DNA barcoding of spider mites (Prostigmata: Tetranychidae) in vegetable crops

By

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THESIS

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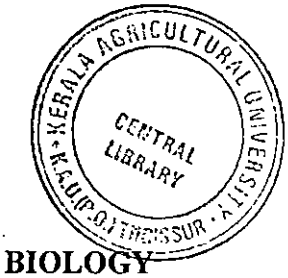

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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DECLARATION

I, here by declare that the thesis entitled **"DNA barcoding of spidermites (Prostigmata : Tetranychidae) in vegetable crops"** is a bonafide record of research work done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"DNA barcoding of spidermites (Prostigmata : Tetranychidae) in vegetable crops"** is a record of research work done independently by **Ms. Shruti Bennur** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to her.

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Shruti Bennur

Dedicated to Lord Shiva, my beloved family and Guide

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ABBREVIATIONS

%	Percentage
@	At the rate
<	Less than
=	Equal to
>	Greater than
µg	Microgram
µl	Microlitre
BLAST	Basic Local Alignment Search Tool
bp	base pair
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
L	Litre
M	Molar
mg	Milligram
ml	Millilitre
mM	Milli mole
NCBI	National Centre for Biotechnology Information
ng	Nanogram
°C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration

ITS2	Second Internal Transcribed spacer
COI	mitochondrial Cytochrome c Oxidase subunit I
RNA	Ribonucleic acid
rpm	Revolutions per minute
TAE	Tris Acetate EDTA
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts
BOLD	Barcode of Life Database System
CBOL	Consortium for the Barcode of Life

Introduction

1. INTRODUCTION

The family Tetranychidae (Acari: Prostigmata), also known as the spider mites, includes the most injurious plant-feeding mites (Helle and Sabelis, 1985; Meyer, 1987; Zhang, 2003). Some infest a wide range of host plants, whereas others are highly specific (Bolland *et al.*, 1998). Pestiferous spider mite biology is characterized by a short generation time, high fecundity, rapid dispersal, effective exploitation of new feeding sites and rapid development of resistance to acaricides (Helle and Sabelis, 1985). The common damage symptoms caused due to these mites are stunting of growth, severe defoliation, reduction in yield and often various types of malformations and deformations of plant parts, etc. Besides causing direct damage, some species are known to transmit plant viral diseases e.g. Potato virus-Y by *Tetranychus urticae* (Schulz, 1963), Dolichos Enation Mosaic virus by *T. ludeni* (Rajagopalan, 1974), Beans Mosaic Virus, Cotton Curliness, etc. (Jeppson *et al.*, 1975). The precise identification of tetranychid taxa (spider mites) is problematic since all the members morphologically look similar. In addition, both sexes of many species are often in order to arrive to precise determinations (Jeppson *et al.*, 1975; Helle and Sabelis, 1985). Hence there is a need of molecular method for taxonomic purposes.

Methods followed currently, for identifying, naming, and classifying organisms are built on the taxonomic system that was developed by Carl Linnaeus, years ago and is mostly based on visible morphology. There are, however, limitations to rely solely or largely on morphology in identifying and classifying the species. A sustainable identification capability lies in the construction of systems that employ DNA sequences as taxon 'barcodes'. DNA sequencing, with key sequences serving as pattern "barcode", has therefore been proposed as a technology that might expedite species identification (Waugh, 2007).

DNA barcoding is a novel system designed to provide rapid, accurate, and automatable species identification using short, standardized gene regions as internal species tags. As a consequence, it will make the Linnaean taxonomic system more accessible, with benefits to ecologists, conservationists, and the diversity. In addition to assigning specimens to known species, DNA barcoding will accelerate the pace of species discovery by allowing taxonomists to rapidly sort specimens and by highlighting divergent taxa that may represent new species (Hebert *et al.*, 2004).

Understanding the extent of natural variation and phylogenetic relationship at molecular level is essential to develop DNA barcode for spider mite species. At present, *COI* have been popularly used as DNA barcodes in animal kingdom (Hebert *et al.* 2003).

Accumulating large number of spider mites sequences from genes such as the mitochondrial cytochrome c oxidase I (*COI*) and the transcribed spacer regions (*ITS1* and *ITS2)* of nuclear ribosomal DNA (Navajas *et al.*, 1996; 1998), can also serve as the scaffold of a molecular method that could simplify spider mites identification. BLAST analysis of barcode sequences from an unidentified sample could tell if there are similar DNA sequences in the database, which were previously obtained from vouchered specimens, therefore leading to simple sample identification. Previous studies on several acarine families such as: Phytoseiidae, Tetranychidae, Listrophoridae and the subfamily Rhizoglyphinae, have indicated that *ITS2* sequence divergence can provide species-level resolution and may be used to differentiate between closely related species, complexes of species and even populations (Navajas, 1998; Navajas *et al.*, 1998; 1999; 2001; Vargas *et al.* 2005; Noge *et al.*, 2005).

Considering the importance of spider mite identification and the accurate

concept of DNA barcoding, the present study was undertaken with the objective to generate DNA barcode for different species of spider mites in vegetable crops and to study the intra and inter species genetic relationship.

Review of Literature

2. REVIEW OF LITERATURE

The present study on "DNA barcoding of spider mites (Prostigmata : Tetranychidae) in vegetable crops" has been executed through the amplification of mitochondrial cytochrome oxidase subunit 1 (*COI*) and second internal transcribed spacer (*ITS2*) gene from different species belonging to family Tetranychidae. Then amplified *COI* and *ITS2* 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 Spider mite taxonomy

Scientific classification of spidermites:

Kingdom: Animilia

Phylum: Arthropoda

Class: Arachnida

Subclass: Acari

Order: Trombidiformis

Sub order: Prostigmata

Family: Tetranychidae

There are 19 genera, 6 tribes and 2 subfamilies in the Tetranychidae family. The subfamilies are Bryobiinae and Tetranychinae. Gupta (1985) in his Handbook Plant mites of India, has included 82 species under 18 genera. The family Tetranychidae comprises 1200 species worldwide (Bolland *et al.*, 1998).

2.2 Identification of spider mites

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).

Identification of tetranychid species is very problematic due to their minute size and the members belonging to this family morphologically look similar. In addition, both sexes of many species, especially in the large and economically-important genera *Oligonychus* and *Tetranychus*, are often needed in order to arrive at precise determination (Jeppson *et al.*, 1975; Helle and Sabelis, 1985).

Expert taxonomists, microscopic slide preparations and comprehensive keys are also essential for the task of species identification. Even though the accepted morphological method of classification and identification of species is indispensable, molecular methods are increasingly being applied for accurate taxonomic purposes as a result of the above reasons (Navajas and Fenton, 2000).

