Substitution rate estimation of molecular markers to evaluate evolutionary aspects in ladybird beetles

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In this study, we examined the ribosomal DNA internal transcribed spacers and mtDNA markers for their use in the prospecting of 480 ladybird species belonging to 14 tribes to assess the evolutionary topology and substitution rates. Substitution patterns of the respective markers were estimated using a cascade of algorithms such as pairwise sequence comparisons, maximum likelihood estimates of the substitution matrix, transitions/transversions (*ti*/*tv*) and gamma parameters with a suitable substitution model. Maximum likelihood (ML) estimates showed that COI ($R = 1.16$) and COII ($R = 1.36$) were more biased towards transitions. COI has a higher *ti*/*tv* ratio indicating more substitutions and less divergence among the species in the phylogenetic tree, though it had moderate bootstrap support. ML and Bayesian analysis were used to construct the morphology character matrix and molecular datasets in order to establish the evolutionary relationship. All the characters of male and female genitalia supported mophyletic topology. The phylogenetic results of molecular datasets suggest that most of the taxa significantly support monophyly. Phylogenetic analysis depict COI consists of more substitution as it shows less divergence among species.

Keywords: Evolutionary topology, ladybirds, molecular markers, phylogenetic analysis, substitution rates.

THE Coccinellidae family comprises over 6000 species, with 360 genera and 25 tribes identified globally[1–4]. Ladybirds have a number of traits that make them an intriguing model from both a biological and an economic perspective[5]. They are widespread, available in a great extent of environments and usually considered as aphid predators, their food is far more varied and frequently consists of various hemipterous insects, moth larvae, pollen, fungal spores and even plant tissues[6–9]. Recent years have witnessed an increase in molecular systematic studies in the Coleoptera, especially in the Cucujoidea, due to the growing viability of large-scale DNA sequencing and computationally intensive phylogenetic analysis[10]. To comprehend the relationships among the constituents at lower and higher taxa of plants and animals have consistently been studied using molecular sequences of mitochondrial and ribosomal markers[11,12]. It has been found that genetic alterations that affect structural genes and their phenotype mostly result from mutations[13]. Depending on their utility and adaptability, many of these modifications are maintained over time and distributed among the related species. The molecular marker that has been most commonly used in the study of genetic variability between genes is the internal transcribed spacer (ITS). It has been reported that some of the extreme length variations in the ITS1 gene region of 12 species comprising five subfamilies of the Coccinellidae were caused by internal repetitions and all the species showed high ITS1 sequence variability[14]. Mutations within the ITS spacers of the rDNA transcript occur with greater frequency; therefore spacers are useful in detecting both genera and species due to sequence heterogeneity[15]. The ITS markers are considered due to their various utilities such as phylogenetics analysis, species identification and particularly for use in parasitology and fungi barcoding[16]. The ITS sequences have been a popular choice as they have more variable nuclear loci and are considered as universal primers that work with most of the organisms, including fungi[16–18], plants[19], digeneans[20], insects[14,21], etc.

Mitochondrial DNA (mtDNA) is considered as a source of species-specific markers, even though it evolves quickly between even closely related species[22]. A few studies of evolutionary history have utilized mtDNA as a molecular marker because of its significant features, and have established that the associations between subfamilies below the tribe level are strongly supported by 16S rDNA in ladybirds[23]. However, its effective linkage with endosymbiont bacteria reported in ladybirds, which is responsible for male–female ratio distortion[24,25], limits mtDNA usability. Additionally, the study of a 658-bp mitochondrial DNA fragment from the 5′-end of the COI gene, revealed the evolutionary relationships within the Coccinellidae[26]. Ghosh *et al.*[27] employed the COI gene of mtDNA for genetic characterization of diverse populations of four species of ladybirds, including *Coccinella transversalis*, *Cheilomenes sexmaculata*, *Micraspis discolor* and *Anisolemnia dilatata* obtained from various habitats. The aim was to establish evolutionary relationships between genetically distinct ladybird species obtained from different environments and to assess the level of genetic diversity. Mitochondrial genes can be useful in phylogenetic studies in insects, as their high substitution rates

