RESEARCH ARTICLE

DNA Barcoding of Six Species of Family Rhopalidae (Insecta: Hemiptera: Heteroptera) from India

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ABSTRACT

Rhopalidae is a small family comprising 18 genera and 209 recognised species all over the world. The Oriental Realm embraces approximately 30 species of about 10 genera. In this study, first ever comprehensive DNA barcode analysis of 17 specimens of six species belonging to five genera of family Rhopalidae from northern parts of India has been presented and compared with worldwide available data. Sequences for all the species but *Leptocoris augur* (Fabricius, 1974) are first submissions from India while six sequences of *Liorhyssus rubicundus* (Signoret, 1859) are the pioneer submissions for this particular species to the GenBank and BOLD.

Both the species of genus *Liorhyssus* viz *L. hyalinus* (Fabricius, 1794) and *L. rubicundus* (Signoret, 1859) show striking phenotypic plasticity but can be easily delineated by distinct barcodes showing minimum interspecific K2P distance of 7.6 % and much lower maximum intraspecific K2P distances of 2.1% and 0.5% respectively. Our two sequences of *Stictopleurus pictus* (Fieber, 1861) show K2P distance of 0.0% but 2.3% with sequences from Germany. The *Rhopalus (Aeschyntelus)* sp. nr. *maculatus* shows average K2P distance of 3.2% with *Rhopalus maculatus* (Fieber,1837) and appears to be a cryptic species. *Corizus sp*. shows distinct barcode which delineates it from its nearest neighbour *Corizus hyocyami* (Linnaeus, 1758).

Keywords: COI gene, Coreoidea, K2P distance, Evolutionary affinities

INTRODUCTION

With most of the studies concentrating on the largest family Coreidae of superfamily Coreoidea, Rhopalidae is its small and marginalized family in terms of taxonomical and molecular studies. Members of this family are small sized plant bugs comprising 18 genera and 209 recognised species classified into two subfamilies, Rhopalinae and Serinethinae, which are distributed in all major faunal regions of both the Old and New Worlds (Schuh and Slater, 1995). The Oriental Realm embraces approximately 30 species of about 10 genera (Distant, 1904; Chopra, 1967; Ahmad, 1978; Ahmad, *et al.*1979; Rizvi, 2000; Hua *et al*., 2008 and Jung *et al*., 2011).

Morphologically, rhopalids are generally small sized bugs of ≤ 10mm and are often misidentified with members of other families like Pyrrhocoridae, Lygaeidae and Coreidae. The small size, inconspicuous morphologically distinguishing features and variable body colorations further make their identifications a tricky affair at generic as well as specific level. Moreover, while working on rhopalids, it is a tough call to differentiate whether morphologically dissimilar specimens belong to two different sibling species or just exhibit phenotypic plasticity.

COI gene based barcoding has been proved to be reliable, cost-effective and readily accessible tool for species identification, and has largely been used to resolve taxonomic ambiguities in gastropods (Remigio and Hebert, 2003), birds (Hebert *et al.,* 2004; Kerr *et al.,* 2007) and fish (Ward *et al*., 2005). However, application of this approach in Heteroptera remained much delayed as initial works, for one reason or the other, undermined the utility of DNA barcoding (Memon *et al*., 2006; Damgaard, 2008). Jung *et al.* (2011), for the first time, strongly advocated the use of COI based barcoding as useful identification tool for Heteroptera which was later used by Park *et al*. (2011), Lis *el al.* (2012), Rebijith *et al*. (2012), Zhou *et al.* (2012), Li *et al*. (2014), Tembe *et al.*(2014), Raupach *et al*.(2014), Gwiazdowski *et al*. (2015) and Kaur and Sharma (2016).