The two most commonly used DNA markers in spider mites are some parts of the coding region of the mitochondrial cytochrome c oxidase sub unit I (*COI*) gene and the Internal Transcribed Spacer 2 (*ITS2*), a nuclear ribosomal DNA fragment. This is a fast and relatively inexpensive technique for spider mite identification. The same approach was utilized in Africa for the discrimination between the invasive pest *T. evansi* and the local closely related species *T. urticae* RF (Knapp *et al.*, 2003).

Molecular methods may also be used to determine the origin of invading spider mites. Navajas *et al.*, (1994) showed that *Mononychellus progresivus* Doreste of Africa has originated in Colombia rather than in Brazil, by similarities of base

composition of *COI* and *ITS2* fragments of specimens from two locations in Africa and from the two South American locations. This information can be used to search for efficient natural enemies of invasive pests in their area of origin.

2.3 General morphological characters of Tetranychidae

General morphological characters of Tetranychidae was given by Gupta and Gupta in 1994 and the characters are listed below:

- Proximal duplex setae of tarsus I of female distal to tactile setae.
- Aedeagus with a knob distally.
- Aedeagal knob with very small anterior and posterior projections, empodium II of male with proximoventral tridigitate spura.
- Aedeagus with tiny knob.
- Aedeagus bent dorsal, posterior angulation absent.
- Female with longitudinal to irregularly longitudinal striae between 3rd pair of dorsocentral hysterosomals and longitudinal between 4th pair of dorsocentrals.
- Female hysterosoma with longitudinal striae between 4th pair of dorsocentral hysterosomal forming a more or less diamond shaped figure between 3rd and 4th pair of dorsocentral hysterosomals.
- Female hysterosoma with longitudinal striae between 3rd pair of dorsocentral setae.
- Upper surface of aedeagal knob straight or rounded with some kind of projection
- Aedeagal knob with anterior projection rounded.
- Terminal sensillum of male palpus about 3-4 times as long as broad, aedeagal knob about 0.33 the length of dorsal margin of shaft
- Empodium I of male with strong mediodorsal spur, female carmine in color.
- Anterior and posterior projection of aedeagal knob acuminate and similar axis of knob parallel with axis of shaft, upper surface of aedeagal knob rounded.

2.4 Spider mite biology

Pestiferous spider mite biology is characterized by a short generation time, high fecundity, rapid dispersal, effective exploitation of new feeding sites and rapid development of resistance to acaricides (Helle and Sabelis, 1985).

Most spider mites are arrhenotokous, where males are haploid and develop from unfertilized eggs, whereas females are diploid and develop from fertilized eggs (Helle and Sabelis, 1985).

Ewing (1914) made a significant contribution to the spider mite biology by recognizing the importance of webbing as a substratum for the attachment of eggs and quiescent individuals.

Yokoyama and Ishii (1934) made a detailed study on the mites attacking mulberry leaves and the morphology and biology of *Panonychus mori.*

Muller (1957) provided information on the morphology, biology and control of the hawthorn spider mite, *T. viennensis*. Iglinsky and Rainwater (1954) studied the life cycle of *T. desertorum* and *T. bimaculatus* and also prescribed chemicals for checking their spread.

In India, Rahman and Sapra (1946) conducted biological studies of the vegetable mite *T. cucurbitae* and reported the association of the species with more than 50 host plants.

2.5 Diversity of spider mites in vegetables

Survey conducted in eight districts of West Bengal for mites associated with vegetable crops in 1985 has revealed a total of 22 species of both phytophagous and

predatory mites. The phytophagous mite fauna included *Tetranychus cinnabarinus* (Boisd.) on cucumber, okra, bitter gourd, brinjal and beans; *Tetranychus neocalidonicus* Andre on brinjal, bottle gourd, okra, ridge gourd, pumpkin and bitter gourd, *Tetranychus macfarlanei* Baker and Pritchard on sponge gourd and pumpkin (Gupta and Gupta, 1985).

According to Gupta (1991), six species of mites *viz*; *T. cinnabarinus*, *T. neocaledonicus*, *Tetranychus ludeni* Zacher, *T. macfarlanei, Aceria lycopersici* (Wolffenstein) and *Polyphagotarsonemus latus* (Banks) were found to be serious pests of vegetables like brinjal, okra, cucurbits, chilli and potato in major parts of India.

The phytophagous mites namely *T. cinnabarinus, Bravipalpus californicus* (Banks) and *Bravipalpus phoenicis* (Geij.) were reported to be associated with brinjal in Punjab (Grewal, 1992).

Vora (1994) gave an account of the phytophagous mite fauna on brinjal in Navasari, Gujarat. *Tetranychus urticae* Koch, *Polyphagotarsonemus latus* (Banks), *Aceria lycopersici* (Wolffenstein) and *Brevipalpus phoenicis* (Geij.) were reported as phytophagous species.

Arbabi *et al.* (1994) reported *T. macfarlanei* as severe pest of brinjal from Varanasi, UP.

A survey conducted in Thiruvananthapuram district, Kerala during 1996 recorded five species of phytophagous mites belonging to the families Tetranychidae, Tenupalpidae and Tarsonemidae in different vegetables. The Tetranychid mite *T. cinnabarinus* was observed on *Amaranthus bicolor* and *A. dubius, T. ludeni* was

associated with *Abelmoschus esculentus* (L.) and *Vigna unguiculata* sub sp. *sesquipedalis* L. (Verdecourt) and *T. neocalidonicus* was found to infest *Cucurbita pepo* L., *Moringa oleifera* Lam. and *Solanum melongena* L. (Sudharma, 1996).

Abd-El-Rahman (1996) reported the occurrence of 42 species of mites belonging to 17 genera and two families (Tetranychidae and Tenuipalpidae) from different vegetables, fruits, ornamental and medicinal plants as well as weeds. According to him, *T. cucurbitacearum* (Sayed), was found in most economic plants while *T. urticae* was found in all kinds of sampled plants.

The tetranychid mites *T. neocalidonicus* on brinjal and *T. ludeni* on bhindi were reported to be the major prey mites of *Cunaxa* sp. and *Amblyseius longispinosus* (Evans) (Sudharma and Nair, 1999).

Zacarias and Moraes (2002) conducted a study on the diversity of mite species on euphorbiaceous plants in three regions of the State of Sao Paulo, Brazil. They reported a total of 31,603 mites belonging to 105 species in 74 genera and 16 families among which 21 species belonged to phytophagous families and 43 to families of predators.

Gulati (2004) reported the distribution and abundance of two spotted mite *Tetranychus urticae* Koch from the vegetable growing tracts of Punjab and Haryana. He also reported the incidence of *T. cinnabarinus* on different varieties of okra.

Zhovnerchuk (2006) made investigations on the tetranychid mites inhabiting green plantings in Kyiv metropolis, Ukraine for the first time and recorded nine species of mites belonging to six genera of the families Tetranychidae and Bryobiidae.

Adango *et al.* (2006) reported *Tetranychus ludeni* Zacher as a pest of two leafy vegetables *Amaranthus cruentus* L. (Caryophyllales: Amaranthaceae) and nightshade, *Solanum macrocarpon* L. (Solanales: Solanaceae) in West Africa.

