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

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Short running title: Substitution rate analysis of molecular markers.

Abstract

Here in this study, we examined the respective accounts of ribosomal DNA internal transcribed spacers and mtDNA markers for their use in prospecting for 480 species of 14 tribes, ladybird species to assess the evolutionary topology and substitution rates. Substitution patterns of respective markers were estimated in a cascade of algorithms such as pairwise sequence comparisons, maximum likelihood estimates of substitution matrix, transitions/transversions (ti/tv) and gamma parameters with a suitable substitution model. Maximum likelihood estimates shows that COI (R=1.16) and COII (R=1.36) are more biased towards transitions. As COI consists more ti/tv ratio depicts more substitutions accumulation and it shows less divergence among species in phylogenetic tree, though it has moderate bootstrap support. Bayesian and maximum likelihood analysis were used to execute morphology character matrix and molecular datasets to established evolutionary relationship. All the characters of male and female genitalia supported mophyletic topology. Our phylogenetic results of molecular datasets suggest that the most of the taxon significantly supporting monophyly. Combined datasets of both markers estimation of phylogeny topology analysis depict COI consists of more substitution as it shows less divergence among species.

Keywords: Ladybirds, Ladybirds, ITS1 and ITS2, COI and COII, substitution rates, phylogenetic analysis

Introduction

Coccinellidae family comprises over 6000 species, with 360 genera and 25 tribes have been identified globally[1,2,3,4]. Ladybirds have a number of traits that makes it 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[6,7,8,9]. Recent years have seen an increase in molecular systematic investigations 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 constituent at lower and higher taxa of plants and animals have consistently been derived using molecular sequences of mitochondrial and ribosomal markers[11,12]. It is rigorously studied that genetic alterations that affect structural genes and their phenotypic most frequently result from mutations[13]. Depending on their utility and adaptability, many of these modifications are kept 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). Extreme length variations in the ITS1 gene region of twelve species comprising five subfamilies of the Coccinellidae and revealed that some of the extreme length variations were caused by internal repetitions and species all showed high ITSI sequence variability[14]. The occurrence of mutations within the ITS spacers of rDNA transcript occurs with greater frequency, therefore spacers are useful in detecting both genera and species due to sequence heterogeneity[15]. ITS markers are considered due to various utilities such as phylogenetics analysis, species identification and it is particularly used for parasitology and fungi barcoding[16]. ITS sequences have been a popular choice as it is more variable nuclear loci and are considered as universal

primers that work with most of the organisms including fungi [16, 17,18] plants[19], digeneans[20], insects[14,21] etc.

Relatively mitochondrial DNA (mtDNA) is considered as a source of species-specific markers compared to ITS, even though mtDNA evolves very quickly between even very closely related species[22]. A few studies of evolutionary history have utilised mtDNA as a molecular marker, because of its significant features and established the associations between the subfamilies below tribe level were 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 its usability. Additionally, based on the investigation of a 658-bp mitochondrial DNA fragment from the 5' end of the COI gene, researchers revealed the evolutionary relationships within the Coccinellidae[26]. Ghosh et al. (2017)[27], employed the COI gene of mtDNA for genetic characterisation of diverse populations of four species of ladybirds, including *Coccinella transversalis*, *Cheilomenes sexmaculata*, *Micraspis discolor*, and *Anisolemnia dilatata* obtained from various habitats. The purpose was to establish evolutionary relationships between genetically distinct of 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[28] render them especially useful to resolve the relationships among closely related taxa[29]. However, assessments conducted on mitochondrial genes typically do not resolve broader evolutionary divisions, and the significant diversity among-site rate variations may be a contributing factor for mitochondrial gene's poor performance 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[32,33] that result in unrelated taxa with high substitution rates erroneously grouping in phylogenetic trees. In this study, we will evaluate the rate of evolution between 480 species belonging to 14 tribes from Coccinellinae, were

inspected along with biogeographical locations. As genital evolution has led to a morphologically and functionally diverse suite of genital traits. A shared number of genital characteristics were assessed in all tribes. Male and female genitalia morphology character matrix were executed 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, 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, to 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 and14 tribes (Table 1). All the unclassified species were eliminated, partial sequences were removed for accuracy with the Sequence Viewer (V 3.33.0). Respective countries of retrieved sequences were recorded to mark the biogeographical locations (Supplementary file 1). Morphology characteristics of male and female genitalia of all 480 species of 128 genera and 14 tribes were manually inspected. Character matrix were assembled with 8 traits (Supplementary file 2) and analysed.

