MOLECULAR PHYLOGENY OF SOUTH INDIAN *APHTHONA* SPP. (COLEOPTREA: CHRYSOMELIDAE)

by

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(2014-09-111)

THESIS

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DECLARATION

I hereby declare that this thesis entitled "**MOLECULAR PHYLOGENY OF SOUTH INDIAN *APHTHONA* SPP. (COLEOPTREA: CHRYSOMELIDAE***)***"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

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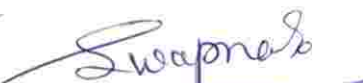Thiruvananthapuram 695522

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF ABBREVATIONS

BLAST	Basic Local Alignment Search Tool
COI	Cytochrome c oxidase I
DNA	Deoxyribonucleic acid
et al.	Co-workers/ Co-authors
g	gram
M	Molar
mg	milligram
min.	minute
mM	Millimolar
NCBI	National Center for Biotechnology Information
ng	nanogram
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
sec.	Seconds
spp.	Species (Plural)
UV	Ultraviolet
V	Volts
μg	microgram
μl	microlitre
%	Percent of
°C	Degree celsius

1. INTRODUCTION

Chrysomelidae, the leaf beetles, are the second biggest animal family containing 37,000 to 40,000 species categorized in 2000 genera (Seeno and Wilcox., 1982). No crop plant is safe from contagion by at least one species of leaf beetle. Other than immediate eating harm, some leaf beetles also function as transmitters of plant pathogens. This family is one of the biggest community of beetles globally with over 50,000 recognized species and many more thousands to be found, particularly from Asia and the Neotropics; 566 species and subspecies are found from Canada and Alaska (Lesage et al.,1996). Every recognized adult is phytophagous. Some adult individuals feed on pollen. Larvae demonstrate a wider ecological variety: Donaciinae are aquatic; Clytrinae reside in ant nests from which they devour on plant debris and on ant larvae or pupae; most Cryptocephalinae nourish on rotting vegetation in leaf litter; Criocerinae, Chlamisinae, Chrysomelinae and Cassidinae and some Galerucinae and Alticinae eat accessible leaves; those of the remaining subfamilies are either root feeders, root miners, stem miners or leaf miners (Lesage et al.,1996). Being important as crop pests, Chrysomelidae are also important subjects of study under various branches of organismic and functional biology. *Aphthona* is an important beetle genus from the Alticinae subfamily of *the* Chrysomelidae family.

The flea beetle genus *Aphthona* is broad and contains over 350 species that are spread across the biogeographic regions of the Palearctic, Oriental, African, and Australian Old World (Konstantinov et al., 2002). *Aphthona* species are weakly recognized taxonomically. *Aphthona* species are generally mesophilous, however they are diverse ecologically, so that they occur in a wide variety of biogeographic regions and inhabit almost all known habitats ranging from low land rainforest to high altitude coniferous mountains and from deserts to sub artic environs. Most frequently, *Aphthona* is discovered in wet meadows or on vegetation along waterways and streams (Konstantinov et al., 2002). As with most flea beetles *Aphthona* species are specialized phytophagous insects. They mostly feed on plants belonging to 11 different families. Most of them are Dicotyledenae

but 3 of them are monocotyledenae. The host specialisation in *Aphthona* is narrower than that of most flea beetles (Konstantinov *et al.*, 1996). This flea beetle genus is important because of the usefulness of some species in controlling invasive weeds (Roehrdanz *et al.*, 2009). The fact that a number of *Aphthona* species feed on Euphorbiacea and the tendency towards monophagy makes them ideal candidates for biological control against weed plants belonging to Euphorbiacea family. In many western countries *Aphthona* beetles are used as biological control agent against leafy spurge (*Euphorbia esula),* which is noxious weed (Lym *et al.*, 2000). Although several Indian workers like Prathapan (2003&2010) have contributed towards classical studies which helped to resolve the taxonomic mystery of Indian *Aphthona* species, information on the molecular systematics of Indian *Aphthona* spp. is scant.

Molecular phylogenetics is a disciplinary research of developmental relations among species using molecular sequences. The analytical methods used in molecular phylogenetics were originally developed to reveal developmental patterns, but now molecular phylogenetics is used in several fields, such as systematic biology and biodiversity, molecular epidemiology, cell function recognition etc. For such purposes, molecular phylogenetics is a basic area of science that most scientists need to comprehend the background (Ajawatanawong *et al.*, 2016). Phylogenetic analysis of DNA sequences has become an important tool for studying the evolutionary history of organisms. Since the rate of sequence evolution varies extensively with gene or DNA segment one can study the evolutionary relationships of virtually all levels of classification of organisms (Kajtoch *et al.*, 2015). Phylogenetic analysis is also important for clarifying the evolutionary pattern of multigene families as well as for understanding the adaptive evolution at the molecular level. The proposed work is intented to resolve the phylogeny of southern Indian *Aphthona spp.* and to provide a better understanding about the intra generic genetic variation among the south Indian *Aphthona* spp.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The present study on "Molecular phylogeny of the South Indian *Aphthona spp.* (Coleoptera: Chrysomelidae)" has been executed through the amplification of mitochondrial cytochrome oxidase subunit 1 (COI) gene from different species belonging to genus *Aphthona* the amplified COI gene fragments were sequenced and the sequence information was further used to identify the species relation. The relevant literature available on various aspects of this study were collected and are reviewed in this chapter, under different heads.

2.1 *Aphthona* taxonamy

Kingdom: Animalia

Phylum: *Arthropoda*

Class: *Insecta*

Order: *Coleoptera*

Family: *Chrysomelidae*

Subfamily: Alticinae

Genus: *Aphthona*

There are 12 described species of *Aphthona* beetles across South India. Southern India, as defined here, includes Andhra Pradesh, Karnataka, Tamil Nadu, and Kerala states (Prathapan *et al.*,2003).

2.2 General biology of *Aphthona* genus

Aphthona species occur in a wide variety of habitats, from high altitude of around 3500m to low land deserts, and forest and grassland biomes in between. Most commonly, *Aphthona* are found in moist meadows or on vegetation along rivers and streams. Adults are active nearly all year, particularly from March through

November (Konstantinov *et al.*, 2002). Early larval instars of the *Aphthona* feed in or on the root hairs of the host plant, whereas later instars feed in or on the yearling roots (Nowierski *et al.*, 2001). Adult beetles feed on the leaves and flower bracts of leaf of the host plant. Members of this genus are small to moderate in size (1-4 mm), oval or oblong-oval and more or less convex in lateral view. Based on previous cladistic study, three synapomorphic characters define *Aphthona*: elytron length/width ratio less than 2.85; anterior part of metanotal ridge e attached below middle of ridge b-1; and setae on ventral side of first metatarsomere sinuate (Konstantinov *et al.*, 1998).

A combination of a few external, more easily observed characters is also useful for recognizing *Aphthona*: frontoclypeal suture with several rows of short setae; sulci surrounding antenna calli well developed; frontal ridge relatively short and convex in lateral view; anterofrontal ridge as low as frontal ridge, lowering laterally, pronotum with or with-out antebasal transverse impression (if im-pression is present, it is shallow and is never limited laterally by longitudinal grooves); procoxal cavity open behind; intercoxal prosternal process and mesosternum narrow; elytral punctation irregular, rarely with irregular striae on disk; dorsal surface of metatibia apically flat, apical spur inserted laterally; first metatarsomere comparatively long, as long as two following tarsomeres combined, but never more than half as long as metatibia.

2.3 Identification of *Aphthona* species

For understanding species diversity, phylogenetic patterns, and evolutionary processes, species identification is the basis. Precise identifications allow for comparisons between studies and the repetition or expansion of earlier experiments. In pest species, accurate identification is of paramount importance for quarantine and management purposes: the development of biological and other control strategies (David *et al.*, 2007).

In outdoor conditions, many leaf beetles can be determined using their size, coloration, and host plants. Coloration is fairly reliable for mature specimens but much practice and good sight (hand lens might be necessary) are essential to distinguish between species in the field. Most tenerals are impossible to recognize externally, and that's why voucher specimens should always be preserved for further identification with the aid of a microscope and genitalic features (LeSage *et al.*, 1996).

Males and females are quite similar in *Aphthona*. The most common characters differentiating males and females in other groups of flea beetles are not useful in case of *Aphthona* (Konstatinov & Langifelter., 2002). Males of *Aphthona* are usually smaller than females. There is a deep apical impression in males which is absent in females. In some oriental species the first metatarsomere is much wider in males than females. Males of other species have antenomers which are flattened dorsoventrally, and strong swollen first antennomere. Males of some species group have antennomer abruptly widened apically (Konstatinov &Langifelter., 2002). The keys for identification of South Indian *Aphthona* species were given by Prathapan and Konstantinov (2003).

2.4 Important species group in *Aphthona* genera

Konstantinov and Lingafelter in 2002 has described about the important species group falling in *Aphthona* genera in their work "The revision of the oriental species of *Aphthona* chevrolat (Coleoptera: Chrysomeliade)". It falls into five species groups. These groups are primarily based on the structure of male and female genetalia and are recognizable only by various combinations of characters of different nature. The following is brief description about these species groups as described them.

Crypta group. – Males of this group possess a median lobe of aedeagus, convex ventrally, ventral side with wide impression, extremely deep basally. The apical part of the lobe bears two shallow and short impressions. The dorsal side of the lobe is usually flat apically, abruptly widened at basal half. The female genetalia

are quite similar to the genetalia of the hmmarstroemi group. The posterior Y-shaped sclerotization of the tignum is moderately short, with posterior branches widening apically. The spermatheca and the vaginal palpus are not distinctive.

Cyparissiae group. – This group includes several large, yellow species and the type species of the genus. They share the following characters: body large, more than 3mm in length, frontal ridge wide anterofrontal ridge thick, especially in the middle: median lobe of aedeagus moderately straight in lateral view, apex usually flat with impression in middle: vaginal palpi usually robust; apical abdominal tergite always lacks basal appendages.

Hammarstroemi group. – The following autapomorphic characters define this group: ventral side of median lobe of aedeagus with two impressions situated lateral to middle ridge, without membranous window; posterior part of the tignum with two branches; dorsal part of the body metallic blue or green, or with strong copper or bras lustre; basal part of pronotum with much coarser punctuation than apical. Unfortunately, none of these characters consistently occurs in all the species of the group; some of them are also observed outside the group. Tis group is much more speciose in oriental region than in the palearctic region.