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render them especially useful to resolve the relationships among closely related taxa[28,29]. However, assessment of mitochondrial genes typically does not resolve broader evolutionary divisions, and the significant diversity in rate variations may be a contributing factor for the poor performance of the mitochondrial genes in comparison to nuclear genes[30,31]. An additional problem with mitochondrial gene sequences is that differences in mitochondrial evolutionary rates among insect lineages can cause long-branch attraction problems that result in unrelated taxa with high substitution rates erroneously grouping together in a phylogenetic tree[32,33]. In this study, we evaluated the rate of evolution between 480 species belonging to 14 tribes from the Coccinellinae in biogeographical locations. Genital evolution has led to a morphologically and functionally diverse suite of genital traits. A shared number of genital characteristics was assessed in all tribes. Male and female genitalia morphology character matrix was constructed to infer the topology and evolutionary mutation analysis. All datasets of molecular markers were assessed under different algorithms such as substitution patterns in pairwise alignment, and mutation rate estimation evidenced with a set of statistical parameters. Bayesian tree evaluation confirmed the number of substitution sites in both morphology and molecular datasets. An extensive survey was conducted to explore the biodiversity of ladybirds and evaluate their biodiversity indices from different countries.

Materials and methods

Taxon sampling

We obtained the sequences of ribosomal internal transcribed spacers (ITS1 and ITS2) and mitochondrial genes (COI and COII) of 480 species from 128 genera and 14 tribes (Supplementary Table 1). All the unclassified species were eliminated and partial sequences were removed for better accuracy using the Sequence Viewer (V 3.33.0). The respective countries of the retrieved sequences were recorded to mark the biogeographical locations (Supplementary material 2). Morphology characteristics of male and female genitalia of all 480 species were manually inspected. The character matrix was assembled with eight traits (Supplementary material 3) and analysed.

Sequence analysis and alignment

Pairwise alignment enables one to recognize regions of similarity that can be used to predict functional, structural and evolutionary relationships. All the retrieved sequences of ITS1 (12), ITS2 (8), COI (458) and COII (28) were simultaneously utilized for evolutionary rate analysis. Sequences were assembled and globally aligned using ClustalW[34] algorithm of MEGAX (V 10.0.5)[35] with default cost matrix (1.0/0.0), gap open penalty (12), gap extension penalty (3) and refinement iteration.

Assessment of substitution rate patterns

Further, the sequences were estimated for maximum likelihood with substitution matrix transition/transversion (*ti*/*tv*) bias and gamma-parameter for site rates. To measure the homogeneity of substitution patterns, the Markov chain Monte Carlo (MCMC) test (100 million) was used to estimate the significant *P*-value. Homogeneity was examined on the basis of base composition differences within molecular markers (ITS1, ITS2, COI and COII) and significance of the *P*-value. Sequences having $P > 0.05$ were considered with significant substitutions. Nucleotide frequency biases and variations of *ti*/*tv* among sites were estimated using the statistical method maximum composite likelihood (MCL) with the substitution model Tamura-Nei model[36,37]. All the positions having gaps and missing data were completely excluded.

Evolutionary analysis

Maximum likelihood (ML) and Bayesian analysis[38] were used to describe the best substitution patterns with a suitable evolutionary model. Phylogenetic evolutionary analysis was done between sequences using MrBayes (V 3.7.2 a)[39] and ML with the general time reversible model (GTR), as it assumes different rates of substitution for each pair of nucleotides. Datasets of both markers clustered simultaneously. The initial tree for the heuristic search was obtained automatically by applying neighbor joining and BioNJ algorithms[40] to a matrix of pairwise distances estimated using the MCL approach[35], and then selecting the topology with the preferred log-likelihood value. The tree was engineered with branch lengths measured in the number of substitutions per site. Character matrix was constructed using Mesquite (V 3.70)[41] to estimate evolutionary rates of eight traits of male and female genitalia of the 480 species.

