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Molecular phylogeny of south Indian *Aphthona* species (Coleoptera: Chrysomelidae: Galerucinae: Alticini) with evidence for colour polymorphism

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ABSTRACT: Phylogenetic relationships among south Indian *Aphthona* species using the cytochrome c oxidase subunit 1 mitochondrial gene (*COX1* or *COI*) is reported. This study confirms colour polymorphism in *Aphthona: A. tamila and A. glochidionae*; *A. marataka* and *A. macarangae,* respectively, could be confirmed as colour morphs. *Aphthona phyllanthae* is the most diverged taxon according to the genetic distance. © 2020 Association for Advancement of Entomology

KEY WORDS: Cytochrome oxidase, south Indian *Aphthona*, Molecular phylogeny

INTRODUCTION

The flea beetle genus *Aphthona* Chevrolat includes over 350 species distributed in the Old World (Konstantinov *et al.,* 2002). The species among this genus are ecologically diversified, occuring in a wide range of bio geographical areas from lowland rainforests to high-altitude coniferous hills and deserts to sub-arctic environments. This flea beetle genus is important in the biological control of invasive weed plants of the spurge family Euphorbiaceae (Roehrdanz *et al.,* 2009). Being monophagous, they are ideal candidates in biological control of these weeds. Most studies on *Aphthona* species have focused on morphological and behavioural data (Prathapan and Konstantinov 2003, 2011), only little molecular work has been published to date.

Only one species-level phylogeny of *Aphthona* has been published so far, that primarily considered the molecular phylogenetic analysis of five *Aphthona* species introduced to North America for biological control of leafy spurge (Roehrdanz *et al.,* 2011). Molecular systematics of Indian *Aphthona* spp. was never attempted. Hence, this study was carried out.

MATERIALS AND METHODS

A total of 25 specimens of adult *Aphthona* belonging to nine species (Fig. 2-10) were collected from different locations in south India among which, two specimens showing slight genital variation were designated as *A. chrozophorae* 1 and *A. chrozophorae* 2 (Table 1). Total genomic DNA was isolated from the collected specimens using DNA easy column method (Cockburn *et al.,* 1996;

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Reineke *et al.,*1998) using Qiagen, DNA easy; Blood and TissueKit as per the manufacturer's instruction. The DNA was quantified using spectrophotometric method (Gallagher *et al.,* 2006).

The cytochrome c oxidase subunit 1 mitochondrial gene (*COX1* or *COI*) was amplified using the forward primer with DNA sequence 5'-CATGGGG AATGCTTAGATGC-3' and reverse primer with DNA sequence 5'-AAACTTTCAGGGTGACC AAAAA-3'. The PCR reaction mixture consisted of 2 nanogram of genomic DNA (1 µl), 1 µl each forward and reverse primers at a concentration of 10 µM, 2.5 µl of dNTPs (2 mM), 2.5 µl 10X reaction buffer, 0.20 µl Taq polymerase (5 U/µl) and 16.8 µl H2O. The PCR profile consisted of an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 10 sec at 95°C, 10 sec at 55°C and 1 min at 72°C and ending with a final phase at 72°C for 3 min. The PCR product was sequenced from both ends using the Sanger's sequencing (Sanger *et al.,* 1975) method at Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum. The forward and reverse sequences were alligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). After removing the forward and reverse primer sequences, the consensus sequence was taken for the analysis. The percentage of each nucleotide in the *COI* codon of *Aphthona* was determined by MEGA X software. A phylogenetic tree was constructed using MEGA software with ten sequences obtained, in order to understand the intrageneric genetic diversity among the species of southern Indian *Aphthona*. For this tree, *Chrysomela aeneicollis* was chosen as the outgroup.

