdominal length (HL/AL)

It is unique in three species of *Acanthaspis*: *A. quinquespinosa* (0.99), *A. pedestris* (0.90) and *A. siva* (0.92).

4. Head length/ Diameter of eye (HL/DE)

It is unique in three species belonging to three genera: *A. pedestris* (0.99), *E. annulata* (0.96) and *V. sinensis* (0.94).

5. Head width/ Width between eyes (HW/WBE)

It is unique in all the six species belonging to four genera: *A. quinquespinosa* (0.98), *E. annulata* (0.97) and *E. slateri* (0.99), *A. pedestris* (0.93), *A. siva* (0.89) and *V. sinensis* (0.84).

6. Head width/ Prothoracic width (HW/PTW)

It is unique in two species belonging to two genera: *E. annulata* (0.98) and *A. siva* (0.89).

7. Entire Antennal length/ Head length (EAL/HL)

It is only in *A. quinquespinosa* (0.97).

8. Entire Antennal length/ Prothorax length (EAL/PTL)

It is unique in three species of *Acanthaspis* viz., *A. quinquespinosa* (0.99), *A. pedestris* (0.97) and *A. siva* (0.95) and the lone species of *Empyrocoris, E. annulata* (0.96) belonging to two genera.

9. Entire Antennal length/Fore tibial length (EAL/FTL)

It unique in four species belonging to three genera: *V. sinensis* (0.99), *A. pedestris* (0.97), *E. annulata* (0.96) and *E. slateri* (0.95).

10. Entire Antennal length/Abdominal length (EAL/AL)

It is unique in three species belonging to three genera: *E. slateri* (0.98), *V. sinensis* (0.96) and *A. pedestris* (0.94).

11. Rostral length/ Head length (RL/HL)

It is unique only in *E. slateri* (0.97).

12. Rostral length/ Prothoracic length (RL/ PTL)

It is unique in all the six species belonging to four genera viz., *A. pedestris* (0.98), *V. sinensis* (0.97), *E. annulata* (0.96), *A. quinquespinosa* (0.92), *E. slateri* (0.90) and *A. siva* (0.89).

13. Rostral length/ Foretibial length (RL/ FTL)

It is unique in three species belonging to three genera: *E. annulata* (0.97), *A. siva* (0.95) and *E. slateri* (0.92).

14. Rostral length/ Abdominal length (RL/ AL)

It is unique in four species belonging to four genera *E. annulata* (0.96), *V. sinensis* (0.95), *A. siva* (0.90) and *E. slateri* (0.89).

15. Prothoracic length/ Prothoracic width (PTL/ PTW)

It is unique only in *V. sinensis* (0.88).

16. Prothoracic length/ Fore tibial length (PTL/ FTL)

It is unique only in *E. slateri* (0.97).

17. Prothoracic length/ Midtibial length (PTL/ MTL)

It is unique in two *Acanthaspis* species viz., *A. pedestris* (0.94) and *A. siva* (0.86).

18. Prothoracic length/ Hind tibial length (PTL/ HTL)

It is unique in two species belonging to two genera: *E. slateri* (0.99) and *A. pedestris* (0.94).

19. Prothoracic length/ Abdominal length (PTL/ AL)

It is unique in four species belonging to three genera viz., *A. siva* (0.99), *A. quinquespinosa* (0.97), *E. slateri* (0.92) and *V. sinensis* (0.90).

20. Prothoracic length/ Abdominal width (PTL/ AW)

It is unique in three species viz., *E. slateri* (0.99), *E. annulata* (0.96) and *V. sinensis* (0.95) belonging to two genera.

21. Abdominal length / Abdominal width (AL/AW)

It is unique in two species viz., *E. slateri* (0.98) and *V. sinensis* (0.99) belonging to two genera.

UPGMA affinity analysis

The dendrogram of linear regression coefficient values (r) of postembryonic developmental morphometry was constructed with Unweighted Pair Group Method with

Arithmetic Mean (UPGMA) clustering revealed intergeneric relationships among six species of Reduviinae. The results showed, that among 21 morphometric characters of all the six species, the highest similarity was observed between EAL/HL and EAL/FTL (99.57%) followed by EAL/PTL and AL/AW (99.57%); EAL/AL and PTL/AW (99.56%); HL/HW and RL/HL (`99.40%); RL/PTL and RL/AL (99.11%); HW/PTW and PTL/ MTL (98.85%) and HL/AL and PTL/ FTL (98.40%) (Table 2) (Figure 1).

Discussion

The twenty one morphometric analyses carried out in six species of Reduviinae and observed under exactly similar, closer and unique (r) values reveal inter- and intrageneric and intraspecific characters. The analyses further reveal no subfamily characteristics supported by exactly similar, closer and unique (r) values. This could be attributed to the lesser number of taxa subjected to the analysis.

Intrageneric affinity could be analysed only in *Acanthaspis* since all other three genera are represented by one species each. In this genus intrageneric affinity is revealed by three similar (r) values: RL/HL, PTL/FTL and PTL/AW. Intergeneric affinity is greater between *Edocla* and *Velitra* and *Empyrocoris* and *Velitra, Acanthaspis* and *Velitra* as evidenced by four similar (r) values: HL/HW, HL/AL, HW/PTW, EAL/HL and RL/FTL and RL/HL, PTL/FTL, PTL/MTL and PTL/HTL respectively. The closer (r) value analysis reveal the greater intergeneric affinity between a species of *Acanthaspis* and *Edocla* and *Edocla* and *Empyrocoris* with seven values followed by two species of *Acanthaspis* and *Edocla,* one species of *Acanthaspis* and *Velitra* and *Edocla* and *Velitra* with six values. Thus, the closer values also reveal the differential level of intergeneric affinity between these species. However, varied levels of affinities observed between the three different species of *Acanthaspis* and the other three genera viz., *Edocla, Empyrocoris* and *Velitra* could not be

correlated and subjected into meaningful analysis. This is due to the fact that the genus *Acanthaspis* alone is represented by multispecies and the other three genera are represented by only one species, each. Hence, this analysis should be analysed by incorporating more number of species in each genus and more genera.

Interspecific affinity could be analysed only among the three members of *Acanthaspis.* The two alate species viz., *A. quinquespinosa* and *A. siva* are very closely related than to the micropterous *A. pedestris.* The alate species *A. quinquespinosa* and *A. siva* share a very high affinity with elven exactly similar (r) values: HL/HW, HL/PTL, HL/DE, EAL/FTL, EAL/AL, RL/HL, PTL/FTL, PTL/HTL, PTL/AL and AL/AW. This greater affinity is in much contrast between the two alate species with the micropterous one. For instance the alate *A. quinquespinosa* shares with micropterous *A. pedestris* by seven similar (r) values, and the alate *A. siva* with micropterous *A. pedestris* with four similar (r) values. It is also revealing that *A. pedestris* is closer to *A. quinquespinosa* than to *A. siva.* It is interesting to report here that both *A. pedestris* and *A. quinquespinosa* live in almost microhabitats, i.e., underneath the boulders and in crevices whereas *A. siva* prefers to live under barks. Hence, not only the morphological characters but also the ecological characteristics have the impact on morphometrics. Hence, a multidisciplinary approach of integrating morphometrical, morpholocical and ecological along with molecular characteristics could lead into a meaningful biosystematic analysis.

The varied levels of interspecific affinity observed between these three species further suggests the genetic variability existing among these species. Such a generic plasticity could be lead not only for species affinity but also leads to speciation.

Intraspecific or species specific (r) values found in *E. slateri* (eleven unique values: (HL/PTL), (HW/WBE), (EAL/FTL), (EAL/AL), (RL/HL), (RL/PTL), (RL/FLTL), (RL/AL),

(PTL/FTL), (PTL/HTL) and (PTL/AL)) followed by *A. pedestris* (ten unique values: (HL/PTL), (HL/AL), (HL/DE), (HW/WBE), (EAL/PTL), (EAL/FTL), (EAL/AL), (RL/PTL), (PTL/MTL) and (PTL/HTL)), *A. siva* (nine unique values: (HL/AL), (HW/WBE), (HW/PTW), (EAL/PTL), (RL/PTL), (RL/FTL), (RL/AL), (PTL/MTL) and (PTL/AL)), *E. annulata* (eight unique values: (HL/HW), (HL/PTL), (HL/DE), (HW/WBE), (HW/PTW), (EAL/PTL), (EAL/FTL) and (RL/FTL)), *V. sinensis* (eight unique values: (HL/PTL), (HL/DE), (HW/WBE), (EAL/FTL), (EAL/AL), (RL/AL), (PTL/PTW) and (PTL/AL)) and *A. quinquespinosa* (five unique values: (HW/WBE), (EAL/HL), (EAL/PTL), (RL/PTL) and (PTL/AL)) could be considered for species specific markers.

Rukmani (1992) first explored the linear regression coefficient (r) values of postembryonic developmental morphometry of life stages of reduviids as a tool in the multidisciplinary biosystematics. She used 36 morphological characteristics of 27 species belonging to 14 genera and three subfamilies and reported their utility in the multidisciplinary biosystematics. Later Ambrose and Ambrose (2009b) reported 36 morphometric indices belonging to 27 species, 14 genera and 3 subfamilies viz., Harpactorinae, Peiratinae and Reduviinae by direct observation as well as unweighted pair group method using arithmetic mean (UPGMA) incorporating Euclidean distances, Nei and Li's genetic distance coefficient and Jaccard's coefficient to understand diagnostic intra- as well as intersubfamilial and generic affinities. George *et al.* (2005) also performed UPGMA cluster analysis of thirty three morphological and biological characteristics of thirty species of reduviids belonging to seventeen genera and three subfamilies and found intra- and intergeneric and interfamilial affinities.

The dendrogram analysis showed the highest affinity between EAL/HL and EAL/FTL (99.57%) followed by EAL/PTL and AL/AW (99.57%); EAL/AL and PTL/AW (99.56%);

HL/HW and RL/HL (`99.40%); RL/PTL and RL/AL (99.11%); HW/PTW and PTL/ MTL (98.85%) and HL/AL and PTL/ FTL (98.40%). The higher similarity values (98.4 to 99.57%)) confirmed the intergeneric affinity of all the four genera of subfamily Reduviinae. Singh *et al.* (2011) analysed the linear regression coefficient values of twenty one morphological characteristics of four *Rhynocoris* species viz., *R. fuscipes*, *R. kumarii*, *R. longifrons* and *R. marginatus* and found interspecific and intraspecific markers.

Conclusion

The above said analysis of linear regression coefficient values (r) of postembryonic developmental morphometry suggests its utility as a biosystematics tool at generic and species levels. However, the sample size taken for the present study is highly inadequate. Hence, further studies with more number of species and genera from different subfamilies are imperative to analyze the multidisciplinary facets of biosystematics with postembryonic developmental morphometry as a tool.

CHAPTER II

PROTEIN PROFILING

Introduction

Proteins do not have a predictable structure as nucleic acids and thus their rates of migration are not similar to each other. They may not migrate when applying an electromotive force (when they are in their isoelectric point). In these cases, the proteins are denatured by adding a detergent such as sodium dodecyl sulfate (SDS) to separate them exclusively according to molecular weight. This technique was firstly introduced by Shapiro *et al*. (1967). Moreover, comparison of protein patterns is widely used for taxonomic purposes (Stephen, 1958; Halliday, 1975; Nunamaker *et al.,*1984; Soares *et al.,* 1998; Suranto, 2002). The value of protein profiling is increasing daily because it provides a much better understanding of an organism (Graves and Haystead, 2002).

Gel electrophoresis is a widely known group of techniques used to separate and identify macromolecules such as DNA, RNA, or proteins based on size, form, or isoelectric point. These techniques have become a main tool in biochemistry, molecular biology, analytical chemistry and proteomics. Gel electrophoresis is usually used for analytical purposes, but may be a preparative technique to partially purify molecules before applying other techniques (Wasinger *et al*., 1995). Recently, they have tended to use a new additional experimental method called electrophoresis. This method has been regarded to be the most useful for resolving taxonomic problems, if comparisons about morphological characters are felt not adequate in helping taxonomists to make decisions (Suranto, 2002). So undoubtedly electrophoresis will play an increasingly important role in taxonomy, and in monitoring for example, the manipulations of crop genetic resources (Brown, 1978) and in the study of origin of species (Gastony, 1986). Earlier, the identification of insects was totally based on morphology but there were some limitations in employing morphology based identification

which may be due to natural phenotypic variation within a species, shortage of experienced taxonomists required for identification (Hebert *et al*., 2003). Subsequently, gel electrophoresis, in recent years has gained immense importance through the introduction of the synthetic polyacrylamide gel. The polyacrylamide gel is considered as one of the best medium which supports for resolving larger number of protein bands and is clearer than with cellulose acetate for paper (Suranto, 2002).

Despite alternative technologies that have emerged, gel electrophoresis of proteins together with mass spectrometry has allowed the interpretation of a great set of data generated by the "omics". Understanding the proteome of an organism, thanks to these techniques, makes available a dynamic picture of all proteins expressed, at any given moment and under specified conditions of time and environment (Laura *et al.,* 2012).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Though many methods exist for the determination of the molecular size of protein complexes, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS –PAGE) is widely used for the study of the homogeneity of protein complexes, as this ionic detergent introduces charge to the proteins (Kantzilakis *et al.,* 2007). In this technique, the charged molecules moves through a gel matrix by means of an electric current and used to determine protein subunit composition, verify homogeneity of the protein sample, and purify proteins for use in other applications and the migration rate of the proteins during SDS-PAGE is determined by the pore size of the gel matrix charge, size and shape of the protein (Manns, 2011).

Methodology

Laemmli (1970) adopted the discontinuous electrophoresis method and the term "Laemmli buffer" is often used to describe the tris-glycine buffer system that is utilized during SDS-PAGE. For instance, a 7% polyacrylamide gel has larger pores than a 12%

polyacrylamide gel. Gels with a low percentage of acrylamide are typically used to resolve large proteins, and high percentage gels are used to resolve small proteins. "Gradient gels" are specially prepared to have low percent-acrylamide at the top and high percent-acrylamide at the bottom, enabling a broader range of protein sizes to be separated (Laura *et al.,* 2012).

Hjelmeland and Chrambach (1981) stated that a source of error in molecular weight estimates, is that protein mobility in the gel is more a function of molecular size (which is a function of both weight and length) than of molecular mass. It is generally assumed that SDS proteins all exist in a random coil form, so the relationship between length and mass should be constant. Even assuming constant charge, if a protein has unreduced disulfide bonds or areas of incompletely disrupted secondary structure, it cannot unfold to full length and, it would tend to run faster than expected in a typical SDS gel (Sallantin *et al.,* 1990).

The polyacrylamide gels are formed by polymerization of acrylamide by the action of a cross-linking agent, the bis-acrylamide, in the presence of an initiator and a catalyst. Persulfate ion (S_2O_8-), that is added as ammonium persulfate (APS) is the gel solidifying initiator and a source of free radicals, while TEMED (N, N, N', N'-tetramethylethylenediamine) catalyzes the polymerization reaction by stabilizing these free radicals. In some situations, for example, isoelectric focusing the presence of persulfate can interfere with electrophoresis, so riboflavin and TEMED are used instead (Laura *et al.,* 2012). The ratio between of acrylamide/bisacrylamide as well as the total concentration of both components, affects the pore size and rigidity of the final gel matrix. These, in turn, affect the range of protein sizes that can be resolved (Laura *et al.,* 2012).

The size of the pores created in the gel is inversely related to the amount of acrylamide used. The SDS is a reducing agent that breaks disulfide bonds, separating the protein into its sub-units and also gives a net negative charge which allows them to migrate through the gel in direct relation to their size. In addition, denaturation makes them lose their

tertiary structure and therefore migration velocity is proportional to the size and not to tertiary structure (Laura *et al.,* 2012).

Application

Gel electrophoresis is used in a wide variety of scientific, criminal, and legal investigations. Although all organisms have a high percentage of genes in common, there are significant genetic differences among members of the same family, even among members of the same species. Analysis of insect protein patterns by gel electrophoresis using polyacrylamide gel is used for taxonomic purposes (Stephen, 1958; Halliday, 1975; Nunamaker and Wilson, 1981; Nunamaker *et al.,* 1984, 1985), recognition of species complexes in various animals (Selander *et al.,* 1971; Mahon, 1974), identification of the origin of species, differentiation of species and understanding the population variations and inter- and intra- genetic relationships (Suranto, 2002).

In addition to that, it has been widely used for the separation of proteins and particularly used to identify the differences and similarity between the species (Sulistyarsi *et al.,* 2012). Moreover, it is a convenient, fast and inexpensive method because it requires only the order of micrograms quantities of protein (Laura *et al.,* 2012).

A combined approach of identifying insects has been developed where both morphology and molecular data are being considered. Here, an attempt was made to document the whole body protein data of the reduviid species to understand the species and generic specificity and interspecies and intergeneric affinity.

Materials and Methods

Collection:

The adults of six species of Reduviinae viz., *Acanthaspis pedestris* Stål and *Acanthaspis quinquespinosa* (Fabricius) were collected from Sivanthipatti (latitude 8^{o}7 "N and longitude77^{o} 21"E), Tirunelveli District and *Acanthaspis siva* Distant*, Empyrocoris*

annulata (Distant)*, Edocla slateri* Distant and *Velitra sinensis* (Walker) were collected from Muppanthal Scrub Jungle (latitude 8° 26"N and longitude 77°54"E), Kanyakumari District, South India. The body protein of six species of reduviids belonging to four genera of subfamily Reduviinae were subjected to electrophoretic protein profiling to evaluate the utility of protein profile as a marker at subfamily, genus and species level.

Sample preparation:

Field collected and laboratory acclimatized entire fresh adults were homogenized by mortar and pestle, separately. The 2X sample buffer (Tris (0.5M, pH 6.8 - 2.5ml), SDS(10%) - 4.0ml, Glyceral(100%) - 2.0ml, β-mercaptoethanol - 0.8ml, Bromophenol blue (0.1%) - 300µl and made up to 10.0ml with dH_2O) was used for homogenization. A standard marker (14.3-97.4 KDa: Geni Bangalore, India) was added in the first well. The homogenized samples were kept in water bath at 90°C for 10 minutes and 10-15µl of the supernatant of six samples was loaded into six different lanes, i.e., 2 to 7 of the vertical electrophoretic unit (Bio-Rad Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels: 165-8005EDU. 2-gel vertical electrophoresis system includes electrode assembly, tank, lid with power cables, mini cell buffer dam).

SDS-PAGE analysis:

The SDS-PAGE was carried out with **10% separating gel** (dH_2O-4.00ml, 30% acrylamide: bisacrylamide-3.33ml, 4xTris (pH 8.8)-2.50ml, TEMED-5µl, 10% SDS-100µl, 10% APS (100mg/ml)-50µl); **4% stacking gel** (dH_2O-3.00ml, 30% acrylamide: bisacrylamide-0.67ml, 4xTris (pH 6.8)-1.25ml, TEMED-2.5µl, 10% SDS-25µl, 10% APS (100mg/ml)-50µl); t**ank buffer** (pH (8.3) (Tris-6.05g, glycine-28.80g, 10%SDS-10.0ml, dH_2O -1000ml) and a mid-range **molecular weight marker** (14.3-97.4 KDa (GENI, Bangalore, India)) was used. The gel was run at 80 volts for 2 to 3 hours. The gel was stained for 2-3 hours in staining solution (coomassie blue-0.3g, methanol (AR)-80ml, glacial acetic

acid-20ml, dH_2O-100ml) and destained for 2-4 hours in destaining solution (acetic acid-100ml, methanol-3.00ml, dH_2O -1000ml).