Results

Biogeographical locations and sequence analysis

In total, 480 species belonging to 14 tribes from subfamily Coccinellinae were studied and 223 species locations were depicted on the map. Inadequate sequences were available for 257, out of the 480 species. Figure 1 shows all the species along with their respective countries. Pairwise sequence identity of COI (66.4%), COII (66.4%), ITS1 (36.5%) and ITS2 (26.2%) varied due to gap penalties and the score of substitution mutations. The calculated G + C content was for ITS1 (50.0%), ITS2 (54.6%), COI (31.0%) and COII (23.4%).

Figure 1. Species distribution of all obtained sequences. The pie diagram is assigned with different colours, indicating the species with the respective countries.

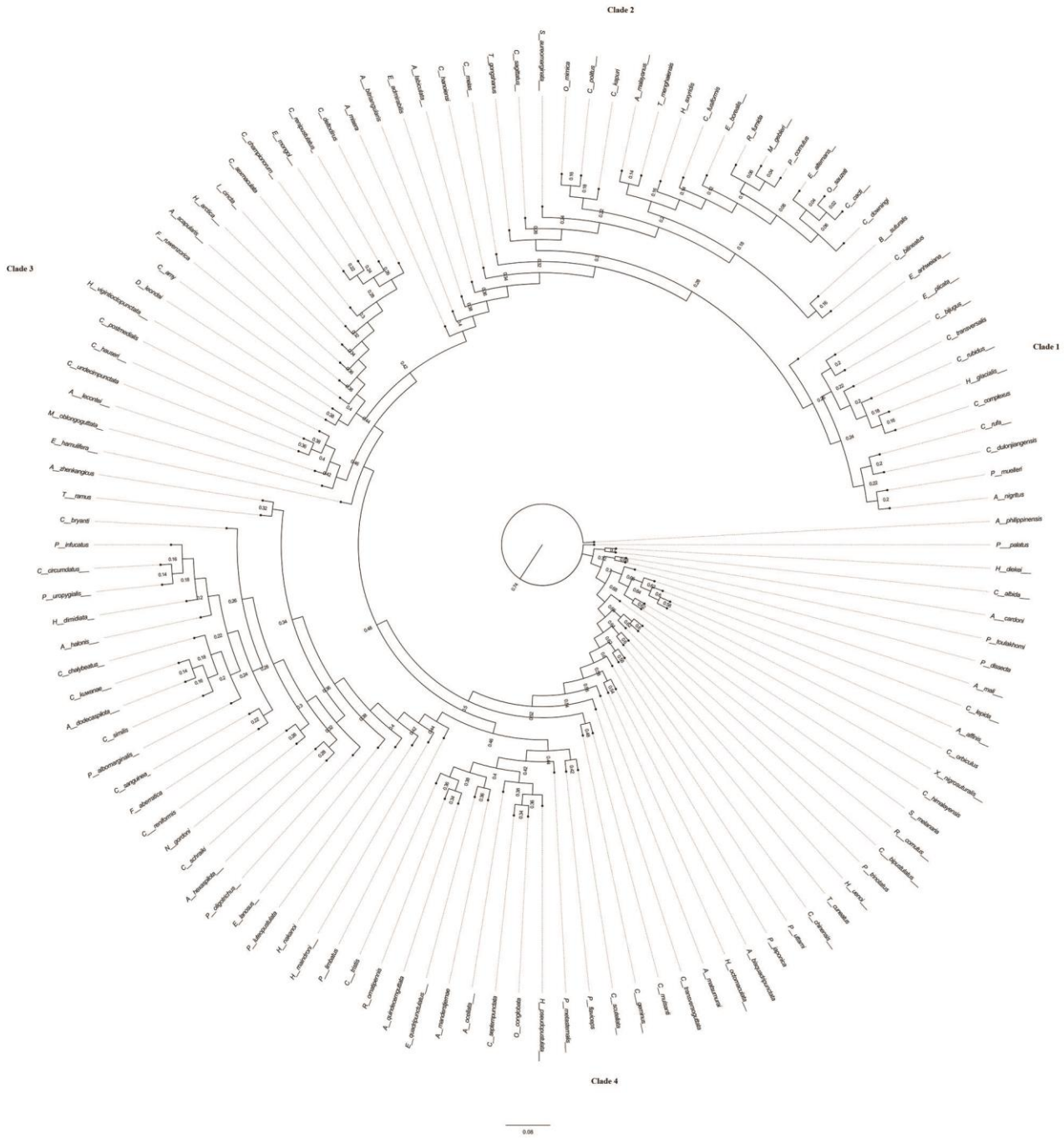
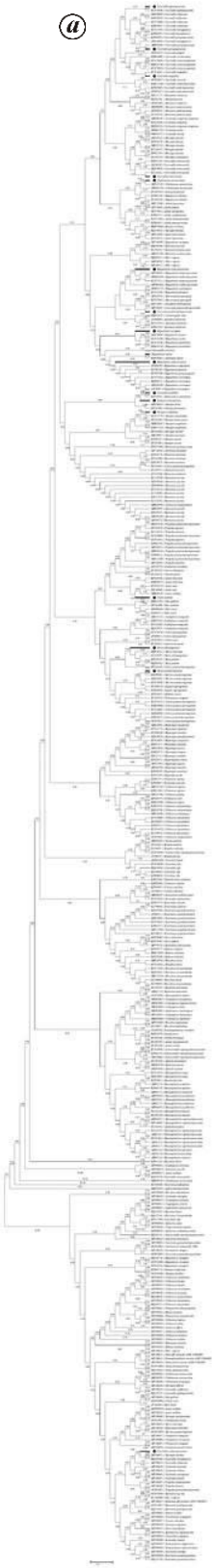