The results of the study made by Prasad (2006) revealed that during hot summer months, *T. urticae* emerged as extremely severe pest of brinjal, okra and cucumber. *T. ludeni* affected cowpea and French bean; *T. macfarlanei* appeared as extremely severe pest on sponge gourd, ridge gourd, bottle gourd and pumpkin.

The red spider mite *Tetranychus evansi* Baker and Pritchard was reported for the first time in Greece on *Solanum nigrum* L. by Tsagkarakou *et al.* (2007). *T. evansi* was a serious pest of tomatoes and other Solanaceae crops (aubergine, potato, tobacco) and also recorded on several other vegetables and ornamental crops as well as on many weeds.

Survey on the diversity of mites in brinjal ecosystem in Dharwad revealed three tetranychid mite species viz., *T. macfarlanei, T. urticae* and *Tetranychus* sp. of which *T. macfarlanei* was the major one (Prasanna, 2007).

Amelia *et al.* (2007) reported four species of phytophagous mites on different host plants in Mozambique, namely *Tetranychus evansi, T. urticae, T. ludeni* Zacher and *Polyphagotarsonemus latus*. They also reported 76 alternative host plants with varied spider mite densities, suggesting preference of mites for some species. The most frequent plant genera found as alternate hosts were tomato, cucurbits, *Datura, Sida, Solanum* and *Vigna.*

The survey conducted in Varanasi and Azamgarh districts of eastern Uttar Pradesh revealed that tetranychid mites, *Tetranychus urticae*, *T. neocalidonicus*

Andre, *T. ludeni* Zacher and *T. macfarlanei* Baker and Pritchard were found infesting many vegetables like okra, brinjal, cowpea, chilli, pumpkin, bitter gourd, cucumber, bottle gourd, sponge gourd, tomato, water melon etc. *Eutetranychus orientalis* Klein was recorded on hyacinth bean (Rai and Indrajeet, 2011).

Study conducted to investigate phytophagous mites associated with vegetable plants in Riyadh, Saudi Arabia reported eight phytophagous mites from 14 species of vegetable crops covering five major production localities (Al-Atawi, 2011).

Predatory mite species on vegetables and fruit trees in Kahramanmara, Turkey were reported by Ozisli and Çobanoglu (2011). Phytophagous mites, *Tetranychus turkestani* (Ugarov and Nikolski) and *T. cinnabarinus* were obtained from egg plant, bean, and cucumber.

2.6 Spatial distribution of mites on host plants

In brinjal, tetranychids were abundant on the middle and bottom leaves. But more number of mites were seen on leaves of upper canopy in June to August raised crop. Whereas, during September to December period, middle canopy leaves harbored more number of mites (Anon., 1994).

According to Rai *et al.* (1995), *T. urticae* on brinjal preferred bottom canopy leaves than middle and top canopy leaves.

Studies of Srinivasa *et al.* (2007) on the distribution of two spotted spider mite on tomato reported that the middle canopy leaves harboured significantly more number of mites followed by leaves in the bottom canopy.

2.7 Damages caused by spider mites

Jeppson *et al.*, 1975 reported that pestiferous spider mites damage agricultural and horticultural crops and cause severe economic losses. Spider mites suck out the content of the plant tissue using their needle like piercing-sucking mouth parts and cause the destruction of mesophyll cells, which then appear as small chlorotic spots. Leaf area for photosynthesis decreases. In a severe attack, leaves and fruit show necrotic surface and defoliation, stunting of growth, plants wilt, the quality of the yield is damaged and even a death of the attacked plant can occur. Some spider mites spin fine webbing on plant parts that may cover them, increasing the direct damage (Jeppson *et al.*, 1975).

Plant pathogens are able to penetrate plant tissue via feeding wounds (Helle and Sabelis, 1985). Besides causing direct damage, some species are known to transmit plant viral diseases e.g. Potato virus-Y by *Tetranychus urticae* (Schulz, 1963), Dolichos Enation Mosaic virus by *T. ludeni* (Rajagopalan, 1974), Beans Mosaic Virus, Cotton Curliness, etc. (Jeppson *et al.*, 1975).

2.8 Concept of DNA barcoding

DNA barcoding is a novel system designed to provide rapid and accurate identification of species using short DNA sequences from a standardized region of a genome. It helps in precise identification of species. This concept was proposed by Hebert *et al.* in 2003 with the description of the first marker as a "barcode", the mithocondrial *COI* gene, for species identification in the animal kingdom.

Valentini *et al.* (2008) reported that the DNA target sequence should be identical among the individuals of the same species, but different between species . However the perfect DNA target region does not exist and more than one marker have been proposed.

The *COI* region has been almost widely accepted for barcoding animals because of its generally conserved priming sites. Moreover the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species (Cox and Herbert, 2001; Wares and Cunningham, 2001).

Wolfe *et al.* (1987) stated that in plants, the mtDNA has low substitution rates and a rapidly changing gene content and structure, which makes *COI* unsuitable for barcoding in plants. For this reason two regions of chloroplast DNA, ribulose-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*) have been recommended for initiating the barcoding process of plant species (CBOL Plant Working group, 2009; Consortium for the barcode of life, 2009). Due to the only 70% species discriminatory power, additional loci need to be used in this field (Vijayan and Tsou, 2010).

On the contrary, Seifert (2009) reported that in case of fungi, the *ITS* of nuclear DNA (nrDNA) has recently been proposed as the official primary barcoding marker, while no standard regions have been found yet for viruses, bacteria and phytoplasma, but studies are ongoing (Contaldo *et al.*, 2011).

The primary intent of DNA barcoding was to use large-scale screening of one or a few reference genes in order to assign unknown individuals to species, and to enhance discovery of new species (Hebert *et al.*, 2003; Stoeckle, 2003).

Valentini *et al.* (2008) and Moritz and Cicero (2004) stated that DNA barcoding is the approach extensively used by taxonomists and ecologists in particular for biodiversity studies in spite of limitations and pitfalls.

2.9 DNA barcoding in systematics

DNA barcoding is a method for identifying the living organisms at species levels, in which a short universal gene sequence taken from a standardized portion of a genome is used. Dayrat (2005) opined that, 'delineating species boundaries correctly and also identifying species - are crucial to the discovery of life's diversity because it determines whether different individual organisms are members of same entity or not'. The identification of species depends on the knowledge held by taxonomists whose work cannot cover all taxon identification requested by non specialists. To deal with these difficulties, the DNA Barcode of Life' project was started, which aims to develop a standardized, rapid and inexpensive species identification method accessible even to non-specialists.

