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Molecular phylogeny of *Scymnus latifolius*, a predator species of mealy bug shows divergent evolution among *Scymnus* species

Ladybird beetles belong to the family Coccinellidae, super family Cucujoidea, suborder Polyphaga of order Coleoptera[1], and consist of more than 360 genera and 6000 species[2]. Many of the coccinellid insects are widely used as predators in the biological control of major agricultural pests. Previously, we have described a novel predator, *Scymnus* (*Pullus*) *latifolius* Poorani belonging to Scymnini tribe of the family Coccinellidae[3]. The *S. latifolius* beetle is found to predate upon all developmental stages of several mealybug species, a major pest with a wide host range, and hence could play a key role in biological control.

Several phylogenetic subdivisions for the family Coccinellidae have been proposed based on conventional morphological observations[2,3] and molecular methods[4,5]. Mitochondrial gene sequences have been widely used to understand the evolutionary history of coleopterans[6,7] and coccinellids in particular[4,5,8,9]. The mitochondrial cytochrome *C* oxidase subunit I (COI) gene nucleotide sequences have been extensively used for phylogenetic analysis and species-level identification of Coccinellidae[9]. The present study was conducted to identify the phylogenetic relationship of *Scymnus* species with other Scymnini and coccinellids through comparative analysis of partial sequences of mitochondrial COI gene.

Insect collections were obtained from mulberry gardens located in Murshidabad, Malda, Birbhum and Nadia districts, West Bengal, India using standard techniques. Adult specimens of *S. latifolius* were positively identified using morphological descriptions[2] and preserved in 85% ethanol in the dark at 4°C until further analysis. DNA was isolated from the hind legs of individual beetles using a DNA isolation kit (Qiagen, Germany) following the manufacturer's protocol and stored at –20°C until use. Polymerase chain reaction (PCR) amplification of partial gene sequences of mitochondrial COI gene was conducted using the universal COI primers[10] following a method described previously[11]. The PCR products were purified using a kit (Qiagen, Germany) and sequenced by Sanger's method at a commercial facility. The nucleotide sequences of COI gene generated have been submitted to the National Center for Biotechnology Information (NCBI) and can be accessed at GenBank (accession number KU512906). Sequence diversity of specific 595 bp fragment of the mitochondrial COI gene (the COI 5′ region) amplified from *S. latifolius* was compared with COI gene sequences of 44 different Coccinellids with one out-group from another subfamily (*Serangium* spp.) available in the NCBI database. The initial multiple alignment and sequence editing were carried out using Molecular Evolutionary Genetics Analysis software (MEGAX)[12]. Later, phylogenetic analysis was carried out employing Bayesian approaches[13] using Mr Bayes programme (Mr Bayes 3.2.7v WIN64) available at https://nbisweden.github.io/MrBayes/download.html. Before constructing the phylogenetic tree, the substitution model was analysed using MEGAX software. The model GTR + I + G was found to fit well with lowest Bayesian inference (BI) index. BI analyses were done using two runs simultaneously, with maximum likelihood starting tree, and four chains were used for the analysis (one cold and three hot) and the temperature set at 0.1. A run was