Kanaraensis group. – Members of this group shares following characters: lateral side of pronotum usually nearly straight, lateral margins mostly narrowly explanate; frontal ridge moderately wide compared to the frontal ridge of species from hummarstroemi group but narrower than in cyparissiae group; anterofrontal ridge usually high in middle and comparatively high laterally, which is unusual for *Aphthona*, but common among species from related genera.

Laeta group. – Several characters of males some species of this group; antennomeres 8 through 11 wides apically, nearly triangular; head and pronotum reddish brown in combination with blue elytron; metatibia (in lateral view) swollen at apical one third; first metatarsal segment greatly enlarge as first pro-tarse segment in neocrepidodera heikertinger (this feature rarely occurs in some Asian *Aphthona* from the hummarstroemi group); median lobe of aedeagus short

and wide , curved ventrally, ventral side with high lateral ridges, sometimes forming denticles.

2.5 Diversity of *Aphthona* across South India

Across South India there are 12 described species of Aphthona. South India as described here include different sites of Andhra Pradesh, Karnataka, Tamil Nadu, and Kerala states (Prathapan *et al.*, 2003). Until 2003 only three species oh *Aphthona* were known. Through a study conducted by Prathapan and Konstantinov, they introduced 7 new species of *Aphthona*. Prior to this study only *A. atripes, A. kanaraensis*, and *A. tamila* were known. The new species introduced were *A. chrozophorae, A. glochidionae, A. macarangae, A. mallotae, A. marataka, A. nandiensis*, and *A. phyllanthae*. Later in 2011 Prathapan and Konstantinov introduced two more species A. bombayensis and *A. yercaudensis*.

Following is a brief description about these species and their host plants as explained by Prathapan and Konstantinov (2003& 2011).

Aphthona chrozophorae:

Host plant. - *Chrozaphora rottleri* Klotzsch. (*Euphorbiaceae*).

Distribution. -India (Tamil Nadu, Kar-nataka).

Description. - Head, labrum, scutellum, suture, apex of metafemur, meso- and meta-thoracic sterna, and abdomen dark brown to piceous. Antennomeres 4 to 7 light brown. Prothorax, elytron, legs (except for meta-femoral apex), antennomeres 1 to 3 and 8 to 11 yellowish.

Aphthona glochidionae:

Host plant. - *Glochidion zeylanicum* A. Juss. *(Euphorbiaceae).*

Distribution. -India (Karnataka).

Description. - Body black, 7 apical antennomeres dark brown or black, metafemur dark brown, bases of pro- and meso-femora light brown. Rest of legs and anten-na yellowish.

Aphthona macarangae:

Host plant. -*Macaranga peltata* Muell. (*Euphorbiaceae*).

Distribution. -India (Tamil Nadu, Kerala).

Description. -Colour yellowish, head and 5 apical antennomeres dark yellow. Meta-sternum and abdomen light piceous.

Aphthona mallotae:

Host plant. - *Mallotus philippinensis* Muell. (Euphorbiaceae).

Distribution. -India (Karnataka).

Description. -Body with metallic green luster. Six apical antennomeres and meta-femur brownish. Rest of antenna and legs yellow.

Aphthona marataka:

Host plant- Unknown.

Description. -Body with metallic green luster. Seven apical antennomeres and apex of metafemur brownish. Rest of antenna and legs yellow.

Aphthona nandiensis:

Host plant. - Unknown.

Distribution. - India (Karnataka).

Description. -Body entirely yellowish except darker 4 or 5 last antennomeres and ventral side of body.

Aphthona phyllanthae:

Host plants. - Phyllanthus polyphyllus Wild. and P. emblica L. (Euphorbiaceae).

Distribution. -India (Karnataka).

Description. -Body yellowish except darker (light brown) 7 last antennomeres and ventral side of body.

Aphthona tamila:

Host plants. -Drypetes sp. (Euphorbiaceae).

Distribution. -India (Tamil Nadu) (Kon-stantinov and Lingafelter 2002).

Description. -Color yellow. Head, 8 apical antennomeres, scutellum, narrow stripe along elytral suture, meso- and metasterna, and apex of metafemur dark brown.

Aphthona bombayensis:

Host plants. Phyllanthus airyshawii Brunel and Roux, Phyllanthus amarus Schum. and Thonn. and Phyllanthus urinaria L. (Euphorbiaceae).

Distribution. -India (Delhi, Karnataka, Kerala, Maharashtra), Nepal, Sri Lanka.

Description - color yellow to dark brown; body extremely small (1.2–1.6 mm); head with supra antennal sulci well-developed, postcallinal sulci poorly developed or absent, and antennal calli not raised; elytral punctures forming nearly regular striae; median lobe of aedeagus ventrally with longitudinal impression and acute apex lacking denticle, with dorsal side membranous and concave; spermatheca with ovoid receptacle and duct making loop away from receptacle; posterior sclerotization of tignum wide, without well-developed arms.

Aphthona yercaudensis:

Host plants. Unknown.

Distribution - India (Tamil Nadu).

Description. - Length 1.22–1.44 mm; width 0.67–0.84 mm. Color yellow brown, distal 2 or 3 antennomeres and metathoracic sternite darker in some specimens. Head moderately flat in lateral view.

Aphthona atripes:

Host plants. - Unknown.

Distribution. - India, Sri Lanka.

Description. - Pronotum and elytron, ex-cept suture, yellow. Facial part of head and basal 3 antennomeres dark yellow. Vertex orange. Labrum, apical 8 antennomeres, scutellum, prosternum, pro- and mesofe-mora, all tibiae and metatarsi brown or dark brown. Elytral suture brownish. Abdomen dark brown almost black. Meso- and me-tasterna, and metafemur black. Elytral su-ture light brown.

Aphthona kanaraensis:

Host plants. -Eucalyptus rostrata Sch.

Distribution. -India (Assam, Bihar, Maharashtra, Karnataka, Himachal Pradesh, Uttar Pradesh).

Description. - Colour yellow. Last 7 an-tennomeres, labrum, metasternum and metafemoral apices brown. Rest of legs and antenna yellow.

2.6 Molecular Phylogeny

Molecular phylogenetics is a disciplinary study of evolutionary relationships between species using molecular sequences. The analytical techniques used in molecular phylogenetics were initially created to disclose evolutionary pathways, but today molecular phylogenetics is used in several areas, such as systematic biology and biodiversity, molecular epidemiology, identification of gene function and microbial identification in microbiome studies. For these reasons, molecular phylogenetics is a fundamental field in science that most biologists need to understand the context (Ajawatanawong *et al.*, 2016). According to them, there are five specific phylogenetic assessment steps: (1) sequence data preparation, (2) sequence alignment, (3) phylogenetic reconstruction method, (4) best tree identification, and (5) tree evaluation.

According to Futuyama (1997) a phylogenetic tree is the estimate of species divergence from a common ancestor. Data gathered by systematists are the information used to suggest how comparable organisms are, particularly morphological characters, comparative embryology, fossil records and comparative anatomy. The cladograms produced by the instruments of bioinformatics are based on sequence information alone. Each position in a sequence is a character with 4 states (nucleotides): A, T, C or G that are obviously

defined; this is a benefit over phenotypic traits such as sizes that sometimes are variable. DNA sequence-relatedness is therefore regarded to be a very strong predictor of species-relatedness (Futuyama, 1997).

2.7 Mitochondrial genes for phylogenetic studies

If someone was to specify the preferred characteristics of an ideal phylogenetic analysis molecular system, the following could be included in the bucket list. The molecule should: (a) be unique, yet omnipresent, so that safe homologous comparisons can be made between a broad variety of species (b) be easy to isolate and test (c) have a straightforward genetic structure that lacks a complicated function such as repetitive DNA, transposable elements, pseudogenes and introns (d) show a straightforward mode of genetic transmission, without recombination or other genetic alterations (e) provide rooms of qualitative states whose phylogenetic interrelationships could be inferred by reasonable parsimony criteria and for microevolutionary evaluation purposes, (f) evolve at a fast rate so that new character states generally emerge within a species ' lifespan. The mitochondrial DNA of higher animals meets all the above requirements to a notable degree (Avise *et al.*, 1987). In recent years, the study of mitochondrial DNA (mtDNA) sequences has become the method of choice for a wide range of animal taxonomic, population, and evolutionary investigations. Many features of mtDNA's structure and evolution have made it a useful evolutionary resource. These include its ease of isolation, high copy number, absence of recombination, conservation of sequence and composition across metazoa, and variety of mutation rates in different areas of the molecule (Moore *et al.*, 1987).

It is evident that the mitochondrial genome of animals is a better target for phylogeny assessment than the nuclear genome due to the fact that the former is characterised with exposure to recombination, and its haploid inheritance mode (Saccone *et al.*, 1999). Robust primers also allow the regular retrieval of particular

sections of the mitochondrial genome (Vrijenhoek *et al.*, 1994; Simmons & Weller 2001). Past phylogenetic research has often concentrated on mitochondrial genes encoding ribosomal (12S, 16S) DNA, but their use in wide taxonomic analysis is restricted by the incidence of insertions and deletions (indels) that complicate sequence alignments (Doyle & Gaut., 2000). Insect mitochondrial cytochrome oxidase I (COI) genes are used as a model to examine the evolutionary rate within gene heterogeneity and its implications for evolutionary analysis.

2.8 Cytochrome oxidase subunit1 gene

The mitochondrial gene coding for subunit I of cytochrome oxidase (COI) has some important properties that make it particularly relevant as a molecular marker for evolutionary studies since it has been comparatively well researched at the biochemical level as the terminal catalyst in the mitochondrial respiratory chain, and its size and structure tends to be conserved across all the aerobic organisms studied (Saraste *et al.*,1990). Cytochrome oxidase I is associated in both electron transport and the related translocation of protons throughout the membrane and has been shown to contain a selection of distinct kinds of functional domain including ligand locations, ion channel elements, structural ahelices and interspersing hydrophilic loops (Saraste, 1990 and Gennis, 1992). Amino acid residues in strongly conserved reaction sites do not control the entire COI molecule, enabling for significant variation in some areas. Such a mixture of extremely preserved and variable areas so tightly connected with a mitochondrial gene makes the COI gene especially beneficial for evolutionary research. COI gene is the biggest of the three subunits of cytochrome oxidase encoded with mitochondria (consisting of 511 amino acids in *D. yakuba* compared to 228 for COII and 261 for COIII; Clary & Wolstenholme., 1985) and is one of the biggest protein encoding genes in the metazoan mitochondrial genome. This allows one to amplify and sequence many more elements (nucleotides) within the same

functional complex than is feasible for almost any other mitochondrial gene (Lunt *et al.*, 1996).