Figure 2. Bayesian tree representing the evolutionary relationship of 120 species. Genitilia traits of male (four characters) and female (four characters) were considered.

Phylogenetic analysis

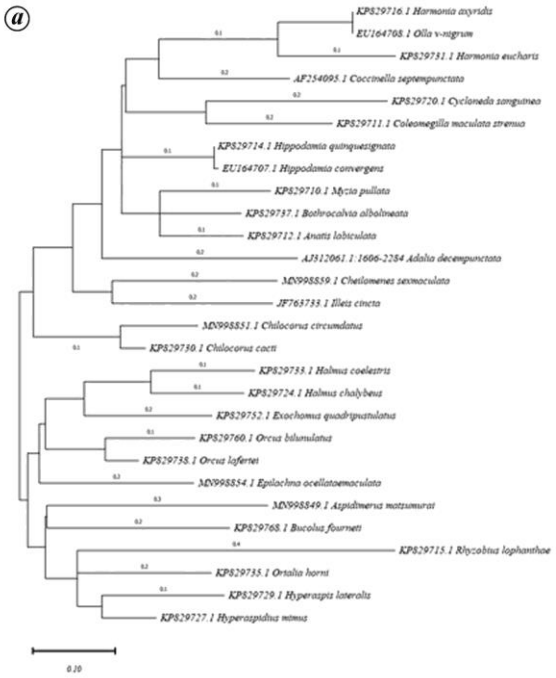
The morphology matrix comprised eight characters of male and female genitalia of 119 ladybird species (Supplementary material 3). ML and Bayesian analysis of the morphology dataset provided an evolutionary relationship. Bayesian tree of the morphological traits was split into four clades. Comprehensively, all the clades showed monophyletic topology with supported branch lengths in the species (Figure 2). Molecular datasets of all the molecular markers were executed for the evolutionary rate analysis. In COI, maximum species showed monophyletic topology and significantly supported branch lengths (Figure 3 *a*). COII (28 sequences) was also found to have monophyletic topology (Figure 4 *a*). ITS1 showed overall monophyletic topology with significantly supported branch lengths (Figure 5 *a*). In ITS2,



(b)

