J. Curr. Crop Sci. Technol., 2022; https://doi.org/10.29321/MAJ.10.000596
(online first draft)

RESEARCH ARTICLE

Molecular Validation of *Aulacophora* Species Complex within the Geographical Limits of Tamil Nadu

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Received: 11th Feburary,2022
Revised : 18 th February,2022
Revised: 23 rd March,2022
Accepted : 04 th April,2022

ABSTRACT

The present study aimed to the delimitation of *Aulacophora* spp. from Tamil Nadu through DNA-barcoding. Samples were collected from three districts of Tamil Nadu and cytochrome c oxidase subunit I (*COI*) gene ofthree species (*A. foveicollis, A. lewisii* and *A. cincta*) was sequenced. Eight specimens were sequenced and was submitted to the Genbank. Molecular delineation of species was done using tree-based (phylogenetic tree), and distance-based (Automatic Barcode Gap Discovery) approaches. The data set consists of 35 sequences, including an out-group. Two phylogenetic trees were constructed using neighborhood joining and maximum likelihood method. Both the trees resulted in six distinct species groups, and the branching topology was also similar for both the trees. *A. foveicollis, A. abdominalis* and *A. relicta* had branched into three distinct groups from a single node, and *A. cincta* and *A. lewisii* sub-branched into different clades from another node. ABGD method gave six identical species groups similar to that of morphological data. The *p*-value for the grouping ranges from 0.001–0.0359, which gave satisfactory results for species delineation. Both tree-based and distance-based approachesenabled the rapid delineation of species with accurate species identification.

Keywords: *Aulacophora; Crop pest; Mitochondrial cytochrome oxidase I; Phylogenetic analysis; Automatic Barcode Gap Discovery; Species delineation*

INTRODUCTION

Genus *Aulacophora* (Coleoptera: Chrysomelidae: Galerucinae) was erected by Chevrolat in 1836. *Aulacophora* comprises 186 species worldwide (Barroga and Mohamedsaid, 2006). In India, 16 species of *Aulacophora* beetles have been documented. Five species are distributed within the geographical limits of Tamil Nadu. Genus *Aulacophora* of the Indian subcontinent, including Sri Lanka and Burma, was divided into three groups based on their elytral color pattern *viz*., section 1: yellowish-brown elytra; section 2: black or blue elytra; section 3: elytra with more than one color (Maulik, 1936). Though the genus *Aulacophora* had been grouped superficially based on their elytral color, identifying individual species within each group needs taxonomic expertise as they look similar in their external morphology. Moreover, some species of *Aulacophora* are non-native to the geographical limits of the study area which also have similar morphological characters similar to native species. Members of the genus *Aulacophora* notoriously feed on cucurbits and cause economic damage, if their populations is left unchecked. *A. foveicollis* has also been reported as vector of Rice Yellow Mottled virus in Benin (Das *et al.,* 2020). A sound understanding of the systematics and phylogeny of the genus *Aulacophora* is necessary because of its significant economic importance and taxonomic complexities. The recent unprecedented invasion of non-native insects warrants the need for rapid identification of the insects encountered in the fields.

DNA-based molecular markers were extensively used to resolve the systematic complexities across the phyla (Salvi *et al*., 2020). Mitochondrial cytochrome oxidase subunit I (*mtCOI*) gene has been used asa suitable candidate for phylogenetic studies in animals due to their high inter-species variability and low

intra-species variation (Tobe *et al.,* 2009). DNA barcoding studies use different methods to identify unknown species by comparing the barcodes of known species. Commonly used methods are similarity-based (Little and Stevenson, 2007), tree-based (Elias *et al*., 2007), distance-based (Bergmann *et al.,* 2013), and diagnostic character methods (Hebert *et al*., 2003a). Phylogenetic trees were constructed using the evolutionary information of the specimen, not the barcode gap. Distance-based methods use inter and intra specific distances between the query and reference sequences to identify the unknown species. The selection of correct method is a crucial factor in DNA barcoding. Inappropriate selection of DNA barcoding methods led to erroneous identification of species.