An appropriate genetic marker is an important prerequisite for achievement in many evolutionary studies. The key feature of choosing such a marker is the region's substitution rate. To a significant extent, it is the wide range of substitution rates that accounts for the prominence of animal mtDNA as a molecular tool, as it allows the resolution of both intra-specific phylogenies (Avise *et al.*, 1987) and the higher-level systematics of anciently deviated taxa (Ballard *et al.*, 1992). It is well understood that distinct genes can evolve at distinct rates, and in distinct lineages the same gene might have distinct rates of evolution. But, the evolution rate within-gene heterogeneity has still not gained sufficient consideration, particularly in the field of phylogenetic research at a smaller taxonomic level. For several situations, it may be inaccurate to consider a gene as quickly or slowly evolving, as this indicates a homogeneity of speed across the entire gene, which is usually true owing to the concentration of functional limitations in particular areas of the DNA sequence. For several situations, it may be inaccurate to consider a gene as quickly or slowly evolving, as this indicates a homogeneity of speed across the entire gene, which is usually true owing to the concentration of functional limitations in particular areas of the DNA sequence (Lunt *et al.*, 1996). Therefore, it is extremely advantageous to have data about the relative substitution rates of distinct gene areas, as this will allow a much more informed sequence selection for specific phylogenetic inquiries. Sequences that evolve too rapidly are known to lose their capacity to explicitly disclose the phylogeny of anciently deviated taxa. Likewise, choosing a sequence that is too conserved to solve intra-specific phylogeography issues, for instance, will not provide sufficient informative traits to evaluate the required interactions (Lunt *et al.*, 1996).

2.9 DNA isolation from insects

DNA isolation from the sample is the key and very important step in molecular phylogeny study. For isolating the gene of interest first we must extract good quality genomic DNA for further downstream processes like PCR. Henry *et al.*, in (1990) introduced a new method of good quality DNA isolation involving an initial nucleus enriching process. Genomic DNA was isolated from solubilized nuclei by separating the organic stage (liquid / liquid extraction). Later in 1996, Cockburn and Fritz presented different isolation for different procedures like restriction digestion, PCR amplification, mitochondrial DNA extraction, Large-Scale Purification of total DNA for genomic libraries etc. Chen *et al.*, (2010) compared five different DNA extraction methods from beetles, which are DNA extractions by the SDS method, CTAB method (Shajahan *et al.*, 1995), DNAzol® reagent (DITTRICH *et al.*, 2012), Puregene® solutions (Zetzsche *et al.*, 2008) and DNeasy® column method (Chen *et al.*, 2010). They concluded that, while all five techniques resulted in appropriate DNA amounts and absorbance proportions, the SDS and CTAB techniques led in greater DNA yields (ng DNA vs. mg tissue) at much reduced price and less degradation as disclosed on agarose gels. The DNeasy ® kit was the most time-efficient, but the most expensive among the techniques tested.

2.10 DNA sequencing

Sequencing is the method of finding out the precise order of the four bases in a specified DNA strand (Sanger and Coulson., 1975). DNA sequencing procedures are important methods in many sectors. A wide range of separate fields are benefiting from these methods, spanning from archaeology, anthropology, genetics, biotechnology, molecular biology, forensic sciences, etc. In many disciplines, a silent and notable revolution is underway; DNA sequencing promotes fresh findings that revolutionize the conceptual bases of many sectors (Franca *et al.*, 2002).

2.10.1 Sanger's method

The very first technique outlined for DNA sequencing was introduced by Sanger and Coulson and was called ' plus and minus method ' (Sanger and Coulson., 1975). This technique used polymerase I and DNA polymerase of E. coli from bacteriophage T4 with distinct restricting nucleoside triphosphates. The polymerase generated products were resolved by gel electrophoresis. After two years, due to the ineffectiveness of the 'plus and minus technique', Sanger and his colleagues outlined a fresh innovative technique for studying oligonucleotides via enzyme polymerization (Sanger *et al.*, 1977). It involves a catalysed enzyme response that polymerizes the DNA pieces complementing the interesting template DNA (unidentified DNA). Briefly, a 32 P-labelled primary (short oligonucleotide with a sequence complementary to the template DNA) was annealed to a particular recognized area on the template DNA, which given a baseline for DNA synthesis. Catalytic polymerization of deoxynucleoside triphosphates (dNTP) on the DNA happened in the existence of DNA polymerases. Reaction was continued until an altered nucleoside [called terminator or dideoxynucleoside triphosphate (ddNTP)] was integrated into the increasing chain by the enzyme. This technique was conducted in four distinct pipes, each carrying the suitable quantity of one of the four terminators. Every piece produced had the same 5«-end, while the residue at the 3«-end was dictated by the dideoxynucleotide used in the response. After all four responses were finished, the combination of different-sized DNA pieces was settled in four simultaneous routes by electrophoresis on a denaturing polyacrylamide gel. The banding pattern indicates the termination propagation in the synthetic DNA strand and autoradiography could read the unidentified sequence.

2.10.2 Maxam and Gilbert Method

Maxam and Gilbert (1977) defined a sequencing technique relying on chemical degradation. Using particular chemical agents, end-labelled DNA pieces are exposed to spontaneous cleavage in adenine, cytosine, guanine, or thymine

locations. The chemical attack is focused on three stages: base modification, extraction of the altered base from its sugar, and splitting of the DNA strand at that sugar place (Maxam and Gilbert., 1977). Using PAGE, the components of these four responses are then segregated. The sequence can be obtained readily from the four concurrent routes in the sequencing gel. The template used for this sequencing procedure can either be double-stranded (ds)DNA or chromosomal DNA ssDNA. The pieces are generally first digested with a suitable restriction enzyme (Maxam and Gilbert., 1980), but they could also be ready from an embedded or reconstructed DNA area (Maxam et al., 1980). Such DNA templates are then labelled on one of strands. Mainly, this labelling was performed with [32P] phosphate or with a nucleotide connected to 32 P and enzymatically integrated into the end piece (Maxam & Gilbert, 1977). Optionally, [35S] dideoxyadenosine 5'-(α-thio) triphosphate ([35S] ddATPαS) and terminal deoxynucleotidyltransferase components were also used (Ornstein & Kashdan, 1985).

2.10.3 Pyrosequencing

Pyrosequencing is a real-time DNA sequencing method based on the detection of the PPi generated during the DNA polymerization response (Nyre!n and Lundin., 1985 ; Hyman, 1988. Ronaghi et al., 1996). There are currently two distinct solutions to pyrosequencing: solid-phase sequencing (Ronaghi et al., 1996) and liquid-phase sequencing (Ronaghi et al., 1998). Solid-phase sequencing (three-enzyme combination) needs a template washing step between nucleotide additions to extract non-incorporated deoxynucleotides and ATP derived from sulphurylase activity. A technique using ssDNA-binding proteins has recently been suggested. Those kinds of proteins displace primers that attach non-specific to the destination DNA template, thus minimizing non-specific signals (Ronaghi et al., 2000). This approach improved enzyme effectiveness, decreased mis-priming, enhanced signal strength, enhanced precision in identifying the amount of identical

neighbouring nucleotides in challenging templates, and gave read-lengths of more than 30 nucleotides.

2.10.4 Single-molecule sequencing with exonuclease

Single-molecule sequencing was originally developed as a laser-based method that enables the rapid sequencing of 40 kb or more DNA pieces at a speed of 100–1000 bases per second (Jett et al., 1989). This method is focused on detecting individual fluorescent nucleotides in a flowing sample flow (Shera et al., 1990; Harding & Keller., 1992). The technique is split into the following stages: fluorescent labelling of DNA in a given piece of DNA, Connection of this labelled DNA molecule to the microsphere, motion of the endorsed DNA molecule into a swirling buffer flow, digestion of the labelled DNA with exonuclease that chronologically cleaves the 3"-end nucleotides, and detection and verification of overall fluorescently labelled bases as they cross a oriented laser beam (Davis et al., 1991; Goodwin et al., 1997). Though a significant single molecule sequencing test has not yet been conducted, a mixture of all experimental processes has been proved (Dörre et al., 2001). Because natural bases in DNA have inherent quantum fluorescence outputs of less than 10-3at ambient temperature, the single-molecule sequencing technique requires the total labelling of each base in one strand. Every nucleotide form must be labelled with a distinctive dye, with big quantum fluorescence returns and recognizable spectral characteristics (Dorre et al., 1997).

2.10.5 Next-generation sequencing

Next-generation sequencing techniques cover a multitude of approaches depending on distinct technology. Though quite varied methods and biology were used in each phase from model library preparing, fragment amplification, to sequencing, they all embraced a huge matrix setup popularized by microarray

assessment – DNA specimens on the grid are concurrently evaluated in conjunction. Though quite varied methods and biology were used in each phase from model library preparing, fragment amplification, to sequencing, they all embraced a huge matrix setup popularized by microarray assessment – DNA specimens on the grid are concurrently evaluated in conjunction (Zhou *et al.*, 2010). Based on the overall outline, several main features can be readily noted. First, huge parallelism can be accomplished by configuring an organized or disordered range that provides a large degree of density of information. Conceptually, this is restricted only by the diffraction limit of light (i.e., half of the wavelength used to detect autonomous optical occurrences. This drastically improves the general sequencing operation throughput. Secondly, no electrophoresis is used, leading in great ease of miniaturization and less sample / reagent usage over 1st-generation technology (Zhou *et al.*, 2010).

2.11 Phylogenetic Reconstruction

Phylogenetic reconstruction techniques can be categorized into two primary strategies: distance-based techniques and character-based techniques. The idea of the former is the conversion of all sequence data into a distance matrix, which will then be evaluated using an algorithm to cluster the taxa. Constructing a tree with such a technique is quick, but in the procedure all sequence data is wasted. The latter technique is time-consuming since all the sequence data is used to evaluate the finest phylogenetic tree. The computation of phylogenetic trees by this technique can be performed using several methods, like those of maximum parsimony (MP), highest probability (ML), or Bayesian analyses (Ajawatanawong *et al.*, 2016).

The main idea of the techniques of distance matrix is the transformation of a pairwise sequence alignment into remote values. Because there should be three or more samples in a multiple sequence alignment (MSA), range values from all feasible pairwise sequences produce a distance matrix. After a matrix has been

created, the alignment is not anymore used for phylogenetic reconstruction. By this stage, the matrix is being used as the tree construction input. Various tree construction strategies used involve unweighted couple group technique with arithmetic mean (UPGMA), weighted couple group technique with arithmetic mean (WPGMA), neighbour-joining (NJ), least square (LS), and minimal development (ME) techniques (Popescu et al., 2012).