RESULTS

A total of 25 *Aphthona* specimens were investigated in this study out of which, nine were distinct morpho-species (Table1; Fig. 2-10). All of the samples yielded good quality DNA having A_{260}/A_{280} ratio in the range of 1.8-2.0, which gave good amplicons in the PCR with a product size of 680bp. The phylogenetic tree illustrating relationships between the species of south Indian *Aphthona* species is provided (Fig.1). Using the Neighbour-Joining technique, evolutionary background was inferred. The optimal tree shows the distance of the branch being equivalent to 3.3. The evolutionary distances have been calculated using the technique Maximum Composite Likelihood and are in the number of base substitutions per unit. There were 11 nucleotide sequences in this study. The inclusion of codon positions was 1st+2nd+3rd+Noncoding. For each sequence couple, all unclear locations were deleted (pairwise deletion option). In the final dataset there were a total of 844 positions. The genetic distance between the species was

Table 1. Details of species collected

Sl. no	Species name	Host Plant	Location
1	*A. bombayensis*	*Phyllanthus amarus*	Vellayani, Kerala
2	*A. chrozophorae 1*	*Croton* sp.	Munnar, Kerala
3	*A. chrozophorae 2*	*Croton* sp.	Munnar, Kerala
4	*A. glochidionae*	*Glochidion zeylanicum*	Ponmudi, Kerala
5	*A. macarangae*	*Macaranga peltata*	Vellayani, Kerala
6	*A. mallotae*	*Mallotus philippinensis*	Dandeli, Karnataka
7	*A. marataka*	*Macaranga peltata*	Mattupettti, Kerala
8	*A. nigrilabris*	*Euphorbia hirta*	Vellayani, Kerala
9	*A. phyllanthae*	*Phyllanthus emblica*	Vellayani, Kerala
10	*A. tamila*	*Glochidion zeylanicum*	Pampadum Shola, Kerala

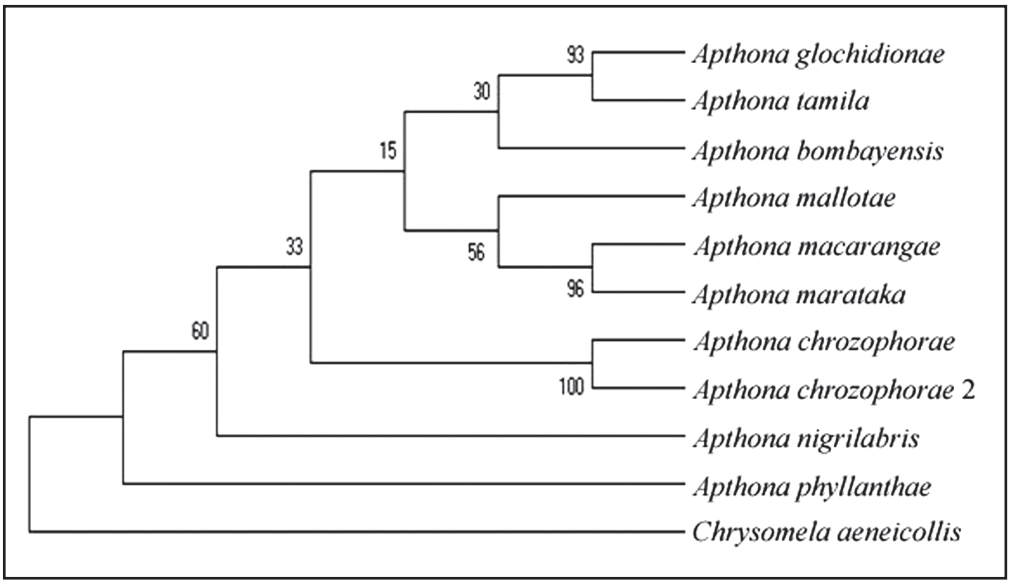


Fig. 1. Intragenomic phylogenetic tree of south Indian *Aphthona* spp.

calculated using MEGA (Tamura *et al.,* 2013) software by kimura 2 modelling (Kimura *et al.,* 1980) as depicted in table 2.