Considering the significance of molecular taxonomy of genus *Aulacophora,* the present study was carried out to resolve the taxonomic complexity of *Aulacophora* spp. from agro-ecosystems of Tamil Nadu based on DNA barcoding and validating different barcoding methods for accurate delineation of species.

MATERIAL AND METHODS

Insect collection

Aulacophora spp. were collected from different agro-ecosystems of Tamil Nadu during 2019-2021 (Table 1). The collected specimens were killed using ethyl acetate and stored in 90 percent ethanol. *Aulacophora* spp. collected were delineated to species level based on morphological keys given by Maulik (1936), and voucher specimens were deposited at TNAU Insect Museum, Department of Agricultural Entomology, TNAU. Further, the species were subjected to molecular validation.

DNA extraction

Total genomic DNA was extracted from individual adults of *Aulacophora* sp. The specimen was taken in a 1.5 mL micro centrifuge tube and added with 300 µL of pre-warmed CTAB buffer (1M Tris HCl (pH 7.5), NaCl 5M, EDTA 0.5 M, ddH_2O, and 20% SDS) and the contents were ground with plastic micro pestle and incubated for 1 hour at 65 °C. Then the contents were centrifuged at 10,000 rpm for 10 minutes and the supernatant was transferred into another tube into which an equal volume of 24:1 Chloroform: Isoamyl alcohol was added, the contents were vortexed and centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a new tube into which ice-cold isopropanol (300 µL) was added, mixed gently, and kept in deep freezer at -20 °C overnight. The next day, the samples were centrifuged at 10,000 rpm for 10 minutes, the supernatant decanted and 70% ice-cold ethanol (300 µL) added into the same tube and centrifugation repeated at 10,000 rpm for 10 minutes. After removing the supernatant, the pellet was air-dried for one hour. Pellet was dissolved in 50 µL of nuclease-free water and stored at -20 °C for further use.

DNA amplification and sequencing

Amplification of mitochondrial cytochrome oxidase I gene (*mtCOI*) was done using universal primers (Folmer *et al*., 1994) LCO1490 (Forward primer: 5'-GGTCAACAAATCAT AAAGATATTGG-3') and HC02198 (Reverse primer: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') in Eppendorf Thermal Cycler Machine, with 25 µL of PCR mix (5 µL Template DNA, 12.5 µL of master mix (ReadyMix, Sigma-Aldrich), 2 µL of 10 µM concentration of each primer, 3.5 µL distilled water). PCR was performed following the conditions given by Hebert *et al.* (2003). Initial denaturation (1 Cycle): 95 °C for 5 minutes, 30 cycles of denaturation 95 °C for 1 minute, annealing 54 °C for 1 minute, extension 72 °C for 1 minute and final elongation (1 cycle) 72 °C for 4 minutes. PCR amplicon was separated by electrophoresis in a 1 % agarose gel with 1 µL ethidium bromide (EtBr; 10 mg/mL) in TAE (pH 8.0) buffer (40 mM Tris acetate, 1 mM EDTA) at 70 volts for 1 hr. DNA bands were visualized under UV transilluminator and documented. The PCR amplicon was sequenced by Eurofins Pvt Ltd, Bangalore, India

Phylogenetic tree construction and species delimitation

The dataset consisted of 35 sequences (eight sequences from present study, twenty six sequences retrieved from NCBI and one outgroup). Sequences were aligned with default settings using MUSCLE (Edgar, 2004) in MEGA X program (Kumar et al., 2018). Two phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) and maximum likelihood method (Tamura *et al.*, 2011) in MEGA X. Neighbor-joining tree was built with a Kimura two-parameter (K2P) evolutionary model (Hebert *et al*., 2003a,b). Branch supports were estimated using 1000 bootstrap replications. All other parameters used default settings. The maximum likelihood tree was also constructed using the Kimura two-parameter (K2P) with 1000 bootstrap replications. The topologies of the two phylogenetic trees were compared and monophyletic groups were inferred to be the same species (Rubinoff, 2006).