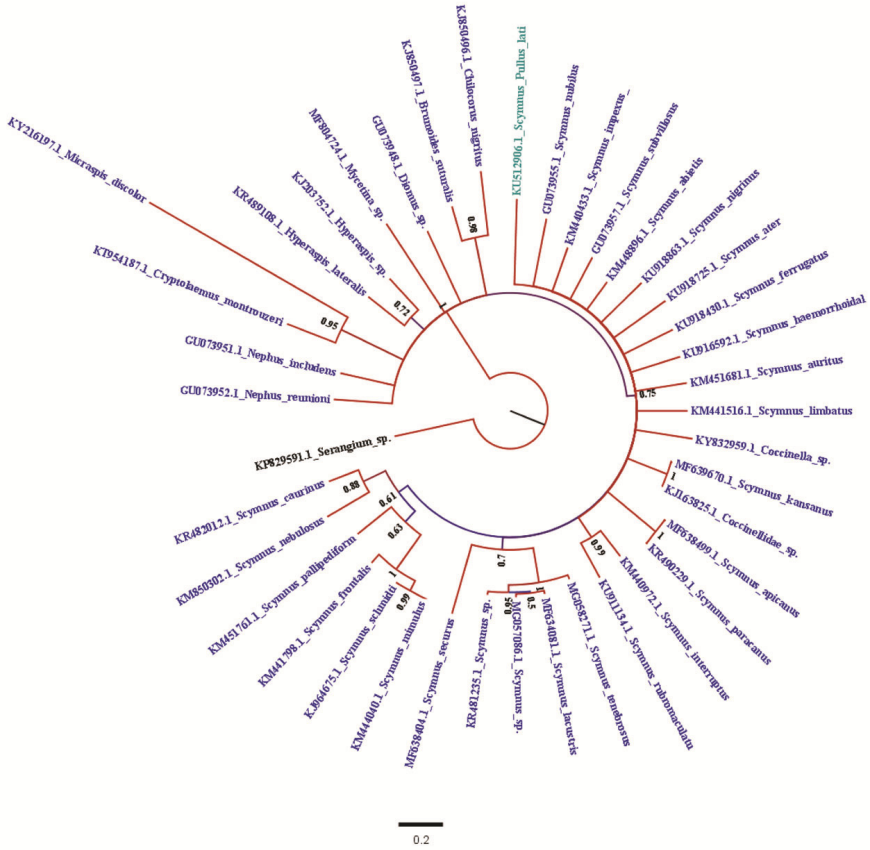

Figure 1. Bayesian inference tree with 50% majority-rule consensus. The posterior probability is given at each node of various *Scymnus* species. *Serangium* spp. is used as outgroup.

set for 10 million generations with sample frequency of 1000. The initial 25% trees were discarded using Burn-in 25%, and a 50% majority rule consensus tree was constructed from the remaining trees and posterior probabilities were estimated for each nodes. The final consensus tree was built using FigTree1.4v available online (http://tree.bio.ed.ac.uk/software/figtree/) by 50% majority-rule consensus. Figure 1 shows the branch length and posterior probability for each node calculated and the consensus tree generated.

The analysis suggests a close phylogenetic association of *S. latifolius* with other *Scymnus* spp. and coccinellids from the same subfamily included in the study. In the phylogenetic tree, all the species belonging to the genus *Scymnus* formed the same clade with further few subclades among themselves, whereas the other genera from the same subfamily branched into major clades with a few subclades. The topology of the phylogenetic tree was in line with the morphological classification and broadly, the results of the present study are in agreement with previously described molecular phylogeny of coccinellids[4,5,14]. Based on branch length, the species *Micrapsis discolor* can be considered to be more diverged from the rest of the species and has close association with *Cryptoleamus montrouzieri*. However, a detailed study is necessary to explain their evolutionary relationship.

The consensus phylogenetic tree is suggestive of *S. latifolius* as a different species, supporting the conventional morphological study[3]. As can be observed from the Bayesian inference phylogenetic tree, the genus *Scymnus* evolved as a single clade in the subfamily Scymninae, forming a single clade separated from all other genera of the subfamily. The genera *Cryptoleamus* and *Micrapsis* form a single clade, which is paraphyletic to other genera such as *Hyperapsis*, *Diomus* and *Nephus* and Chilocorini (*Chilocorus* and *Brumoides* spp.). The consensus tree also suggest a co-lineage for *Cryptolaemus montrouzieri* along with *S. latifolius*. The present study is in agreement with earlier observations that the subfamily Scymninae, the genera *Nephus*, *Scymnus* and *Diomus* are considered monophyletic with different lineages within the subfamily, with *Nephus* and *Scymnus* spp. as sister taxa[5].

Even though the evolution of different species of Scymninae, Coccinellinae, Chilocorinae and Sticholotidinae could not be specifically described, a clear trend of mixed lineages could be seen among these subfamilies, suggesting a polyphyletic origin. However, the present study does not find supporting results against the monophyletic origin of Scymninae, which needs further examination.

The morphological observations described earlier[2] and molecular phylogenetic approaches were completely consistent with each other. The close evolutionary relationship of *S. latifolius* with various Scymnini (*Scymnus* spp., *Nephus* spp. and *Cryptolaemus* spp.), Diomini (*Diomus* spp.) and Hyperapsidini (*Hyperapsis* spp.) in Chilorinae, the tribe Chilocorini (*Chilocirus* and *Brumoides* spp.) in

Coccinellinae, the tribe Coccinellini (*Coccinella*, *Micraspis*, *Harmonia*, *Cheilomenes*, *Anatis* spp.) that was observed during morphological studies[3] was further confirmed through sequence diversity analysis of the COI gene.

In biological speciation, differences in shape of the genitalia[4,15] is one of the most important natural mechanisms that results in lineage divergences and evolution in two or more descendant species[16]. Evolution of reproductive isolation within an ancestral species causes genetic, physiological and behavioural differences that result in lineage divergences. The presence of a unique male genitalia of *Scymnus latifolius* that is different from other *Scymnus* species has been reported earlier[3]. Thus, species divergence of *S. latifolius* observed in the molecular phylogenetic analysis is supported by morphological studies[3]. To conclude, a lineage divergence of *S. latifolius* from other Scymninae could be seen during molecular phylogenetic analysis, confirming its distinctiveness as a separate species and its taxonomic position. Apart from the phylogenetic perspective, application of a suitable molecular marker such as the COI gene can be useful to identify insects during their morphologically indistinct immature stages before emergence of the adults.

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