Though several universal systems have been employed for molecular based identification of lower taxa (Floyd *et al.*, 2002), they are not successfully implemented for higher taxa. The Barcode of Life project aims to create a universal system or inventory for eukaryotic species, based on standard molecular approaches. It has been initiated in 2003 by researchers at university of Guelph, Ontario, Canada (http://www.barcoding.si.edu) and was promoted in 2004 by the international initiative 'Consortium for the Barcode of Life' (CBOL). The DNA barcode project attempts to produce a simple diagnostic tool based on strong taxonomic knowledge that is collated in the DNA barcode reference library (Schindel and Miller, 2005; Gregory, 2005). The DNA barcode of Life Data System (BOLD, http://www.boldsystems.org) has progressively been developed since 2004 and was officially established in 2007 (Ratnasingham and Hebert, 2007). This data enables the acquisition, storage, analysis and publication of DNA barcode records.

The DNA barcode project was initially conceived as a standard system for fast and accurate identification of animal species and presently all eukaryotic species are

within its scope (Hebert *et al.,* 2003; Miller, 2007). DNA barcoding is based on the postulate that every species will most likely have a unique DNA barcode among the 4^{650} possible ATGC-combinations (Wilson, 2004) and that genetic variation between species exceeds variation within species (Hebert *et al.,* 2003; Hebert *et al.,* 2004). The two main ambitions of DNA barcoding are to (i) assign unknown specimens to species and (ii) enhance the discovery of new species and facilitate identification, particularly in cryptic, microscopic and other organisms with complex or inaccessible morphology (Hebert *et al.,* 2003).

DNA barcoding acquired the sequences of identified barcoding locus and based on this, the species delimitation will be computed, in compression with the existing DNA barcodes. Furthermore, in three important situations, relevant species identification must necessarily be molecular based. First, in determining the taxonomic identity of damaged organisms or fragments. The DNA barcoding tool is thus potentially useful in the food industry, diet analyses, forensic sciences and in preventing illegal trade and poaching of endangered species (e.g. Fisheries, trees, bush meet). Second, molecular-based identification is necessary when there are no obvious means to match adults with immature specimens. The third case is when morphological traits do not clearly discriminate species, especially when size precludes visual identification (Blaxter *et al.,* 2005; Webb *et al.,* 2006).

2.10 Efficacy of DNA barcoding

Efficacy of DNA barcoding to recover biologically significant groupings or species have been convincingly demonstrated by few recent studies. Within a single morphologically identified skipper butterfly species, DNA barcoding separated ten cryptic species (Hebert *et al.* 2004). These cryptic species differ in larval appearance, food plant or habitat preference.

Recently, barcoding study carried out by Smith *et al.* (2006) discriminated amongst all 17 highly host-specific morphospecies of the tropical genus Belvosia (Diptera: Tachinidae), and also revealed that each of the three generalist species are actually arrays of highly host-specific cryptic species

Results of the study, carried out by Hajibabaei *et al.* (2006) revealed that *COI* DNA barcodes effectively discriminated among species in three lepidopteran families from northwestern Costa Rica. 97.9% of the 521 species that were recognized by prior taxonomic work were shown to possess a distinctive *COI* barcode.

Previous studies on several acarine families such as: Phytoseiidae, Tetranychidae, Listrophoridae and the subfamily Rhizoglyphinae, have indicated that *ITS2* sequence divergence can provide species-level resolution and may be used to differentiate between closely related species, complexes of species and even populations (Navajas 1998; Navajas *et al.* 1998, 1999, 2001; Vargas *et al.* 2005; Noge *et al.* 2005).

2.11 Storage of spider mites for molecular work

Evidence from insects suggests that the most effective method for preserving specimens for molecular work is ultracold freezing (-80°C) of live specimens (Post *et al.*, 1993; Reiss *et al.*, 1995; Dillon *et al.*, 1996), however, this is not always practicable.

Cruickshank (2002) suggested that an acceptable alternative is storage in 100% ethanol. Mites from other media should be transferred to 100% ethanol and stored at -20°C as soon as possible. Lower concentrations of ethanol give variable results and should be avoided if at all possible, however, mites stored in ethanol as low

as 70% for many years can give good yields of DNA. Drying is not generally good for DNA and therefore parasitic mites collected from museum specimens may not be suitable for molecular work (Cruickshank, 2002).

2.12 DNA isolation from spider mites

Klompen (2000) found it necessary to use 2-5 individuals in each extraction whereas Anderson and Trueman (2000) used only leg tissue dissected from individual mites. If possible it is preferable to extract DNA from single individuals to prevent mixing of distinct genotypes, particularly if there is any question about the identity of the mites. This should be possible using all but the very smallest of mites. Extracted DNA should be stored at -80°C. When extracting DNA it is usual to slide mount a few individuals as voucher specimens so that they are available for examination subsequent to DNA sequencing (Ruedas *et al.*, 2000). Ideally the voucher specimen should be the individual that is sequenced but this individual is usually completely destroyed during the extraction process. However, non-destructive methods of DNA extraction are available (Rose *et al.*, 1994; Phillips and Simon, 1995). Cruickshank *et al.* (2001) have adapted the DNeasy Tissue Kit from Qiagen (http://www.qiagen.com/) for non-destructive DNA extraction of lice by increasing the duration of the lysis step. This method has also been applied to midges (*Culicoides* sp.) and specimens have been successfully identified to species after DNA has been extracted (Cruickshank, 2002). The method has also been tested on mites and complete grinding of the specimen was only found necessary for the very small mites, although there was some loss of yield in larger mites (Cruickshank, 2002).

2.13 *ITS2* locus

Cruickshank (2002) reviewed that the non-coding region which has been most widely used in mites is the second internal transcribed spacer region of the nuclear ribosomal gene cluster (*ITS2*). This cluster consists of three genes (18S rDNA, 5.8S

rDNA and 28S rDNA) which are transcribed into RNA but not translated into protein. These RNA molecules form parts of the ribosome, a cellular structure involved in making proteins (Cruickshank, 2002). The three rDNA genes are transcribed as a single transcript of RNA transcript separated by two regions (*ITS1* and *ITS2*) which are subsequently spliced out and serve no further purpose (Cruickshank, 2002). Since they have no other function these *ITS* sequences are under very little selection pressure and can accumulate substitutions very quickly. This can be very useful for distinguishing between closely related species. By designing primers within the slowly evolving genes that span the rapidly evolving spacers, primers have been developed which work well in a wide variety of taxa (Cruickshank, 2002).

First ever use of molecular phylogenetics in mites, Navajas *et al.* (1992) used primers in the 5.8S and 28S rDNA genes to amplify the *ITS2* region in several species of tetranychid mites and showed that this region is suitable for examining phylogenetic relationships within genera, but that differences between genera were too great to enable correct alignment of the sequences. The relationships within the genus *Tetranychus* agreed closely with morphology but sequences from two sympatric species of *Eutetranychus* from different hosts, which appeared morphologically very similar except for a slight difference in the shape of the peritreme, showed substantial genetic divergence (Cruickshank, 2002).