Sequence analysis and alignment

Pairwise alignment enables to recognize regions of similarity that can be used to predict functional, structural and evolutionary relationships. All retrieved sequences of ITS1 (12 sequences), ITS2 (8 sequences), COI (458 sequences) and COII (28 sequences) were simultaneously executed for evolutionary rates 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 sequences were estimated for maximum likelihood substitution matrix, transition/transversion bias and gamma-parameter for site rates. To measure the homogeneity of substitution patterns with a Markov Chain Monte-Carlo (MCMC) test (100 million) applied to estimate the significant P-value. Homogeneity was examined on basis of base composition differences within molecular markers (ITS1, ITS2, COI and COII) and the basis of significance of P- value. Sequences having $P>0.05$ considered with significant substitutions. Nucleotide frequency biases, variations of transition/transversion (ti/tv) among sites were estimated with statistical method maximum composite likelihood (MCL)[36] the substitution model Tamura-Nei model[37]. All the positions having gaps and missing data were excluded with complete deletion.

Evolutionary analysis

Maximum likelihood (ML) and Bayesian analysis[38] for 480 sequences of molecular markers to describe the best substitution patterns with a suitable evolutionary model. Phylogenetic evolutionary analysis was inferred between sequences by using the 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 were obtained automatically by applying neighbour-join and BioNJ algorithms[40] to a matrix of pairwise distances estimated using the MCL approach[35] and then selecting the topology with preferred log likelihood value. The tree is engineered to a scale, with branch lengths measured in the number of substitutions per site. Character matrix were constructed using Mesquite (V 3.70)[41] to estimate evolutionary rates of 8 traits of male and female genitalia of 480 species.

Results

Biogeographical locations and sequence analysis

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

Phylogenetic analysis

The morphology matrix comprised 8 characters of 119 species male and female genitalia (Supplementary file 2). Bayesian and maximum likelihood analysis of the morphology dataset achieved an evolutionary relationship. Bayesian tree of morphological traits splitted into 4 clades. Comprehensively, all clades are depicting monophyletic topology with supported branch lengths in species (Figure 2). Along with these molecular datasets of all the molecular markers were executed for the evolutionary rate analysis. In COI, maximum species were found with monophyletic topology and significantly supported branch lengths (Figure 3A). COII (28 sequences), also assessed for evolutionary analysis and found with monophyletic topology (Figure 4A). On assessment of ITS1 (Figure 5A), dataset overall monophyletic topology with significantly supported branch lengths. In ITS2 (Figure 6A), *Epilachna borealis and Harmonia axyridis* were sister taxon with significant supported branch lengths. Topology between *Henosepilachna diekei* and *Adalia frigida* were found as sister taxon.

Estimation of substitution patterns

Probability of substitutions were evaluated with molecular markers *i. e.* ITS1, ITS2, COI and COII respectively using maximum likelihood estimates of substitution matrix, transition/transversion 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 the shape parameter for the discrete gamma distribution is 5.8975. Mean evolutionary rates in these categories were 0.59, 0.95, 1.47 substitutions per site. Frequency of transition substitutions were more than transversions, overall transition/transversion bias was R = 1.13 (Figure 3B). In ITS2, nucleotide frequencies were 22.41% (A), 22.97% (T/U), 25.74% (C), and 28.87% (G). The estimated value of the shape parameter for the discrete Gamma Distribution is 37.7806. The overall transition/transversion bias was R = 1.11 (Figure 4B).

In COI, the nucleotide frequencies were 31.16% (A), 37.60% (T/U), 15.40% (C), and 15.83% (G). The estimated value of the shape parameter for the discrete Gamma Distribution is 0.4953. The overall transition/transversion bias was R = 1.66 (Figure 5B), whereas in COII nucleotide frequencies were 35.56% (A), 41.07% (T/U), 13.88% (C), and 9.45% (G). The estimated value of the shape parameter for the discrete Gamma Distribution is 0.4256**.** The overall transition/transversion bias was R = 1.36 (Figure 6B).