In 2003, Hebert *et al.* underlined the need to advance the COI databases comprehensively for a Global Identification System for taking objective taxonomic decisions, quantifying the boundaries of intraspecific diversity and recognizing the sibling species. This Global Identification system was later realised as BOLD (Herbert *et al.,* 2003; Ratnasingham and Hebert, 2013). Hebert *et al.* (2003) had suggested the thresholds of COI for species diagnosis to be greater than 3%. But Li *et al.* (2014), while studying two species of genus *Nezara* of family Pentatomidae, stressed upon the need to have molecular data of a species from different geographical regions to know the worldwide sequence diversity and determine the species delineating thresholds. In this study, first ever comprehensive DNA barcodes of seventeen specimens of six species belonging to five genera of family Rhopalidae of India have been compared with worldwide data available and their phylogenetic relationships have been discussed. The data includes the species which are either morphologically highly similar or show phenotypic plasticity and thus tend to be taxonomically problematic.

MATERIALS AND METHODS

Adult specimens of six species of Rhopalidae referable to five genera were collected from northern parts of India (Fig. 1 & Fig 2). Table 1 enlists the collected taxa along with the place and date of their collection. A few specimens were pinned for identification while others were preserved in ethanol on the spot. The preliminary identification was done in the laboratory with the help the literature procured from different taxonomists of Rhopalidae and the data available online (Distant, 1904; Chopra, 1967; Rizvi, 2000; Steill and Meyer, 2003; websites www.britishbugs.org.uk; www.biolib.cz; www.coreoidea.speciesfile.org) and then got rechecked and confirmed from Professor Emeritus (Dr.) C.A. Viraktamath of Department of Entomology, University of Agricultural Sciences, Bangalore. The specimens of bugs were stretched and photographed using Nikon AZ 100 Stereo Zoom binocular.

Molecular analysis was carried out for 17 specimens in molecular laboratory of the Department of Zoology and Environmental Sciences, Punjabi University Patiala. These include 1 each of *Leptocoris augur* (Fabricius, 1781) and *Corizus* sp, 2 of *Stictopleurus pictus* (Fieber,1861), 3 of *Rhopalus sp.*, 4 of *Liorhyssus hyalinus* (Fabricius,1794) and 6 of *Liorhyssus rubicundus* (Signoret, 1859). The voucher specimens were preserved and submitted with the collection code PUP/2012 to the Museum of the Department of Zoology and Environmental Sciences, Punjabi University, Patiala. All relevant voucher information, taxonomic classifications, pictorial illustrations, trace files and DNA barcodes have been submitted to Barcode of Life Datasystems (BOLD; www.boldsystem.org) under the project RHOAL. DNA was extracted from ethanol preserved specimens by following the method suggested by Kambhampati and Rai (1991) with minor modifications. A region ranging from 580bp - 650 bp of COI gene was amplified using 1µl each 10µM of primers LCO 1490 and HCO 2198 (Folmer *et al.*, 1994), 12.5 µl of ready to use master mix (containing *Taq* polymerase, 400µM of each dNTP, 5.5mM of $MgCl_2$ and reaction buffer) and 1µl of template DNA. The PCR conditions used for amplification were: Initial denaturation at 95°C for

5min, 35 cycles of denaturation at 95°C for 1 min, annealing at 45 - 50^{0}C for1 min and elongation at 72^{0}C for 90 sec followed by a final elongation at 72 ^{0}C for 7 min. The amplified COI gene was run on EtBr stained 1% agarose gel along with 100bp ladder to confirm the length of gene segment amplified. The gene segments were got sequenced from Yaazh Xenomics, Mumbai. 17 COI gene sequences varying from 580 to 650 bp for six species were submitted to BOLD and GenBank (Table 1). Species identification was attempted by conducting BOLD SEARCH and NCBI BLAST. The sequences of conspecific or congeneric specimens submitted by other workers from different regions were retrieved from GenBank (Table 2) to calculate intraspecific and interspecific K2P divergence. The sequence alignment and intra and interspecific variability analysis using Kimura-2-Parameter was done with help of MEGA 7.0.14 (Kumar *et al*. 2015). All barcodes were subjected to Barcode Index Number (BIN) system of BOLD. To derive evolutionary affinities, a total of 78 sequences were analysed and Neighbour-Joining tree was inferred using K2P model of substitution with the help of MEGA 7.0.14 (Kumar *et al*., 2015). These include 17 sequences of six species of present study, 59 sequences of fourteen species of Rhopalidae and 2 sequences of Pentatomidae taken as the outgroup 1 each of *Dalpada nigricollis* (Westwood) - Acc no. KU377166 and *Halyomorpha picus* (Fabricius) -Acc no. KM226884) retrieved from GenBank.