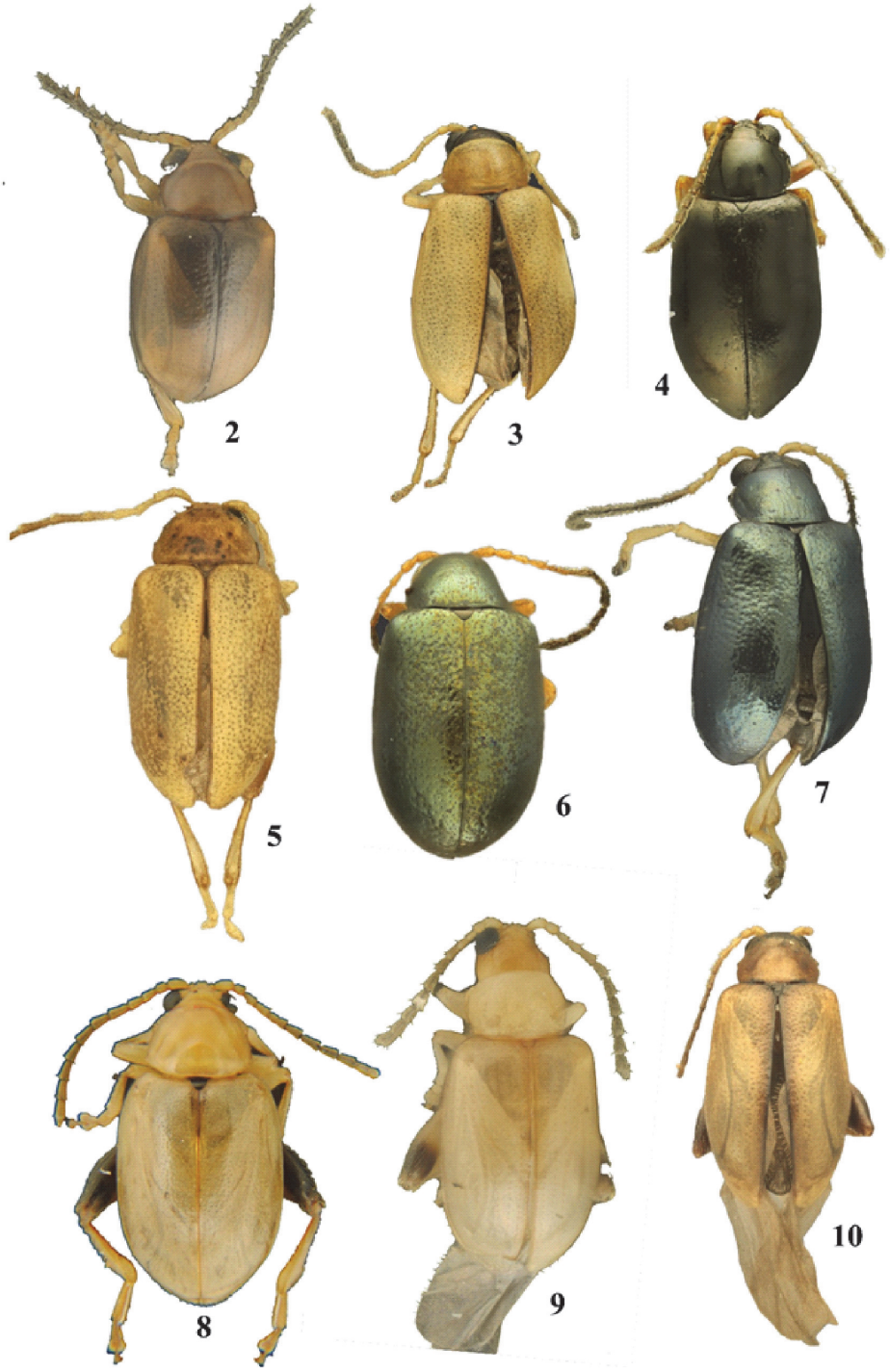
DISCUSSION

Based on prior cladistic research, *Aphthona* is defined by three synapomorphic characters: elytron length / width ratio below 2.85; anterior portion of the metanotal ridge connected below the centre of ridge b-1; and setae on the ventral side of the first sinuate metatarsomere (Konstantinov, 1998). *Aphthona* species are specialized phytophagous insects, as with most flea beetles. Most of them feed on crops from 11 distinct families (Nowierski *et al*., 2002).