Automatic Barcode Gap Discovery (ABGD) is an approach that automatically find the distance where the barcode gap is located and delineates the data set into different species (Puillandre *et al.,* 2012). The fasta sequences of *Aulacophora* spp. were submitted to the ABGD online website

(https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). One of the critical parameters in the ABGD method is the prior maximum divergence of intraspecific diversity (P). When the value is set too high (0.1), the whole data set will be considered as a single species, and if set too low (0.001), only identical sequences will be considered as part of the same species (Puillandre *et al*., 2012). So, the prior P value was set up in the range from 0.001 to 0.1 and Steps set to 10; X (minimum relative gap width) set to 1.5; Nb bins (for distance distribution) set to 20; we selected the Kimura (K80) model and set TS/TV to 2.0.

RESULTS AND DISCUSSION

Aulacophora foveicollis (Lucas, 1849)*, A. lewisii* Baly, 1866 and *A. cincta* (Fabricius, 1775) were the three species of genus *Aulacophora* collected during the present study within the geographical limits of Tamil Nadu. After the morphological confirmation, individuals of each species from all the locations were successfully sequenced for the barcode portion of the COI gene. The resultant COI sequence was deposited in NCBI GenBank with accession numbers OL853840, OL851821 and OM757834 - OM757839 (Table 1). In the present study, two methods were used (tree-based and distance-based) to assign species identity to the query sequence.

Phylogenetic analysis (Tree-based approach)

Both neighbor-joining and maximum likelihood phylogenetic trees resulted in six distinct species groups (Figure 1 & 2). From the six species group, *A. foveicollis, A. lewisii* and *A. cincta* were recorded from our study area, whereas *A. abdominalis* (Fabricius, 1781), *A. relicta* (Boisduval, 1835) and *A. nigripennis* (Motschulsky, 1857) were non-native to this faunal limits. The topology of the phylogenetic trees obtained from both methods yielded a similar branching pattern. *A. foveicollis, A. abdominalis* and *A. relicta* were grouped into two separate clades branching from a single node. On the other hand, *A. cincta* and *A. lewisii* formed two different clades from a single node separately. *A. foveicollis, A. abdominalis* and *A. relicta* originate from a single node due to their high nucleotide similarity. Morphologically *A. foveicollis* and *A. abdominalis* were similar (yellowish-brown elytra) and it is difficult to distinguish without taxonomic expertise. *A. foveicollis* was geographically limited to North of Africa and Asia, whereas in the Malay Archipelago and Oceania it was habituated by *A. abdominalis.* From the phylogenetic analysis, it was evident that both *A. foveicollis* and *A. abdominalis* had common point of origin but are two different species. *A. foveicollis* of our study population (Salem, Madurai and Coimbatore) showed close proximity with *A. foveicollis* population of Bangalore (Karnataka) and Kerala, whereas *A. foveicollis* population of Meghalaya and Punjab were grouped together separately. This indicates homogeneity of the population in the southern part of the country. Further, *A. cincta* and *A. lewisii* formed separate species clusters under single node as they had different elytral pattern. Madurai and Coimbatore populations of *A. lewisii* showed closer proximity with Meghalaya population, while Kerala population was similar to the population from China and Bangladesh. The shortfall of reference sequences in NCBI genbank for *A. lewisii* made it difficult to substantiate the population diversity at the molecular level. *A. cincta* and *A. lewisii* were found in the geographical limits of India and East and South-East Asian countries. Moreover, *A. intermedia* Jacoby, 1892 described from British India, became a junior synonym of *A. lewisii* (Lee, 2015).

It was found that two different tree construction methods viz., neighbour r-joining and maximum likelihood method, had produced phylogenetic trees with minimal topological differences and a similar pattern of species grouping. Though the results of both the methods were similar, the computational efficiency of the two methods varies. The Neighbor joining method was the most rapid compared tothe maximum likelihood method.