Figure 1: Photographs showing Phenotypic plasticity in *Liorhyssus hyalinus* (a,b.c) and *Liorhyssus rubicundus* (d,e,f)

Figure 2 – Photographs of: **a)** *Stictopleurus pictus* **b)** *Rhopalus* sp. **c)** *Corizus* sp. **d)** *Leptocoris augur*

Table 1: The specimen voucher details including place and date of collection with accession no. and BIN assigned by GenBank and BOLD respectively.

S. No.	Taxa	Place of Collection	Date of Collection	Voucher ID	Length of gene segment (bp)	GenBank Accession No.	BIN
1.	*Liorhyssus hyalinus* (Fabricius)	Bilaspur (Himachal Pradesh)	21.04.2013	7-PUP-2012	654	KU234088	AAG8881
		Bilaspur (Himachal Pradesh)	21.04.2013	7.2-PUP-2012	639	KU234089	AAG8881
		Shimla (Himachal Pradesh)	16.06.2016	22-PUP-2012	655	KY126825	AAG8881
		Shimla (Himachal Pradesh)	16.06.2016	23-PUP-2012	627	KY126828	AAG8881
2.	*Liorhyssus rubicundus* (Signoret)	Patiala (Punjab)	11.04.2013	6-PUP-2012	591	KU234092	ACB2310
		Patiala (Punjab)	11.04.2013	C6-PUP-2012	636	KX380794	ACB2310
		Patiala (Punjab)	01.05.2013	R2-PUP-2012	612	KX380795	ACB2310
		Solan (Himachal Pradesh)	26.04.2016	PDRED-PUP-2012	612	KY126824	ACB2310
		Solan (Himachal Pradesh)	24.04.2016	WH-PUP-2012	612	KY126826	ACB2310
		Solan (Himachal Pradesh)	24.04.2016	DYP-PUP-2012	612	KY126823	ACB2310
3.	*Stictopleurus pictus* (Fieber)	Solan (Himachal Pradesh)	20.04.2015	13-PUP-2012	644	KU234084	ACQ3402
		Solan (Himachal Pradesh)	20.04.2015	CN2-PUP-2012	641	KU234085	ACQ3402
4.	*Rhopalus* sp.	Palampur (Himachal Pradesh)	25.05.2015	11-PUP-2012	651	KU234086	AAZ6796
		Palampur (Himachal Pradesh)	25.05.2015	RHT-PUP-2012	651	KU234087	AAZ6796
		Palampur (Himachal Pradesh)	25.05.2015	SP-PUP-2012	623	KY126827	AAZ6796.
5.	*Corizus* sp.	Bilaspur (Himachal Pradesh)	20.04.2015	CM-PUP-2012	580	KU234091	ADF9671
6.	*Leptocoris augur* (Fabricius)	Dehradun (Uttrakhand)	06.08.2012	S-PUP-2012	624	KU234090	ACD8243

RESULTS AND DISCUSSION

Out of 543 positions of 76 sequences belonging to twenty species of Rhopalidae analysed, 209 sites are variable of which 196 are parsimony informative sites. The detailed Maximum Composite Likelihood Estimate of pattern of nucleotide substitution has been presented in Table 3. The overall transition/ transversion bias is R = 3.212 indicating transitions to be more prevalent. The nucleotide composition shows bias towards A+T content (66.99%) with T (36.43%) as the most represented base which is a general observation made for class Insecta (Hebert *et al*., 2003; Habeeb and Sanjayan, 2011; Raupach *et al*., 2014).

The details of molecular analyses of six species of present study are discussed as follows:

Liorhyssus (Stål)

The COI gene sequences of two species of *Liorhyssus* viz. *L. rubicundus* (Signoret) and *L. hyalinus* (Fabricius) have been analysed in the present study. Both the species show remarkable phenotypic variations. For *L. hyalinus*, the colouration varies from stramineous to piceous with many gradations of ferruginous tinge (Fig 1). In field, the extreme colour morphs appear to be belonging to two different species. Same is the case with *L. rubicundus* where ground colour varies from scarlet red with black spots to sorrelaceous and to piceous (Fig 1). In the present study, six COI gene sequences of *L. rubicundus* have been furnished which are first and the only submissions with GenBank and BOLD for this species. For *L. hyalinus*, four sequences have been submitted which are first records from India. The CO I gene based barcodes well delineates the two species with a minimum K2P distance of 7.6% (Table 6).