	A	T/U	C	G
A	-	*11.25*	*0.83*	**5.34**
T/U	*9.32*	-	**15.29**	*0.30*
C	*1.68*	**37.33**	-	*3.76*
G	**10.51**	*0.72*	*3.66*	-

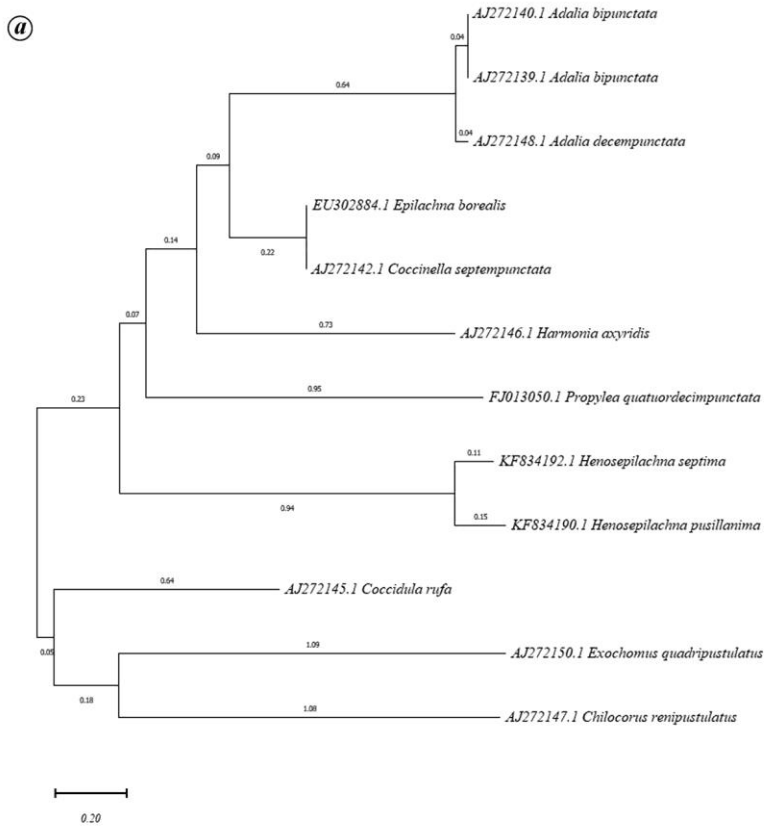
Figure 3. ***a***, Phylogenetic tree constructed using ML method of COI (458 sequences). Totally 1051 positions were used final dataset for analysis. Branch lengths are shown above the branches. All the nodes have been collapsed and indicated with a symbol (♦) with zero branch length. Scale bar shown below the phylogenetic tree depicts the number of substitutions per site. ***b***, Discrete gamma distribution used to model evolutionary rate differences among sites (three categories, [+*G*], parameter = 0.5017). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 1% sites). Rates of different transitional substitutions as shown in bold and those of transversionsal substitutions in italics.



b

	A	T/U	C	G
A	-	*11.30*	*1.38*	**3.55**
T/U	*9.78*	-	**13.07**	*0.50*
C	*3.54*	**38.68**	-	*1.11*
G	**13.30**	*2.16*	*1.62*	-