There are many techniques created from character-based approaches, such as maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference techniques. These methods strive to reconstruct a phylogeny straight from the information of the series, without any conversion (Fujita et al., 2012). They give highly slow calculations, but it is said that the ultimate tree is very precise (Maddison et al., 2014). Fleetingly, the algorithm used in such starts by ranking all feasible phylogenies that can be produced from the n taxa. Therefore, the ideal tree is presumed to be the highest rating tree. Even so, it would be almost absurd to score all individual trees once the number of taxa is larger than 20 (as this means the number of actual possible trees is greater than 2,211018) using a covetous method that scans for all possible trees (Fujita et al., 2012).

MATERIALS AND METHODS

3.MATERIALS AND METHODS

The study on "Molecular phylogeny of South Indian *Aphthona spp.*" was carried out at the Department of Plant Biotechnology and Department of Agricultural Entomology, College of Agriculture, Vellayani, Kerala Agricultural University during the period 2018-2019. The materials used and methodologies adopted are presented in this chapter.

3.1 Sample collection and Identification

Different species of *Aphthona* were gathered from multiple places such as Vellayani, Ooruttabalam, Munnar, Ponmudi, Mattupeti, Pampadumchola from different host plant belonging to the family *Euphorbiacea* like *Macrangae*, *Euphorbia hirtae*, Phyllanthus *emblica* etc. Some samples were gathered from the private collection of Dr. K. D. Prathapan, Assistant Professor, Department of Agricultural Entomology, College of Agriculture Vellayani. The collected specimens were identified with the help of Dr. K. D. Prathapan using the morphological keys described by him.

3.2 DNA isolation

Modified CTAB method detailed by Rogers and Bendich (1994) was used for the extraction of total genomic DNA from identified specimens. For large beetles only, hind legs were used and for small beetles, whole organism was used for isolation of total genomic DNA. For isolation of genomic DNA from old dried insect samples Quiagen tissue and blood DNA isolation kit was used with slight modification of the manufactures protocol. The protocol is given below.

3.2.1 Procedure

* ❖ Either the whole specimen (in case of small beetles) or hind legs (in case of large beetles) were taken in an autoclaved 1.5ml Eppendorf tube containing 20μl CTAB buffer (2X) and crushed using micro pestle and then remaining 80μl of CTAB buffer was added.

* ❖ The Eppendorf tube was then kept in water bath at 56-65^{0}C for one hour after vertexing for two minutes.

* ❖ Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently for two minutes. Spun at 10000 rpm for 15 minutes at 4^{0}C.

* ❖ After centrifugation, the contents got separated into three distinct phases.

  Aqueous topmost layer - DNA and RNA

  Middle layer - fine particles and proteins

  Lower layer - Chloroform, pigments and cell debris

* ❖ The 85μl of top aqueous layer was transferred to a clean centrifuge tube. 200μl of chilled 96% alcohol and 30μl sodium acetate was added.

* ❖ Mixture was kept for incubation at -20^{0}C for 1hour.

* ❖ Mixture was centrifuged at 13000 rpm for 10 minutes at 4^0 C and remove the supernatant.

* ❖ Five hundred microliter of 70% alcohol was added and centrifuged at 13000 rpm.

* ❖ Alcohol was removed slowly and kept in dry bath at 60^{0}C for 15 minutes.

* ❖ DNA was dissolved in 15μl of double distilled water.

3.2.2 Procedure for isolation of DNA from dried samples

- ❖ Added 180µl of buffer ATL (tissue lysis buffer) to labelled microcentrifuge tubes.

- ❖ Using sterile forceps placed the beetle sample in the corresponding labelled 1.5ml microcentrifuge tube.

- ❖ Using a sterile micro pestle, the tissue was homogenized.

- ❖ Added 20µl proteinase K to the microcentrifuge tube.

- ❖ Mixed the contents very well by vortexing.

- ❖ Incubated the mixture in water bath set at 56^0C with gentle agitation for several hours (4-5 hours). Completion of lysis can be affirmed by observation of digested inner tissue as well as enhanced brownish colour of lysate solution.

- ❖ Added 200µl buffer AL to the digestion buffer (lysate) and was vortexed.

- ❖ Added 200µl 100% ethanol to the digestion buffer (lysate) and was vortexed.

- ❖ Pipetted out the lysate mixture from step 8 into the DNeasy Mini spin column placed in a 2 ml collection tube.

- ❖ Centrifuged the lysate at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

- ❖ Placed the DNeasy Mini spin column from step 9 into a new 2 ml collection tube, add 500µl buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.

- ❖ Placed the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

- ❖ Placed the DNeasy Mini spin column in a clean, labelled 1.5 ml microcentrifuge tube and pipet 100 µl warmed buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 15min, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.

* Repeated the elution step with the elutate obtained in previous step using same centrifuge tube same column, for increasing yield and final concentration of DNA.
* The final elutate was stored in -20^{0}C freezer.

3.3 Agarose Gel Electrophoresis for DNA Quality check

The quality of the isolated DNA was verified using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was mixed with 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) solution comprising 0.5 µg/ml ethidium bromide. Electrophoresis was performed at 75 V with 0.5X TBE until the front of the bromophenol dye migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the picture was caught using the Gel documentation system (Bio-Rad) under UV light.

3.3.1 Procedure

* For casting the gel, the gel tray was prepared by sealing the ends with tape. Comb was placed vertically such that the teeth are about 1 to 2mm above the surface of the tray.
* Prepared 2% agarose in a glass conical flask by dissolving 1.2g agarose in 60ml 1X TBE buffer (1.2ml TBE from stock which was made up to 100ml). The contents were heated for 45 to 60 seconds until agarose was dissolved and solution was clear.
* Solution was allowed to cool to 42^0 C to 45^0 C under room condition and at this point 4µl ethidium bromide was added to a concentration of 10µg/ml and mixed well.
* This warm gel solution was poured into the tray to a depth of about 5mm and the gel was allowed to solidify for about 30 to 45 minutes at room temperature.

*   After the expiry of time, the comb and the tape used for sealing the gel tray along with the gel were gently removed and the tray was placed in electrophoresis chamber and covered (just until wells are submerged) with 1X electrophoresis buffer.

*   Samples for electrophoresis were prepared by adding 1µl of 6X gel loading dye for every 10µl of DNA and by mixing them well. Loaded 6µl DNA sample per well.

*   The electrophoresis was carried at 100 volts until dye has migrated two third of the length of the gel.

*   The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein. The intact DNA has appeared as orange fluorescent bands and the degraded one appears as a smear, because of the presence of a large number of bands, which differed in few base lengths. The presence of thick white patches, trapped in the well was taken as protein contamination whereas, thick bases below 100bp was understood as RNA contamination

3.4 Quantification of DNA

Quantification of DNA was carried out using UV visible spectrophotometer (Systronics). The optical density of the DNA samples was recorded at both 260 nm and 280 nm wave lengths. The concentration of DNA was calculated using the following formula:

Amount of DNA (µg/µl) = A260* dilution factor/1000

Where A260 = absorbance at 260 nm

The quality of the DNA was judged from the ratio of the OD values recorded at 260 nm and 280 nm. The A260/A280 value 1.8 shows the best quality of DNA.

3.5 Amplification of cytochrome oxidase 1 gene

Complete gene sequence of cytochrome oxidase gene of *Aphthona* beetles were retrieved by sequencing the amplified CO1 gene using universal Folmer primer (Folmer *et al.*, 1994). Based on the sequence information, specific primer for *Aphthona* beetles was designed with the help of Primer 3 software. Alignment and melting temperature, secondary structure formation of designed primer was checked using Primer express software. The sequence of the primers used for the amplification of cytochrome oxidase gene is shown below.

Folmer primer – Forward- 5'-ggtcaacaaatcataaagatattgg-3'

Reverse- 5'-taaacttcagggtgaccaaaaaatca-3'

Aphthona specific- Forward- 5'-CATGGGGAATGCTTAGATGC-3'

Reverse- 5'- AAACTTTCAGGGTGACCAAAAA-3'

A standard PCR mix was prepared for 25µl total volume containing 100ng of template DNA, 200µM dNTPs, 10pM of primers, 1 unit of Taq DNA polymerase, and 1X Taq polymerase buffer. The DNA was amplified using a thermal cycler (Biorad-thermal cycler) using programme shown in the table below.

Table 1: Details of PCR programme used for CO1 amplification.

Step	Stage	Cytochrome oxidase 1 primers	
		Temperature (°C)	Duration
1	Initial denaturation	94	4 minutes
2	Denaturation	94	30 sec

3	Annealing		45	30 sec
		Folmer primer		
		Aphthona specific primer	55	30 sec
4	Extension		72	45 sec
5	Final extension		72	7 minutes

The steps 2-4 were allowed to repeat 32 times.

The PCR product was electrophoretically analysed in agarose gel (1%) in a horizontal gel electrophoresis unit (as explained above). One of the wells was loaded with 5 µl of the 100bp molecular weight marker (Merc Genei) with required volume of the gel loading dye reached three fourth length of the gel and the gel was documented.

3.6 Gel documentation

Gel documentation was done with BioRad Gel Documentation System using 'Image lab' software. Image lab is a software package for imaging, analysing, and databasing the electrophoresed gels. The gel containing DNA was viewed under UV light due to ethidium bromide dye. The image of a gel is captured using the Image lab controls in the imaging device window and band size was confirmed.

3.7 PCR product sequencing

The PCR products amplified using ITS2 and COI primers were run on 1 per cent agarose gel. The PCR product, confirmed to yield only single band on electrophoresis, was sequenced with specific set of primers. The sequencing was carried out at RGCB, Trivandrum.

3.7.1 Sequencing

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer	-	3.2 pM (Forward or Reverse)
Sequencing Mix	-	0.28 µl
DMSO	-	0.30 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96oC for 2 minutes followed by 30 cycles at 96oC for 30 sec, 50oC for 40 sec and 60oC for 4 minutes.

3.7.2 Post Sequencing PCR Clean up

❖ Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared.

❖ 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed.

❖ 52 µl of master mix II was added to each reaction.

❖ Contents were mixed by inverting and incubated at room temperature for 30 minutes

❖ Spun at 14,000 rpm for 30 minutes

❖ Decanted the supernatant and added 100 µl of 70% ethanol

- ❖ Spun at 14,000 rpm for 20 minutes.
- ❖ Decanted the supernatant and repeated 70% ethanol wash
- ❖ Decanted the supernatant and air dried the pellet.

The cleaned-up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.7.3 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.*, 2010).

3.8 Phylognetic analysis

The partial CO1 gene sequences obtained were aligned and combined with the CO1 gene sequences of the same genus, class, order (Details are given in the table). Phylogenetic tree using the Maximum Likelihood method in MEGA software using the above dataset. For genetic distance calculation between the south Indian *Aphthona spp.*, kimura based modelling in the MEGA software was used.