All the six sequences of *L. rubicundus*, have been clustered in a single BIN (Table 1). Among them, intraspecific K2P distance ranges from 0.0% - 0.5% with an average of 0.3% ± 0.2. With BOLD, there are total of eight sequences (including the present six) put in the same BIN BOLD: ACB2310. The other two are unidentified species from Pakistan. Therefore, DNA barcode for this species is distinct and is useful for its identification. For *L. hyalinus*, the K2P distance for four sequences of the present study ranges from 0.5%-1.2% with an average of 1.1% ±0.2 (Table 4). In earlier studies, average K2P distance has been reported to be < 1%, being 0.9% ± 0.00 for two sequences from National Park of Mercantour, France (Dousoulier *et al.* Unpubl.*), 0.8% ± 0.3 for three sequences from Germany (Raupach *et al.* 2014) and 0.2% ± 0.2 for four sequences from Canada (Gwaiazdowski *et al*., 2015). A comparison of present four sequences with nine sequences from other countries (Table 2) shows average intraspecific K2P divergence to be ≤ 1% with sequences from France and Germany but >1% with Canadian sequences. The latter show >1% intraspecific divergence with sequences from France and Germany as well (Table 4). These thirteen sequences of this species have been assigned a common BIN URI BOLD: AAG8881.

Stictopleurus pictus (Fieber)

The morphological identification of this species is based upon fine details on pronotum and scutellum and these characters are not always easy to discern. Two COI gene sequences of *S. pictus* of the present study show K2P distance of 0.0% but when compared with two corresponding sequences from Germany procured from GenBank (Table 2), the K2P widens to 2.3%. The German sequence pair shows K2P of 0.0% (Raupach *et al*., 2014). A wide K2P distance of 2.3% between the sequences from India and Germany may be due to phylogeographical separation of two populations.

Amongst congeneric species of *Stictopleurus*, *S. abutilon* (Rossi) has been observed to be nearest to *S. pictus* showing minimum interspecific distance of 6.5% (Table 6).

Rhopalus sp.

This species is identified prima facie as *Rhopalus maculatus* as per the specific key (Rizvi, 2000). But when COI sequence of 3 specimens of the present study and 3 sequences of *R. maculatus* retrieved from GenBank (submitted by Raupach *et al.* 2014 from Germany) were compared, the K2P divergence range from 3.1%-3.3% with an average of 3.2% (Table 6) which is slightly above the threshold suggested by Herbert (2003) and this led to the predicament. A number of specimens were thoroughly examined morphologically in the lab and were compared with photographs of *R. maculatus* uploaded by Raupach in the Barcode Of Life Datasystems (www.boldsystems.org) under the project GEBUG. Although, differences in coloration were observed but nothing could be concluded. Then Prof. C.A Viraktamath and his team analysed the specimens and found morphological

features and genetalia to be largely similar to *R. maculatus* but on the basis of some differences in maculae on the dorsum of abdomen, he described it as "*Rhopalus (Aeschyntelus)* sp. nr. *maculatus*". BOLD has put these three sequences of the present study in a separate BIN from that of *Rhopalus maculatus*. All these facts indicate the present species to be a cryptic species reported for the first time.

***Corizus* sp.**

Till date, 2 valid species of this genus have been reported from Indo-pakistan subcontinent viz. *C. hyocyami* and *C. baluchistanensis*. The species description of the specimen with us does not corroborate with any of them. Morphologically it is close to *C. hyocyami* with which it shows minimum interspecific K2P distance of 7.9% (Table 6). This

Table 2: COI gene sequences retrieved from GenBank used for inter- and intraspecific K2P analyses in present study.