Navajas *et al.* (1998) used *ITS2* sequences in combination with *COI* to investigate intraspecific variation in *Tetranychus urticae* and found species-wide homogeneity of *ITS2* sequences but extensive *COI* polymorphism (reviewed in Navajas, 1998). Navajas *et al.* (2000) subsequently added more taxa to this data set and these revealed very low levels of variation at the *ITS2* locus in this species.

Navajas *et al.* (1994) found a similar pattern of variation in the cassava green

mite (*Mononychellus progresivus*) with intraspecific diversity being low for both *ITS2* and *COI,* but lower in *ITS2*.

Essig *et al.* (1999) used *ITS2* to investigate relationships within the genus *Chorioptes*. Their sequences fell into two distinct groups that correlated well with morphology but not with host suggesting that the sequences in their study represent two species both with low host specificity.

Zahler *et al.* (1999) used *ITS2* sequences to investigate the relationships within the genus *Sarcoptes*. Although sequence variation was found, this did not correlate well with morphology, host or geography suggesting that all of the sequences in their study belong to a single polymorphic species (Cruickshank, 2002). Zahler *et al.* (1998) performed a similar analysis for the genus *Psoroptes* but with less conclusive results.

2.14 *COI* locus

COI sequence variation within tetranychid species is relatively high, not linked to geographical location nor associated with the host plant (Navajas, 1998; Navajas *et al.*, 1998). The use of mitochondrial genes for delineating and classifying biological species should be done cautiously. *COI* has a similar range of uses to *ITS2* but appears to evolve slightly faster (Cruickshank, 2002).

Navajas *et al.* (1998) found extensive *COI* polymorphism (but species-wide homogeneity of *ITS2*) in *Tetranychus urticae*. Anderson and Trueman (2000) used *COI* to show that *Varroa jacobsoni* is a complex of two morphologically indistinguishable species infesting *Apis cerana*, only one of which (*Varroa destructor*) has been transferred to *Apis mellifera*.

Toda (2000) used a 546bp portion of *COI* to investigate the phylogenetic relationships of Japanese *Panonychus* species and Salomone *et al.* (2002) used a 488bp portion of this gene to examine the relationships between the different members of the oribatid genus *Steganacarus* on the Canary Islands.

Navajas *et al.* (1996) used *COI* gene to resolve relationships between genera in two subfamilies of the Tetranychidae. This is despite a very high AT content. This contrasts with the poor resolution of 12S and 16S (which are similarly AT rich) at this level in ticks. *COI* is likely to be on its limit of resolution here. An alternative approach to using *COI* for deeper level phylogeny is to eliminate the hypervariable third codon positions from the analysis (Cruickshank, 2002). This was the approach taken by Soller *et al.* (2001) who used only the first and second codon positions of a 408bp fragment of *COI* to investigate the phylogeny of the Parasitengona, however, they found that even after removal of third codon positions considerable homoplasy remained and this lead to an incompletely resolved phylogeny with low bootstrap values. They also had trouble amplifying all of their taxa, presumably due to mutations at the primer site.

2.15 *ITS1* locus

While not as widely used as *ITS2*, *ITS1* has similar properties to *ITS2* and has been used at the population level to study *Ixodes scapularis* (McLain *et al.*, 1995).

Navajas *et al.* (1999) compared the sequences of *ITS1, ITS2* and 5.8S rDNA in several species of phytoseiids and concluded that *ITS1* is more variable than *ITS2* and also more difficult to align. *ITS1* was also the only region that showed polymorphism within species, although this was low. 5.8S rDNA was substantially more conserved than either *ITS*.

In a study of the coevolution of *Cecidophyopsis* mites and their *Ribes* hosts,

Fenton *et al.* (2000) used primers in 18S and 28S rDNA to amplify a 1629bp fragment of the nuclear ribosomal gene cluster spanning both of the internal transcribed spacers and 5.8S. They also sequenced the homologous region in the hosts and found that the phylogeny of the mites does not closely follow the phylogeny of their hosts. The problems associated with internal transcribed spacers are those typical of rapidly evolving genes. Failure to amplify some species with universal *ITS2* primers has been reported for phytoseiids (Yli-Mattila *et al.*, 2000) and eriophyiids (Fenton *et al.*, 1997). With these problems under consideration, Cruickshank (2002) suggested that internal transcribed spacers should be used only for studies of intraspecific variation or phylogenies of very closely related species. It may be necessary to clone PCR products and sequences should be treated as haplotypes rather than individuals.

2.16 Phylogeny

According to Futuyama (1997) a phylogenetic tree is the estimate of species divergence from a common ancestor. Data collected by systematists, especially morphological characters, comparative embryology, fossil records and comparative anatomy are the data used to suggest how similar organisms are. The cladograms generated by bioinformatics tools are based on sequence data alone. Each position in a sequence is a character with 4 states (nucleotides): A, T, C or G, which are clearly defined; this is an advantage over morphological traits such as dimensions, which are sometimes variable. Hence, DNA sequence relatedness is considered to be a very powerful predictor of the relatedness of species (Futuyama, 1997). Barrett and Hebert (2005) suggested using a 0.02 divergence as the threshold for species diagnosis with mtDNA-*COI* sequences.

2.17 Speciation and molecular divergence

DNA sequences evolve at roughly constant rates over time, and might thereby

provide internal biological timepieces for dating past evolutionary events. The concept of molecular clocks fits well with neutrality theory because the rate of neutral evolution in genetic sequences is, in principle, equal to the mutation rate to neutral alleles. Nevertheless, different DNA sequences evolve at different rates e.g. introns and pseudogenes evolve faster than non-degenerate sites in protein coding genes, and mtDNA in many vertebrate animals evolve 5-10 times faster than single-copy nuclear DNA. Data from fossils and biogeography evidence gives the conventional mtDNA clock calibration of 2% sequence divergence per million years between recently separated lineages in mammals whereas evolutionary rate of 1% per 50 million years is estimated in 16S rDNA of eubacteria. The branching orders in phylogenies can be inferred directly from distributions of qualitative character states, using cladistic, parsimony or maximum likelihood analyses, which are valid irrespective of whether molecules evolve in strictly time-dependent fashion. The correlation between speciation rates and genetic divergence is not universal. If genetic divergence is proportional to time, than mean genetic distance among extent species should be similar in rapidly speciating (species-rich) and slowly speciating (species-poor) clades of similar evolutionary age, whereas if genetic divergence is a function of the number of speciation events, mean genetic distance among extent forms should be obviously greater in a species-rich clade. Empirically this link was rejected when mtDNA sequences were studied in pairs of temperate and tropical avian taxa, but was accepted from studies of allozyme genetic distances within 111 17 vertebrate genera. At least 50 percent of phylogenetic trees showed no significant correlation between rates of molecular evolution and apparent rate of speciation (Avise, 2004).

Materials and Methods

3. MATERIALS AND METHODS

The study on "DNA barcoding of spider mites (Prostigmata : Tetranychidae) on vegetable crops" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Agricultural Entomology, College of Horticulture, Kerala Agricultural University during the period 2013-2015. The materials used and methodologies adopted are presented in this chapter.