Table 2: Details showing the gene sequences used in phylogenetic analysis with their accession number.

Sl no	Species name	Accession no
1	*Polyommatus icarus*	HM874671.1
2	*Acalymma albidovittatum*	AY242447.1

3	*Agelastica coerulea*	KU188468.1
4	*Galerucella calmariensis*	KR488823.1
5	*Chrysomela aeneicollis*	AY243662.2
6	*Donacia provostii*	EF532509.1
7	*Eumolpinae sp*	HF921986
8	*Altica aenescens*	KU917046.1
9	*Amphizoa lecontei*	AY071797.1
10	*Capraita obsidiana*	AF479453.1
11	*Chaetocnema concinna*	MH407426.1
12	*Crepidodera aurata*	MH020470.1
13	*Dibolia borealis*	KR121416.1
14	*Disonycha latifrons*	KM850626.1
15	*Epitrix brevis*	JQ947980.1
16	*Glyptina cyanipennis*	FJ977951.1
17	*Hippuriphila modeeri*	HQ954160.1
18	*Kuschelina rugiceps*	AF479480.1
19	*Longitarsus aeneicollis*	MH323185.1

20	*Orthaltica copaline*	AY171410.1
21	*Phyllotreta albionica*	KM850883.1
22	*Podagrica malvae*	MH323322.1
23	*Psylliodes affinis*	KR126815.1
24	*Sphaeroderma testaceum*	HQ953592.1
25	*Systena blanda*	FJ973986.1
26	*Eriphia scabricula*	KC771023.1
27	*Aphthona euphorbiae*	KU918471.1
28	*Aphthona herbigrada*	MH322715.1
29	*Aphthona venustula*	MH020465.1
30	*Aphthona atrovirens*	KU915359.1
31	*Aphthona sp*	KX943353.1

RESULTS

4. RESULTS

The study 'Molecular phylogeny of the South Indian *Aphthona spp.* (Coleoptera: *Chrysomelidae*)' has been carried out at the Department of Agricultural Entomology and Department of Plant Biotechnology, College of Agriculture Vellayani, Kerala Agricultural University. The objective of the study was to identify the genetic structure of South Indian *Aphthona spp.* using mitochondrial DNA markers and to identify patterns of intra generic genetic diversity among South Indian *Aphthona spp.* The results of various aspects of the investigation are detailed in this chapter.

4.1 Purposive survey

A total of ten species of *Aphthona* were gathered from multiple places such as Vellayani, Ooruttabalam, Munnar, Ponmudi, Mattupeti, Pampadumchola and Dandeli from different host plant belonging to the family *Euphorbiaceae* viz., *Macarangae*, *Euphorbia hirta* and *Phyllanthus* spp. etc. All the samples were collected in rainy seasons. The beetles were seen in moist cool climate condition. Collection of samples between 7.30 am-8.30 am during morning and between 4.30-6.00 resulted in giving maximum number of beetles from a given site. The freshly collected specimens were immediately transferred to 70% ethanol and were kept in -20^0C refrigerator till molecular analysis. Some samples were gathered from the private collection of Dr. K. D. Prathapan, Assistant Professor, Department of Agricultural Entomology, College of Agriculture Vellayani. The dried specimens thus obtained were kept in autoclaved distilled water overnight to increase the translucency of the tissue. Otherwise the tissue was found to be hard to lyse. The following table shows the details of specimens collected. Images of the specimens are shown in plates (Plate 1- Plate 4).

Table 3: Details of sample collected

Sl no	Species name	Host Plant	Location
1	*Aphthona nigrilabris*	*Euphorbia hirta*	College of Agriculture, Vellayani, Kerala
2	*Aphthona phyllanthae*	*Phyllanthus emblica*	College of Agriculture, Vellayani, Kerala
3	*Aphthona macarangae*	*Macaranga peltata*	College of Agriculture, Vellayani, Kerala
4	*Aphthona bombayensis*	*Phyllanthus* species.	College of Agriculture, Vellayani, Kerala
5	*Aphthona tamila*	*Glochidion zeylanicum*	Pampadumchola, Kerala
6	*Aphthona marataka*	*Macaranga peltata*	Mattupettti, Kerala
7	*Aphthona glochidionae*	*Glochidion zeylanicum*	Ponmudi, Kerala
8	*Aphthona mallotae*	*Mallotus philippinensis*	Dandeli, Karnataka
9	*Aphthona chrosophorae*	*Croton* species.	Munnar, Kerala
10	*Aphthona chrosophorae* 2	*Croton* species.	Munnar, Kerala

A.*Aphthona chrozophorae 2*

B.*Aphthona marataka*
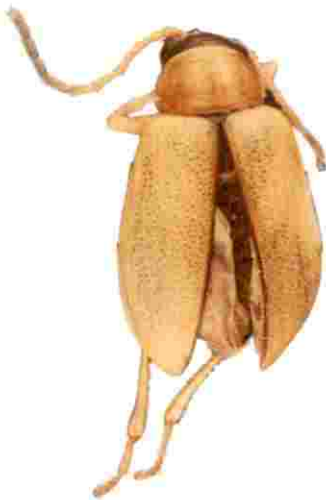
C.*Aphthona chrozophorae*

D.*Aphthona tamila*

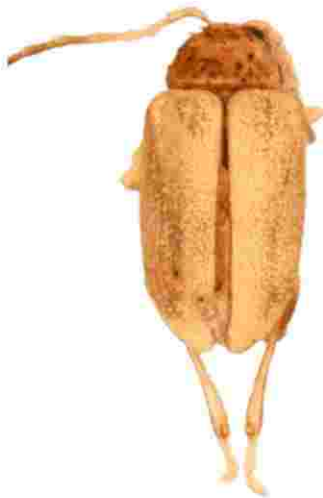**Plate1: Images of specimens collected from Munnar**

A. *Aphthona nigrilabris*

B. *Aphthona phyllanthae*

C. *Aphthona macarangae*

D. *Aphthona bombayensis*

Plate 2: Images of specimens collected from College of Agriculture, Vellayani.

Aphthona mallotae

Plate 3: Image of specimen collected from Dandeli

Aphthona glochidionae

Plate 4: Image of specimen collected from Ponmudi hills

4.2 Molecular analysis

4.2.1 DNA Isolation

Total genomic DNA was isolated from all eleven species using modified CTAB method (Rogers and Benedich, 1994). The subsequent agarose gel electrophoresis has indicated clear bands without contamination. Image of agarose gel showing DNA bands is shown in plate 5. Spectrophotometric analysis gave ratio of UV absorbance ratio (A260/280) 1.8 to 2.0.

4.2.2 Assessing the quality of DNA using spectrophotometer

The purity of DNA was checked using spectrophotometer. Nucleic acid shows absorption maximum at 260 nm whereas protein shows peak absorbance at 280 nm. Absorbance has been recorded at both wavelengths and the purity was indicated by the ratio OD260/OD280. A value 1.8 indicated that the DNA is pure and free from proteins and RNA. Spectrophotometric analysis gave ratio of UV absorbance ratio (A260/280) around 1.8. Quality and quantity of DNA isolated from *Aphthona* species assessed by spectrophotometer method are presented in Table below.

Table 4: Quality and quantity of DNA isolated from *Aphthona* species assessed by spectrophotometer method

Species name	UV absorbance at 260 nm (A260)	UV absorbance at 280 nm (A280)	A260/A280	Quantity (μg/μl)
A. nigrilabris	2.072	1.129	1.84	103.6

|				
A. phyllanthae	2.586	1.348	1.82	129.3
A. macarangae	1.567	0.789	1.86	78.35
A. bombayensis	4.343	2.308	1.88	217.15
A. tamila	5.529	2.838	1.86	44.25
A. marataka	4.956	2.588	1.82	514.7
A. glochidionae	2.220	1.220	1.80	111
A. mallotae	2.056	1.145	1.79	102.8
A. chrozophorae	2.345	1.289	1.81	117.2
A. chrozophorae 2	4.390	2.008	2.180	219.5

4.2.3 Amplification of Cytochrome oxidase subunit 1 loci with selected primers

Using the initially reported primers, high quality total genomic DNA was used to amplify the CO1 loci. Cytochrome c Oxidase subunit I gene, was successfully amplified using the selected primers. Both primers gave amplicons in the size range of 680-700 bp. From these products, species-specific sequences were generated. The PCR assay was standardized, which gave intended results. Candidate locus amplification was assessed through 1 percent agarose gel electrophoresis. The barcoding primers used resolved the COI gene into specified clear bands without contamination when visualised under the gel documentation system (Plate 6& Plate 7).

Lane 1: *A. bombyensis*, Lane 2: *A. nigrilabris*, Lane 3: *A. phyllanthae*, Lane 4: *A. macarangae*,Lane 5: *A. marataka*, Lane 6: *A. chrozophorae*, Lane 7: *A. tamila*, Lane 8: *A. glochidionae*, Lane 9: *A. chrozophorae*, Lane 10: *A.mallotae*.