S No.	Place	Accession No	Authors
I.	***Liorhyssus hyalinus* (Fabricius)**		
1.	France (National Park of Mercantour)	KJ541520	Dousoulier *et al.*, Unpubl.
2.	France (National Park of Mercantour)	KJ541521	Dousoulier *et al.*, Unpubl.
3.	Germany	KM023097	Raupach *et al.*, 2014
4.	Germany	KM021653	Raupach *et al.*, 2014
5.	Germany	KM022038	Raupach *et al.*, 2014
6.	Canadian National Collection of Insects	HQ105841	Park *et al*., 2011
7.	Canada	KR034668	Gwaiazdowski *et al.*, 2015
8.	Canada	KR038380	Gwaiazdowski *et al.*, 2015
9.	Canada	KR041512	Gwaiazdowski *et al.*, 2015
II.	***Stictopleurus pictus* (Fieber)**		
10.	Germany	KM022183	Raupach *et al.*, 2014
11.	Germany	KM022691	Raupach *et al.*, 2014
III.	***Stictopleurus abutilon* (Rossi)**		
12.	Germany	KM022638	Raupach *et al.*, 2014
13.	Germany	KM021500	Raupach *et al.*, 2014
14.	Germany	KM022342	Raupach *et al.*, 2014
15.	Germany	KM022743	Raupach *et al.*, 2014
IV.	***Rhoplalus maculatus* (Fieber)**		
16.	Germany	KM 021523	Raupach *et al.*, 2014
17.	Germany	KM 021712	Raupach *et al.*, 2014
18.	Germany	KM 023131	Raupach *et al.*, 2014
V.	***Rhopalus sapporensis* (Matsumura)**		
19.	South Korea	GQ292285	Jung *et. al.,* 2011
VI.	***Corizus hyocyami* (Linnaeus)**		
20.	Germany	KM021674	Raupach *et al.*, 2014
21.	Germany	KM021784	Raupach *et al.*, 2014
22.	Germany	KM021889	Raupach *et al.*, 2014
23.	Germany	KM023093	Raupach *et al.*, 2014
24.	Germany	KM022350	Raupach *et al.*, 2014
25.	Germany	KM022552	Raupach *et al.*, 2014
26.	Germany	KM021975	Raupach et al., 2014
27.	France	KJ541534	Dousoulier *et al.*, Unpubl.
VII.	***Leptocoris augur* (Fabricius)**		
28.	South India	HQ 236473	Tembe *et al.,* 2014
29.	South India	HQ236472	Tembe *et al.,* 2014
VIII.	***Boisea trivittata* (Say)**		
30.	Canada	KR035746	Gwaiazdowski et al., 2015
31.	Canada	KR035416	Gwaiazdowski et al., 2015

Table 3: Maximum composite likelihood Estimate of the pattern of substitution shown by probability of substitution (r) from one base in row to another base in the column out of 100 events

	A	T	C	G
A	-	03.92	01.84	15.42
T	03.29	-	10.69	01.72
C	03.29	22.83	-	01.72
G	29.52	03.92	01.84	-

Table 4: The intraspecific distance of sequences

S. No.	Region	Range of intraspecific K2P distance	Average K2P
1)	***Liorhyssus hyalinus* (13 sequences of 585 bp)**		
1.	India – India	0.5% - 1.2%	1.1% ± 0.2
2.	India-France	0.2% - 1.2%	0.8% ± 0.1
3.	India-Germany	0.3% - 1.7%	1.0% ± 0.3
4.	India-Canada	1.4% - 2.1%	1.6% ± 0.3
5.	France-France	-	0.9%
6.	France-Germany	0.3% -1.2%	0.8% ± 0.3
7.	France-Canada	1.5% -1.7%	1.6% ± 0.1
8.	Germany-Germany	0.5% -1.0%	0.8% ± 0.3
9.	Germany- Canada	1.2% -2.1%	1.7% ± 0.3
10.	Canada-Canada	0.0% -0.3%	0.2% ± 0.2
		Overall Average Intraspecific K2P Distance	**1.2%± 0.3**
2)	***Liorhyssus rubicundus* (6 Sequences of 591 bp)**		
1.	India-India	0.0%- 0.5%	0.3% ± 0.2
		Overall Average Intraspecific K2P Distance	**0.3% ± 0.2**
3)	***Stictopleurus pictus* (4 sequences 624 sites)**		
1.	India-India	0.0%	-
2.	India-Germany	2.3%	-
3.	Germany-Germany	0.0%	-
		Overall Average Intraspecific K2P Distance	**1.5% ± 0.4**
4)	***Rhopalus* sp. (3 seqences of 606 bp)**		
1.	India- India	0.0%-0.2%	**0.1%±0.1**
		Overall Average Intraspecific K2P Distance	**0.1%±0.1**
5)	***Leptocoris augur* (3 sequences of 549 bp)**		
1.	India- India	0.2% - 1.5%	1.0% ± 0.3
		Overall Average Intraspecific K2P Distance	**1.0% ± 0.3**