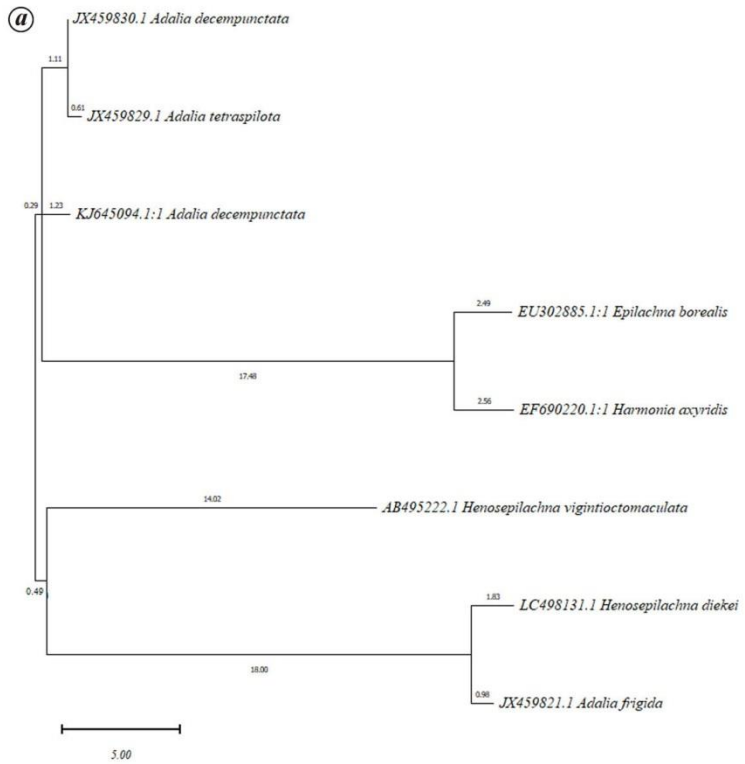
Figure 4. ***a***, Evolutionary analysis conducted with 28 COII sequences. Branch lengths are shown next to the branches. Codon positions included were 1st+2nd+3rd+noncoding. There was a total of 570 positions in the final dataset. ***b***, Discrete gamma distribution used to model evolutionary rate differences among sites (three categories, [+*G*], parameter = 1.0287). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 30% sites). Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions in italics. The nucleotide frequencies are *A* = 35.56%, *T/U* = 41.07%, *C* = 13.88% and *G* = 9.49%.

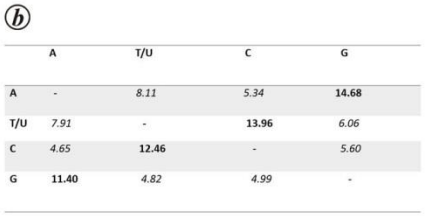


b

	A	T/U	C	G
A	-	*7.95*	*6.41*	**13.48**
T/U	*7.88*	-	**13.05**	*5.74*
C	*6.87*	**14.12**	-	*3.73*
G	**12.32**	*5.29*	*3.18*	-

Figure 5. ***a***, All the 12 sequences of ITS1 obtained and used for evolutionary relationships. There was a total of 1244 positions in the final dataset. ***b***, Transition/transversion bias estimated with ML. The nucleotide frequencies are *A* = 24.78%, *T/U* = 25.01%, *C* = 23.11% and *G* = 27.10%. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions in italics.





	A	T/U	C	G
A	-	*8.11*	*5.34*	**14.68**
T/U	*7.91*	-	**13.96**	*6.06*
C	*4.65*	**12.46**	-	*5.60*
G	**11.40**	*4.82*	*4.99*	-

Figure 6. ***a***, A total eight sequences of ITS2 were considered for evolutionary analysis with ML. There was a total of 601 positions in the final dataset. ***b***, Totally 573 positions were involved in the final data assessment. The nucleotide frequencies are $A = 22.41\%$, $T/U = 22.97\%$, $C = 25.74\%$, and $G = 28.87\%$. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions in italics.

Epilachna borealis and *Harmonia axyridis* were sister taxa with significantly supported branch lengths (Figure 6 *a*). *Henosepilachna diekei* and *Adalia frigida* were also found to be sister taxa.

Estimation of substitution patterns

The probability of substitutions was evaluated with molecular markers ITS1, ITS2, COI and COII using ML estimates of the substitution matrix, *ti*/*tv* and gamma parameter for site rates. Nucleotide frequencies of ITS1 (12 sequences) were 24.78% (A), 25.01% (T/U), 23.11% (C) and 27.10% (G). The estimated value of shape parameter for the discrete gamma distribution was 5.8975. Mean evolutionary rates in these categories were 0.59, 0.95 and 1.47 substitutions per site. Frequency of transition substitutions was more than transversions; overall *ti*/*tv* bias was $R = 1.13$ (Figure 3 *b*). In ITS2, nucleotide frequencies were 22.41% (A), 22.97% (T/U), 25.74% (C) and 28.87% (G). The estimated value of shape parameter for the discrete gamma distribution was 37.7806. The overall *ti*/*tv* bias was $R = 1.11$ (Figure 4 *b*).