From the genomic DNA, mitochondrial genes were amplified using selected primer. The amplification product was 700 bp in size; the same was used to generate sequences. The sequences were analyzed using BLAST (Altschul *et al.,* 1990). The genetic

Table 2. Genetic distance between south Indian *Aphthona* species.

Chrysomela_ aeneicollis										
A. bombayensis	0.295185									
A. chrozophorae_2	0.306005	0.22637								
A. chrozophorae_1	0.309262	0.226399	0.00341							
A. glochidionae	0.32423	0.20086	0.25555	0.25384						
A. mallotae	0.310312	0.211074	0.209836	0.214217	0.20297					
A. nigrilabris	0.279839	0.217638	0.195807	0.215064	0.196102	0.204721				
A. macarangae	0.320594	0.221219	0.197176	0.208616	0.195192	0.090091	0.21095			
A. phyllanthae	3.65206	3.6038	3.45497	3.45377	3.33880	2.84769	2.98418	3.0598		
A. tamila	0.267043	0.18270	0.23447	0.24313	0.13205	0.21412	0.21983	0.20680	3.21757	
A. marataka	0.32281	0.22121	0.19883	0.20861	0.19696	0.21317	0.21947	0.091659	3.0598	0.20836



Figs. 2-10. *Aphthona* spp. 2. *A. bombayensis;* 3. *A. chrozophorae;* 4. *A. glochidionae;* 5. *A. macarangae;* 6. *A. mallotae;* 7. *A. marataka*; 8. *A. nigrilabris;* 9. *A. phyllanthae;* 10. *A. tamila*

distance in the specified matrix ranges from 0.003 to 3.652. It is confirmed that, *A. phyllanthae* (Fig.9) is the most diverse species among the southern Indian *Aphthona*. The genetic distance between the species that created the same clade is as follows: the genetic distance between *A. glochidionae* and *A. tamila* is 0.132; genetic distance between *A. chrozophorae* and *A. chrozophorae 2* is 0.003 and distance between *A. marataka* and *A. macarangae* is 0.090. If genetic distance between two sequences is less than 0.2, then those species are considered as same (Vogle *et al.,* 1993). Hence, based on these observations of genetic distance, the aforesaid species can be considered as synonymous as they exhibit 99 percent sequence similarity. In *Aphthona*, the species group is determined by morphological characteristics, predominantly based on the morphological colour. The colours range from non-metallic yellow to metallic bright shades (Fig.2-10). There are no intermediate colouring patterns. Based on the genetic distance between the southern Indian *Aphthona* spp. calculated by kimura2 model, the species *A. marataka* (Fig.7) which is a bright metallic green coloured beetle, found in higher altitude habitats is identified to be synonymous with *A. macarangae* (Fig.5) which is yellow non-metallic in colour and inhabits lower altitude regions. The same applies to the metallic black species *A. glochidionae* (Fig.4) which inhabits high altitude areas, which is synonymous with the yellow non-metallic species, *A. tamila* (Fig.10) These species also share a significant quantity of resemblance morphologically. The presence of colour morphs within the *Aphthona* genus was not recognized until now. Similar kind of polymorphism in the subfamily Chrysomelinae of Chrysomelidae was reported by Van Noort, 2013. Thus, these species are recognized as colour morphs depending on morphological analysis and molecular information gathered from this study.

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