Data analysis:

The gels were photographed and analysed with TotalLab 100 software to determine banding pattern and molecular weight. A dendrogram was constructed with the SAHN program using the UPGMA in NTSYS version 2 (Rohlf, 1993). Band sharing coefficient between individuals was calculated by employing the following formula (Wetton *et al.*, 1987).

$D = 2\ N_{ab}/(N_a+N_b)$

D = Band sharing coefficient

N_{ab} = Number of bands shared by individuals a and b

N_a = Number of bands obtained by a

N_b = Number of bands obtained by b

Results

Total number of bands

The total number of protein bands observed in *A. pedestris, A. quinquespinosa, A. siva, E. slateri, E. annulata* and *V. sinensis* was 14, 6, 17, 11, 10 and 12 (Table 3) suggesting the uniqueness among species, i.e., species specific characters (Plate 1).

Total Band Volume

Each species had an unique total band volume. The highest band volume was observed in *A. siva* (5511319) and the lowest in *E. slateri* (3310743) and the band volumes of remaining species were: *A. pedestris* (5097651), *A. quinquespinosa* (1921035), *E. annulata* (4088102) and *V. sinensis* (3647999) (Table 3).

Banding pattern

The highest band volume observed in the six reduviine species is as follows: *A. pedestris* (972414)> *A. siva* (783452)> *E. annulata* (661677)> *E. slateri* (472061)> *A.quinquespinosa* (361164)> *V. sninensis* (263130) at 16th, 13th, 16th, 16th and 13th position respectively. The lowest band volume observed is as follows: *V. sinensis* (125016)<*E. slateri* (168207)<*A. siva* (178990<*A. pedestris* (228439)<*A. quinquespinosa* (235943)<*E. annulata* (295545) at 13th, 13th, 14th , 6th, 19th and 4th position respectively. Thus, as observed for total number of bands and the total band volume, the highest and the lowest band volumes also exhibit uniqueness among the six reduviine species (Table 4).

Common protein band

The protein profile shows the common protein bands at 13th and 22nd position for all the six reduviine species. Similarly, common protein bands were observed in *A. pedestris, A. quinquespinosa* and *A. siva* at 13th and 19th banding position and in *E. annulata* and *E. slateri* at 7th, 8th, 11th, 12th, 13th, 16th, 21st and 22nd banding positions.

Peak height of band

The highest peak observed for the six reduviine species is as follows: *A. pedestris* (101.26)> *A. quinquespinosa* (93.31)> *A. siva* (102.36)> *E. slateri* (111.54)> *E. annulata* (105.85) > *V. sinensis*(109.59) at 3rd ,14th , 4th ,3rd , 4th and 1st position respectively . The lowest peak observed is as follows: *A. pedestris* (66.91) > *A. quinquespinosa* (76.93)> *A. siva* (76.63)> *E. slateri* (79.98)> *E. annulata* (86.30)> *V. sinensis* (95.90) at 6th, 22nd, 17th, 16th, 21st and 16th position respectively. The highest and the lowest peak heights also exhibit uniqueness among the six reduviine species (Table 5) (Figure 2-8).

Molecular Weight of bands

The highest molecular weight band observed in the six reduviine species is as follows: *A. pedestris* (80363.175)> *A. quinquespinosa* (35082.317)> *A. siva* (74696.088)> *E. slateri*

(75614.023)> *E. annulata* (69179.310) and *V. sinensis*(98351.515) at 3rd, 13th 3rd, 3rd, 4th and 1st position respectively. The lowest peak observed is as follows: *A. pedestris* (18122.222)> *A. quinquespinosa* (18486.508)> *A. siva* (18546.032)> *E. slateri* (16568.254)> *E. annulata* (18757.937)> *V. sinensis*(16992.063) at 22nd position. Thus, each species exhibits uniqueness for molecular weight (Table 6).

The electropherogram of all the six species of Reduviinae examined with similar band at 29kDa shows the intergeneric affinity. Similar bands are observed between *E. slateri* and *V. sinensis* (16kDa); *A. pedestris, A. quinquespinosa, A. siva* and *E. slateri* (18kDa) ; *A. quinquespinosa* and *E. slateri* (20kDa); *A. siva* and *E. slateri* (21kDa); *A. pedestris, A. quinquespinosa, A. siva* (24kDa); *A. siva* and *E. slateri* (25 and 48kDa); *A.siva* and *V. sinensis* (32kDa), *A. pedestris, A. quinquespinosa, A. siva, E. slateri* (35kDa); *A. siva, E. slateri, E. annulata,* and *V. sinensis* (38kDa); *E. slateri, E. annulata* and *V. sinensis* (42kDa); *A. siva* and *E. annulata* (45kDa); *A. pedestris* and *E. slateri* (46kDa); *A. pedestris* and *E. annulata* (69kDa) and *E. slateri* and *V. sinensis* (75kDa) suggesting intrageneric, intergeneric and interspecies affinities at different levels (Table 7).

Similar Molecular Weight bands

The protein profile shows the similar molecular weight band of 18, 29 and 34-36 kDa for all the six reduviine species. Likewise, similar molecular weight bands of 35, 29, 24 and 18 kDa were uniformly observed for genus *Acanthaspis* and molecular weight bands of 29, 38 and 42 kDa were observed for *E. annulata* and *E. slateri*.

Similarity coefficient and species affinity:

Similarity coefficient matrix calculated using the UPGMA of the NTSYS version 2 shows that all the three *Acanthaspis* species viz., *A. pedestris, A. quinquespinosa* and *A. siva* together constitute a cluster suggesting the intrageneric affinity. Another cluster is constituted

by *E. slateri* and *E. annulata* together whereas the *V. sinensis* stood independently suggesting again generic specificity and intergeneric variability (Figure 9).

Discussion

The morphologically similar species with similar distribution complicates their determination. In reduviids, Kumaraswamy (1991) used the salivary gland proteins of three *Rhynocoris* species to differentiate the morphologically similar species. Soares *et al.* (2000) also used the salivary heme proteins as markers in the determination of two *Rhodnius* species. But in this analysis, the whole body protein profile of six reduviine species were used to differentiate the species. The electropherogram shows the difference in total number of protein bands, i.e., *A. pedestris* (14)*, A. quinquespinosa* (6)*, A. siva*(17)*, E. slateri*(11)*, E. annulata* (10) and *V. sinensis* (12). Hence, the electrophoretic banding profiles could be used as a potential marker in determination of species and to understand the phylogenetic relationship at species and generic levels (Jariyapan *et al.,* 2006). Hence, the analysis of whole body protein may help in the determination of species.

Subfamily characters

A common band observed at 13^{th} and 22^{nd} position, a similar molecular weight band observed at 18, 29 and 34-36 kDa could be used as a marker for reduviine species.

Generic characters

The common protein bands at 13^{th} and 19^{th} banding position, a closer peak height at 22^{nd} and 19^{th} positions and similar molecular weights of 35, 29, 24 and 18 kDa bands uniformly observed in three species of *Acanthaspis* viz., *A. pedestris, A. quinquespinosa* and *A. siva* suggest the intrageneric affinity. Thus, these common values could be used as a generic marker for *Acanthaspis* species. This is further reiterated by the clustering of all the three *Acanthaspis* species in the dendrogram analysis. Likewise, common protein bands at 7^{th}, 8^{th}, 11^{th}, 12^{th}, 13^{th}, 16^{th}, 21^{st} and 22^{nd} banding positions, a closer peak height at 7^{th} and

12th banding positions and similar molecular weights of 29, 38 and 42 kDa bands observed for *Empyrocoris annulata* and *Edocla slateri* reveal intergeneric affinity. The intergeneric affinity between these two species is further emphasized by clustering together. The separate lineage shown by *V. sinensis* shows the unique character of this genus.

Species specific characters

A higher concentration band at 16th position and unique molecular weight bands of 80, 61, 56, 52 43, 39 and 31 kDa of *A. pedestris* could be used as its species specific markers.

A higher concentration band at 16th banding position and unique molecular weight band of 33 kDa of *A. quinquespinosa* could be used as its species specific markers.

A higher concentration band at 13th position and unique molecular weight bands of 74, 66, 59, 53, 41 and 23 kDa of *A. siva* could be used as its species specific markers.

A higher concentration band at 9th and 7th position and unique molecular weight bands of 75 and 55 kDa of *E. slateri* could be used as its species specific markers.

A higher concentration band at 16th banding position and unique molecular weight bands of 54, 49 and 34 kDa of *E. annulata* could be used as its species specific markers.

A higher concentration band at 13th position and unique molecular weight bands of 87, 57, 36 and 19 kDa of *V. sinensis* could be used as its species specific marker for this species. The findings corroborate with that of Brodie and Ryckman (1967) who used electrophoretic analysis of haemolymph proteins of 18 species of kissing bugs as a tool to differentiate within each of the complexes and distinctive differences between species groups.

Clustering of *A. pedestris, A. quinquespinosa* and *A. siva* together in the dendrogram revealed the intrageneric affinity. The other cluster constituted by *E. slateri* and *E. annulata* together shows the intergeneric affinity between these two genera whereas *V. sinensis* standing independently suggests its generic specificity. Intergeneric variability is also

observed while considering the overall distribution of all the four genera. Baskar (2010) and Singh (2012) observed intrageneric affinity in four *Rhynocoris* species viz., *R. kumarii* Ambrose and Livingstone*, R. marginatus* (Fabricius)*, R. fuscipes* (Fabricius) and *R. longifrons* Stål. Singh (2012) also observed intraspecific affinity in four ecotypes of *R. kumarii* and three morphs of *R. marginatus*.

Hence, the electrophoretic profiles could be used as a potential marker not only in determination of species but also to understand the phylogenetic relationship at species and genus levels as suggested by Jariyapan *et al.* (2006). Furthermore, similar study would be helpful for taxonomic separation that had been carried out earlier by Adams and Ryckman (1969) in *Triatoma rubida* (Uhler) population.

Conclusion

The whole body electrophoretic profiling could be used as an identification marker at subfamily, generic and species level. It could help not only in the determination of these species but also differentiates one species from another. The dendrogram analysis clearly shows the intra- and intergeneric affinity and species specificity of these species. Such analysis could improve the quality and authenticity of species determination based on molecular data. Though protein profiling could be used as identification markers for these species, better results could be observed with more number of species to understand the phylogenetic affinity. Hence, further work is needed with more number of species belonging to this family.

CHAPTER – III

BIOSYSTEMATICS OF CHOSEN REDUVIINE SPECIES BASED ON MITOCHONDRIAL CYTOCHROME C OXIDASE SUBUNIT I GENE SEQUENCES

Introduction

The genetic diversity of life underpins all biological studies, but it is also a harsh reality. In fact, since few taxonomists can critically identify more than 0.01% of the estimated 10-15 million species (Hammond, 1992; Hawksworth and Kalin-Arroyo, 1995), a community of 15000 taxonomists will be required, in perpetuity, to identify life if our reliance on morphological diagnosis is to be sustained.

Moreover, this approach to the task of routine species identification has significant limitations. Both phenotypic plasticity and genetic variability in the characters employed for species recognition can lead to incorrect identifications. This approach overlooks morphologically cryptic taxa, which are common in many groups (Knowlton, 1993; Jarman and Elliott, 2000). Since morphological keys are often effective only for a particular life stage or gender, many individuals cannot be identified. Finally, although modern interactive versions represent a major advance, the use of keys often demands such high levels of expertise that wrong diagnoses are common.

The limitations inherent in morphology-based identification systems and the dwindling pool of taxonomists signal the need for a new approach to taxon recognition. Microgenomic identification systems, which permit life's discrimination through the analysis of a small segment of the genome, represent one extremely promising approach to the diagnosis of biological diversity. This concept has already gained broad acceptance among those working with the least morphologically tractable groups, such as viruses and bacteria (Nanney, 1982; Pace, 1997; Allander *et al*., 2001; Hamels *et al*., 2001). However, the

problems inherent in morphological taxonomy are general enough to merit the extension of this approach to all life. In fact, there are a growing number of cases in which DNA-based identification systems have been applied to higher organisms (Brown *et al*., 1999; Bucklin *et al*., 1999; Trewick, 2000; Vincent *et al*., 2000).

Miotochondria

The **mitochondrion** (plural **mitochondria**) is a membrane-bound organelle found in most eukaryotic cells (the cells that make up plants, animals, fungi, and many other forms of life (Henze, 2003). Mitochondria range from 0.5 to 1.0 micrometer (μm) in diameter. These structures are sometimes described as "cellular power plants" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy (Campbell, 2006). In addition to supplying cellular energy, mitochondria are involved in other tasks such assignaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth (McBride, 2006)

Mitochondria are known as energy suppliers for the cell, as it is in the mitochondria that the transfer of electrons down an electrochemical gradient to the final acceptance of oxygen occurs with the subsequent production of energy. The cytochrome, large oligometric protein, is embedded in the inner lipid bilayer membrane of the mitochondrion and acts as a carrier of the electrons (Keeton and Gould, 1985).

Mitochondrial DNA

Mitochondrial DNA (**mtDNA** or **mDNA**) is the DNA located in organelles called mitochondria, structures within eukaryotic cells that convert chemical energy from food into a form that cells can use, adenosine triphosphate (ATP). Mitochondrial DNA is only a small portion of the DNA in a eukaryotic cell; most of the DNA can be found in the cell nucleus, and in plants, thechloroplast as well (Sykes, 2003).

Nuclear and mitochondrial DNA are thought to be of separate evolutionary origin, with the mtDNA being derived from the circular genomes of the bacteria that were engulfed by the early ancestors of today's eukaryotic cells. This theory is called the endosymbiotic theory. Each mitochondrion is estimated to contain 2-10 mtDNA copies (Wiesner, 1992).

Due to its ease of enzymatic amplification with conserved primers, mtDNA is frequently used to infer genetic and biogeography subdivision within species (Kaplan *et al.,* 1989). Mitochondrial genes are viewed as advantageous for phylogenetic analysis for several reasons. The mitochondrial genes are generally easier to amplify than nuclear genes (Simon *et al*., 1994), clonally inherited and non-recombining (Sunnucks and Hales, 1996; Zhang and Hewitt, 1996). It is also estimated to evolve 2–9 times faster than nuclear protein-coding genes (Monteiro and Pierce, 2001). Mitochondrial genes have been used as the source of data for studies of insect molecular phylogeny, genetic variation and phylogeography (Avise, 2000; Caterino *et al*., 2000; Simmons and Weller, 2001).

Among the mitochondrial genes the COI gene possesses additional interesting characteristics which make it particularly suitable as a molecular marker for evolutionary and genetic studies. A combination of highly conserved and variable region is so closely associated in mitochondrial genes (Gennis, 1992). The COI gene is the largest of the three mitochondria-encoded cytochrome oxidase subunits and one of the largest protein-coding genes in the mitochondrial genome (Clary and Wolstenholme, 1985; Morlais and Severson, 2002).

COI, COII, COIII genes

Cytochrome c oxidase I (COX1) is a large transmembrane protein complex found in bacteria and the mitochondrion of eukaryotes (Kosakyan, *et al.,* 2012)and it is the main subunit of the cytochrome c oxidase complex (Rumbley, 1994).

Cytochrome c oxidase subunit II, also known as cytochrome c oxidase polypeptide II, is a protein that in humans is encoded by the MT-CO2 gene which is abbreviated as

COXII, COX2, COII, or MT-CO2, is the second subunit of cytochrome c oxidase (Capaldi, *et al.,* 1983). Cytochrome c oxidase subunit II is an oligomeric enzymatic complex which is a component of the respiratory chain and is involved in the transfer of electrons from cytochrome c to oxygen. In eukaryotes this enzyme complex is located in the mitochondrial inner membrane; in aerobic prokaryotes it is found in the plasma membrane. The enzyme complex consists of 3-4 subunits in prokaryotes to up to 13 polypeptides in case of mammals. In Leigh's disease, there may be an abnormality or deficiency of cytochrome oxidase (Rumbley *et al.,* 1994).

Cytochrome c oxidase subunit III is one of the main transmembrane subunits of cytochrome c oxidase which is an oligomeric enzymatic complex that is located in the mitochondrial inner membrane of eukaryotes and in the plasma membrane of aerobic prokaryotes. The core structure of prokaryotic and eukaryotic cytochrome c oxidase contains three common subunits, I, II and III. In prokaryotes, subunits I and III can be fused and a fourth subunit is sometimes found, whereas in eukaryotes there are a variable number of additional small subunits (Mather, *et al.,* 1993).

Cytochrome Oxidase Subunit I:

The COI gene is one of the best-known mitochondrial genes. It contains both highly conserved and variable regions. The sequence of this gene is potentially useful for phylogenetic analysis over a wide taxonomic range and has been explored for that purpose. This COI gene sequence has been generally useful for reconstructing phylogenetic relationships among more closely related groups (Willis *et al.,* 1992; Beckenbach *et al.,* 1993; Brown *et al.,* 1994; Sperling and Hickey, 1994; Emerson and Wallis, 1995; Spicer, 1995; Smith and Bush, 1997). When compared to other cytochrome subunits, COI has a relatively large size and can therefore present the researcher with both highly conserved and

variable regions making it an especially valuable tool in molecular genetic studies (Morlais and Severson, 2002).

COI gene sequences have been used to address phylogenetic problems at a wide range of hierarchical levels, from species to orders (Caterino and Sperling, 1999). Furthermore, Gaunt and Miles (2002) found that the COI gene is better suited to conduct studies based on the molecular genetics assumption compared to other gene sequences, such as 16S rRNA and 18S rRNA. The mtDNA is the most commonly sequenced region in studies involving insect genetic variation and COI is one of the most frequently sequenced segments within the mtDNA.

Applications: Although COI gene may be matched by other mitochondrial genes in resolving such cases of recent genetic divergence, this gene is more likely to provide deeper phylogenetic insights than alternatives such as Cyt-b (Simmons and Weller, 2001), because changes in its amino acid sequence occur more slowly than those in this, or any other, mitochondrial gene (Lynch and Jarrell, 1993). Nonetheless, COI gene has proved to be a versatile tool with a variety of applications, for example, by facilitating the association between different developmental stages in insects (Ahrens *et al.,* 2007). The approach has also proved to be an effective auxiliary tool in forensic science (Dawnay *et al.,* 2007), on feeding ecology (Bourlat *et al.,* 2008; Garros *et al.,* 2008; Kuusk and Agusti, 2008) and habitat conservation intiative (Neigel *et al.,* 2007; Ward *et al.,* 2008) among other applications. Most importantly, the COI gene has proved to be especially useful in the study of taxonomically challenging taxa, where morphology-based identifications are frustrated due to genetic diversity (Hebert *et al.,* 2004b; Smith *et al.,* 2006; Quicke *et al.,* 2006; Witt *et al.,* 2006; Yassin *et al.,* 2008).

Merits: The 13 protein-coding genes in the animal mitochondrial genome are better targets because indels are rare since most lead to a shift in the reading frame. There is no prior

reason to focus analysis on a specific gene, but the COI does have two important advantages. The universal primers for this gene are very robust, enabling recovery of its 5' end from representative of most, if not all, animal phyla (Folmer *et al.,* 1994; Zhang and Hewitt, 1997). The COI gene appears to possess a greater range of phylogenetic signal than any other mitochondrial gene.

In fact, the genetic variation of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogenetic groups within a single species (Cox and Hebert, 2001; Wares and Cunningham, 2001).