3.1 Purposive survey

Spider mite infected leaf samples were collected from various locations *viz*; Alathur, Anthikad, Elenad, KVK (Krishi Vigyana Kendra) Thrissur and Vellanikkara from different vegetable crops *viz*; Amaranthus, brinjal, cowpea, cucumber, dolichos bean, okra and ridge gourd separately in polythene bags from each locality and brought to the laboratory. In the laboratory, the leaves were observed under stereo-microscope. Spider mite infected leaves collected from different vegetable crops are presented in plate 1 and 2. Different locations from which spider mites were collected are presented in plate 3 and 4.

3.2 Maintenance of an isoline

Single gravid female was placed on a fresh leaf. This fresh leaf carrying gravid female was then placed on a wet cotton pad in Petriplate. Gravid female laid many eggs which hatched and adults were obtained subsequently. These were regarded as individuals of an isoline. Materials used during spider mite collection and rearing are given in annexure I.

3.3 Preparation of permanent microscopic slides and identification of mite specimens

Few males and females from an isoline were separately used for permanent

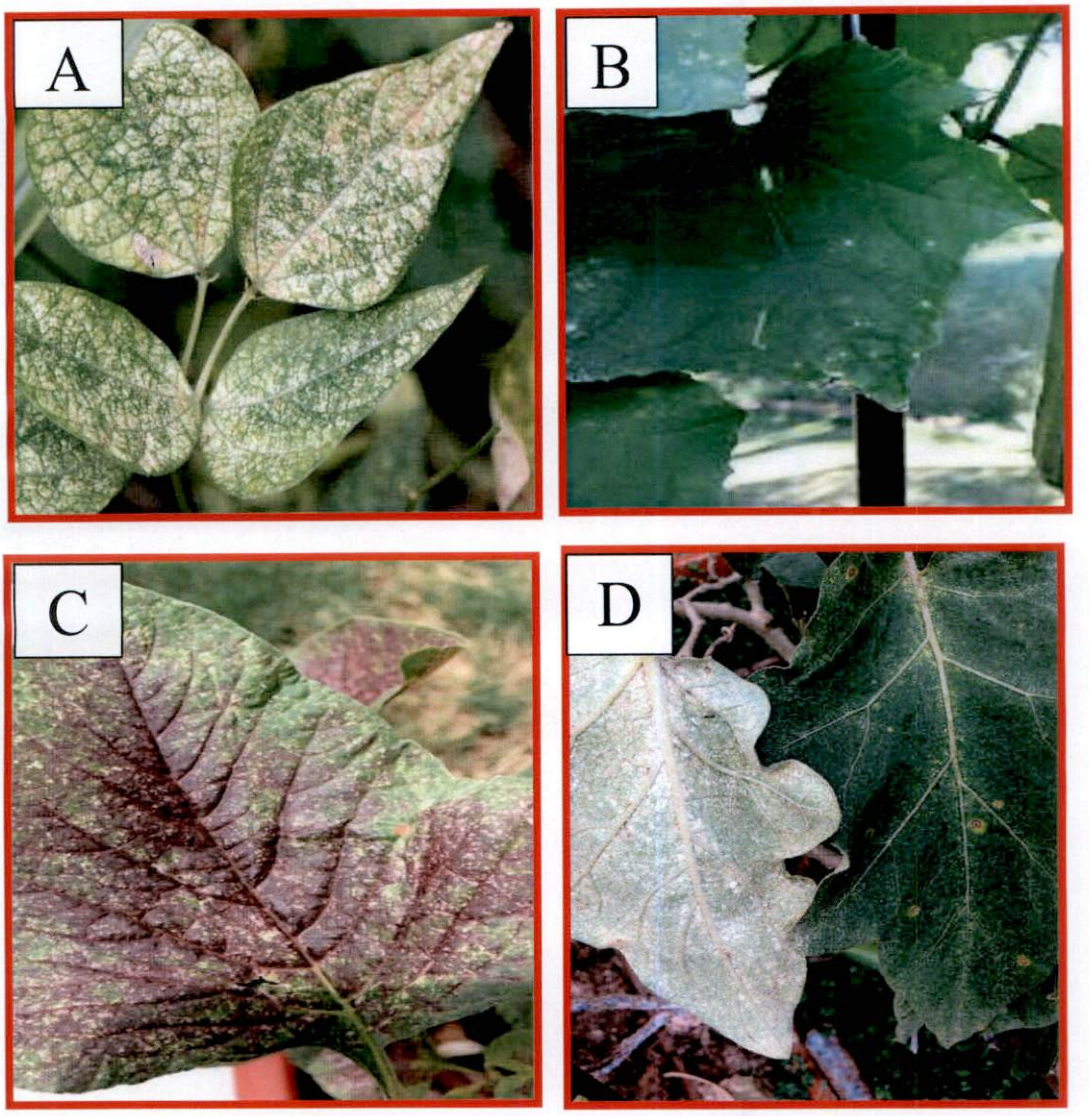
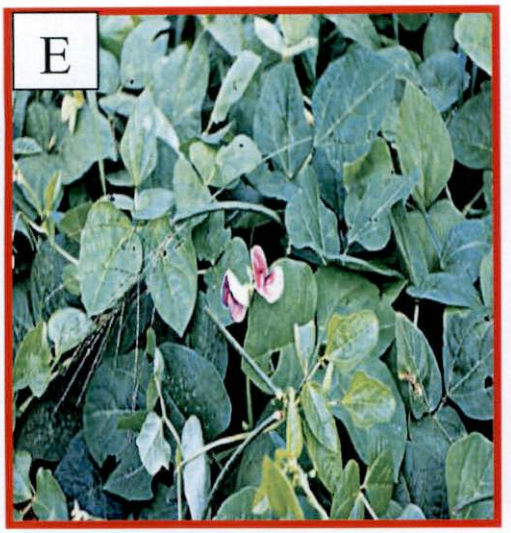
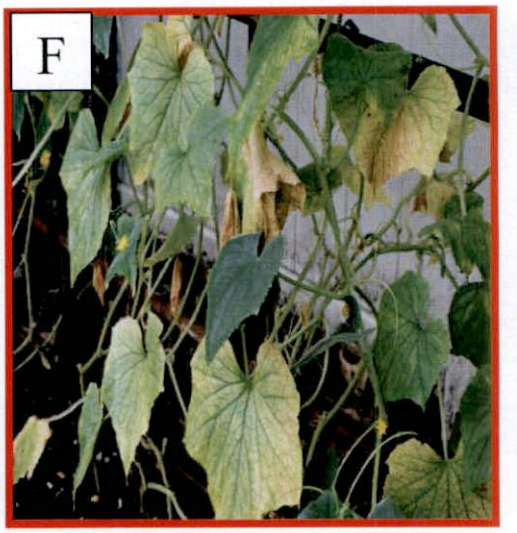