Plate 5: Image: 0.8% Agarose gel image of genomic DNA isolated
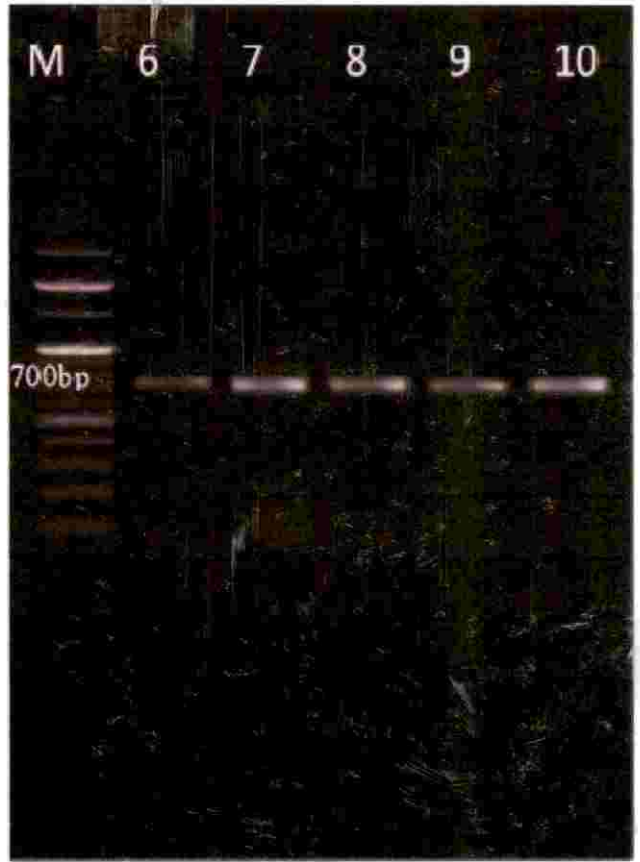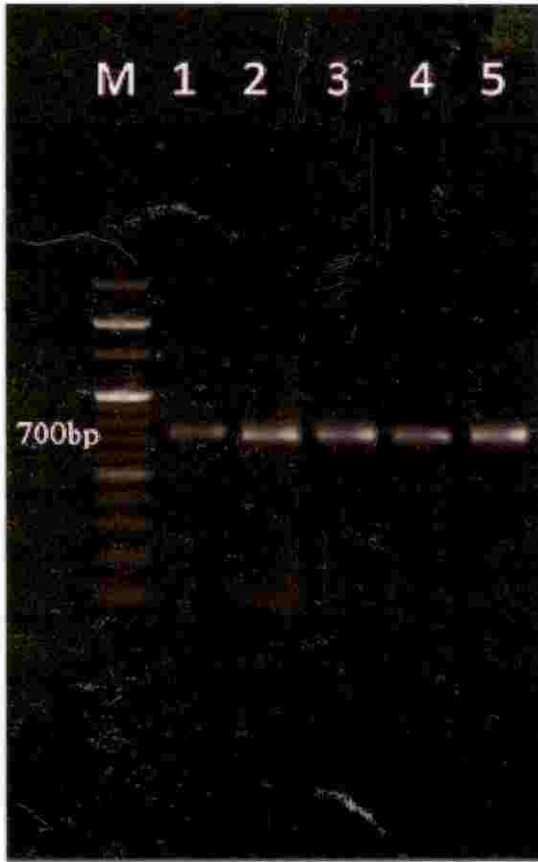
Lane 1: *A. bombyensis*, Lane 2: *A. nigrilabris*, Lane 3: *A. phyllanthae*, Lane 4: *A. macarangae*, Lane 5: *A. marataka*, Lane 6: *A. chrozophorae*, Lane 7: *A. tamila*, Lane 8: *A. glochidionae*, Lane 9: *A. chrozophorae*, Lane 10: *A.mallotae*.

Plate 6: Amplification profile of PCR with Folmer primer on 1.2% of agarose gel.
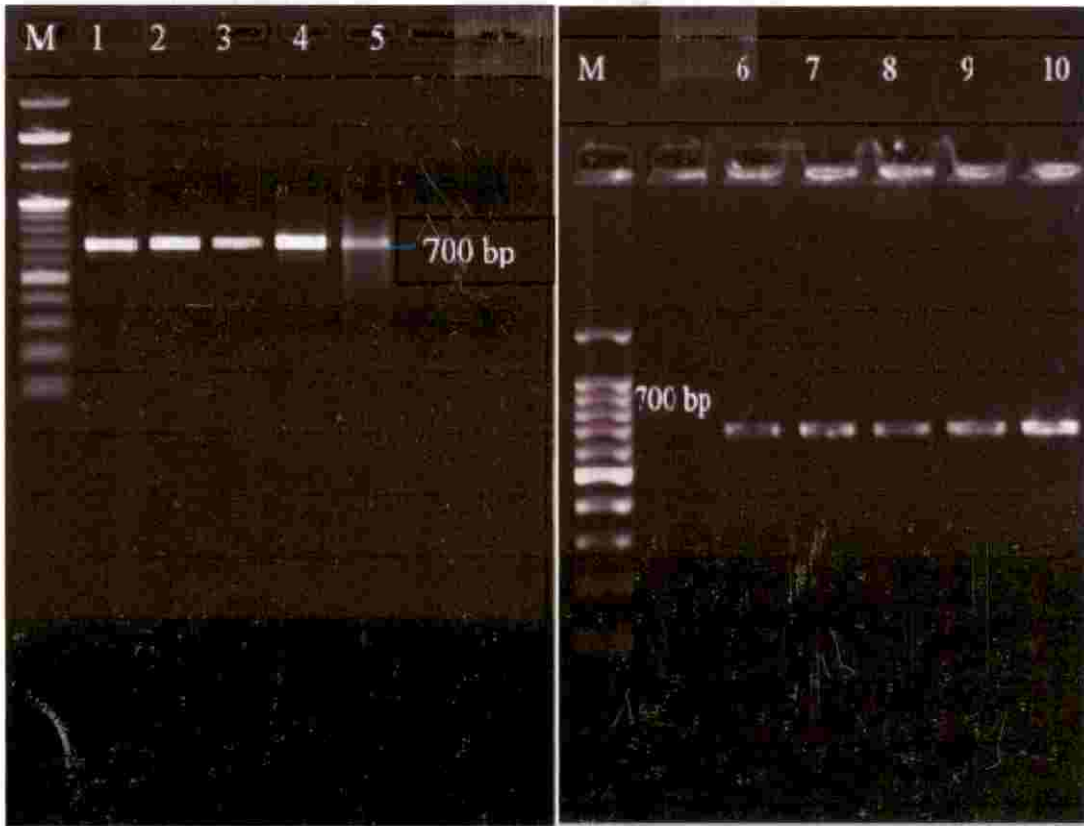
Lane 1: *A. bombyensis*, Lane 2: *A. nigrilabris*, Lane 3: *A. phyllanthae*, Lane 4: *A. macarangae*, Lane 5: *A. marataka*, Lane 6: *A. chrozophorae*, Lane 7: *A. tamila*, Lane 8: *A. glochidionae*, Lane 9: *A. chrozophorae*, Lane 10: *A.mallotae*.

Plate 7: Image of amplification profile of *Aphthona* specific primer on 1.2% agarose gel.

4.3 Phylogenetic Analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-5887.30) is shown in figure. The phylogenetic tree generated with the help of COI sequences from the collected samples (represented as *Aphthona*) and the already published Gen bank sequence of *Aphthona* formed a single clade which reveals that, all collected samples belongs to the desired genus. The tree thus drawn is shown in the Plate 8.

The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. This analysis involved 42 nucleotide sequences. There was a total of 415 positions in the final dataset per site. This analysis involved 42 nucleotide sequences.

To understand the intra generic genetic diversity among south Indian *Aphthona* species, a phylogenetic tree was drawn using MEGA software with ten sequences obtained by sequencing the samples collected from different locations at Kerala and Karnataka. *Chrysomela aeneicollis* is selected as the outgroup for this tree. The phylogenetic tree showing relationship among south India *Aphthona* spp. is given in the plate 9.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.32240403 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each

sequence pair (pairwise deletion option). There was a total of 844 positions in the final dataset. Evolutionary analyses were conducted in MEGA. The genetic distance between the species calculated using MEGA software is shown in the below table 5.

Table 5: Genetic distance between south Indian *Aphthona spp.*

	Chrysomela_ aeneicollis	A._bombaye nsis	A._chrosoph orae_2	A._chrosoph orae	A._glochidio nae	A._mallotae	A._nigrilabri s	A._macarang ae	A. phyllanthae	A. tamila
Chrysomela_ aeneicollis										
A._bombaye nsis	0.29 5185									
A._chrosoph orae_2	0.30 6005	0.22 637								
A._chrosoph orae	0.30 9262	0.22 6399	0.00 341							
A._glochidio nae	0.32 423	0.20 086	0.25 555	0.25 384						
A._mallotae	0.31 0312	0.21 1074	0.20 9836	0.21 4217	0.20 297					
A._nigrilabri s	0.27 9839	0.21 7638	0.19 5807	0.21 5064	0.19 6102	0.20 4721				
A._macarang ae	0.32 0594	0.22 1219	0.19 7176	0.20 8616	0.19 5192	0.09 0091	0.21 095			
A. phyllanthae	3.65 206	3.60 38	3.45 497	3.45 377	3.33 880	2.84 769	2.98 418	3.05 98		
A. tamila	0.26 7043	0.18 270	0.23 447	0.24 313	0.13 205	0.21 412	0.21 983	0.20 680	3.21 757	

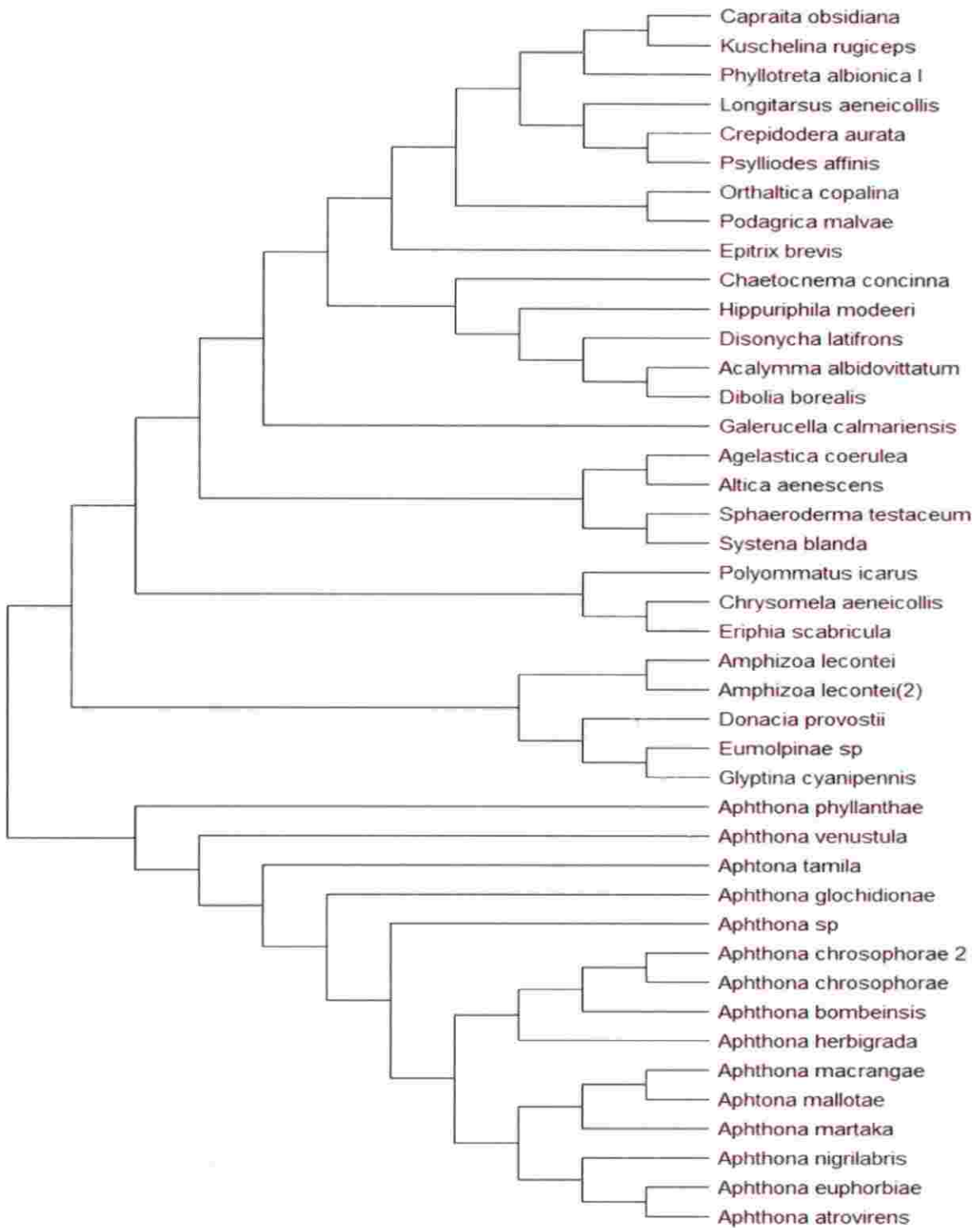| A. marataka | 0.32 281 | 0.22 121 | 0.19 883 | 0.20 861 | 0.19 696 | 0.21 317 | 0.21 947 | 0.09 1659 | 3.05 98 | 0.20 836 |