The mitochondrial COI gene is the most popular marker in molecular genetics-partitions of this gene are frequently employed for higher arthropods phylogeny. In addition, COI is currently in the focus of special interest, its 5' partition is used for the molecular genetics initiative (Hebert *et al.,* 2003b; Stoeckle, 2003). The nucleotide sequence of this particular 640 nucleotide region (Folmer *et al.,* 1994) shall be the unique identification code for all species to facilitate the correct determination of species and the discovery of new species (Moritz and Cicero, 2004). The DNA sequences of the mitochondrial COI gene can serve as a molecular marker for identifying all kinds of animals (Hebert *et al.,* 2004a), especially cryptic species in tropical regions (Wilcox *et al.,* 1997; Berkov, 2002; Monaghan *et al.,*2005; Hajibabaei *et al.,* 2006).

Demerits: While some ''COI-like'' sequences may represent simple errors in manual editing and lack of quality control, others may actually be nuclear copies of mitochondrial derivedgenes that crossed into the nuclear genome and became non-functional and therefore, non-coding (Lopez *et al.,* 1994). The rate of evolution of *cox1* is very slow (Hebert *et al.,* 2003a).

The aim of the present study is thus, to compare the nucleotide sequences from the mitochondrial COI gene of six species of Reduviinae belonging to four genera viz.,

Acanthaspis pedestris Stål, *Acanthaspis quinquespinosa* (Fabricius), *Acanthaspis siva* Distant*, Empyrocoris annulata* (Distant)*, Edocla slateri* Distant and *Velitra sinensis* (Walker) encompassing an attempt to analyze the generic as well as inter- and intraspecific genetic variations to understand the phylogeny of the sub family Reduviinae.

Materials and Methods

DNA isolation

The adults of above said six species were morphologically identified. The body tissues (100mg) from each species were selected as the source of genomic DNA, stored in 90% ethyl alcohol and washed in ddH_2O and 750µl of 1X suspension buffer was added to the sample such that the final volume did not exceed 750µl. Then 5µl RNAse was added and mixed for 5-6 times. It was kept at 65^oC for 10 min and the mixture was separated into two tubes. Thereafter, 0.5µl of lysis buffer was added to each tube and mixed for 5-6 times. It was kept at 65^oC for 15min and cooled to room temperature. Centrifugation was carried out at 10,000 rpm for 1min at room temperature. The supernatant was collected and equal volume of isopropanol was added and mixed well. Centrifugation was carried out at 10,000 rpm for 15 min at room temperature and the supernatant was discarded. One ml of 70% ethanol was added to the pellets and again centrifuged at 10,000 rpm for 15minutes and the supernatant was discarded. Ethanol wash was repeated. Pellets were dried at 57^oC for 10min and 20µl of glass-distilled water was added and kept at 65^oC for 15 minutes. The DNA sample thus obtained was preserved and used for further analysis.

PCR amplification

PCR was carried out to amplify the partial mitochondrial COI gene. It was amplified using COI gene forward primer **LCOF:** 5’ GGT CAA CAA ATC ATA AAG ATA TTG G 3’and reverse primer **HCOR:** 5’ TAA ACT TCA GGG TGA CCA AAA AAT CA 3’. The primer combination yielded a fragment size of ~700 bp, in all the species. The PCR was

performed on a BioRAD Thermal Cycler PTC-100 in a reaction mixture containing genomic DNA : ~20ng , dNTP mix (2.5mM each) : 1µl , forward primer : 100ng , reverse primer : 100ng 10XAssay buffer for Taq DNA polymerase: 1X, Taq DNA polymerase enzyme : 3units. The final volume of 50µl was made using glass distilled water. Thereafter, amplification at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 72^{o}C for 40 seconds and final extension at 72^{o}C for 10min were performed.

Gel electrophoresis and PCR product purification

The amplified PCR products were separated on 1.8% agarose gel along with 100 bp ladder and stained using ethidium bromide (0.5 mg/ml) and visualized under ultraviolet light. The PCR products were column purified by GeneiPure^{TM} gel extraction kit.

Gene sequence analysis

The purified amplicons were sequenced using Ampli Taq Fs dye terminator cycle sequencing reaction kit (Applied Biosystems Inc., USA) in the ABI prism 3100 genetic analyzer. Each run is termed as a 'Single Pass Analysis' where the electropherogram represents a multicolour picture of sequence showing coloured peaks that indicate the corresponding bases. The ABI Genetic analyser uses the sequencing analysis software V.5.1 with kilobasecaller which displays the quality values for pure and mixed bases. The partial nucleotide sequence of partial COI gene obtained for each of the species was confirmed for its identity by Blast analysis at NCBI (http://www.ncbi.nlm.nih.gov/blast/). The representative sequences were submitted to NCBI GenBank database and accession numbers are given in table 8.

Gene sequence data analysis

The DNA sequences of the six species were aligned by ClustalW program and imported into MEGA version 5.1 (Tamura *et al.,* 2011) (Figure 10) for phylogenetic reconstruction. The A+T and G+C composition of the nucleotide sequences of six species

and the codon frequencies were calculated by MEGA 5.1 (Tamura *et al.,* 2011). These sequences were translated by ExPASy server http://www.expasy.org/translate (Gasteiger *et al.,* 2003) and amino acid compositions were analyzed by statistical analysis of protein sequence server http://www.ebi.ac.uk/tools/saps (Brendel *et al.,* 1992) and the structure of transmembrane protein segments was predicted using Hidden Markov Models by server http://www.cbs.dtu.dk /services/TMHMM (Krogh *et al.,* 2001). The protein secondary structure was predicted by PSIPRED server http://www.bioinf.cs.ucl.ac.uk/psipred (Jones, 1999). The homology modeling was built using SWISS-MODEL workspace server http://www.swissmodel.expasy.org/workspace (Arnold *et al.,* 2006). This model was validated with RAMPAGE server www.mordred.bioc.cam.ac.uk/~rapper/rampage.php (Lovell *et al.,* 2003). The dihedral angles phi against psi of amino acid residues in protein structures were visualized with the help of Ramachandran Plot. It shows the possible conformation of phi and psi angles for a polypeptide chain (Lovell *et al.,* 2003). The SWISS-MODEL Server produces theoretical models for proteins. The possible 3D structure was generated from theoretical model protein using Deep View-Swiss Pdb Viewer ver.4.0.3 software http://www.expasy.org.org/spdbv (Guex and Peitsch, 1997).

Homology modeling

To determine the quarternary structure of proteins present in six species homology modeling was done using SWISS-MODEL workspace server http://www.swissmodel.expasy.org/workspace. The model was developed based on experimentally determined structures of related family members as templates. The template structure was selected based on Blast E-value limit. A single chain model of quarternary structure was built based on the selected template, 1v54N (1.80 A) (Guex and Peitsch, 1997; Schwede *et al.,* 2003; Arnold *et al.,* 2006). The evaluation of the quality of structure was carried out by calculating QMEAN 4 score (Benkert, 2011). (The QMEAN4 score is a

composite score consisting of a linear combination of four statistical potential terms that estimates model reliability between 0-1). This score helps to determine the quality of the model obtained.

Phylogenetic analysis

The analysis involved nucleotide sequences of six species. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA ver.5.1 (Tamura *et al.,* 2011) in three different methods namely, Maximum Likelihood, Maximum Parsimony and Neighbour-Joining Distance methods as described below.

Maximum Likelihood method

The evolutionary history was inferred from the phylogenetic tree constructed using the Maximum Likelihood method, based on the Tamura 3-parameter model (Tamura, 1992). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method is used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (16 categories (+*G,* parameter = 1.3705). The rate variation model allowed for some sites is evolutionarily invariable ([+*I*], 40.1177% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Maximum Parsimony method

The evolutionary history was inferred from the phylogenetic tree constructed using the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange

algorithm (Nei and Kumar, 2000) with search level 3 in which the initial trees were obtained with the random addition of sequences (1000 replicates). Branch lengths were calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence.

Neighbor-Joining method

The evolutionary history was inferred from the phylogenetic tree constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length=0.75503880 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic relationship. The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1000).

Results

Basic Sequence Statistics

In the study of molecular evolution, it is necessary to know some basic statistical quantities, such as nucleotide composition (Table 9), codon frequencies (Table 10) and transition/transversion ratios (table 11) (Software MEGA ver 5.1) (Tamura *et al.,* 2011).

Nucleotide composition

In *A. siva*, the nucleotide bases were found AT rich (70.0%) followed by *E. annulata* (69.4%), *E. slateri* (65.5%), *V.sinensis* (64%), *A. pedestris* (61.9%) and *A. quinquespinosa* (59.5). The average AT composition of nucleotide bases of the six reduviids was 65.1%. *A. quinquespinosa* was found GC rich (40.5%), followed by *A. pedestris* (38%), *V. sinensis*

(35.9%), *E. slatari* (34.6%), *E. annulata* (30.7%) and *A. siva* (29.9%). The average GC composition of six reduviids was 34.9%.

The nucleotide composition of first codon of *A. siva* had the highest percentage of AT (70.9%) followed by *E. annulata* (70.5%), *E. slateri* (67.8%), *A. pedestris* (64.3%), *V. sinensis* (64.1%) and *A. quinquespinosa* (63.6%). The average AT composition of first codon of six reduviids was 66.9%. The first codon nucleotide composition of *A. quinquespinosa* was found GC rich (36%) followed by *A. pedestris* (35.6%), *V. sinensis* (35.4%), *E. slateri* (32.2%), *E. annulata* (29.7%) and *A. siva* (29.4%). The average GC composition of six reduviids was 33.1%.

The nucleotide composition of second codon of *A. siva* was found AT rich (65.8%) followed by *E. annulata* (63.6%), *V. sinensis* (62.2%), *E. slateri* (61.1%), *A. pedestris* (57.1%) and *A.quinquespinosa* (55.3%). The average AT composition of six reduviids was 60.85%. The second codon of *A. quinquespinosa* was found GC rich (45.1%), followed by *A. pedestris* (42.8%), *E. slateri* (39%), *V. sinensis* (38.3%), *E. annulata* (36.3%) and *A. siva* (33.8%). The average GC composition of six reduviids was 39.2%.

The nucleodite composition of third codon of *E. annulata* (74.5%) followed by *A. siva* (73.2%), *E.slateri* (67.5%), *V. sinensis* (65.8%), *A. pedestris* (64.8%) and *A. quinquespinosa* (59.8%) was found AT rich. The average AT composition of third codon of six reduviids was 67.6%. The third codon of *A. quinquespinosa* was found GC rich (40.6%), followed by *A. pedestris* (35.7%), *V. sinensis* (34.2%), *E. slateri* (32.5%), *A. siva* (26.6%) and *E. annulata* (26%). The average GC composition of third codon of six reduviids was 32.6%. (Table 9)

Codon frequencies

Many amino acids are coded by more than one codon, thus multiple codons for a given amino acid are synonymous. However, many genes display a non-random usage of synonymous codons for specific amino acids. A measure of the extent of this non-

randomness is given by the Relative Synonymous Codon Usage (RSCU) (Sharp *et al.,* 1986). The codon frequencies of six reduviids are given in table 4. The frequently used codons were AUU (14.5), UUU (11.7), AAU (10.5) and AUA (9.2) (table 10).

Transition/Transversion ratios (R=si/sv)

This is the ratio of the number of transitions to the number of transversions for a pair of sequences. Transition/Transversion ratio test of six reduviids is given in table 11.

The average transition/transversion ratio of six reduviids studied was 0.79. The average identical pair of six reduviids was 408, transitional pairs was 102 and transversional pairs was 130.

The first codon transition/transversion ratio of six reduviids analysed was 0.68. The average identical pairs of first codon nucleotides was 136, transitional pairs was 31 and transversional pairs was 46.

The second codon transition/transversion ratio of six reduviids was 0.93. The average identical pairs of second codon nucleotides was 141, transitional pairs was 35 and transversional pairs was 37.

The third codon transition/transversion ratio of six reduviids was 0.78. The average identical pairs of third codon nucleotides was 131, transitional pairs was 36 and transversional pairs was 46.

Genetic distance estimation

Estimation of pairwise distance

The evolutionary divergence between nucleotide sequences of six reduviids was estimated by MEGA 5.1(Tamura *et al.,* 2011) (Table 13). The longest evolutionary distance was observed between *A. quinquespinosa* and *V. sinensis* (1.182) followed by *E. slateri* and *V. sinensis* (1.180), *A. siva* and *V. sinensis* (1.165), *E. annulata* and *V. sinensis* (1.079) and *A. pedestris* and *V. sinensis* (1.025) and the shortest between *E. slateri* and *E. annulata* (0.088)

followed by *A. quinquespinosa* and *A. pedestris* (0.211), *A. siva* and *E. annulata* (0.269), *A. pedestris* and *E. annulata* (0.306), *A. siva* and *E. slateri* (0.334), *A. pedestris* and *E. slateri* (0.363), *A. quinquespinosa* and *E. slateri* (0.393), *A. siva* and *A. pedestris* (0.543) and *A. siva* and *A. quinquespinosa* (0.566).

Models for estimating distances

Evolutionary distances are fundamental for the study of molecular evolution and are useful for phylogenetic reconstruction and the estimation of divergence times (Nei and Kumar, 2000). The evolutionary distance between a pair of sequences is usually measured by the number of nucleotide substitutions occurring between them. Models with the lowest Bayesian Information Criterion (BIC) scores are considered to describe the substitution pattern, the best. The Tamura 3-parameter is the best-fit substitution model for estimating maximum likelihood distance and the estimated distances are given in table 12. The lowest BIC scores were observed in Tamura 3-parameter model (6313.7) followed by Tamura-Nei model (6316.4), General Time Reversible model (6318.3) and Kasegawa-Kishino-Yano model (6318.6).

Substitution Pattern Disparity

(i) Homogeneity test for substitution pattern

Disparity index measures the observed difference in substitution patterns for a pair of sequences. It works by comparing the nucleotide frequencies in a given pair of sequences and using the number of observed differences between sequences. The disparity index test showed homogeneity between *A. siva* and *E. annulata* (0±0.824) followed by *A. siva* and *A. pedestris* (0±2.474), *E. slateri* and *V. sinensis* (0.004±2.958), and *E. annulata* and *V. sinensis* (0±3.368) *A. siva* and *A. quinquespinosa* (0±3.391), *A. pedestris* and *E. slateri* (0±4.240), *A. quinquespinosa* and *E. slateri* (0±4.706), *A. pedestris* and *E. annulata* (0±5.297), *A. quinquespinosa* and *E. annulata* (0±6.172), *A. siva* and *E. slateri* (0.016±0.863), *A. siva* and

V. sinensis (0.016±1.332), *A. quinquespinosa* and *V. sinensis* (0.020±1.361), *E. slateri* and *E. annulata* (0.102±0.105), *A. quinquespinosa* and *A. pedestris* (0.126±0.162) and *A. pedestris* and *V. sinensis* (0.274±0.167) (Table 14).

(ii) Composition distance

Composition distance is a measure of the difference in nucleotide composition for a given pair of sequences. The MEGA software computes and presents the composition distance per site, which is given by the total composition distance between two sequences divided by the number of positions compared, excluding gaps and missing data. The composition distance analysis showed maximum similarity between *E. slateri* and *E. annulata* (0.105) followed by *A. quinquespinosa* and *A. pedestris* (0.162), *A. pedestris* and *V. sinensis* (0.167), *A. siva* and *E. annulata* (0.824), *A. siva* and *E. slateri* (0.863), *A. siva* and *V. sinensis* (1.332), *A. quinquespinosa* and *V. sinensis* (1.361), *A. siva* and *A. pedestris* (2.474), *E. slateri* and *V. sinensis* (2.958), *E. annulata* and *V. sinensis* (3.368). *A. siva* and *A. quinquespinosa* (3.391), *A. pedestris* and *E. slateri* (4.240), *A. quinquespinosa* and *E. slateri* (4.706), *A. pedestris* and *E. annulata* (5.297) and *A. quinquespinosa* and *E. annulata* (6.172) (Table 15).

Test of neutral evolution

This provides a test of selection based on the comparison of the number of synonymous (d_S) and nonsynonymous (d_N) substitutions between sequences. The variance of difference was computed using the bootstrap method (100 replicates). Analyses were conducted using the Nei-Gojobori method (Nei and Gojobori, 1986). The test of neutral evolution revealed minimum variation between *A. quinquespinosa* and *E. annulata* (0±3.625) followed by *A. quinquespinosa* and *E. slateri* (0±3.648), *E. slateri* and *E. annulata* (0.001±3.306), *A. quinquespinosa* and *A. pedestris* (0.003±2.994), *A. siva* and *A. quinquespinosa* (0.007±2.758), *A. pedestris* and *E. slateri* (0.060±1.900), *A. pedestris* and *E.*

annulata (0.091±1.704), *A. siva* and *E. slateri* (0.116±1.585), *A. siva* and *V. sinensis* (0.214±1.250), *A. siva* and *A. pedestris* (0.307±1.027), *E. annulata* and *V. sinensis* (0.372±0.895), *A. siva* and *E. annulata* (0.387±0.868), *A. pedestris* and *V. sinensis* (0.421±0.808), *E. slateri* and *V. sinensis* (0.661±0.440) and *A. quinquespinosa* and *V. sinensis* (0.825±0.222). They are significant at $P<0.05$ level (Table 16).

Phylogenetic analysis

The phylogenetic analysis of six reduviids namely, *A. siva, A. quinquespinosa, A. pedestris, E. slateri, E. annulata* and *V. sinensis* by three methods such as Maximum Likelihood, Maximum Parsimony and Neighbor-Joining was carried out. The phylogenetic tree branch lengths were also calculated.

In Maximum Likelihood method, the branch lengths of the phylogenetic tree observed were: *A. siva* (0.2490)*, A. quinquespinosa* (0.1314)*, A. pedestris* (0.0801)*, E. slateri* (0.0789)*, E. annulata* (0.0092)*,* and *V. sinensis* (0.6855). *Acanthaspis pedestris* and *A. quinquespinosa* share a common node of branch length (0.436) and *E. slateri* and *E. annulata* share a common node of branch length (0.0206). The longest length observed in *V. sinensis* (0.6855) diverged independently as a separate node and the shortest length was observed in *A. pedestris* (0.0801) (Figure 11).

In Maximum Parsimony method, the branch lengths of the tree observed were: *A. pedestris* (46.917)*, A. quinquespinosa* (68.417), *A. siva* (94.333)*, E. slateri* (38.958)*, E. annulata* (11.708)*,* and *V. sinensis* (206.583). *Acanthaspis pedestris* and *A. quinquespinosa* share a common node of branch length (63. 792) and *E. slateri* and *E. annulata* share a common node of branch length (36. 042). The longest length observed in *V. sinensis* (206.583) diverged independently as a separate node and the shortest length was observed in *A. pedestris* (46.917) (Figure 12).

In Neighbour-Joining method, the branch lengths of the tree observed were: *A. pedestris* (0.0789), *A. quinquespinosa* (0.1292), *A. siva* (0.2290), *E. slateri* (0.0824), *E. annulata* (0.0046), and *V. sinensis* (0.6428). *Acanthaspis pedestris* and *A. quinquespinosa* share a common node of branch length (0.0613) and *E. slateri* and *E. annulata* share a common node of branch length (0.0341).The longest length observed in *V. sinensis* (0.6428) diverged independently as a separate node and the shortest length was observed in *A. pedestris* (0.0789) (Figure 13).

COI genes

A. pedestris

The partially sequenced COI gene amplicon of *A. pedestris* is given in figure 14. revealed an average size of 668bp. The A+T percentage was 61.9% and G+C percentage was 38%. The analysis also revealed the nucleotide frequencies: A-31.7%, T-30.2%, G-17.5%, C-20.5% (Table 9).