Plate 1 : Spider mite infected leaves collected from different vegetables crops
A- Cowpea, B-Ridge gourd, C-Amaranthus, D-Brinjal





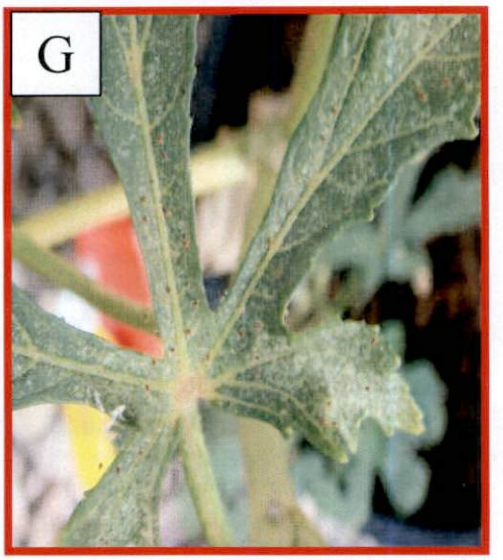


Plate 2 : Spider mite infected leaves collected from different vegetables crops
E- Dolichos bean, F-Cucumber, G-Okra

Plate 3: Survey done at various locations
A- Elenad, B-Alathur

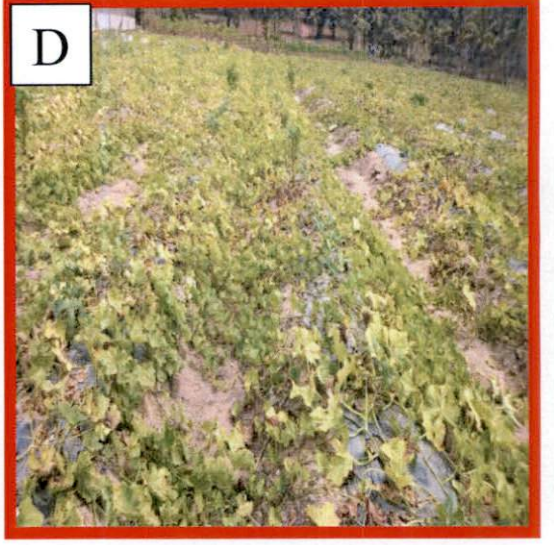


Plate 4: Survey done at various locations,
C- KVK Thrissur, D-Anthikad and E-Vellanikkara

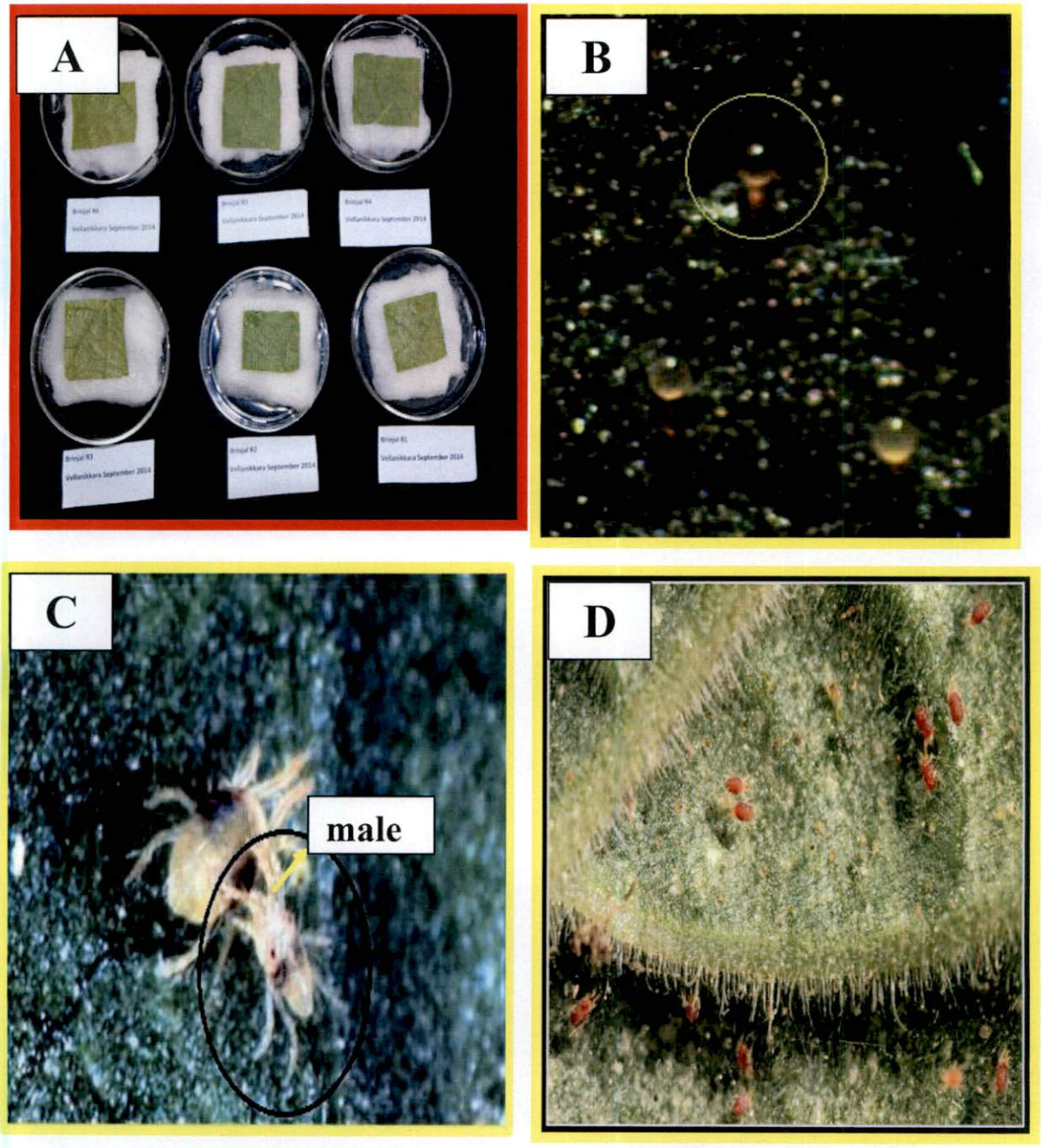


Plate 5: Maintenance of isolines and various stages of spider mites
A-isolines,
B-Eggs, C-Adult male and female, D- Adults