Plate 8: Phylogenetic tree showing relationship of *Aphthona* group with higher level organism
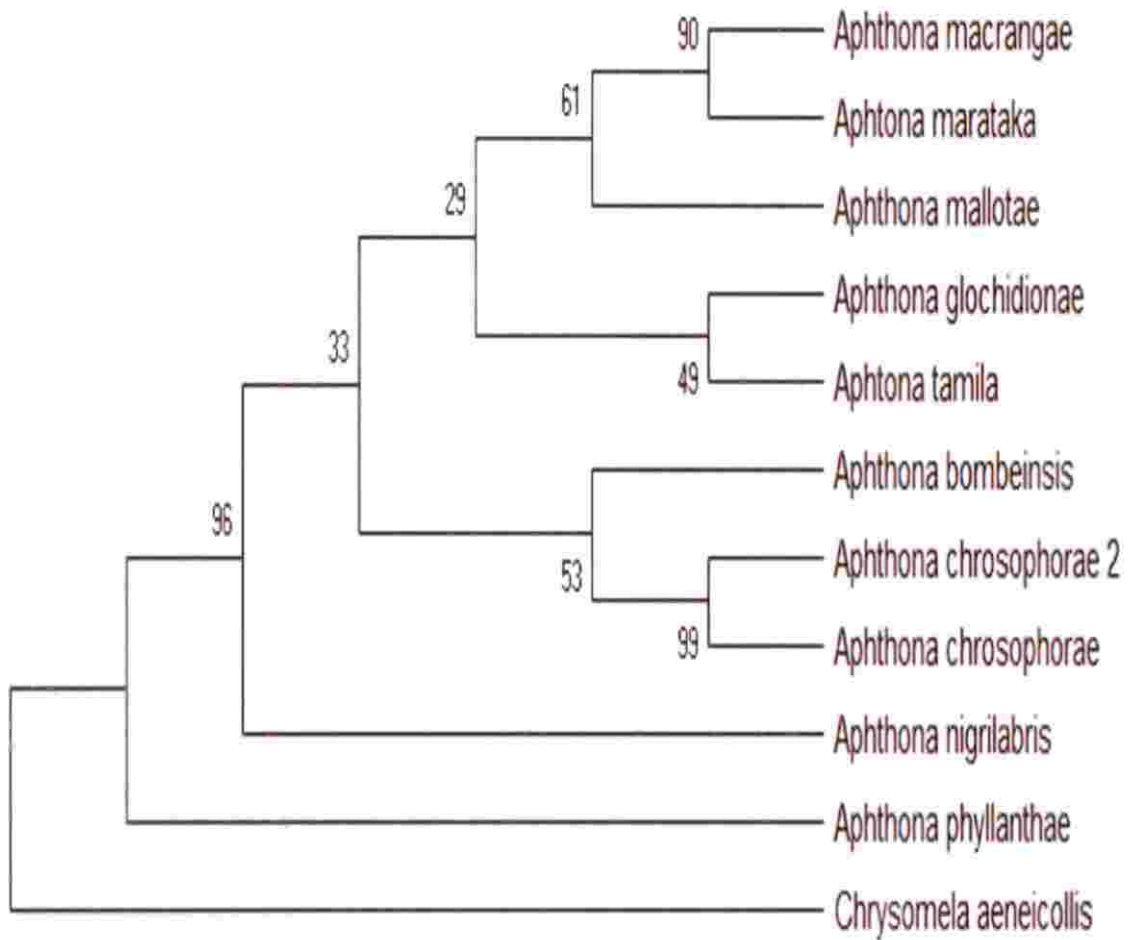
Plate 9: Intrageneric phylogenetic tree of south Indian *Aphthona spp.*

DISCUSSION

5. DISCUSSION

The flea beetle genus *Aphthona* is broad and contains over 350 species that are spread across the biogeographic regions of the Palearctic, Oriental, African, and Australian Old World (Konstantinov *et al.,* 2002). *Aphthona* species were weakly recognized taxonomically. *Aphthona* species are generally mesophilous, however they are diverse ecologically, so that they occur in a wide variety of biogeographic regions and inhabit almost all known habitat ranging from low land rainforest to high altitude coniferous mountains and from desert s to sub artic environs. Most frequently, *Aphthona* is discovered in wet meadows or on vegetation along waterways and streams (Konstantinov *et al.,* 2002). As with most flea beetles, *Aphthona* species are specialized phytophagous insects. They mostly feed on plants belonging to 11 different families. Most of them are Dicotyledenae but 3 of the are monocotyledenae. The host specialisation in *Aphthona* is narrower than that of most flea beetles (Konstantinov *et al.,* 1996). Members of this genus are small to moderate in size (1-4 mm), oval or oblong-oval and more or less convex in lateral view. Based on previous cladistic study, three synapomorphic characters define *Aphthona*: elytron length/width ratio less than 2.85; anterior part of metanotal ridge e attached below middle of ridge b-1; and setae on ventral side of first metatarsomere sinuate (Konstantinov *et al.,* 1998). Although several Indian workers like Prathapan (2003&2010) have contributed towards classical studies which helped to resolve the taxonomic mystery of Indian *Aphthona* species, information on the molecular systematic of Indian *Aphthona spp.* is scant. This study was carried out to resolve the intra generic genetic diversity of study the intra generic genetic variation of south Indian *Aphthona spp.* and thus reveal its phylogenetic status.

A total of ten different species native to south India were collected from different locations across Kerala and Karnataka. The collected samples were identified using morphological keys given by Prathapan (2003). *Aphthona nigrilabris, Aphthona phyllanthae, Aphthona bombyensis, Aphthona macarangae, Aphthona marataka, Aphthona glochidionae, Aphthona tamila,*

Aphthona mallotae, Aphthona chrozophorae, Aphthona chrozophorae 2 were the collected specimens. All of the beetles collected were from plants belonging to the Euphorbiaceae family. The distribution of *Aphthona* spp. and host plants are same as described by Prathapan (2003). The species *A. nigrilabris* is widely distributed. *A. macarangae, A. phyllanthae, A. chrozophorae, A. tamila* and *A. bombyensis* are more abundant in low altitude regions. *A. marataka, A. mallotae* and *A. glochidionae* are more frequently found in high altitude regions. The morphological keys described by Prathapan (2003) was used to identify and confirm the species. *Aphthona* species are more active during rainy seasons. There is a better chance of collection of beetles during June- July months.

The *Aphthona* species were characterized with two different set of primers, each amplifying the mitochondrial cytochrome oxidase subunit 1 gene. The candidate loci used in the study lays in the mitochondrial region. After the total genomic DNA isolation using specific primers mitochondrial cytochrome oxidase subunit 1 gene was amplified. DNA isolation was done using modified CTAB method (Rogers and Benedich, 1994). For dried insect tissue sample, commercial kit was used. Legs of male beetles were used for DNA isolation, as there is less chance of DNA contamination with gut micro fauna. Since beetle lack phenols, browning was not a problem and the use of antioxidant such as PVP was not necessary. The quality of the isolated DNA was checked using spectrophotometric method. A DNA sample is said to be of high quality if it is free from RNA and protein contamination (Wettasing and Peffley, 1998). In spectrophotometric method, the DNA is said to be good quality when ratio between the absorbance 260 nm and absorbance at 280 nm is between 1.8-2.0. The optical density at the given wavelengths were worked out for samples and majority of the samples recorded ratio between 1.8-2.00. A total of 10 sequences of *Aphthona* species were amplified with two selected primers and sequenced by outsourcing (Rajiv Gandhi Centre for Biotechnology). Cloning was not carried

out in the study since there are specific set of primers. Both forward and reverse sequencing was done for all samples using Sanger's platform.

The sequence homology was checked using BLAST (Basic Local Alignment Search Tool) tool. It takes the query (DNA or protein sequence) and searches either DNA or protein databases for levels of identity that range from perfect matches to very low similarity. Using statistics, it reports back to you what it finds, in order of decreasing significance, and in the form of graphics, tables, and alignments (Altschul *et al.*, 1990). It is also helpful in annotating the sequences. Sequence annotation is the method with which coding and non-coding areas and other particular positions that are relevant to the DNA sequence are recognized. There is a possibility that the sequences acquired may be in the wrong direction. Checking the alignment of sequences using BLASTn makes the structure appropriate for further evaluation. In BLASTn analysis of the south Indian *Aphthona*, species having range of similarity from very high similarity to low level similarity could be observed. The high similarity sequences were of already sequenced *Aphthona spp.* The sequences were used to find out the interspecific genetic distance using the kimura 2 model with the help of MEGA software. The overall mean distance between the south Indian *Aphthona spp.* was found to be 0.39. The distance indicates the conspecific mean genetic divergence of the CO1 sequences. A similar distance calculation in *Aphthona* group was done Roehrdanz *et al.*, (2011) which included only 5 species of *Aphthona* species which were introduced to Canada for the biological control of leafy spurge.