In-silico translation with the ExPASy translate tool (Gasteiger *et al.,* 2003) revealed a peptide of 218 amino acid sequences for *A. pedestris* with four TGA stop codons. The translation of partial nucleotide sequence and its deduced amino acid sequences are given in Figure 14. Because of the codon preference, the A+T composition in *A. pedestris* was particularly biased at the third codon position, which accounts for 64.8%. The A+T content at first and second codon positions were 64.3% and 57.1% respectively. The G+C composition in *A. pedestris* was the highest in second codon position, which accounts for 42.8%. The G+C content at first and third codon positions were 35.6% and 35.7% respectively (Table 18).

The amino acid compositions analysed by Statistical Analysis of Protein Sequence (Brendel *et al.,* 1992) revealed that *A. pedestris* had higher percentage of isoleucine (13.3%) followed by leucine (11.5%), glycine (9.2%), serine (8.3%), proline (7.3%), valine (7.3%), phenylalanine (6.9%), threonine (6.9%), alanine (6.4%), asparagine (6%), arginine (5%),

aspartic acid (4.1%), tyrosine (1.8%), histidine (1.4%), methionine (1.4%), glutamic acid (0.9%), glutamine (0.9%), cysteine (0.5%), tryptophan (0.5%) and lysine was completely absent (Table 19).

The transmembrane prediction using Hidden Markov Models (Krogh *et al.,* 2001) of *A. pedestris* COI gene sequence of 218 amino acid residues revealed four transmembrane segments between amino acids 25-70, 80-110, 125-150 and 160-190 (Figure 20).

The secondary structure of protein predicted by PSIPRED server (Jones, 1999) from the translated amino acid sequences of *A. pedestris* had the following composition: strands (0%), alpha helix (61.5%) and 3,10 helix (2.3%) (Figure 26).

Distribution of amino acid residues analysed by Ramachandran Plot is given in tables 20 & 21 (Lovell *et al.,* 2003). It revealed that *A. pedestris* had a number of residues: favoured region 174 (93%), allowed region 9(4.8%) and outlier region 4(2.1%) (Figure 32).

The amino acid residues ranging from 22 to 210 were subjected to homology modeling (Figure 44 & 45). The quaternary structure was determined based on template 1v54 (1.80A): HETERO 26-mer and the identity of the sequence with this template was 75.52% and E-value obtained was 1.16823e-60. The quality of the model was determined by QMEAN z-score and the score obtained was -3.888 and the model built was single chain (Figure 38).

A. quinquespinosa

The partially sequenced COI gene amplicon of *A. quinquespinosa* is given in figure 15 revealed an average size of 649bp. The A+T percentage was 59.5% and G+C percentage was 40.5% (Table 17). The analysis also revealed the nucleotide frequencies: A-29.6%, T-29.9%, G-17.1% and C-23.4% (Table 9).

In-silico translation with the ExPASy translate tool (Gasteiger *et al.,* 2003) revealed a peptide of 192 amino acid sequences for *A. quinquespinosa* with twenty four TGA stop

codons. The translation of partial nucleotide sequence and its deduced amino acid sequences are given in figure 15. Because of the codon preference, the A+T composition in *A. quinquespinosa* was particularly biased at the first codon position, which accounts for 63.6%. The A+T content at second and third codon positions were 53.3% and 59.8% respectively. The G+C composition in *A. quinquespinosa* was the highest in second codon position, which accounts for 45.1%. The G+C content at first and third codon positions were 36% and 40.6% respectively (Table 18).

The amino acid compositions analysed by Statistical Analysis of Protein Sequence (Brendel *et al.,* 1992) revealed that *A. quinquespinosa* had higher percentage of leucine (16.1%) followed by glutamic acid (9.9%), serine (9.4%), glutamine (8.9%), proline (7.8%), tyrosine (7.3%), threonine (7.3%), phenylalanine (6.2%), histidine (6.2%), aspartic acid (5.2%), isoleucine (4.2%), valine (2.1%), glycine (2.6%), asparagine (2.1%), alanine (1.0%), methionine (0.5%), cysteine (1.6%), lysine (1.0%), tryptophan (0.5%) and arginine was completely absent (Table 19).

The transmembrane prediction using Hidden Markov Models (Krogh *et al.,* 2001) analysis of *A. quinquespinosa* COI gene sequence of 218 amino acid residues revealed the presence of transmembrane segments between the amino acids 25-60 (Figure 21).

The secondary structure of protein predicted by PSIPRED server (Jones, 1999) from the translated amino acid sequences of *A. quinquespinosa* had the following composition: strand (3.1%), of alpha helix (33.3%) and 3,10 helix (2.3%) (Figure 27).

Distribution of amino acid residues analysed by Ramachandran Plot is given in tables 20 & 21 (Lovell *et al.,* 2003). It revealed that *A. quinquespinosa* had a number of residues: favoured region 165 (86.8%), allowed region 18(9.5%) and outlier region 7(3.7%) (Figure 33).

The amino acid residues ranging from 22 to 210 were subjected to homology modeling (Figure 44 & 45). The quarternary structure was determined based on template 1v54 (1.80A): HETERO 26-mer and the identity of the sequence with this template was 75.52% and E-value obtained was 1.16823e-60. The quality of the model was determined by QMEAN z-score and the score obtained was -4. 274 and the model built was single chain (Figure 39).

A. siva

The partially sequenced COI gene amplicon of *A. siva* given in figure 16 revealed an average size of 644bp. The A+T percentage was 70% and G+C percentage was 29.9% (Table 17). The analysis also revealed the nucleotide frequencies: A-32.6%, T-37.4%, G-13%, C-16.9% (Table 9).

In-silico translation with the ExPASy translate tool (Gasteiger *et al.,* 2003) revealed a peptide of 214 amino acid sequences for *A. siva* with eight TGA stop codons. The translation of partial nucleotide sequence and its deduced amino acid sequences are given in figure 16. Because of the codon preference, the A+T composition in *A. siva* was particularly biased at the third codon position, which accounts for 73.2%. The A+T content at first and second codon positions were 70.9% and 65.8% respectively. The G+C composition in *A. siva* was the highest in second codon position, which accounts for 33.8%. The G+C content at first and third codon positions were 29.4% and 26.6% respectively (Table 18).

The amino acid compositions analysed by Statistical Analysis of Protein Sequence (Brendel *et al.,* 1992) revealed that *A. siva* had higher percentage of leucine (17.5%) followed by isoleucine (10.7%), phenylalanine (8.7%), tyrosine (8.3%), serine (7.8%), lysine (7.8%), asparagine (5.3%), glutamine (4.9%), proline (3.9%), histidine (3.4%), arginine (2.9%), glutamic acid (2.9%), aspartic acid (2.9%), cysteine (2.4%), threonine (2.4%), valine (2.4%), tryptophan (2.4%), glycine (1.9%), alanine (1.0%) and methionine (0.5%) (Table 19).

The transmembrane prediction using Hidden Markov Models (Krogh *et al.,* 2001) analysis of *A. siva* COI gene sequence of 214 amino acid residues revealed three transmembrane segments between amino acid 50-100, 125-150 and 160-200 (Figure 22).

The secondary structure of protein predicted by PSIPRED server (Jones, 1999) from the translated amino acid sequences of *A. siva* had the following composition: strand (0%), alpha helix (55.3%), 3,10 helix (1.5%) (Figure 28).

Distribution of amino acid residues analysed by Ramachandran Plot is given in tables 20 & 21 (Lovell *et al.,* 2003). It revealed that *A. siva* had a number of residues: favoured region 181(88.7%), allowed region 15(7.4%) and outlier region 8(3.9%) (Figure 34).

The amino acid residues were subjected to homology modeling (Figure 44 & 45). The quarternary structure was determined based on template 1v54 (1.80A):HETERO 26-mer . The quality of the model was determined by QMEAN z-score and the score obtained was -4. 982 and the model built was single chain (Figure 40).

E. slateri

The partially sequenced COI gene amplicon of *E. slateri* given in figure 17 revealed an average size of 729bp (Table 17). The A+T percentage was 65.5% and G+C percentage was 34.6%. The analysis also revealed the nucleotide frequencies: A-26.5%, T-39%, G-15.8%, C-18.8% (Table 9).

In-silico translation with the ExPASy translate tool (Gasteiger *et al.,* 2003) revealed a peptide of 220 amino acid sequences for *E. slateri* with twenty three TGA stop codons. The translation of partial nucleotide sequence and its deduced amino acid sequences are given in figure 17. Because of the codon preference, the A+T composition in *E. slateri* was particularly biased at the first codon position, which accounts for 67.8%. The A+T content at second and third codon positions were 61.1% and 67.5% respectively. The G+C composition

in *E. slateri* was the highest in second codon position, which accounts for 39%. The G+C content at first and third codon positions were 32.2% and 32.5% respectively (Table 18).

The amino acid compositions analysed by Statistical Analysis of Protein Sequence (Brendel *et al.,* 1992) revealed that *E. slateri* had higher percentage of serine (14.1%) followed by phenylalanine (13.2%), tyrosine (11.4%), isoleucine (10.5%), asparagine (7.3%), arginine (6.8%), threonine (5.5%), cysteine (5.5%), leucine (5.0%), tryptophan (4.5%), proline (4.1%), lysine (3.2%), histidine (2.7%), glycine (1.8%), alanine (1.4%), valine (1.4%), aspartic acid (0.9%) and methionine (0.9%). Glutamic acid and glutamine were completely absent (Table 19).

The transmembrane prediction using Hidden Markov Models (Krogh *et al.,* 2001) analysis of *E. slateri* COI gene sequence of 220 amino acid residues revealed six transmembrane segments between amino acids 25-50, 60-160 and 165-200 (Figure 23).

The secondary structure of protein predicted by PSIPRED server (Jones, 1999) from the translated amino acid sequences of *E. slateri* had the following composition: strand (25%), alpha helix (1.8%) and 3,10 helix (0%) (Figure 29).

Distribution of amino acid residues analysed by Ramachandran Plot is given in tables 20 & 21 (Lovell *et al.,* 2003). It revealed that *E. slateri* had a number of residues: favoured region 170(78%), allowed region 27(12.4%) and outlier region 21(9.6%) (Figure 35).

The amino acid residues were subjected to homology modeling (Figure 44 & 45). The quarternary structure was determined based on template 1v54 (1.80A):HETERO 26-mer. The quality of the model was determined by QMEAN z-score and the score obtained was -4. 462 and the model built was single chain (Figure 41).

E. annulata

The partially sequenced COI gene amplicon of *E. annulata* given in figure 18 revealed an average size of 646bp (Table 17) The A+T percentage was 61.4% and G+C

percentage was 30.7%. The analysis also revealed the nucleotide frequencies: A-28.2%, T-41.2%, G-14.4%, C-16.3% (Table 9).

In-silico translation with the ExPASy translate tool (Gasteiger *et al.,* 2003) revealed a peptide of 211 amino acid sequences for *E. annulata* with four TGA stop codons. The translation of partial nucleotide sequence and its deduced amino acid sequences are given in figure 18. Because of the codon preference, the A+T composition in *E. annulata* was particularly biased at the third codon position, which accounts for 74.5%. The A+T content at first and second codon positions were 70.5% and 63.6% respectively. The G+C composition in *E. annulata* was the highest in second codon position, which accounts for 36.3%. The G+C content at first and third codon positions were 29.7% and 26% respectively (Table 18).

The amino acid compositions analysed by Statistical Analysis of Protein Sequence (Brendel *et al.,* 1992) revealed that *E. annulata* had higher percentage of isoleucine (16.1%), leucine (14.7%), serine (9.5%), glycine (8.5%), proline (7.6%), phenylalanine (7.1%), alanine (6.6%), threonine (6.2%), asparagine (5.2%), valine (4.7%), arginine (4.3%), glutamine (1.9%), histidine (1.9%), tyrosine (1.4%), cysteine (0%), aspartic acid (3.3%), glutamic acid (0.9%) and lysine, methionine and tryptophan were completely absent (Table 19).

The transmembrane prediction using Hidden Markov Models (Krogh *et al.,* 2001) analysis of *E. annulata* COI gene sequence of 211 amino acid residues revealed four transmembrane segments between amino acids 25-75, 80-120 and 125-175 (Figure 24).

The secondary structure of protein predicted by PSIPRED server (Jones, 1999) from the translated amino acid sequences of *E. annulata* had the following composition: strand (0%), alpha helix (60.7%) and 3,10 helix (5.2%) (Figure 30).

Distribution of amino acid residues analysed by Ramachandran Plot is given in tables 20 & 21 (Lovell *et al.,* 2003). It revealed that *E. annulata* had a number of residues: favoured region 201(96.2%), allowed region 7(3.3%) and outlier region 1(0.5%) (Figure 36).

The amino acid residues were subjected to homology modeling (44 & 45). The quarternary structure was determined based on template 1v54 (1.80A):HETERO 26-mer . The quality of the model was determined by QMEAN z-score and the score obtained was -3.933 and the model built was single chain (Figure 42).

V. sinensis

The partially sequenced COI gene amplicon of *V. sinensis* given in figure19 revealed an average size of 687bp (Table 17) The A+T percentage was 64% and G+C percentage was 35.9%. The analysis also revealed the nucleotide frequencies: A-31.7%, T-32.3%, G-18.9%, C-17% (Table 9).

In-silico translation with the ExPASy translate tool (Gasteiger *et al.,* 2003) revealed a peptide of 223 amino acid sequences for *V. sinensis* with six TGA stop codons. The translation of partial nucleotide sequence and its deduced amino acid sequences are given in figure 19. Because of the codon preference, the A+T composition in *V. sinensis* was particularly biased at the third codon position, which accounts for 65.8%. The A+T content at first and second codon positions were 64.1% and 62.2% respectively. The G+C composition in *V. sinensis* was the highest in second codon position, which accounts for 38.3%. The G+C content at first and third codon positions were 35.4% and 34.2% respectively (Table 18).

The amino acid compositions analysed by Statistical Analysis of Protein Sequence (Brendel *et al.,* 1992) revealed that *V. sinensis* had higher percentage of isoleucine (13.0%), proline (9.0%), leucine (8.5%), serine (7.6%), valine (7.2%), asparagine (6.7%), alanine (5.8%), lysine (5.8%), arginine (5.4%), threonine (5.4%), glycine (5.4%), methionine (3.6%), aspartic acid (3.6%), phenylalanine (2.7%), glutamic acid (2.7%), glutamine (1.8%), histidine (1.8%), tryptophan (1.3%), tyrosine (1.3%) and cysteine (1.3%) (Table 19).

The transmembrane prediction using Hidden Markov Models (Krogh *et al.,* 2001) analysis of *V. sinensis* COI gene sequence of 223 amino acid residues revealed two transmembrane segments between amino acids 10-30 and 160-175 (Figure 25).

The secondary structure of protein predicted by PSIPRED server (Jones, 1999) from the translated amino acid sequences of *V. sinensis* had the following composition: strand 6.3%, alpha helix (30%) and 3,10 alpha helix (0%) (Figure 31).

Distribution of amino acid residues analysed by Ramachandran Plot is given in tables 20 & 21 (Lovell *et al.,* 2003). It revealed that *V. sinensis* had a number of residues in favoured region 190(86%), allowed region 20(9%) and outlier region 11(5%) (Figure 37).

The amino acid residues were subjected to homology modeling (Figure 44 & 45). The quarternary structure was determined based on template 1v54 (1.80A): HETERO 26-mer. The quality of the model was determined by QMEAN z-score and the score obtained was -4. 6574 and the model built was single chain (Figure 43).

Discussion

The sequence length of around 639-724 bp observed for the six species of Reduviinae is closer to that of the COI gene sequences of different species of Reduviinae (NCBI database, Genbank accession no: GU12616, GU012616.1 and GU012616.1). The length of this region is highly variable among different insects due to its high rate of nucleotide substitution, insertions/deletions and the presence of a variable number of tandem repeats (Fauron and Wolstenholme, 1980; Inohira *et al*., 1997).

The mitochondrial genome has become not only a major resource for comparative genomics but also play an important role in metabolism, apoptosis, disease and aging (Boore, 1999). A similar overall A+T richness (65%) was reported for the family Reduviidae (Muraji *et al*., 2001; Pfeiler *et al*., 2006; Liu *et al*., 2009; Li *et al*., 2011) and for oriental *Rhynocoris* species (Baskar, 2010 and Singh, 2012). However, the richness of the A+T% ranging from

59.5% (*A. quinquespinosa*) to 70% (*A. pedestris*) recorded for the reduviine species is lower than that of Apidae (80%) and Coleoptera (79.8%) (Jermiin and Crozier, 1994).

Subfamily characters

Though the six reduviine species have their own specific molecular characteristics, the data obtained from the molecular studies suggest that the following characters could be used as subfamily markers.

(i) AT composition

The highest percentage of AT composition observed for six reduviine species (61 to 70%) could be a diagnostic marker of the family Reduviinae. The richness of AT composition has been used as a marker in Reduviidae (Muraji *et al*., 2001; Pfeiler *et al*., 2006; Liu *et al*., 2009; Li *et al*., 2011; Singh, 2012).

(ii) GC composition

A narrow range of GC composition (29.9% to 40.5%) observed for six species of Reduviinae could be again considered as a subfamily character.

(iii) Amino acid composition

The least amount of methionine observed in all the six species of Reduviinae could be also considered as a subfamily character.

(iv) Codon frequencies

The most frequently occurred codon, AUU in six reduviine species could be again considered as a subfamily character.

Generic characters

Each genus has its own specific molecular characteristics. However, the data obtained for three species of *Acanthaspis* alone could be analysed for this purpose because all the other three genera are represented by a lone species.

(i) AT composition

The highest percentage of AT composition of three *Acanthaspis* species ranging from 63 to 70.9% could be used as a generic marker as it was used as a marker for *Rhynocoris* species (Singh, 2012).

(ii) GC composition

A narrow range of GC composition (29.4% to 35.6%) of three *Acanthaspis* species could be treated as a generic character.

(iii) Amino acid composition

The following closer values of amino acid, serine in *A. pedestris* (8.3), *A. quinquespinosa* (9.4) and *A. siva* (7.8) could be considered as a generic character for *Acanthaspis*. The highest amino acid residues were observed in favoured region for all the three species.