Species delineation using ABGD (Distance-based approach)

Aulacophora nucleotide dataset (35 sequences) were partitioned into different groups based on barcode gap on the ABGD website. ABGD outputs had two partitions: the initial and recursive partitions. The results from the initial partitions showed that the number of groups were six. The *P* value for the large range of 0.001–0.0359 gives six groups close to the morphological data (Figure 3a). In case of recursive partition, the number of groups ranges from one (when P = 0.059) to 18 (when P = 0.001), the latter corresponding to groups of identical sequence (Figure 3b). Generally, recursive partitions have more groups than initial partitions. However, recursive partitions expose the heterogeneities in the data set better. In contrast, initial partitions are typically stable on a wider range of prior values (P) and are usually close to the number of groups described based on morphological characters (Puillandre *et al*., 2012). The results revealed that there were six distinct species found in our data set. It coincides with the morphological differentiation of the specimens. This is possible only when the intra specific genetic distance is lesser than inter specific genetic distance. Threshold value between intra specific and inter specific genetic distance was 3% of divergence (Smith *et al*., 2005) or the 10 times rule (Hebert *et al.,* 2004). Apart from several attempts that were made to establish a standard threshold value between intraspecific and interspecific divergence, none of them can be generalized to all groups of organisms (Hebert *et al*., 2003b). Sometimes intra and

interspecific distances frequently overlap, and hinder the process of species delineation (Hebert *et al.,* 2003b). But ABGD proposes a standard definition of the barcode gap and can be used even when the two distributions overlap (Puillandre *et al.,* 2012).

CONCLUSION

The present study validated DNA barcoding of *Aulacophora* spp. and different barcoding methods to ensure the precise and fast identification of species. Scanty reference barcodes in public domains (NCBI and BOLD) make it difficult to establish a strong population structure of *Aulacophora* spp. Morpho and molecular taxonomy of other species of *Aulacophora* from India remain to be studied.

Funding and Acknowledgment

The authors acknowledge facilities provided by Department of Agricultural Entomology to carry out the research work.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There were no conflict of interest in the publication of this content

Ethics statement

No specific permits were required for the described field studies because no human or animal subjects were involved in this research.

Originality and plagiarism

This is original research work and any work and/or words of others, has been appropriately cited.

Data availability

All the data of this manuscript are included in the MS. No separate external data source is required.

Author contributions

Idea conceptualization – NC, DS, Experiments - DS, Guidance – VB, RA, RK, Writing original draft – DS, NC; Writing- reviewing & editing – VB, RA, NC..

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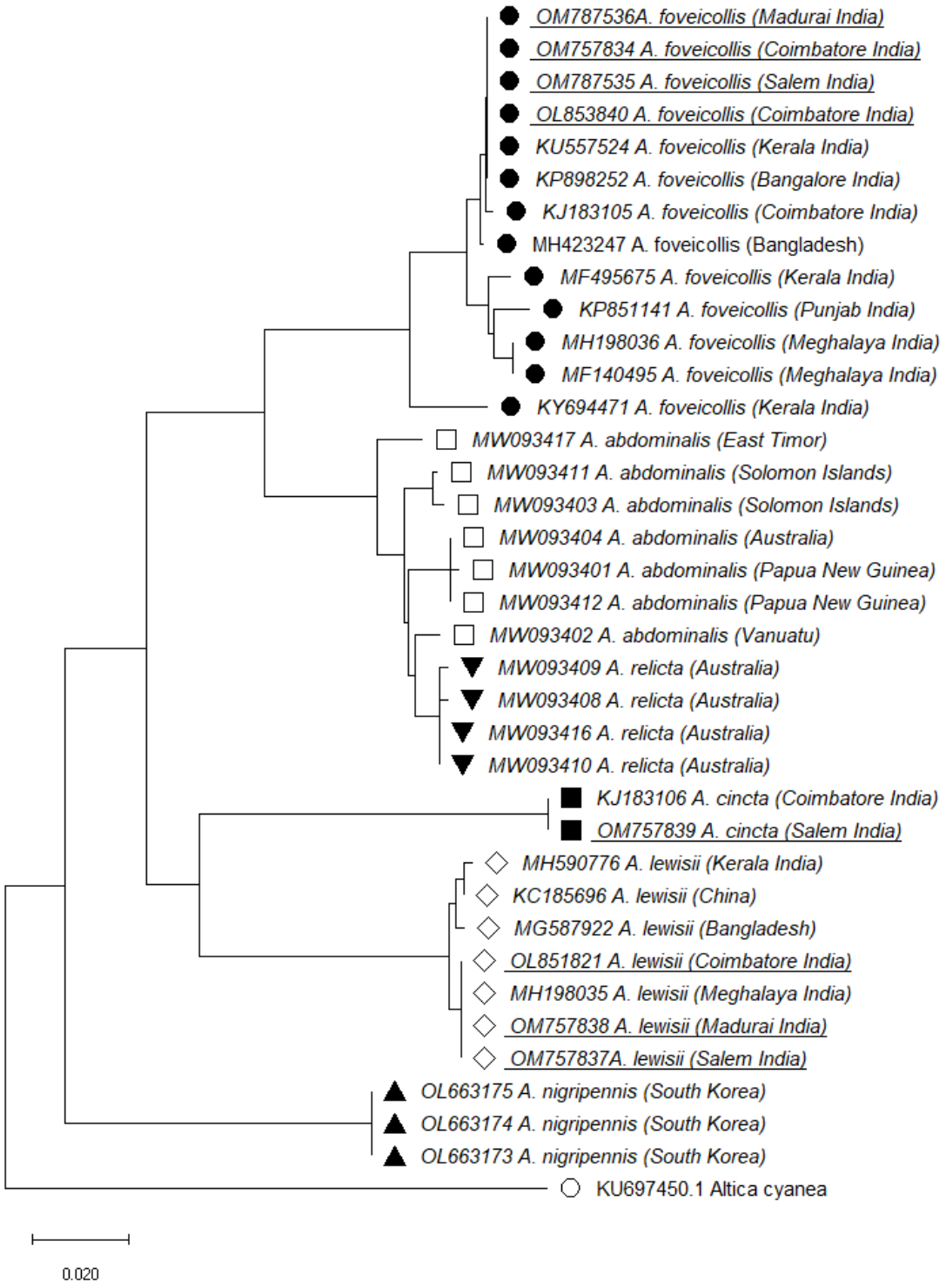