Phylogenetic analysis was carried out using MEGA software to determine the genetic relation between the species. It is advocated through the creation of phylogenetic tree. Two trees were constructed using the CO1 sequences. The first tree was constructed with 42 sequences, which consists of all the south Indian *Aphthona* sequence along with sequences of *Aphthona* beetles taken from NCBI (National Centre for Biotechnology Information) and sequences of organism belonging to higher taxonomic levels such as subfamily, family and

order to form an intergeneric phylogenetic tree which is depicted in plate 8. In the tree, all the *Aphthona* species have formed a separate clade, confirming the genus. To understand the intrageneric genetic variation between the south Indian *Aphthona spp.* All the ten CO1 sequence were used to construct the tree keeping *Chrysomela aeneicollis* as the outgroup. The tree is shown in plate 7. In the tree, species *A. glochidionae, & A. tamila, A. chrozophorae & A. chrozophorae2, A. marataka & A. macarangae* have come together under the same clade, indicating that they are closely related. To have a better understanding of genetic variation, the pairwise genetic distance between the CO1 sequences of *Aphthona* species were calculated using kimura 2 parameter under 1000 bootstrap. The pairwise genetic distance value is shown in table 5. The pairwise genetic distance within the CO1 sequences was calculated using kimura 2 parameter, with MEGA software. If genetic distance between two sequences is less than 0.2, then those species are considered as same (Vogle *et al.*, 1993). The genetic distance in the given matrix range between 0.003 to 3.652. From the data it is clear that *A. phyllanthae* is the most diverged species among south Indian *Aphthona*. The genetic distance between species that have formed the same clade are as follows, genetic distance between *A. glochidionae* and *A. tamila* is 0.132, distance between *A. chrozophorae* and *A. chrozophorae 2* is 0.003 and distance between *A. marataka* and *A. macarangae* is 0.090. So, based on these observations of genetic distance, the afore mentioned species can be considered as synonymous.

A. chrozophorae and A. chrozophorae 2 was believed to be sister species but through this study that assumption is ruled out. From the genetic distance it is clear that they are the same species having 99% sequence similarity. In *Aphthon*a, the species group is determined by the morphological colours. The colours range from yellow non-metallic to bright metallic. There are no intermediate colouring patterns. Based on the genetic distance between south Indian *Aphthona* spp. calculated using kimura 2 model, the species *A. marataka* which is a bright metallic green coloured beetle, found in higher altitude habitats is identified synonymous with *A. macarangae* which is yellow

non-metallic in colour and inhabits lower altitudes. Similarly, the species *A. glochidionae* which is metallic black in colour which inhabits high altitude regions, is found to be synonymous with a yellow non-metallic low altitude inhabiting species, *A. tamila*. These species also share a considerable amount of morphological similarity. Similar colour polymorphism was reported in *Oriena* genus; *Chrysomeliade* by Van noort (Van noort *et al.*, 2013). Prior to this study, the existence of colour morphs in the group was not known. Thus, based on morphological observation and molecular data, these species are designated as colour morphs.

SUMMARY

6. SUMMARY

The study entitled "Molecular phylogeny of South Indian *Aphthona* spp. (*Coleoptera: Chrysomelidae*)" was carried out at the Dept. Plant Biotechnology and Department of Agricultural Entomology, College of Agriculture, Vellayani, during the period 2018-2019. The objective of the study was to identify the genetic structure of South Indian Aphthona spp. using mitochondrial DNA markers and to identify patterns of intra generic genetic diversity among South Indian *Aphthona* spp. Cytochrome oxidase subunit 1 (COI) candidate loci was selected for the study. Aphthona beetles were collected from various parts of western ghats including different sites of Kerala and Karnataka. Plants from which Aphthona beetles were collected belong to the family *Euphorbiaceae*.

The protocol for genomic DNA isolation from *Aphthona* beetles was standardised. The protocol suggested by Rogers and Benedict (1994) with slight modification made after crushing the sample was found to be most appropriate for isolation of DNA from spider mites. A protocol for DNA isolation from dried insect samples, using Qiagen Blood and Tissue kit was also standardised. The quality and quantity of DNA was analysed using spectrophotometric method. The absorbance ratio varied from 1.8 to 1.9, indicating excellent DNA quality and heightened recovery. This DNA was appropriate for the PCR evaluation. A specific primer was developed for the amplification of selected mitochondrial region. Folmer primer (Folmer *et al.*, 1994) was also used for amplifying. Both the primers generated the expected molecular weight bands which were suitable for sequencing. The sequences generated through Sanger sequencing (Sanger *et al.*, 1975) was submitted to NCBI successfully. All the 10 sequences were used to generate an intragenomic phylogenetic tree using MEGA software. The phylogenetic tree differentiated all the 10 species. Out of the ten species, *A. glochidionae & A. tamila, A. chrozophorae & A. chrozophorae 2, A. marataka & A. macarangae* were found to be closely related.

From the genetic distance calculated based on the CO1 sequences the species *A. tamila* & *A. glochidionae*, *A. marataka* & *A. macarangae*, *A. chrozophorae* & *A. chrozophorae 2* were found to be synonymous respectively. The morphological and molecular results correlated in confirming the colour polymorphism among the south Indian *Aphthona* spp. The species *A. tamila* & *A. glochidionae*, *A. marataka* & *A. macarangae* were confirmed as colour morphs. Prior to this study colour polymorphism in south Indian *Aphthona* spp. was not reported so this is a major finding of the study. In future further study may carried out to bring out and characterize the factor causing the colour polymorphism.

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ANNEXURES

ANNEXURE 1

Materials used during collection pf specimens

Materials

- ❖ Sweep net.
- ❖ 70% Ethanol.
- ❖ 50 ml Screw cap bottle.
- ❖ Paint brush (Zero size).
- ❖ Microscope 10x, 8x.

ANNEXURE

Reagents used for DNA isolation

Reagents:

1. 2X CTAB extraction buffer (100 ml)

        CTAB (Cetyl trimethyl ammonium bromide): 2g

        Distilled water: 54ml

        Tris HCL (1M) pH=8: 1 0ml

        EDTA (0.5M) pH=8: 2ml

        NaCl (5M): 30ml

2. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

3. Sodium acetate (3M)

40.8g of Sodium acetate (Molecular weight = 136.08) in 100ml distilled water was mixed

and stored in refrigerator at 90C and was used for study.

4. Ethanol (70%)

To the 70 parts of absolute ethanol (100%), 30 parts of sterile distilled water was added to

make 70 per cent ethanol

ANNEXURE III

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

Tris base: 242 g

Glacial acetic acid: 57.1ml

0.5 M EDTA (pH=8): 100ml

2. Loading Dye (6X)

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of Ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml (SRL) and was stored at room temperature in a dark bottle.

4. Agarose - 0.8 per cent (Genomic DNA)- 1.5 per cent (for PCR samples).

5. Electrophoresis unit- Bio-Rad power PAC 1000, gel casting tray, comb.

ANNEXURE 1V

List of laboratory equipment's used for the study

Refrigerated centrifuge: Hermle Z326k

Waterbath

Microwave oven: LG

-20 Refrigerator: Labline

Gel documentation system: Bio rad

Thermal cycler: Biorad T100 Thermal cycler

Spectrophotometer: Systronics AU 2701

MOLECULAR PHYLOGENY OF SOUTH INDIAN *APHTHONA* SPP. (*COLEOPTERA: CHRYSOMELIDAE*)

by

VISHNU G M.

(2014-09-111)

Abstract of the thesis

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ABSTRACT

Molecular phylogenetics is a disciplinary research of developmental relations among species using molecular sequences. The analytical methods used in molecular phylogenetics were originally developed to reveal developmental patterns, but now it is used in several fields, such as systematic biology and biodiversity, molecular epidemiology, cell function recognition etc. For such purposes, molecular phylogenetics is a basic area of science that most scientists need to comprehend the background. Phylogenetic analysis of DNA sequences has become an important tool for studying the evolutionary history of organisms. Since the rate of sequence evolution varies extensively with gene or DNA segment; one can study the evolutionary relationships of virtually all levels of classification of organisms. Phylogenetic analysis is also important for clarifying the evolutionary pattern of multigene families as well as for understanding the adaptive evolution at the molecular level.

The flea beetle genus *Aphthona* is broad and contains over 350 species that are spread across the biogeographic regions of the Palearctic, Oriental, African, and Australian Old World. *Aphthona* species are generally mesophilous, however they are diverse ecologically, so that they occur in a wide variety of biogeographic regions and inhabit almost all known habitat ranging from low land rainforest to high altitude coniferous mountains and from desert s to sub artic environs. Most frequently, *Aphthona* is discovered in wet meadows or on vegetation along waterways and streams. This flea beetle genus is important because of the usefulness of some species in controlling invasive weeds. The fact that a number of *Aphthona* species feed on *Euphorbiacea* and the tendency towards monophagy makes them ideal candidates for biological control against weed plants belonging to *Euphorbiacea* family. Although several Indian workers like Prathapan

(2003&2010) have contributed towards classical studies which helped to resolve the taxonamic mystery of Indian *Aphthona* species, information on the molecular systematic of Indian *Aphthona* spp. is scant. Through this study the phylogenetic status of the south Indian *Aphthona* spp. is investigated.

The study entitled Molecular phylogeny of south Indian *Aphthona* spp. was conducted at Dept. Plant Biotechnology and Dept. Agricultural Entomology, College of Agriculture, Vellayani. The objective of the study was to identify the genetic structure of South Indian Aphthona spp. using mitochondrial DNA markers and to identify patterns of intra generic genetic diversity among South Indian *Aphthona* spp. For this *Aphthona* beetles were collected from various parts of western ghats including different sites of Kerala and Karnataka. Plants from which *Aphthona* beetles were collected belong to the family *Euphorbiaceae*.

 The ten collected specimens were identified using the morphological keys. From the identified specimen's total genomic DNA was isolated using the Rogers and Benedict (1994) protocol. The DNA was used to amplify the cytochrome oxidase subunit 1 gene, using the selected primers. Both the primers gave amplicons in the size range 650-680. These amplicons were successfully sequenced. All the 10 sequences were used to generate an intragenomic phylogenetic tree using MEGA software. The phylogenetic tree differentiated all the 10 species. Out of the ten species, *A. glochidionae* & *A. tamila*, *A. chrozophorae* & *A. chrozophorae 2*, *A. marataka* & *A. macarangae* were found to be closely related. From the genetic distance calculated based on the CO1 sequences the species *A. tamila* & *A. glochidionae*, *A. marataka* & *A. macarangae*, *A. chrozophorae* & *A. chrozophorae 2* were found to be synonymous respectively. The morphological and molecular results correlated in confirming the colour polymorphism among the south Indian *Aphthona* spp. The species *A. tamila* & *A. glochidionae*, *A. marataka* & *A. macarangae* were confirmed as colour morphs.