Species specific characters

(i) *Acanthaspis pedestris*

The AT composition (61.9%) biased at the third codon (64.8%), the GC composition (38%) biased at the second codon (42.8 %), higher percentage of isoleucine (13.3%), absence of lysine, four specific transmembrane segments between amino acids 25-70, 80-110, 125-150 and 160-190 with specific percentage of alpha helix (61.5 %), 3,10 helix (2.3%), absence of coil and the highest amount of amino acids residues (93%) found in favoured region by Ramachandran Plot could be considered as specific markers for *A. pedestris.*

(ii) *Acanthaspis quinquespinosa*

The AT composition (59.5%) biased at the first codon (63.6%), the GC composition (40.5%) biased at the second codon (45.1%), higher percentage of leucine (16.1%) and absence of arginine, a specific transmembrane segment between amino acids 25-60 with specific percentage of strand (3.1%), alpha helix (33.3%) and 3,10 helix (2.3%) and the

highest amount of amino acids residues (86.8%) found in favoured region by Ramachandran Plot could be considered as specific markers for *A. quinquespinosa.*

(iii) ***Acanthaspis siva***

The AT composition (70%) biased at the third codon (73.2%), the GC composition (29.9%) biased at the second codon (33.8 %), higher percentage of leucine (17.1%), three specific transmembrane segments between amino acids 50-100, 125-150 and 160-200 with specific percentage of alpha helix (55.3%) and 3,10 helix (1.5%), and the highest amount of amino acids residues (88.7%) found in favoured region by Ramachandran Plot could be considered as specific markers for *A. siva.*

(vi) ***Edocla slateri***

The AT composition (65.5%) biased at the first codon (67.8%), the GC composition (34.6%) biased at the second codon (39 %), higher percentage of serine (14.1%), absence of glutamic acid and glutamine, six specific transmembrane segments between amino acids 25-50, 60-160 and 165-200 with specific percentage of strand (25%), alpha helix (1.8%) and 3,10 helix (0%) and the highest amount of amino acid residues (78%) found in favoured region by Ramachandran Plot could be considered as specific markers for *E. slateri.*

(v) ***Empyrocoris annulata***

The AT composition (61.4%) biased at the third codon (74.5%), the GC composition (30.7%) biased at the second codon (74.5%), higher percentage of the isolucine (16.1%), absence of lysine, methionine and tryptophan, four specific transmembrane segments between amino acids 25-75, 80-120 and 125-175 with specific percentage of strand (0%), alpha helix (60.7%) and 3,10 helix (5.2%) and the highest amount of amino acids residues (96.2%) found in favoured region by Ramachandran Plot could be considered as specific markers for *E. annulata.*

(v) *V. sinensis*

The AT composition (64%) biased at the third codon (65.8%), the GC composition (35.9%) biased at the second codon (38.3%), higher percentage of the isolucine (13%), two specific transmembrane segments between aminoacids 10-30 and 160-175 with specific percentage of strand (6.3%), alpha helix (30%) and 3,10 helix (0%) and the highest amount of amino acids residues (86%) found in favoured region by Ramachandran Plot could be considered as specific markers for *V. sinensis.*

Intergeneric and Intrageneric affinity

(i) Evolutionary distance

The order of affinity between any two species based on evolutionary distance between sequences observed: *E. slateri* and *E. annulata* (0.088±0.245) > *A. quinquespinosa* and *A. pedestris* (0.211±0.099) > *A. siva* and *E. annulata* (0.269±0.132) > *A. siva* and *E. slateri* (0.334±0.112) > *A. quinquespinosa* and *E. annulata* (0.352±0.146) > *A. pedestris* and *E. annulata* (0.306±0.132) >*A. pedestris* and *E. slateri* (0.363±0.112) > *A. quinquespinosa* and *E. slateri* (0.393±0.157) > *A. siva* and *A. pedestris* (0.543±0.021) > *A. siva* and *A. quinquespinosa* (0.566±0.099) > *A. pedestris* and *V. sinensis* (1.025±0.068) > *E. annulata* and *V. sinensis* (1.079±0.079) > *A. siva* and *V. sinensis* (1.165±0.068) > *E. slateri* and *V. sinensis* (1.180±0.068) > *E. slateri* and *V. sinensis* (1.182±0.082) suggests affinity between species showing the inter- and intragenric affinity among these species.

(ii) Homogeneity of substitution patterns between sequences

The order of affinity between any two species based on homogeneity of substitution patterns between sequences obtained from disparity index test: *A. siva* and *A. pedestris* (0±2.474) > *E. annulata* and *V. sinensis* (0±3.368) > *A. siva* and *A. quinquespinosa* (0±3.391) > *A. pedestris* and *E. slateri* (0±4.240) > *A. quinquespinosa* and *E. slateri* (0±4.706) > *A. pedestris* and *E. annulata* (0±5.297 > *A. quinquespinosa* and *E. annulata* (0±6.172) > *E.*

annulata and *V. sinensis* (0±3.368) > *E. slateri* and *V. sinensis* (0.004±2.958) > *A. siva* and *E. slateri* (0.016±0.0.863) > *A. siva* and *V. sinensis* (0.016±1.332) > *A. quinquespinosa* and *V. sinensis* (0.020±1.361) > *A. quinquespinosa* and *A. pedestris* (0.126±0.162) > *A. pedestris* and *V. sinensis* (0.274±0.167) also reveals the inter- and intragenric affinity among these species.

(iii) Nucleotide base composition

The order of affinity between any two species based on the distance in composition of nucleotide bases: *E. slateri* and *E. annulata* (0.105±0.099) > *A. quinquespinosa* and *A. pedestris* (0.162±0.042) > *A. pedestris* and *V. sinensis* (0.167±0.691) > *A. siva* and *E. annulata* (0.824±0.078) > *A. siva* and *E. slateri* (0.863±0.512) > *A. siva* and *V. sinensis* (1.332±0.023) > *A. quinquespinosa* and *V. sinensis* (1.361±0.791) > *A. siva* and *A. pedestris* (2.474±0.031) > *E. slateri* and *V. sinensis* (2.958±0.481) > *E. annulata* and *V. sinensis* (3.368±0.312) > *A. siva* and *A. quinquespinosa* (3.391±0.12) > *A. pedestris* and *E. slateri* (4.240±0.069) > *A. quinquespinosa* and *E. slateri* (4.706±0.245) > *A. quinquespinosa* and *E. annulata* (6.172±0.137) further shows the inter- and intrageneric affinity among these species.

(iv) Neutrality test

From the codon-based Z-test of neutrality between sequences of six rduviine species, the highest neutrality (affinity) observed between *A. quinquespinosa* and *E. annulata* (0±2.703) followed by *E. slateri* and *E. annulata* (0.001±1.714), *A. quinquespinosa* and *A. pedestris* (0.003±2.092), *A. siva* and *A. quinquespinosa* (0.007±2.557), *A. pedestris* and *E. slateri* (0.060±1.413),*A. pedestris* and *E. annulata* (0.091±1.604), *A. siva* and *A. pedestris* (0.307±1.002), *A. siva* and *V. sinensis* (0.214±1.100), *E. annulata* and *V. sinensis* (0.372±0.896), *A. siva* and *E. annulata* (0.387±0.740), *A. pedestris* and *V. sinensis*

(0.421±0.715), *E. slateri* and *V. sinensis* (0.661±00.410) and *A. quinquespinosa* and *V. sinensis* (0.825±0.153) again reiterates inter- and intrageneric affinity among these species.

(iv) Transmembrane segments

The affinity among *A. pedestris, A. quinquespinosa, E. slateri* and *E. annulata* as evidenced by the presence of closer value transmembrane segments between aminoacids 25-75; between aminoacids 125-190 among *A. pedestris, E. slateri, E. annulata* and *V. sinensis* and between aminoacids 125-150 between *A. pedestris* and *A. siva* reveal the inter- and intrageneric affinity among these species.

(v) Phylogenetic analysis

Divergence of *A. siva* diverges in a separate clade and *A. pedestris* and *A. quinquespinosa* grouping together in a single cluster in phylogenetic tree determined using Neighbour-Joining method; clustering of *A. pedestris* and *A. quinquespinosa* and *E. annulata* and *E. slateri* uniformly in all the three phylogenetic trees analysed, revealing intergeneric affinity. The intergeneric specificity is also revealed by the independent divergence of *V. sinensis* as a separate clade in all the three phylogenetic trees. Baskar (2010) also reported the intrageneric affinity of *R. marginatus*, *R. fuscipes*, *R. kumarii* and *R. longifrons* by Neighbour-Joining method. Furthermore, Singh (2012) also reported the inter- and intrageneric affinity of three morphs of *R. kumarii* and four ecotypes of *R. marginatus.* Such analysis was carried out for other members of the family Reduviidae with different genes namely COI and Cyt-b genes (Pfeiler *et al*., 2006; Liu *et al*., 2009). The greater intrageneric and interspecific affinity between *A. pedestris* and *A. quinquespinosa* and between *E. slateri* and *E. annulata* as evidenced by the disparity index analysis and supported by evolutionary distance analysis is worth mentioning. The study could be further extended analyzing other mitochondrial genes and by analyzing a reasonable number of reduviine species.

Conclusion

The parameters like AT composition, GC composition, codon frequencies, amino acid composition, transmembrane pattern, protein secondary structure, dihedral angles between amino acids and protein quarternary structure of mitochondrial cytochrome C oxidase subunit I gene analysis could be used either as a generic marker or species specific marker based on the common and unique characters observed.

Greater affinity between *A. pedestris* and *A. quinquespinosa* and *E. slateri* and *E. annulata* and lesser affinity between *V. sinensis* and rest of the species revealed by Maximum Likelihood, Maximum Parsimony and Neighbour-Joining distance methods and supported by neutrality test and evolutionary distance analysis is interesting.

CHAPTER IV

RESTRICTION FRAGMENT LENGTH POLYMORPHISM

Introduction

The restriction fragment length polymorphism, or RFLP (commonly pronounced "rif-lip"), is a molecular technique that exploits variations in homologous DNA sequences. It refers to the difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated. Several molecular techniques are available for identification such as polymerase chain reaction (Rodriguez *et al.,* 1991), randomly amplified polymorphic DNA (RAPD), DNA hybridization (Ebbehoj & Thomson,1991), gene sequencing (Chikuni *et al.,* 1994) and DNA fingerprinting (Ganai *et al.,* 2000) which have been tried elsewhere for identification methods but they have their own limitations. The PCR-RFLP of the mitochondrial gene is a very good molecular marker because it is highly repeatable, cheaper and quicker than the methods cited above (Meyer *et al.,* 1995). Although, DNA sequencing and analysis is accurate and authentic, it is costly, time consuming and not suitable for routine species identification studies. In contrast, the PCR-RFLP has been proven to be a practical, simple and rapid technique (Meyer *et al.,* 1995; Partis *et al.,* 2000).

In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. Although now largely obsolete due to the rise of inexpensive DNA sequencing technologies, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. In addition to genetic fingerprinting, RFLP was an important tool in genome analysis (Gormus *et al.,* 2012).

The RFLPs are inherited as codominant Mendelian traits and can be detected by Southern blotting methods. Subsequent to restriction endonuclease cleavage, genomic fragments of differing size are separated according to their molecular weight and transferred to solid support. DNA hybridization with radio labelled probes produces distinct and specific patterns on autoradiograms (Botstein *et al.,* 1980).

Applications

Restriction fragment length polymorphisms (RFLPs) provide a very large number of genetic markers for detecting and analyzing genetic diversity in plants (Helentjaris *et al.,* 1985; Zhang *et al.,* 1993; Dubreuil and Charcosset, 1998). It has much greater power and was originally developed for mapping human genes than anything previously available (Botstein *et al.,* 1980).

The RFLP detects the difference in homologous DNA sequences in the form of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. Thus, this technique is a combination of PCR amplification and RFLP analysis, in which the desired amplified product is digested with one or more restriction enzymes. Banding patterns that are specific for each species produced from the restriction digestion can be used for species identification (Dowling *et al.,* 1996; Arens, 1999; Chua *et al.,* 2012).

The identification of species and members of species complexes based on morphology, isozyme analysis, karyotyping of polytene chromosomes and cross-mating experiments possess several limitations (Norris, 2002). Molecular markers have proven useful in a wide variety of applications including molecular taxonomy, evolutionary systematics, population genetics, genetic mapping, and a variety of molecular diagnostics (Collins and Paskewitz, 1996; Norris, 2002). In view of its simplicity and rapidity, the PCR-RFLP method for 16s rDNA analysis may be one of the most promising tools for routine use

in systematic and ecological studies (Hiraishi, *et al.,* 1995). Recently, several molecular methods, especially those based on the polymerase chain reaction have been developed for the diagnosis and the identification of species (de Brujin and Barker, 1992; Cupolillo *et al.,* 1995; Noyes *et al.,* 1998; de Andrade *et al.,* 2001) and to differentiate morphologically indistinguishable species (Mitchell *et al.,* 1992). PCR-RFLP, together with morphological and pathobiological characters, elucidates the taxonomic groupings within the genus (Jensen *et al.,* 2001)

Mertis

The RFLPs are generally found to be moderately polymorphic. It is robust and easy. In addition to their high genomic abundance and their random distribution, RFLPs have the advantages of showing co-dominant alleles and having high reproducibility (Neale and Williams, 1991).

The PCR procedures coupled with restriction endonuclease analysis of the amplified product provide a rapid diagnostic tool for genus level (de Moraes and Maruniak, 1997), species specific studies (Christian *et al.,* 2001) and inter-specific variation (Otranto *et al.,* 2000). PCR-RFLP technique is the most popular to apply in biology, medicine and food science (Sato *et al.,* 1998; Kurihara *et al.*, 1999; Russell *et al.,* 2000). Restriction pattern analysis of the coding regions using PCR-RFLPs has been performed to confirm the high degree of polymorphism within species (Sturm *et al.,* 2003; Rozas *et al.,* 2007).

Demerits

The demertis of RFLPs are the requirement of laborious and technically demanding methodological procedures and high expense (Castiglione *et al.,* 1993). Moreover, large quantities of purified, high molecular weight DNA are required for each DNA digestion. Larger quantities are needed for species with larger genomes, and for the greater number of

times needed to probe each blot. There is a lack of automation and collaboration among researchers in the distribution of discovered RFLP probes (Karp *et al.,* 1997).

In the present chapter, an attempt was made to document the RFLP as an additional marker for species identification of six species of Reduviinae belonging to four genera viz., *Acanthaspis pedestris* Stål, *Acanthaspis quinquespinosa* (Fabricius), *Acanthaspis siva* Distant*, Empyrocoris annulata* (Distant)*, Edocla slateri* Distant and *Velitra sinensis* (Walker) encompassing an attempt to analyze the generic, subfamily and species specific as well as inter- and intrageneric genetic variations to understand the phylogeny of the sub family Reduviinae.

Materials and Methods

DNA isolation

The adults of the above said six species were morphologically identified. The 100mg insect body tissues from each species were selected as the source of genomic DNA, stored in 90% ethyl alcohol and washed in ddH_2O and 750µl of 1X suspension buffer was added to the sample such that the final volume did not exceed 750µl. Then 5µl RNAse was added and mixed for 5-6 times. It was kept at 65^{o}C for 10 min and the mixture was separated into two tubes. Thereafter, 0.5µl of lysis buffer was added to each tube and mixed for 5-6 times. It was kept at 65^{o}C for 15min and cooled to room temperature. Centrifugation was carried out at 10,000 rpm for 1min at room temperature. The supernatant was collected and equal volume of isopropanol was added and mixed well. Centrifugation was carried out at 10,000 rpm for 15 min at room temperature and the supernatant was discarded. One ml of 70% ethanol was added to the pellets and again centrifuged at 10,000 rpm for 15minutes and the supernatant was discarded. Ethanol wash was repeated. Pellets were dried at 57^{o}C for 10min and 20µl of glass-distilled water was added and kept at 65^{o}C for 15 minutes. The DNA sample thus obtained was preserved and used for further analysis.

PCR amplification

The PCR was carried out to amplify the partial mitochondrial COI gene. It was amplified using COI gene forward primer **LCOF:** 5' GGT CAA CAA ATC ATA AAG ATA TTG G 3'and reverse primer **HCOR:** 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'. The primer combination yielded a fragment size of ~700 bp, in all the species. The PCR was performed on a BioRAD Thermal Cycler PTC-100 in a reaction mixture containing genomic DNA : ~20ng , dNTP mix (2.5mM each) : 1µl , forward primer : 100ng , reverse primer : 100ng 10XAssay buffer for Taq DNA polymerase: 1X, Taq DNA polymerase enzyme : 3units. The final volume of 50µl was made using glass distilled water. Thereafter, amplification at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 72^{o}C for 40 seconds and final extension at 72^{o}C for 10min were performed.

Gel electrophoresis and PCR product purification

The amplified PCR products were separated on 1.8% agarose gel along with 100 bp ladder and stained using ethidium bromide (0.5 mg/ml) and visualized under ultraviolet light. The PCR products were column purified by GeneiPure^{TM} gel extraction kit.

Gene sequence analysis

The purified amplicons were sequenced using Ampli Taq Fs dye terminator cycle sequencing reaction kit (Applied Biosystems Inc., USA) in the ABI prism 3100 genetic analyzer. Each run is termed as a 'Single Pass Analysis' where the electropherogram represents a multicolour picture of sequence showing coloured peaks that indicate the corresponding bases. The ABI Genetic analyser uses the sequencing analysis software V.5.1 with kilobasecaller which displays the quality values for pure and mixed bases. The partial nucleotide sequence of partial COI gene obtained for each of the species was confirmed for its identity by Blast analysis at NCBI (http://www.ncbi.nlm.nih.gov/blast/). The

representative sequences were submitted to NCBI GenBank database and accession numbers are given in table 8.

WEB cutter V2.0

The compiled query sequences were loaded with online tool WEB cutter V2.0 to know the appropriate restriction enzymes of query sequences and their corresponding restriction sites (Vincze *et al.,* 2003). Commercially available *Sau* 3AI restriction enzyme was selected for enzyme digestion.

RFLP of Cytochrome oxidase Gene with *Sau* 3AI restriction enzyme

The PCR KlenZol purified fragment of Cytochrome oxidase gene was digested with *Sau* 3AI restriction enzyme. Briefly, enzyme-buffer mix was prepared by mixing 2µl of restriction enzyme with 8 µl of the respective buffer. Reaction mix was prepared by mixing 10µl PCR product with 2µl of enzyme buffer mix. Volume was made up to 20 µl with autoclaved MilliQ water and incubated overnight at 37^{o}C. Digested product was visualized by electrophoresis in 12% native PAGE for RFLP profiling (Bangalore Genei).

Results

The enzyme digested PCR amplicons of the six species generated different RFLP fragments (Plate 2). A total of 24 restriction fragments were detected in all the six reduviine species, surely they were polymorphic. The generated fragments of the six reduviine species are given as follows: 20,85,102 and 508 bp in *A. pedestris,* 81, 462 and 504 bp fragments in *A. quinquespinosa,* 84, 363 and 605bp in *A. siva,*74, 359, 475, 574 and 619bp in *E. slateri,* 79, 96, 364, 460, 606, and 625bp in *E. annulata* and 144, 186 and 567bp in *V. sinensis.*

Restriction map of sequenced amplicons of six reduviids of mitochondrial COI partial sequences along with selected restriction sites are given in Table 22. Of these, the specific fragments were observed in *V. sinensis* (Plate 2).

Unique fragments

The unique fragments observed were : i.e., 20, 102 and 508bp in *A. pedestris*; 462 bp in *A. quinquespinosa*; 363bp in *A. siva*; 74, 475, 574 and 619 bp in *E. slateri*; 79, 96 and 625 bp in *E. slateri* and 144, 186 and 567 bp in *V. sinensis.*

Closer fragments

The closer fragments observed were: *A. pedestris* (85bp), *A. quinquespinosa* (81bp) and *A. quinquespinosa* (84bp). Similarly, the closer fragments were observed in *E. slateri* (74bp) and *E. annulata* (79bp) and *A. siva* (605bp) and *E. annulata* (606bp).

Subfamily characters

No such common fragments were observed for all the six species.

Generic characters

The closer fragments in three *Acanthaspis* species observed: *A. pedestris* (85bp), *A. quinquespinosa* (81bp) and *A. quinquespinosa* (84bp) could be used as a genetic marker. Though *E. annulata* and *E. slateri* belong to two different genera, they are having closer fragments, i.e., *E. slateri* (74bp) and *E. annulata* (79bp) suggesting intergeneric affinity. Similarly, *A. siva* (605bp) and *E. annulata* (606bp) show the intergeneric affinity.

Species specific characters

The unique fragments observed: 20, 102 and 508bp in *A. pedestris*; 462 bp in *A. quinquespinosa*; 363bp in *A. siva*; 74, 475, 574 and 619 bp in *E. slateri*; 79, 96 and 625 bp in *E. slateri* and 144, 186 and 567 bp in *V. sinensis* could be considered as species specific markers.