Figure 1. Phylogenetic tree (Neighbor joining method) of 37 sequences of *Aulacophora* spp. Clades with different symbols indicate different species. Underlined *Aulacophora* sequences indicate DNA barcodes of present study. O Symbol indicate out-group.

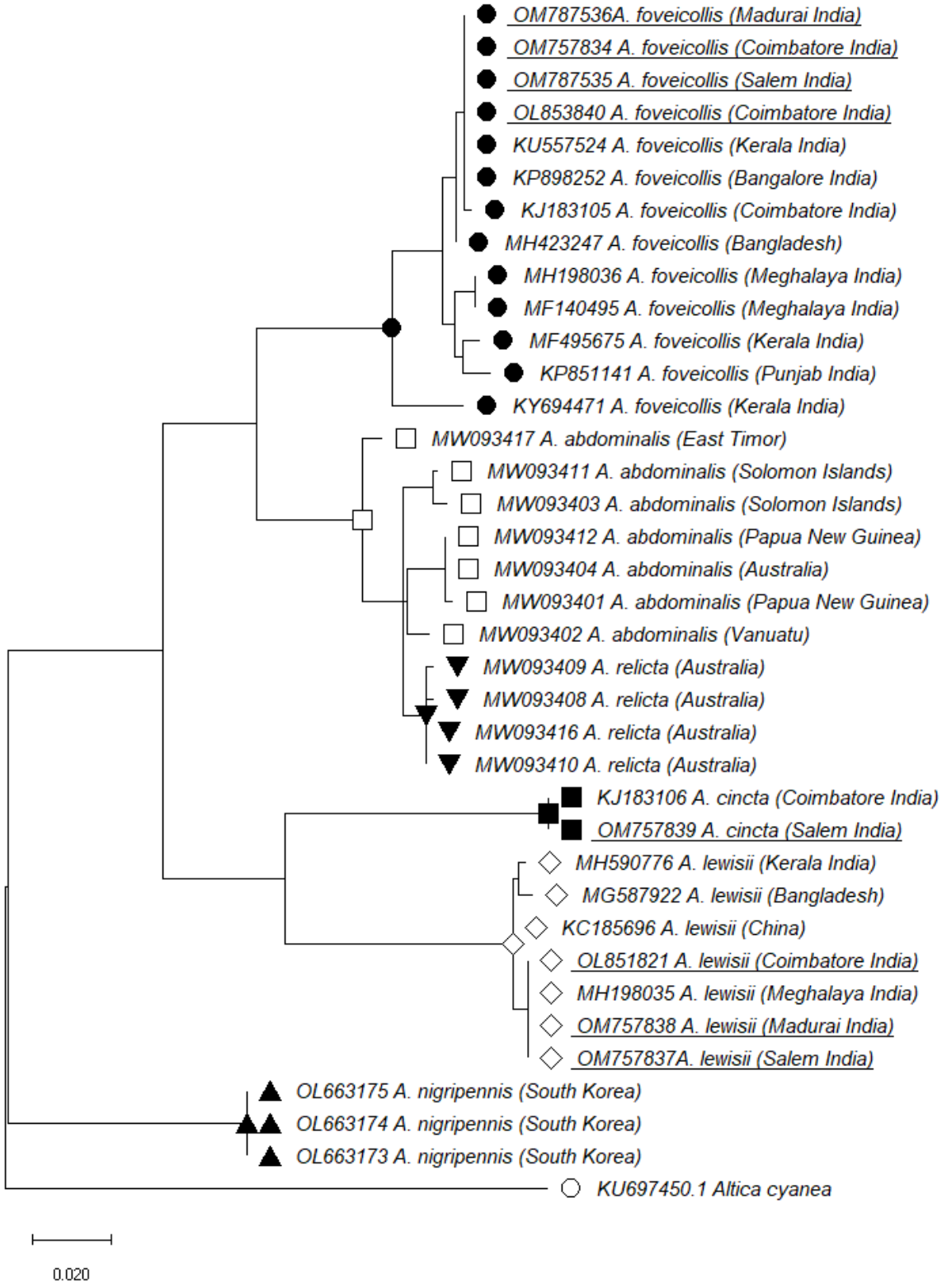
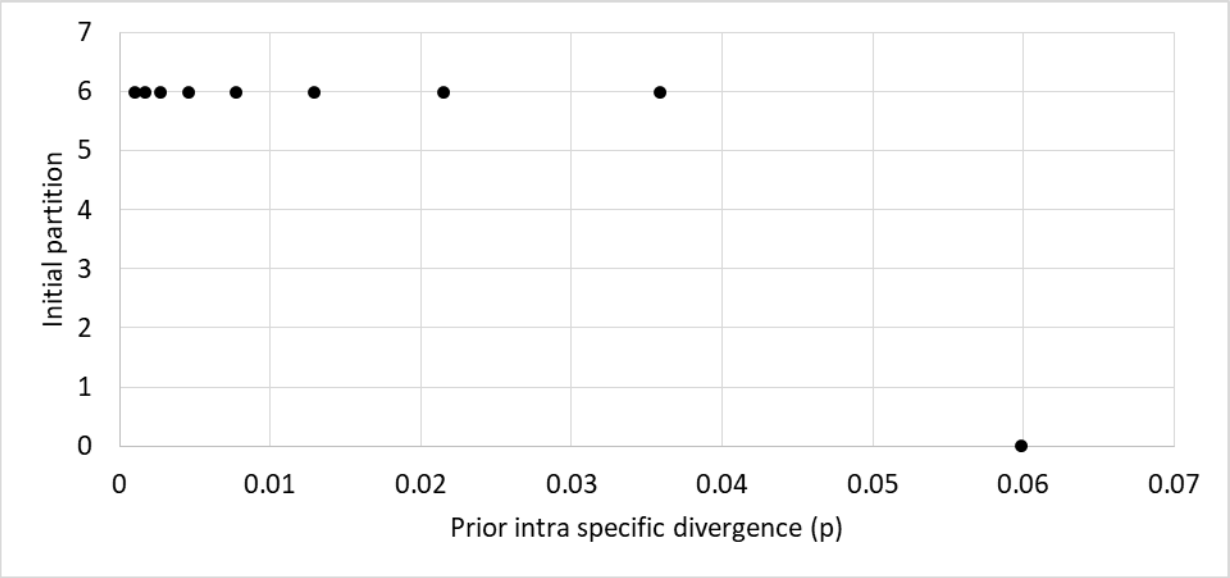
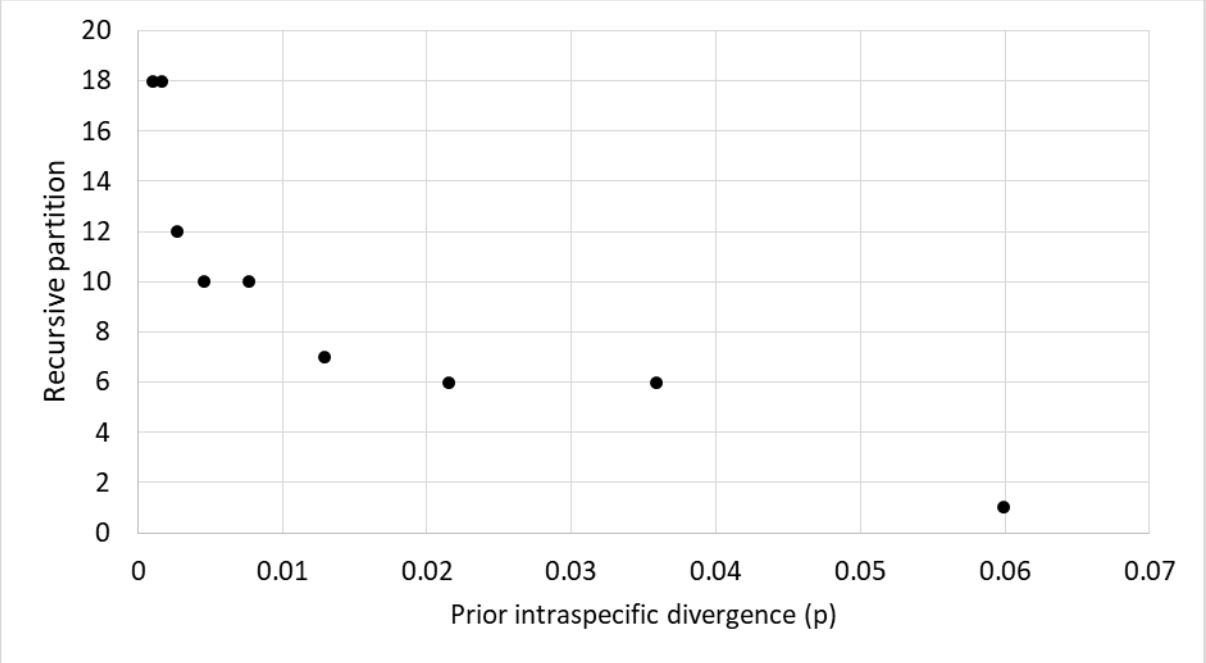