Table 5: The interspecific distance of *Rhopalus* sp. with congeners

S No.	Species	Minimum K2P Distance
1.	*Rhopalus maculatus* (Fieber)	03.1%
2.	*Rhopalus sapporensis* (Matsumura)	13.9%
3.	*Rhopalus conspersus* (Fieber)	14.2%
4.	*Rhopalus subrufus* (Gmelin)	14.6%
5.	*Rhopalus parumpunctatus* (Schilling)	15.1%
6.	*Rhopalus tigrinus* or *Brachycarenus tigrinus* (Schilling)	16.1%

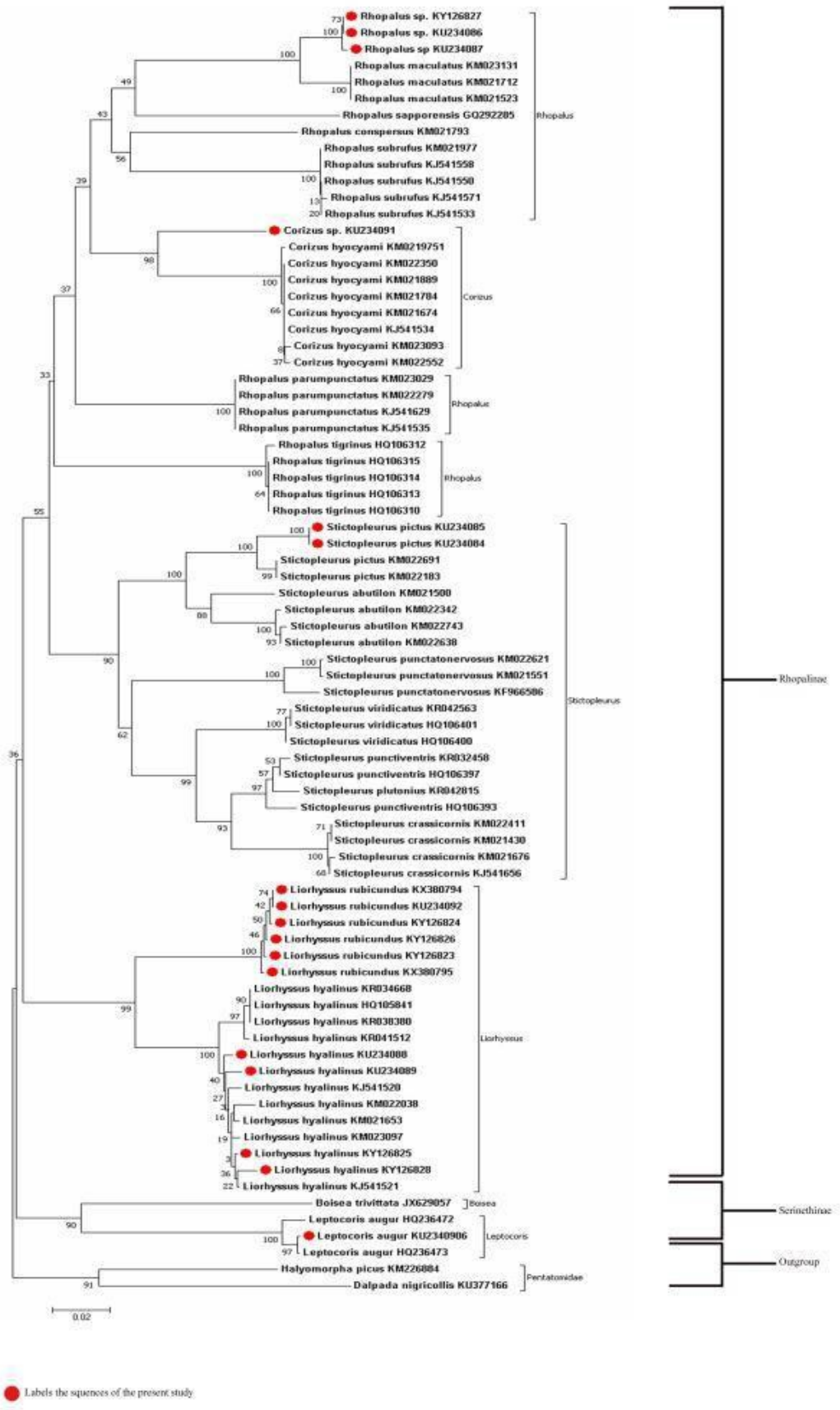

Figure 3: Neighbour Joining (NJ) tree depicting the evolutionary affinities of the six species of present study with other species of Rhopalidae

indicates that this is a new species still to be taxonomically described. A new BIN BOLD: ADF9671 has been created for this species.

***Leptocoris augur* (Fabricius)**

A stretch of 549 bp COI gene of *Leptocoris augur* specimen of the present study was aligned with two corresponding sequences from South India (Tembe *et* al., 2014) and one from Germany retrieved from GenBank database (Table 2). German sequence (Accession no. KP142936*) was found to create gaps within all other sequences, so was excluded from the analysis. The intraspecific K2P distances between the present sequence and two sequences submitted from South India have been found to be 1.3% and 1.5%. However, the South Indian sequence pair shows only 0.2% K2P distance (Table 4).

Sequences for none of the congeneric species of *Leptocoris augur* are available with GenBank. Amongst the data available, the first affinity is shown with *Boisea trivittata* (Say, 1825), also a member of subfamily Serinethinae, with minimum interspecific distance of 15.5% (Table 6).

Evolutionary affinities