Discussion

The *Sau 3AI* restriction enzyme digestion revealed different levels of polymorphism among all the six species of Reduviinae. The PCR-RFLP provides unambiguous identification and disposition of *A. pedestris, A. quinquespinosa, A. siva, E. slateri, E.*

annulata and *V. sinensis* since moderate to high genetic differentiation was observed among the species. The results confirm the specificity and reliability of markers among the species. These diagnostic tools can also be substituted with morphological observations for accurate identification. Different RFLP patterns observed among the species which could be utilized as good molecular markers for identification. The PCR-RFLP pattern was suggested as an identification tool for *Rhodnius* species (Triatominae) and also RFLP pattern to differentiate the members of the same family (Naegele, *et al* 2006). Dissimilarity in RFLP profile pattern of six reduviids shows the genetic variation among the species which contributes to the knowledge about evolutionary relationships as suggested in triatomines (Tartarotti and Ceron, 2005). The different banding pattern observed in the six species supports the earlier findings that RFLP pattern could be used to analyze the genetic relationship and variability in *Rhodinus* species (Lopez *et al.,* 2007)

The fragments obtained were species specific since both the total number of fragments and the size of the fragments were not found to be the same for any two species analysed. Though *A. quinquespinosa, A. siva* and *V. sinensis* have uniformly generated a total number of three fragments they have different-sized fragments like 81, 462 and 504 bp in *A. quinquespinosa,* 84, 363 and 605bp in *A. siva* and 144, 186 and 567bp in *V. sinensis* suggesting polymorphism. A similar kind of polymorphism was observed in target gene sequences when digested with the enzymes *Bsh1236I, HpaII, HaeIII*, and *TaqI* in *Trypanosoma cruzi* Chagas strains, parasite of triatomine bugs (Marlene, *et al.,* 2007).

On the other hand, similar sized fragment of around 81-85 bp was observed in the three *Acanthaspis* species which shows their species closeness. Similar trend was seen in both *E.annulata* and *E. slateri*, where fragment size of 74 bp for *E. slateri* and 79 bp for *E. annulata* was observed. Likewise, *A. siva* and *E. annulata* showed similar banding pattern at 363 bp and 364 bp revealing intergeneric affinity between them. Inter- and intragroup

differences and conserved patterns were evidenced by RFLP method in various *Trypanosoma cruzi* strains (Marlene *et al.,* 2007). Nevertheless this study has been useful in understanding the pattern of molecular differentiation and interpreting the affinities and systematic position of *A. pedestris, A. quinquespinosa, A. siva, E. slateri, E. annulata* and *V. sinensis*.

Conclusion

The PCR-RFLP with *Sau 3AI* enzyme pattern showed polymorphism among all the species and hence their differentiation into respective species or taxa.

This study shows that PCR-RFLP pattern could be used to identify genetic variation among species and inter- and intrageneric variability. This study is the first of its kind on Indian reduviids and a more comprehensive understanding on the systematic and phylogeny of reduviids would require further detailed studies based on an expanded taxa sampling and analysis of more genes with different restriction enzymes through different molecular methods.

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Intrageneric phylogenetics based on mitochondrial DNA variation among fifteen harpactorine assassin bugs with four ecotypes and three morphs (Hemiptera: Reduviidae: Harpactorinae)

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Abstract

Available mitochondrial DNA sequences viz., 16S, Cyt b, Cyt c oxidase subunit – I, and Cyt c subunit-like – I gene of *Rhynocoris* (Kolenati) species were subjected to phylogenetic analysis to understand the intrageneric and intraspecific variations and the role of geographical isolation on speciation; using CLUSTAL W in MEGA version 5.1. This analysis includes fifteen species and four ecotypes of *R. kumarii* Ambrose and Livingstone and three morphs of *R. marginatus* (Fabricius) from four countries viz., Canada, China, Korea, and South Africa. The pairwise genetic distances were calculated and phylograms were constructed using Maximum Likelihood, Maximum Parsimony, and Neighbor-Joining methods. These preliminary analyses not only demarcated the fifteen species of *Rhynocoris,* the four ecotypes of *R. kumarii,* and the three morphs of *R. marginatus,* but also revealed phylogenetic relationships and the role of geographical isolation and polymorphism on speciation.

Keywords: *Rhynocoris,* assassin bugs, biocontrol agents, molecular biosystematics, phylogenetic relationship, speciation, ecotypism, polymorphism, geographical isolation.

Introduction

Assassin bugs have different morphs, biotypes, and ecotypes with various colours and shapes which often mislead a museum entomologist in recognizing the morphs and ecotypes of a particular species.

Hence, classifications of Reduviidae based on morphological characters (Usinger, 1943; Putshkov & Putshkov, 1985; Maldonado, 1990; Schuh & Slater, 1995) may at times become insufficient, and there is an urgent need for a cohesive meaningful classification of Reduviidae based on ecological, morphological, behavioural, cytological, and biochemical data. Moreover, a multidisciplinary biosystematics understanding is imperative to accurately identify reduviids and employ them against a particular insect pest (Ambrose, 1999, Ambrose & Ambrose, 2003, 2009). Although multidisciplinary biosystematics including molecular tools has been attempted on Oriental reduviids (Weirauch, 2008), such an analysis is wanting on non-Oriental reduviids.

This study was undertaken based on available mitochondrial sequences of fifteen species of *Rhynocoris* Kolenati (Table 1), four ecotypes of *R. kumarii* Ambrose and Livingstone, and three morphs of *R. marginatus* (Fabricius). The inclusion of both Indian and non-Indian species of *Rhynocoris* with ecotypes and morphs will further enhance the scope of the work at the intraspecific level and the understanding on the role of geographical isolation in biosystematics.

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Material and methods

Taxon sampling. To understand the intrageneric biosystematics and phylogenetics through molecular markers (viz., 16S, mtCyt b, Cyt c oxidase subunit I gene, and Cyt c oxidase subunit I-like gene), DNA sequences of these species of *Rhynocoris* (Table 1) were subjected to phylogenetic analysis. The sequences of Indian species including ecotypes and morphs obtained from our work are deposited in the National Centre for Biotechnology (NCBI). The sequences of non-Indian species were retrieved from NCBI (Table 2) and all these sequences were taken into consideration.

Phylogenetic analysis. The DNA sequences were subjected into pairwise distance analysis and the following phylogenetic trees were constructed: Maximum Parsimony, Maximum Likelihood and Neighbor-Joining with MEGA 5 software (Tamura *et al.,* 2011).

Pairwise alignment. Pairwise distances were carried out with gap opening penalty 15 and gap extension penalty 6.66 (Clustal W) (Thompson *et al.,* 1994).

Maximum Parsimony. The Maximum Parsimony analyses were analysed with MEGA5 (Tamura *et al.,* 2011). Bootstrap method was used with 100 replications and gap/missing data treatment by complete selection and the search method was Subtree-Pruning-Regrafting (SPR) and substitution based on nucleotide sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) was used (Felsenstein, 1985). The Maximum parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei& Kumar, 2000) with search level 1. The substitution type based nucleotide sequences and the codon positions included were 1st+2nd+3rd+Noncoding and all the positions containing gaps and missing data were eliminated.

TABLE 1. Fifteen *Rhynocoris* species, four ecotypes, and three morphs were subjected to phylogenetic analyses.

Species	Locality
**R. altaicus* (Kiritshenko)	Xiaowutai Mt., Hebei, China
△*R. fuscipes*(Fabricius)	Tamil Nadu, India and Suixi, Zhanjiang, Guangdong, China
**R. hoffmanni* Hungerford	Nanjian, Dali, Yunnan, China
**R. incertus* (Distant)	Dongtang, Maolan, Guizhou, China
**R. iracundus* (Poda)	Tianshan, Xinjiang, China
R. kumarii Ambrose and Livingstone	Tamil Nadu, India
R. longifrons (Stål)	Tamil Nadu, India
R. marginatus (Fabricius)	Tamil Nadu, India
**R. marginellus* (Fabricius)	Dawei Mt., Pingbian Yunnan, China
**R. mendicus* (Stål)	Suixi, Zhanjiang, Guangdong, China
**R. monticola* (Oshanin)	Nileke, Xinjiang, China
**R. ornatus* (Uhler)	GG, Icheon-si, Korea
**R. rubromarginatus* (Jakovlev)	Yanqing, Beijing, China
**R. segmentarius* (Germar)	Limpopo, South Africa
**R. ventralis* (Say)	British Columbia, Inkaneep, OsoyoosIR1,Canada
Ecotypes of *R. kumarii*	
THE	Tamil Nadu, India
KAZ	
MUP	
MAR	
Morphs of *R. marginatus*	
Unknown	Tamil Nadu, India
Niger	
Nigrosanguineous	

Species denoted by * are non-Indian species and △ is Indian as well as non-Indian. The rest of the species are Indian species.

Maximum Likelihood. Maximum Likelihood analyses were run in MEGA 5 (Tamura *et al.,* 2011). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura &Nei, 1993). Initial tree for the heuristic search was obtained automatically by applying Neighbor-Joining 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. The substitution type based nucleotide sequences and the codon positions included were 1st+2nd+3rd+Noncoding and all the positions containing gaps and missing data were eliminated.

TABLE 2. Mitochondrial DNA sequences of fifteen *Rhynocoris* species, four ecotypes, and three morphs subjected to phylogenetic analysis.

Mitochondrial genes	Species	Genbank accession number
16S ribosomal RNA gene, partial sequence; mitochondrial	*Rynocoris fuscipes* (Fabricius)	HM038433.1
	R. marginatus (Fabricius)	HM038434.1
	**R. segmentarius* (Germar)	FJ230384.1
Cytochrome b (cyt b) partial cds; mitochondrial	**R. altaicus* (Kiritshenko)	EU286540.1
	**R. fuscipes*	EU286543
	R. fuscipes	GU225701.1
	**R. hoffmanni* Hungerford	EU286542.1
	**R. iracundus* (Poda)	EU286547.1
	**R. incertus* (Distant)	EU286548.1
	R. kumarii Ambrose and Livingstone	GU225702.1
	R. longifrons (Stål)	GU225703.1
	R. marginatus	GU225700.1
	**R. marginellus* (Fabricius)	EU286544.1
	**R. mendicus* (Stål)	EU286545.1
	**R. monticola* (Oshanin)	EU286546.1
	**R. rubromarginatus* (Jakovlev)	EU286541.1
Cyt c oxidase subunit I- like gene, partial sequence; mitochondrial	*R. fuscipes*	GU967411.1
	*R. kumarii*ecotype – KAZ	HM768317.1
	*R. kumarii*ecotype- MAR	HQ846916.1
	*R. kumarii*ecotype – MUP	HQ846917.1
	*R. kumarii*ecotype- THE	HM768318.1
	R. longifrons Stål	HQ245922.1
	R. marginatus- Unknown morph	HM768319.2
	R. marginatus– Niger morph	JN634062.1
	R.marginatus–Nigrosanguineous morph	JN634063.1
Cyt c oxidase subunit I gene, partial sequence; mitochondrial	**R. ventralis* (Say)	HQ106316.1
	R. longifrons	GQ229412.1
	**R. ornatus* (Uhler)	GQ292194.1
	R. marginatus	GQ229415.1
	R. fuscipes	GQ229414.1
	R. kumarii	GQ229413.1

Species denoted by * are non-Indian species. The rest of the species are Indian species.

Neighbor-Joining. Neighbor-Joining analyses were determined with MEGA5 (Tamura *et al.,* 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) was used (Felsenstein, 1985). The evolutionary distances were computed using the Tajima-Nei method (Tajima & Nei, 1984). Codon positions included were 1st+2nd+3rd+Noncoding and all positions containing gaps and missing data were eliminated.

Results and discussion

Based on 16 S, mtCyt b, Cyt c subunit I gene, and Cyt c subunit like I gene sequences, three phylograms were constructed for each set. The results of Maximum Parsimony, Maximum Likelihood, and Neighbor-Joining trees were analyzed based on the arrangement of each species on the tree.

16S. The Maximum Likelihood tree constructed based on 16 S gene (Fig.1) with two parallel clusters; one with the Indian *R. marginatus* that evolved as a separate lineage, and the second cluster with Indian *R. fuscipes* and *R. segmentarius* of South Africa with the branch length of 0.06 and 0.05, respectively exhibits their closeness. It is interesting that the Indian *R. fuscipes* exhibits closeness to the *R. segmentarius* of South Africa instead of the *R. fuscipes* of China. A similar kind of phylogeny is also observed in Neighbor-Joining (Fig.2) tree. The results reveal monophyly (Cui & Huang, 2012) through phylogenetic relationship not only among the native species but also between Indian and non-Indian species. But *R. marginatus, instead* of clustering with *R. fuscipes,* diverges in a separate lineage exhibiting phylogenetic disparity. The Indian species *R. fuscipes* and *R. segmentarius* of South Africa exhibit affinity despite biogeographical isolation (Mahendran *et al.,* 2006).

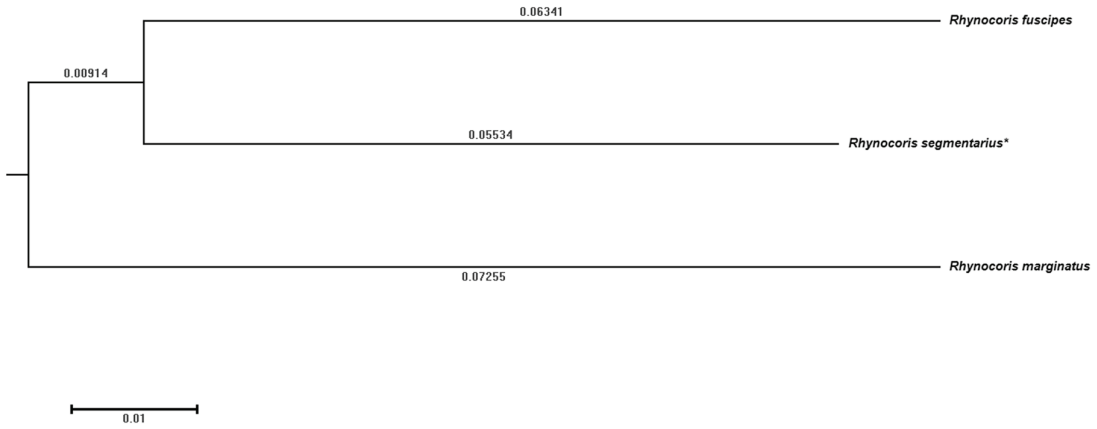


FIGURE 1. Maximum Likelihood tree based on 16S gene variations showing the relationships of two Indian and one Chinese species of *Rhynocoris*

Cyt b. The Maximum Likelihood tree (Fig.3) shows that all the four Indian species are in two clusters. One cluster consists of *R. kumarii* and *R. longifrons* whereas another cluster contains *R. marginatus* and *R. fuscipes.* But they share a common ancestral node suggesting an affinity between these four sympatric species. Among them, the two morphologically related *R. marginatus* and *R. fuscipes* share a common node exhibiting their affinity. Although *R. kumarii* and *R. longifrons* are morphologically quite dissimilar, they share a common node which shows their closeness. Baskar (2010) also observed affinity between *R. longifrons and R. kumarii* and *R. marginatus* and *R. fuscipes.* Another cluster consists of eight non-Indian species. These two clusters, one exclusively Indian and another non-Indian, emerge from the same evolutionary rate as a separate lineage that might be due to allopatric speciation with a closer affinity with *R. marginellus* of China. The line of phylogeny observed for the four Indian species in the Maximum Liklihood tree (Fig.3) is also repeated in Neighbor-Joining and Maximum Parsimony

trees. However, the phylogenetic hierarchy of non-Indian species observed in the Maximum Likelihood tree differs from that of Neighbor-Joining tree (Fig. 4): *R. iracundus* followed by *R. monticola,* followed by a cluster consisting of *R. marginellus, R. mendicus, R. altaicus, R. rubromarginatus,* and *R. incertus*; and a cluster consisting of *R. hoffmanni* and *R. fuscipes.* A similar trend is observed in Maximum Parsimony tree (Fig. 5): *R. marginellus* and *R. mendicus* cluster followed by *R. iracundus, R. incertus, R. monticola,* and *R. alticus, R. rubromarginatus* and *R. hoffmani*. But the affinity between any two species is similar in all three phylograms revealing uniform phylogenetic relationship and suggesting monophyly.

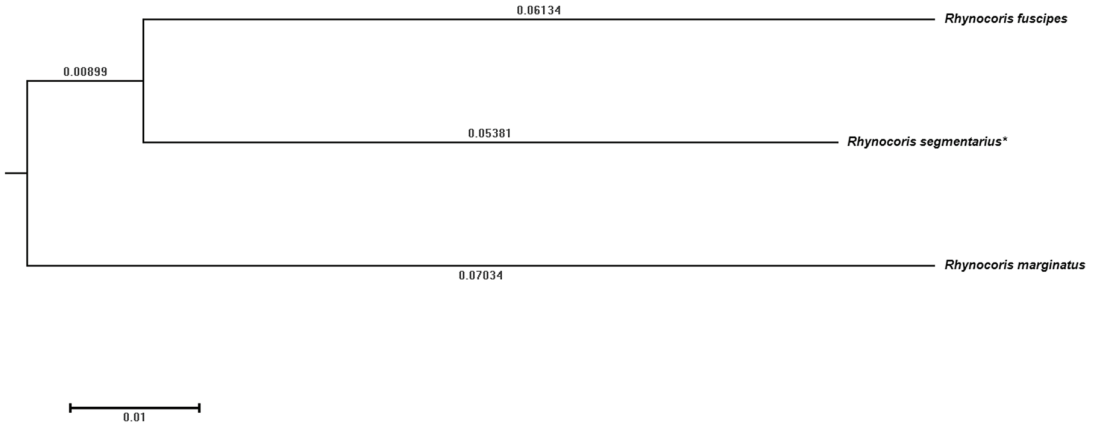
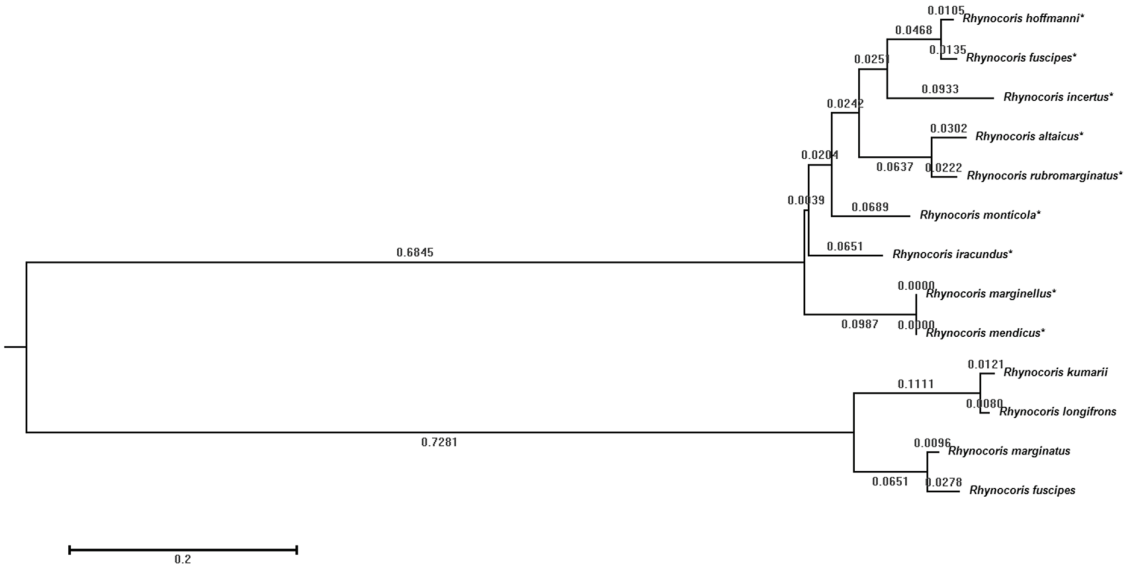