Figure 2. Phylogenetic tree (Maximum likelihood method) of 37 sequences of *Aulacophora* spp. Clades with different symbols indicate different species. Underlined *Aulacophora* sequences indicate DNA barcodes of present study. O Symbol indicate out-group.

Figure 3. The automatic partition results by ABGD for *Aulacophora* sp.



3a. Initial partition of *Aulacophora* spp. mtCOI nucleotide data set



3b. Recursive partition of *Aulacophora* spp. *mtCOI* nucleotide data set

Table 1. Location details of *Aulacophora* spp. collected from different location of Tamil Nadu

District	Geographical coordinates	Species	NCBI accession no
Coimbatore	11.016111 N, 76.937222 E	*A. foveicollis*	OL853840
	11.016111 N, 76.937222 E	*A. lewisii*	OL851821
	10.985757 N, 76.817268 E	*A. foveicollis*	OM757834
Madurai	9.967637 N, 78.201670 E	*A. foveicollis*	OM757836
	9.967637 N, 78.201670 E	*A. lewisii*	OM757838
Salem	11.597432 N, 78.015250 E	*A. foveicollis*	OM757835
	11.597432 N, 78.015250 E	*A. cincta*	OM757839
	11.597432 N, 78.015250 E	*A. lewisii*	OL757837