Discussion and conclusions

Tribes consists diverse genitalia structures, however very few traits were common, as it plays an important role in establishing copulatory interactions between male and female[42]. Sego et al. (2011)[2], analyses both molecular and morphological datasets and confirms the monophyly of Coccinellinae subfamily. A study was performed with ITS1 and ITS2 dataset sequences, explored, aligned, and evaluated conserved motifs in folding patterns of stable consensus secondary structure in six subfamilies, including Epilachaninae, Chilocorinae, Coccinellinae, Coccidulinae, Scymininae, Ortaliinae, and Sticholotidinae. The sister taxon relationship

between *Epilachna 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 and phylogenetic analysis confirms that Coccinellini are monophyletic[44]. Due to inadequate information of morphology traits, it is uncertain to confirm the evolutionary topology of morphology supported the previous studies[2,44]. Inadequate datasets of ITS1 impacted the analysis. Contrasting ti/tv bias (R) of COI (R=1.66) and COII (R=1.36) depicts variations occurs with the population sampling. Estimating the ratio in datasets of ITS1 (R=1.13) and ITS2 (R=1.11) display the substitution bias. As ti/tv ratio are more than 0.05 (significant) in both mitochondrial markers it correlates that in mitochondrial marker retain more substitution than the rDNA marker. Stoltzfus and Norris (2016)[45] suggests that transitions are more conservative yet in few aspects. Substitution rates are not equally probable in COI and COII, as well as in ITS1 and ITS2, they were biased towards ti/tv substitutions. Furthermore, ITS1 and ITS2 have lesser R ratio but were 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 shift tends to be more significant[46]. Therefore, ITS and mtDNA genes were subjected to different degrees of discriminatory algorithms, directing to measurable tv/ti bias in evolutionary rates. In a phylogenetic analysis of ITS2 and COI of *Opisthorchis noverca* (Braun Braun) attributed to the difference in the rate of evolution, mtDNA shows a higher rate of evolution compared to nuclear rDNA[47]. As COI consists more ti/tv ratio depicts more substitutions accumulation and it shows less divergence among species[48,49] in phylogenetic tree, though it has moderate bootstrap support. Studies suggest that an easy way to conclude the topology among species is to estimate the branch lengths, trees having long branch lengths are representing a number of genetic changes[50]. Several studies manifest that species lineage of organisms with a high rate of evolution in mtDNA[51] occurs due to gene arrangement. Substitution patterns of rDNA and

mtDNA markers were estimated with pairwise sequence comparisons, disparity test and maximum composite likelihood in a cascade of algorithms with a suitable substitution model which enable for various ti/tv ratios among evolutionary lineage[52]. With this study further we can elaborate on that estimation of substitution patterns on species fitness. Increasing the sampling size will contribute to better understanding and determining substitution bias and evolutionary topology[53,54].

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Conflict of interest: Authors have contributed equally and there is no conflict of interest.

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Table1 All the retrieved sequences used in this analysis are listed with their accession numbers. Species belonging from 14 tribes are listed.

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

Figure 2 Bayesian tree representing the evolutionary relationship of 120 species. Genitilia traits of male (4 characters) and female (4 characters) were considered.

Figure 3 (A) Phylogenetic tree were constructed with maximum likelihood method of COI (458 sequences). Total 1051 positions were used final dataset for analysis. Branch lengths are shown above the branch. All the nodes were collapsed and indicated with a symbol (♦) with zero branch lengths. A scale bar shown with branch lengths measured in the number of substitutions per site.

Figure 3 (B) A discrete Gamma distribution was used to model evolutionary rate differences among sites (3 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 (bold) and those of transversionsal substitutions are shown in *italics*.

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.

Figure 4 (B) A discrete Gamma distribution was used to model evolutionary rate differences among sites (3 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 are shown in *italics*. The nucleotide frequencies are A = 35.56%, T/U = 41.07%, C = 13.88%, and G = 9.49%.

Figure 5 (A) All the 12 sequences of ITS1 were obtained and executed for evolutionary relationships. There were a total of 1244 positions in the final dataset.

Figure 5 (B) Transition/transversion bias were estimated with maximum likelihood**.** 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 are shown in *italics*.

Figure 6 (A) A total 8 sequences of ITS2 were considered for evolutionary analysis with maximum likelihood. There was a total of 601 positions in the final dataset.

Figure 6 (B) There were a total of 573 positions involved in 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 are shown in *italics*.