An attempt has been made to study evolutionary affinities of the rhopalid species by constructing NJ tree based on present sequences and sequences retri-eved from GenBank (Fig. 3). All the species are distinc-tly clustered in two groups representing subfamilies Rhopalinae and Serinethinae. Rhopalinae splits into two groups, one containing all the species of the single genus *Liorhyssus* and the second containing rest of the genera of Rhopalinae. Within the second group, one line of divergence contains all the species of *Stictopl-eurus*. The second line of divergence is para-phyletic which includes different species of *Rhopalus* forming distinct lineages, and one of these lines leads to *Corizus.* The affinities of six species of present study depicted in the NJ tree support the data presented in Table **6**. *Rhopalus* sp. forms sister group with *R. maculatus*. and the two together share a common ancestor with *R. sapporensis*. *Corizus* sp. is closest to *C. hyosyami*, *Liorhyssus hyalinus* to *L. rubicundus* and *Stictopleurus pictus* to *S. abutilon*.

CONCLUSION

Sequences for all the species but *Leptocoris augur* are first submissions from India while six sequences for *Liorhyssus rubicundus* are pioneer submissions to the GenBank and BOLD. Distinct barcodes of *Liorhyssus hyalinus* and *Liorhyssus rubicundus* resolve the problem of phenotypic plasticity in them. A K2P distance of 2.3% between Indian and German sequences of *Stictopleurus pictus* indicates phylogeographical separation of the two populations. The *Rhopalus (Aeschyntelus)* sp. nr. *maculatus* of the present study seems to be forming species complex with *Rhopalus maculatus* (3.2% K2P distance) and calls for attention of taxonomists for morphological description. Molecular data indicates *Corizus* sp. to be a new one which too needs to be taxonomically described. The taxonomy of small sized bugs of Rhopalidae is a challenging task and DNA barcoding can be of great aid for scientists working on them in terms of identifying the polymorphic species and discovering the potential cryptic species.

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