FIGURE 2. Neighbor-Joining tree based on 16S gene variations showing the relationships of two Indian and one Chinese species of *Rhynocoris*



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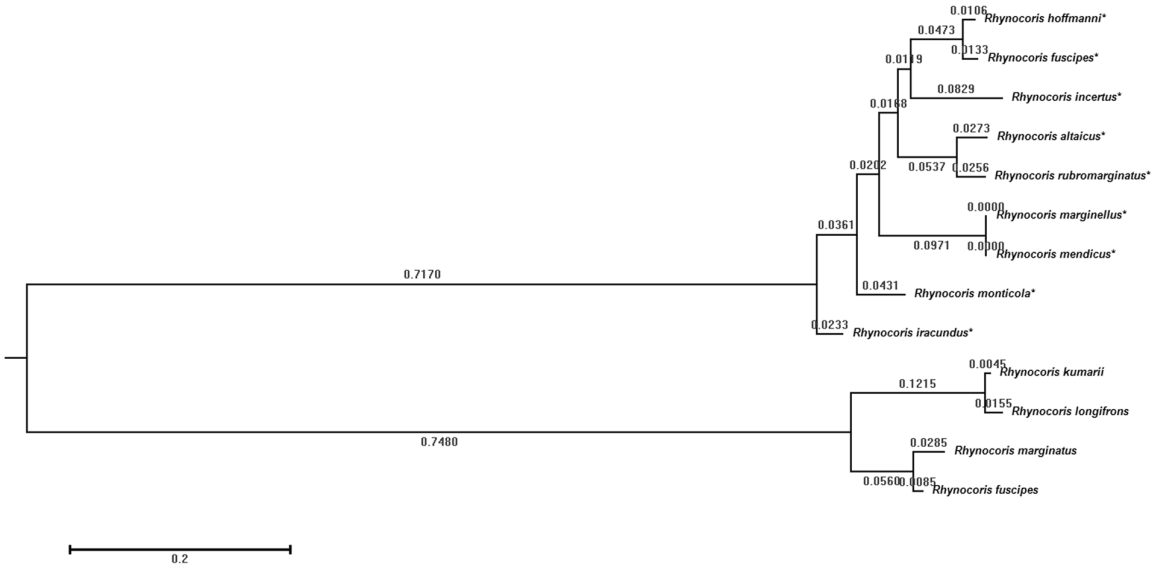

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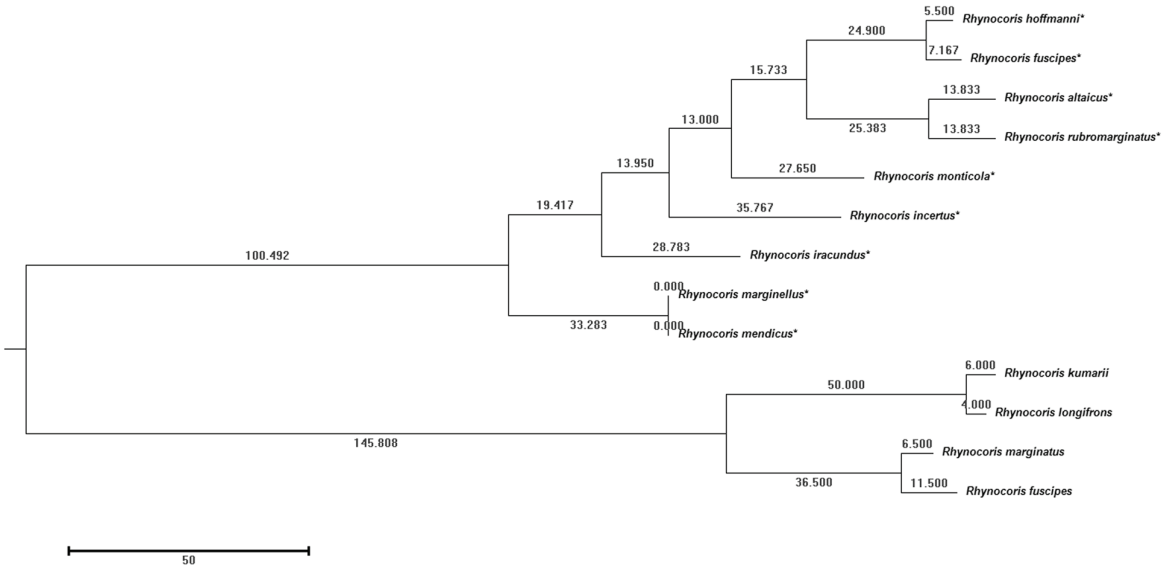


FIGURE 5. Maximum Parsimony tree based on Cyt b gene variations showing the relationships of four Indian and eight non-Indian species of *Rhynocoris*

Interestingly, the non-Indian *R. fuscipes,* instead of clustering with Indian *R. fuscipes,* shows its affinity towards other non-Indian species suggesting the role of geographical isolation at the species level. Thus, the intraspecific genetic variations because of geographical locations or as in distantly located ecotypes are revealed (Almeida *et al.,* 2008, and Naranjo *et al.,* 2010).

Cyt c subunit I. The Maximum Likelihood, Neighbor-Joining, and Maximum Parsimony trees based on Cyt c gene (Fig. 6,7&8) showed the population of Indian *R. marginatus* formed one cluster and populations of Indian *R. fuscipes, R. longifrons,* and *R. kumarii* are together in another cluster with genetic similarity between the Indian *R. kumarii* and *R. longifrons* as observed in the Maximum Likelihood tree for Cyt b gene (Fig.3). But the Indian *R.*

fuscipes diverges independently as a separate clade in this cluster. Another phylogenetic lineage shows two non-Indian species, *R. ventralis* and *R. ornatus,* converging together as a cluster, whereas the Indian *R. marginatus* stands independently, but shares a common phylogenetic lineage and exhibits its affinity with them. All three phylograms obtained based on Cyt c gene (Fig. 6,7&8) show a similar phylogenetic relationship and suggest a monophyly despite geographical isolation. However, Baskar (2010) observed the existence of two main clusters and a single sub-cluster among the four Indian *Rhynocoris* species.

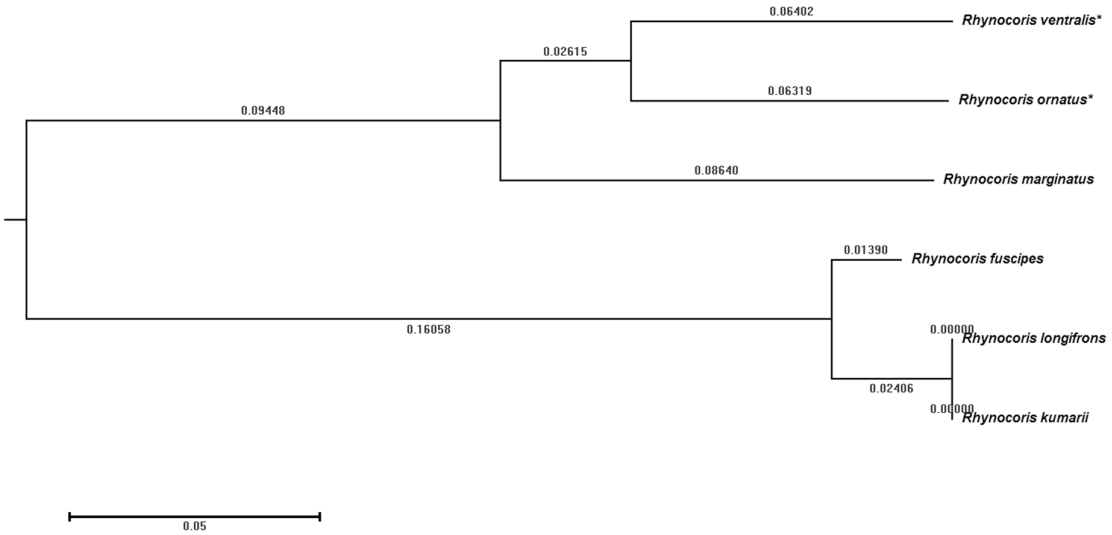
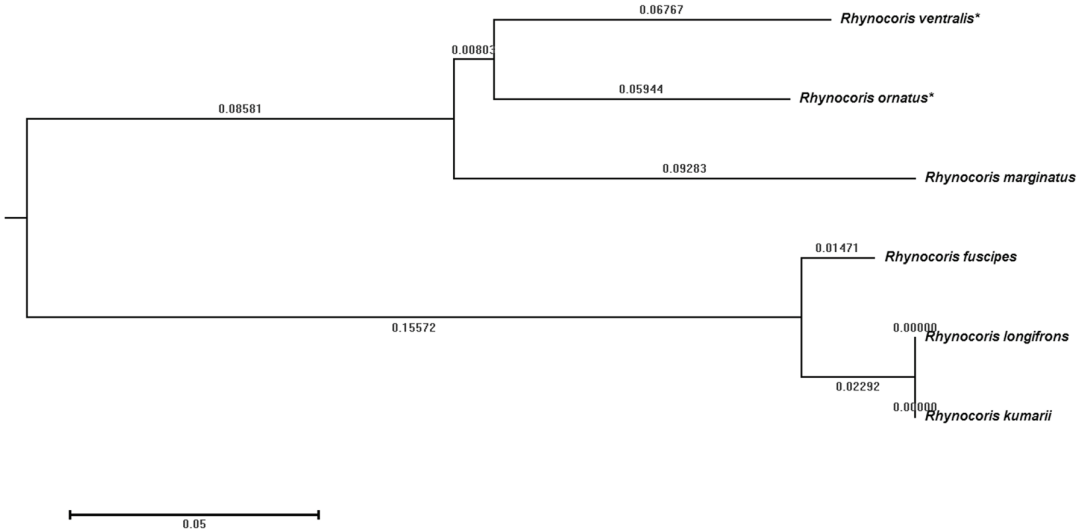


FIGURE 6. Maximum Likelihood tree based on Cyt c gene variations showing the relationships of four Indian and two non-Indian species of *Rhynocoris*



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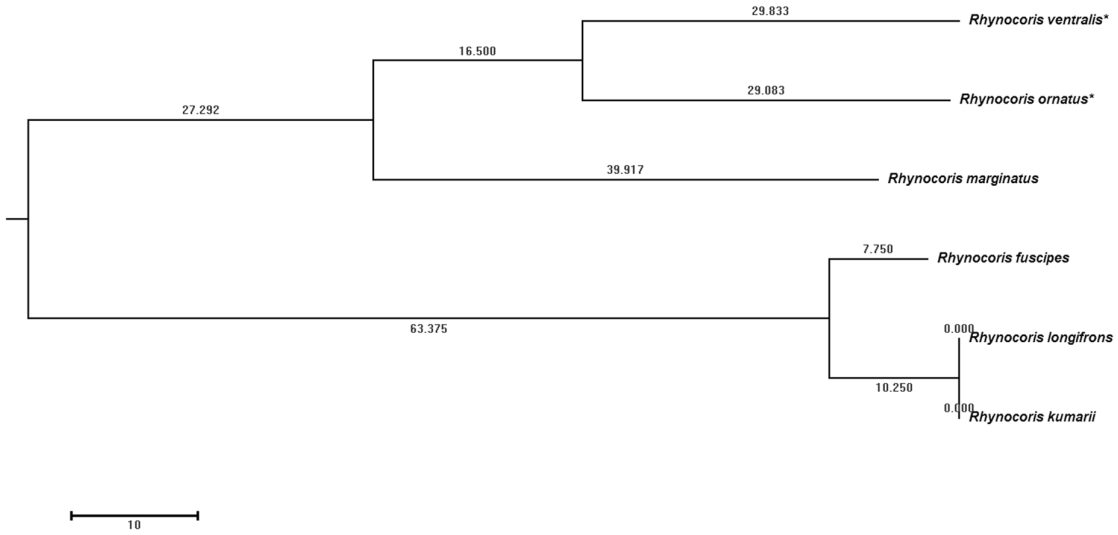

FIGURE 8. Maximum Parsimony tree based on Cyt c gene variations showing the relationships of four Indian and two non-Indian species of *Rhynocoris*

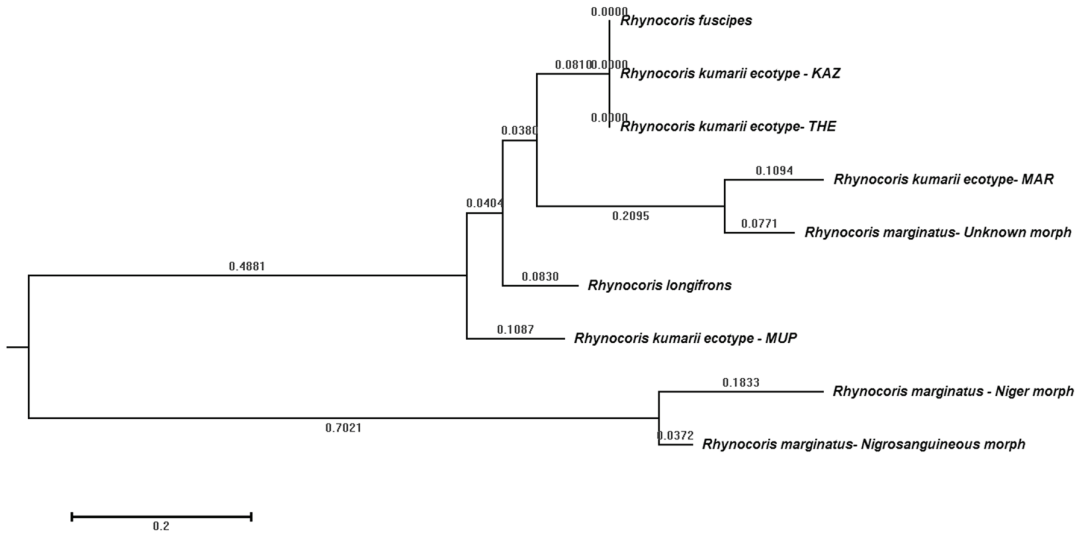

FIGURE 9. Maximum Likelihood tree based on Cyt c subunit like I gene variations showing the relationships of four species of *Rhynocoris,* four ecotypes of *R. kumarii* and three morphs of *R. marginatus* of India.

Cyt c subunit like I. All the three phylograms (Fig.9,10&11) show two separate lineages of which *R. marginatus*-Niger morph and *R. marginatus*–Nigrosanguineous morph stand independently as a separate cluster. Another line of phylogeny shows two separate clusters of which *R. kumarii* -MAR ecotype and *R. marginatus*-unknown morph share a common cluster. Interestingly, the niger and nigrosanguineous morphs of *R. marginatus* instead of clustering together with the unknown morph of *R. marginatus* (intraspecific affinity) as expected, cluster with *R. kumarii*–Mar ecotype (interspecific affinity). Baskar (2010) and Baskar *et al.* (2012a,b,c) reported genetic diversity among the ecotypes of four Indian *Rhynocoris* species, *R. kumari, R. marginatus, R. longifrons,* and *R. fuscipes* based on mitochondrial genes and correlated with the ecological diversity of semiarid, scrub jungle, and tropical rainforest habitats. The present results and the findings of Baskar (2010) & Baskar *et al.* (2012a,b,c) suggest the existence of genetic diversity, gene flow with low level of genetic differentiation among the morphs

and the ecotypes of *Rhynocoris* species as Zaho & Zhu (2011) observed in *Branchiostoma japanicum* Lonnberg. The findings further suggest that the Cyt b fragment is a useful marker to describe the genetic structure of morphs at a particular habitat and ecotypes of closely related habitats (Naranjo, *et al.,* 2010). These observations are contrary to those of Giordana *et al.* (2005) in *Triatoma infestans* (Klug). This contradiction might be the result of the non-dispersal haematophagus feeding behaviour of *Triatoma* in contrast to the dispersal predatory behaviour of *Rhynocoris*.

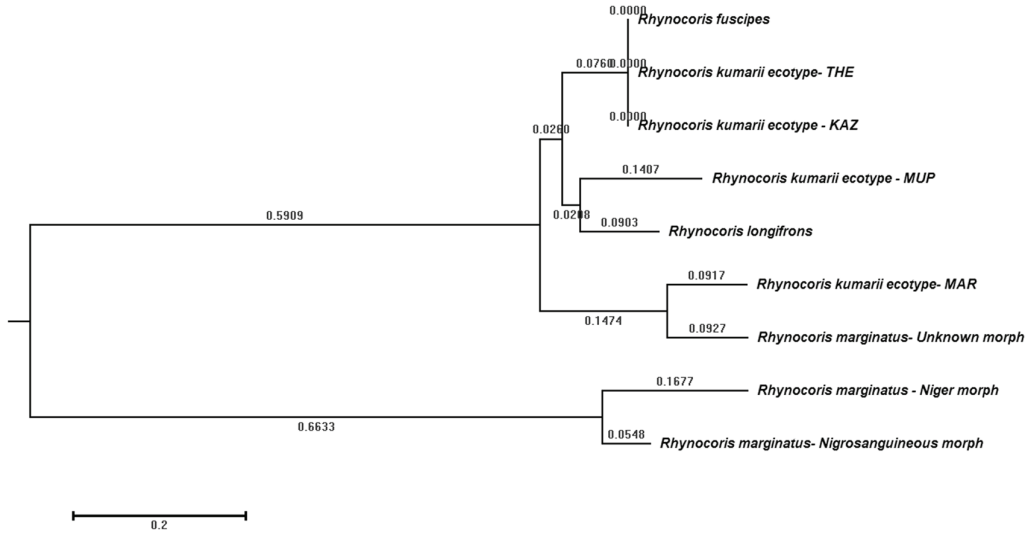

FIGURE 10. Neighbor-Joining tree based on Cyt c subunit like 1 gene variations showing the relationships of four species of *Rhynocoris,* four ecotypes of *R. kumarii* and three morphs of *R. marginatus* of India.

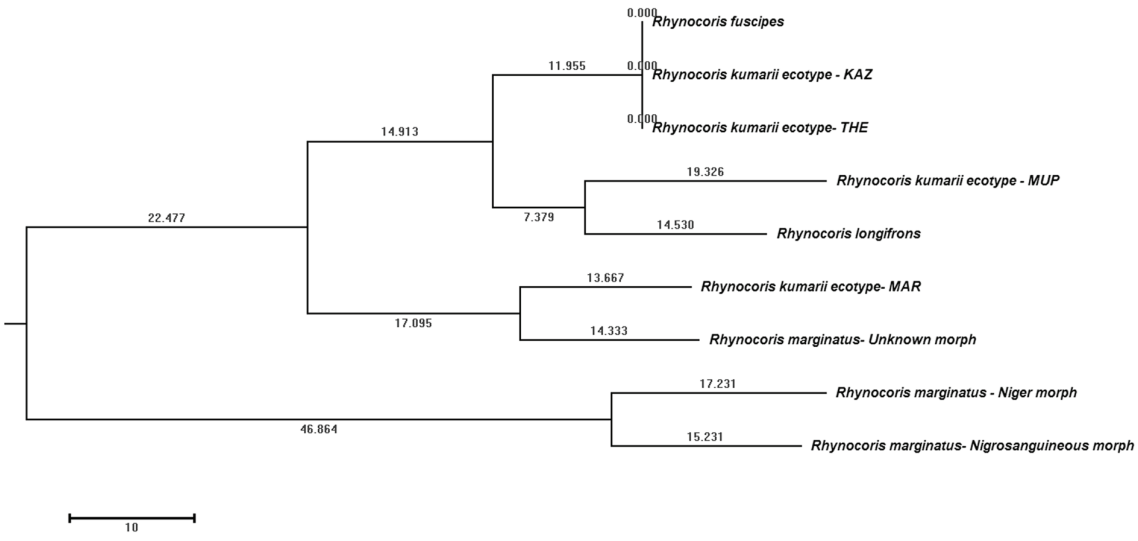

FIGURE 11. Maximum Parsimony tree based on Cyt c subunit like 1 gene variations showing the relationships of four species of *Rhynocoris,* four ecotypes of *R. kumarii* and three morphs of *R. marginatus* of India.