In COI, the nucleotide frequencies were 31.16% (A), 37.60% (T/U), 15.40% (C) and 15.83% (G). The estimated value of shape parameter for the discrete gamma distribution was 0.4953. The overall *ti*/*tv* bias was $R = 1.66$ (Figure 5 *b*). In COII nucleotide frequencies were 35.56% (A), 41.07% (T/U), 13.88% (C) and 9.45% (G). The estimated value of shape parameter for the discrete gamma distribution was 0.4256. The overall *ti*/*tv* bias was $R = 1.36$ (Figure 6 *b*).

Discussion and conclusion

Tribes consisted of diverse genitalia structures; however only a few traits were common, as they play an important role in establishing copulatory interactions between male and female[42]. Seago *et al.*[2] analysed both molecular and morphological datasets and confirmed monophyly in the Coccinellinae subfamily. A study was performed with ITS1 and ITS2 dataset sequences, to explore, align and evaluate conserved motifs in folding patterns of stable consensus secondary structures in six subfamilies, including Epilachaninae, Chilocorinae, Coccinellinae, Coccidulinae, Scymininae, Ortaliinae and Sticholotidinae. The sister taxon relationship between *E. borealis* and *Coccinella septempunctata* was confirmed with a strong bootstrap support[43]. The morphological studies included 61 discrete features and 301 terminals, which were parsimoniously analysed; phylogenetic analysis confirmed that the Coccinellini are monophyletic[44]. Due to inadequate information of morphology traits, it is uncertain to confirm the evolutionary topology on the basis of morphology characteristics[2,44]. Inadequate datasets of ITS1 have impacted the analysis. Contrasting *ti*/*tv* bias of COI ($R = 1.66$)

and COII ($R = 1.36$) depicts variations in population sampling. This ratio in the datasets of ITS1 ($R = 1.13$) and ITS2 ($R = 1.11$) displays substitution bias. As the *ti*/*tv* ratio is more than 0.05 (significant) in both mitochondrial markers, it indicates that these markers show more substitution rates than the rDNA markers. Stoltzfus and Norris[45] suggested that transitions are more conservative yet differ in few aspects. Substitution rates are not equally probable in COI and COII as well as in ITS1 and ITS2; they are biased towards *ti*/*tv* substitutions. Furthermore, ITS1 and ITS2 have less *R* ratio but are not equally probable in both markers, perhaps due to inadequate species sequence availability. Mutations occurring within small populations have neutralized effects on insect fitness; thus the shift is more significant[46]. Therefore, ITS and mtDNA genes were subjected to different degrees of discriminatory algorithms, to measurable *ti*/*tv* bias in evolutionary rates. In phylogenetic analysis of ITS2 and COI of *Opisthorchis noverca* (Braun Braun) attributed to the difference in the rate of evolution, mtDNA showed a higher rate of evolution compared to nuclear rDNA[47]. As COI has a greater *ti*/*tv* ratio, it depicts more substitution and less divergence among species in the phylogenetic tree[48,49], though it has moderate bootstrap support. Studies suggest that an easy way to confirm the topology among species is to estimate the branch lengths; trees with long branch lengths represent numerous genetic changes[50]. Several studies demonstrate that species lineage of organisms with a high rate of evolution in mtDNA occurs due to gene arrangement[51]. Substitution patterns of rDNA and mtDNA markers were estimated with pairwise sequence comparison, disparity test and MCL in a cascade of algorithms with a suitable substitution model which derive *ti*/*tv* ratios between evolutionary lineages[52]. With this study further we can evaluate substitution patterns on species fitness. Increasing the sampling size will contribute to a better-understanding, and also determine substitution bias and evolutionary topology[53,54].

Conflict of interest: The authors declare no conflict of interest.

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