The Indian *R. fuscipes*, *R. kumarii* - KAZ ecotype, and *R. kumarii*–THE ecotype lie in the same line of phylogeny as a separate cluster evolved at a uniform rate indicating their similar rate of evolution. But *R. kumarii* - MUP ecotype and *R. longifrons* have evolved at slightly different evolutionary rates, suggesting the intraspecific genetic variations due to ecotypism. Although the interspecific affinity between the morphs and ecotypes has been

revealed, the genetic diversity among them suggests ongoing speciation among them. The present findings on the affinity of *Rhynocoris* species do not corroborate with the existing literature on identification of these *Rhynocoris* species based on morphological characters, as Garcia *et al.* (2001) observed in *Triatoma* species.

Conclusion

The results obtained not only have enriched our knowledge on biosytematics but have also supplemented multidisciplinary data. The results further reveals the utility of 16 S, mtCyt b, Cyt C oxidase subunit I, and Cyt c oxidase subunit I-like DNA sequences in phylogenetic analysis. The findings clearly suggest the intraspecific and interspecific phylogenetic affinity and diversity not only in the Indian and non-Indian species of *Rhynocoris* but also among the ecotypes of *R. kumarii* and the morphs of *R. marginatus.* Moreover, the genetic diversity observed among ecotypes and morphs suggesting progression of speciation warrant further studies in this direction that could lead to meaningful revision, regrouping, or replacement of species with new revelations through molecular analysis.

Acknowledgments

The authors are grateful to the authorities of St. Xavier's College (Autonomous), Palayamkottai, Tamil Nadu, India for facilities. We are grateful to the Council of Scientific and Industrial Research (CSIR), Government of India, New Delhi, for financial assistance (Ref. No. 21(0865)/11/EMR-II, 2012-2013 dated 28.12.2011). The authors thank Prof. Carl W. Schaefer, University of Connecticut, USA, who reviewed the manuscript, and Prof. Wanzhi Cai, China Agricultural University, China for his timely support.

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Acanthaspis pedestris cytochrome oxidase subunit 1-like (COI) gene, partial sequence; mitochondrial

GenBank: KF443083.1

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            Reduviinae;
            Edocla.
REFERENCE   1  (bases 1 to 724)
AUTHORS   Ambrose,D.P., Lenin,A.E., Manimuthu,M. and Kiruba,A.D.
TITLE     Phylogeny of species of Reduviinae (Hemiptera: Reduviidae) based
          on mitochondrial DNA sequences
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 724)
AUTHORS   Ambrose,D.P., Lenin,A.E., Manimuthu,M. and Kiruba,A.D.
TITLE     Direct Submission
JOURNAL   Submitted (05-NOV-2012) Entomology Research Unit, St. Xavier's
College, Palayamkottai, Tamil Nadu 627002, India
            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     taaacttcagggtgaccaaaaaatca"
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      121 tcatgcttta ttataatttt cttcatagtt ataccaatta taattggggg atttggaaat
      181 tgattagtcc ctttaatatt aggagcccct gatatagctt ttcctcgtat aaataatata
      241 agtttttgat tattcctcct tctttaattt tattaatttc tagtagtaat gatgaaaatg
      301 gagcaggaac tggatgaact gtttatcctc cattatcctc taatattgct catggtggat
      361 catcaggaga tttagcaatt tttctcttca tcttgcaggt atttcttcta ttttaggtgc
      421 tattaatttt attactacaa ttattaatat acgccctaat aatatatctt ttgatcatcc
      481 acctttattt gtttgagctg ttggaattct gcattattat tacttctttc tttacctgaa
      541 tcagcgggag ctattaccat acttcttact gatcgtaact taaatacttc tttttttgct
      601 cctgcaggag gagcagatcc cattttacat cagcattatt ttgatttttt ggtcaccctg
      661 aaagtttaac aatatcttta tgggaccgac cgaccaccac aggacagccc cgcgcaggcc
      721 cagg
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Empyrocoris annulata cytochrome oxidase subunit I-like gene, partial sequence; mitochondrial

GenBank: KC130940.1

FASTA Graphics

Go to:

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LOCUS       KC130940            641 bp    DNA     linear   INV 21-JAN-2013
DEFINITION  Empyrocoris annulata cytochrome oxidase subunit I-like  gene,
            partial sequence; mitochondrial.
ACCESSION   KC130940
VERSION     KC130940.1  GI:442569690
SOURCE      mitochondrion Empyrocoris annulata
ORGANISM  Empyrocoris annulata
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
            Pterygota; Neoptera; Paraneoptera; Hemiptera; Euhemiptera;
            Heteroptera; Panheteroptera; Cimicomorpha; Reduviidae;
            Reduviinae;Empyrocoris.
REFERENCE   1  (bases 1 to 641)
AUTHORS   Ambrose,D.P., Lenin,A.E., Manimuthu,M. and Kiruba,A.D.
TITLE     Phylogeny of species of Reduviinae (Hemiptera: Reduviidae) based
           on mitochondrial DNA sequences
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 641)
AUTHORS   Ambrose,D.P., Lenin,A.E., Manimuthu,M. and Kiruba,A.D.
TITLE     Direct Submission
JOURNAL   Submitted (05-NOV-2012) Entomology Research Unit, St. Xavier's
          College, Palayamkottai, Tamil Nadu 627002, India

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..641
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                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:1276646"
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                     ggtcaacaaatcataaagatattgg, rev_name: hcor, rev_seq:
                     taaacttcagggtgaccaaaaaatca"
     misc_feature    <1..>641
                     /note="similar to cytochrome oxidase subunit I"
ORIGIN
        1 caaactttta gttcggattt gagcaggata gtaggaacat ctttaagatt actaattcga
       61 actgaattag gaactccagg atcattaatt ggagatgatc aaatttataa tactattgtt
      121 acagctctgc ttttattata attttcttca tagttatacc aattataatt gggggatttg
      181 gaaattgatt agttccttta atattaggag ctcctgatat agcttttcct cgtataaata
      241 atataagttt ttgatattac ctccttcttt aattttatta atttctagta gtattgttga
      301 aaatggagca ggaactggat gaactgttta tcctccatta tcctctaata ttgctcatgg
      361 tggatcatca gtagatttag catttttttct cttcatcttg caggtatttc ttctatttta
      421 ggtgctatta attttattac tacaattatt aatatacgaa ttaataatat atcttttgat
      481 caattacctt tatttgtttg agctgttgaa ttactgcatt attattactt ctttctttac
      541 ctgtattagc aggagctatt accatacttc ctactcatcg aaacttacaa tacctctttt
      601 ttgatcctgc aggacggagg agatctccat ttttcccccc t
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Velitra sinensis cytochrome oxidase subunit 1-like (COI) gene, partial sequence; mitochondrial

GenBank: KF443084.1

FASTA Graphics

Go to:

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LOCUS       KF443084            687 bp    DNA     linear   INV 24-AUG-2013
DEFINITION  Velitra sinensis cytochrome oxidase subunit 1-like
            (COI) gene, partial sequence; mitochondrial.
ACCESSION   KF443084
VERSION     KF443084.1  GI:530891382
SOURCE      mitochondrion Velitra sinensis
ORGANISM    Velitra sinensis
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
            Pterygota; Neoptera; Paraneoptera; Hemiptera; Euhemiptera;
            Heteroptera; Panheteroptera; Cimicomorpha; Reduviidae;
            Reduviinae; Velitra.
REFERENCE   1  (bases 1 to 687)
AUTHORS   Ambrose,D.P., Lenin,A.E., Kiruba,A.D. and Manimuthu,M.
TITLE     Direct Submission
JOURNAL   Submitted (22-JUL-2013) Entomology Research Unit, St. Xavier's
           College, Palayamkottai, Tamil Nadu 627002, India

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:1387357"
                     /PCR_primers="fwd_name: lcof, fwd_seq:
                     ggtcaacaaatcataaagatattgg, rev_name: hcor, rev_seq:
                     taaacttcagggtgaccaaaaaatca"
     gene            complement(<1..>687)
                     /gene="COI"
     misc_feature    complement(<1..>687)
                     /gene="COI"
                     /note="similar to cytochrome oxidase subunit 1"
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       61 gtttcggtcg gttaataata ttgtaatatg ctcctgctaa tacaggtaat tgaaagaagt
      121 aataggaggg cagtaattcc tactgatcat acaaatagtg gaattcgttc tggggttatt
      181 ccagttgatc gtatattaat aattgttgaa ataaaattga ctgctcctaa aattgatgaa
      241 atacctgcta aatgtattga aaaaattgct aggtctacag aggctcctct gtgggcaatg
      301 ttgcttgata gggggggata aactgttcat ccagttcctg cccctctttc tacaattcta
      361 ctgatgaaga acaaggttaa tgatggtgga agtaatcaga atcttatatt atttattcgt
      421 gggaatgcca tgtctggggc tccaatcatt aatgggacaa gtcagttccc aaaacctcca
      481 attataatag gcataactat aaagaaaatt atgatgaatg catgggcggt tactactaca
      541 ttgtaagttt ggtcatctcc aatgaaagat cctggttgtc ctaattcaat tcgaattaat
      601 catctgagag atgtgcctac tattccagct catgcaccca atatgaaata aagagtccca
      661 ttatctttat attttttttt aaacaaa
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Catamiarus brevipennis cytochrome oxidase subunit I-like gene, partial sequence; mitochondrial

GenBank: KF056931.1

FASTA Graphics

Go to:

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LOCUS       KF056931        659 bp    DNA     linear   INV 01-JUL-2013
DEFINITION  Catamiarus brevipennis cytochrome oxidase subunit  I-like gene,
            partial sequence; mitochondrial.
ACCESSION   KF056931
VERSION     KF056931.1  GI:514830660
SOURCE      mitochondrion Catamiarus brevipennis
ORGANISM    Catamiarus brevipennis
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
            Pterygota; Neoptera; Paraneoptera; Hemiptera; Euhemiptera;
            Heteroptera; Panheteroptera; Cimicomorpha; Reduviidae;
            Peiratinae; Catamiarus.
REFERENCE   1  (bases 1 to 659)
AUTHORS   Ambrose,D.P., Manimuthu,M., Kiruba,A.D. and Lenin,A.E.
TITLE     Direct Submission
JOURNAL   Submitted (17-MAY-2013) Entomology Research Unit, St. Xavier's
            College, Palayamkottai, Palayamkottai, Tamil Nadu 627002, India

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..659
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                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
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                     taaacttcagggtgaccaaaaaatca"
     misc_feature    complement(<1..>659)
                     /note="similar to cytochrome oxidase subunit I"
ORIGIN      `
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       61 gtttcggtcg gttaataata ttgtaatatg ctcctgctaa tacaggtaat tgaaagaagt
      121 aataggaggg cagtaattcc tactgatcat acaaatagtg gaattcgttc tggggttatt
      181 ccagttgatc gtatattaat aattgttgaa ataaaattga ctgctcctaa aattgatgaa
      241 atacctgcta aatgtattga aaaaattgct aggtctacag aggctcctct gtgggcaatg
      301 ttgcttgata ggggggggata aactgttcat ccagttcctg ccccctcttc tacaattcta
      361 ctgatgaaga acaaggttaa tgatggtgga agtaatcaga atcttatatt atttattcgt
      421 gggaatgcca tgtctggggc tccaatcatt aatgggacaa gtcagttccc aaaacctcca
      481 attataatag gcataactat aaagaaaatt atgatgaatg catgggcggt tactactaca
      541 ttgtaagttt ggtcatctcc aatgaaagat cctggttgtc ctaattcaat tcgaattaat
      601 catctgagag atgtgcctac tattccagct catgcaccca atatgaaata aagagtccc
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Ectomocoris tibialis cytochrome oxidase subunit I-like gene, partial sequence; mitochondrial

GenBank: KF056932.1

FASTA Graphics

Go to:

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LOCUS       KF056932            664 bp    DNA     linear   INV 01-JUL-2013
DEFINITION  UNVERIFIED: Ectomocoris tibialis cytochrome oxidase subunit I-
            like gene, partial sequence; mitochondrial.
ACCESSION   KF056932
VERSION     KF056932.1  GI:514830661
SOURCE      mitochondrion Ectomocoris tibialis
ORGANISM  Ectomocoris tibialis
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
            Pterygota; Neoptera; Paraneoptera; Hemiptera; Euhemiptera;
            Heteroptera; Panheteroptera; Cimicomorpha; Reduviidae;
            Peiratinae; Ectomocoris.
REFERENCE   1  (bases 1 to 664)
AUTHORS   Ambrose,D.P., Manimuthu,M., Kiruba,A.D. and Lenin,A.E.
TITLE     Direct Submission
JOURNAL   Submitted (17-MAY-2013) Entomology Research Unit, St. Xavier's
            College, Palayamkottai, Palayamkottai, Tamil Nadu 627002, India

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     ggtcaacaaatcataaagatattgg, rev_name: hcor, rev_seq:
                     taaacttcagggtgaccaaaaaatca"
     misc_feature    <1..>664
                     /note="similar to cytochrome oxidase subunit I"
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      121 actgcccacg ccttcgtaat gatcttcttt atagttatac caattataat tgggggattc
      181 ggaaattgat tagttccact aataattgga gcccctgaca tagcattccc tcgaataaat
      241 aacataagat tctgattatt acccccatcc ctgacactat tattagtaag aagaatcgtg
      301 gaaagagggg caggaacagg atgaacagtt taccccccctc tttccagaaa tattgcccac
      361 agaggagcat ccgtagatct tgccatcttc tcattacact tggctggtgt gagatcaatc
      421 ctaggagcag ttaactttat ctcaacaatt atcaatatac gaccaaaagg aataaccata
      481 gaacgaattc ctctatttgt atgatcagta ggaattactg ccctcctgct gctactgagt
      541 ctaccggtcc tggccggagc aatcacaata ctattaactg atcgcaattt caacacaaca
      601 ttttttgacc ccgcaggggg tggggatcca ctcctgacca acatctctta ataccattcc
      661 cggt
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Ectomocoris cordiger cytochrome oxidase subunit I-like gene, partial sequence; mitochondrial

GenBank: KF056933.1

FASTA Graphics

Go to:

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LOCUS       KF056933       661 bp    DNA     linear   INV 01-JUL-2013
DEFINITION  Ectomocoris cordiger cytochrome oxidase subunit I-like gene,
            partial sequence; mitochondrial.
ACCESSION   KF056933
VERSION     KF056933.1  GI:514830662
SOURCE      mitochondrion Ectomocoris cordiger
ORGANISM    Ectomocoris cordiger
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
            Pterygota; Neoptera; Paraneoptera; Hemiptera; Euhemiptera;
            Heteroptera; Panheteroptera; Cimicomorpha; Reduviidae;
            Peiratinae; Ectomocoris.
REFERENCE   1  (bases 1 to 661)
AUTHORS   Ambrose,D.P., Manimuthu,M., Kiruba,A.D. and Lenin,A.E.
TITLE     Direct Submission
JOURNAL   Submitted (17-MAY-2013) Entomology Research Unit, St. Xavier's
            College, Palayamkottai, Palayamkottai, Tamil Nadu 627002, India

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /organelle="mitochondrion"
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                     ggtcaacaaatcataaagatattgg, rev_name: hcor, rev_seq:
                     taaacttcagggtgaccaaaaaatca"
     misc_feature    <1..>661
                     /note="similar to cytochrome oxidase subunit I"
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      121 agttaccgcc cacgcattca ttataatttt cttcatagtt atacctatta taattggagg
      181 atttggaaat tgattggtac ctttaataat tggagcccca gatatggcct tccctcgaat
      241 aaataacata agattctgat tactgccacc atccctaaca ctattattaa ccagtagaat
      301 tgtcgaaaga ggggcaggaa ctggatgaac agtttatccc cccctatcaa gaaatattgc
      361 ccacagagga gcatcggtag acttagcaat cttttcatta cacctagcag gtatgtcatc
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      481 tccagaacga attccactat ttgtatgatc agtaggaatc actgccctac ttttacttct
      541 ttcacttacc tgtactagca ggagcaatta caatctacta accgaccgaa acttcaacac
      601 ctcatttttg acccagcagg aggggtggac caatcataac aacaagcgac aacaccgtag
      661 a
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Ectomocoris quadriguttatus cytochrome oxidase subunit I-like gene, partial sequence; mitochondrial

GenBank: KF056934.1

FASTA Graphics

Go to:

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LOCUS       KF056934             649 bp    DNA     linear   INV 01-JUL-2013
DEFINITION  Ectomocoris quadriguttatus cytochrome oxidase subunit
            I-like gene, partial sequence; mitochondrial.
ACCESSION   KF056934
VERSION     KF056934.1  GI:514830663
SOURCE      mitochondrion Ectomocoris quadriguttatus
ORGANISM    Ectomocoris quadriguttatus
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
            Pterygota; Neoptera; Paraneoptera; Hemiptera; Euhemiptera;
            Heteroptera; Panheteroptera; Cimicomorpha; Reduviidae;
            Peiratinae;Ectomocoris.
REFERENCE   1  (bases 1 to 649)
AUTHORS   Ambrose,D.P., Manimuthu,M., Kiruba,A.D. and Lenin,A.E.
TITLE     Direct Submission
JOURNAL   Submitted (17-MAY-2013) Entomology Research Unit, St. Xavier's
            College, Palayamkottai, Palayamkottai, Tamil Nadu 627002, India

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     ggtcaacaaatcataaagatattgg, rev_name: hcor, rev_seq:
                     taaacttcagggtgaccaaaaaatca"
     misc_feature    <1..>649
                     /note="similar to cytochrome oxidase subunit I"
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       61 cgaatcgaac tcggtcaacc aggatctttt attggagatg atcaaacata taatgttgta
      121 gtaactgccc atgcattcgt aataattttc ttcatagtaa taccaatcat aattggagga
      181 ttcggtaact gactagtacc tctaataatt ggagcacctg atatagcatt cccacgaata
      241 aataatataa gattctgatt attaccccca tccctcacac tattactagt aagaagtatt
      301 gttgaaagag gagcagggac tggatgaaca gtttatccac cattatctag aaatgttgcc
      361 cacagagggg cctcagttga tttagcaatc ttttcattac atctagcagg tgtaagatca
      421 attctaggag cagtaaactt tatctccaca attattaata tacgaccaaa gggaataact
      481 atagaacgaa ttcccttatt tgtatgatct gtaggaatta cagcacttct cctcctgtta
      541 agattacctg ttctagcggg agctattacc atattattga ccgatcgaaa cttcaatacc
      601 acatttttg atcccgccgg agggggagat ccaattctta ccaaacata
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