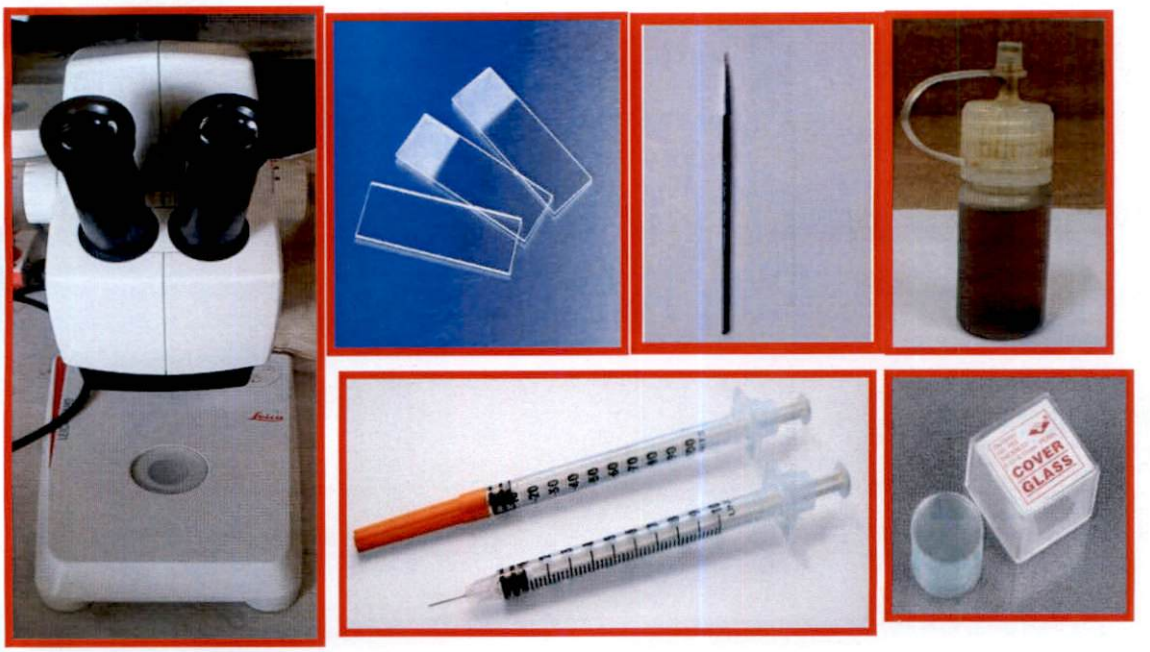


Plate 6: Materials used in permanent microscopic slide preparation

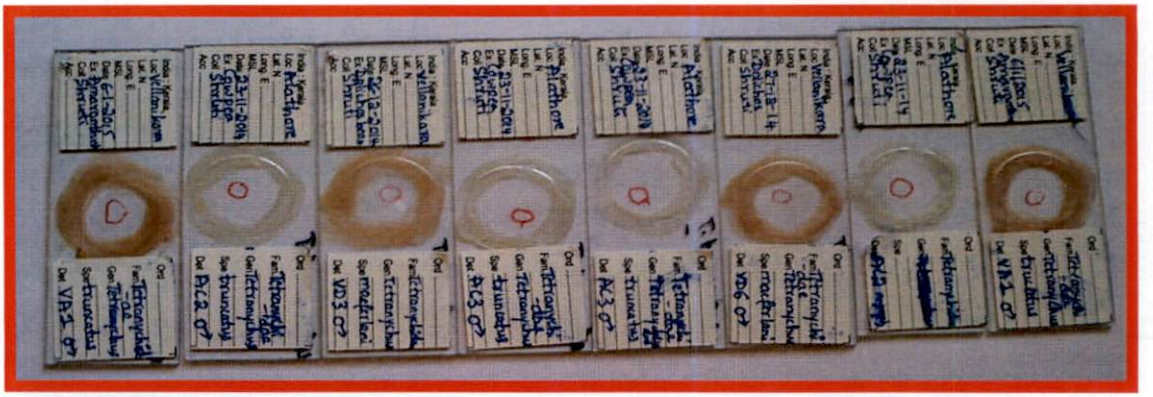


Plate 7: Permanent microscopic slides of spider mites for identification using taxonomic keys

slide preparation using Hoyer's medium. The composition of Hoyer's medium is given in Annexure I. Specimens were placed on a drop of a media on the glass slide in the dorsal view and pressed to the bottom of the slide to spread out the legs and a cover glass was placed on the top of the specimen without air bubbles. The male tetranychid mites were mounted in the lateral position also to ensure the better orientation of the genital structures which were very important for species determination. The mounted specimens were kept in an oven at 40^0C for seven to ten days to get cleared and dried specimens. The slides were then labelled for identification. Details of host, locality, date of collection and collector's name were given on the label. The edges of the cover glass were sealed with nail polish to avoid damage of the specimen due to excessive moisture after the specimens were properly cleared and dried. The permanent slides were used for taxonomic studies using a Leica DM 500 phase contrast microscope.

Morphological characters of different spider mites species were identified by experts in Tetranychid mite taxonomy using keys given by Gupta and Gupta (1994). The major characters used in the morphological analysis were:

Taxonomic keys to identify *Tetranychus truncatus* (Ehara, 1956)

Female:

- Empodia with 6 proximoventral hairs.
- Empodia I-IV each with a minute spur (<2 μm long or absent).
- Tarsus I with sockets of 4 tactile setae proximal to proximal pair of duplex setae.

Male:

- Empodium I claw like (uncinate), empodium II-IV with proximoventral hairs free and long.
- Aedeagus with small knob, anterior projection rounded, short, posterior

projection pointed, short, dorsal surface flat to slightly, convex, with medial indentation.

Taxonomic keys to identify *Tetranychus macfarlanei* (Baker and Pritchard, 1960)

Female:

- Empodia with 6 proximoventral hairs
- Tarsus I with the sockets of 3 tactile setae overlapping with the socket of the proximal duplex setae, socket of 1 tactile seta proximal to socket of proximal duplex setae.
- Pregenital stria almost entire but weak and/or broken medially.

Male:

- Empodia I-IV each with obvious dorsal spur 4 μm long.
- Empodia I claw-like (uncinate), empodia II-IV each with proximoventral hairs long and free.
- Aedeagus with small anvil-shaped knob, anterior and posterior projections tiny, dorsal surface flat to slightly convex.

Taxonomic keys to identify *Tetranychus okinawanus* (Ehara, 1995)

Female:

- Body, including rostrum, 476μm long , 266"μm wide, red in color.
- Dorsal setae on idiosoma slender, pubescent, much longer than distance between consecutive setae.
- Hysterosoma with longitudinal stria between pair of setae C3 and between pair of setae C4, forming a daimond shaped figure between these setae, lobes on dorsal striae very variable in shape, mostly rounded.
- Peritremes strongly hooked distally.
- Pregenital area with longitudinal striae, the straie broken medially, solid laterally,

genital flap with longitudinal striae on anterior part, with transverse striae on posterior part.

- Palpus with spinneret slightly longer than broad, dorsal sensilum fusiform, approximately as long as spinneret.
- The number of setae on leg segments: Tarsus I with 3 tactile setae proximal to proximal set of duplex setae, with 1 tactile setae and 1 solenidion at or near the level of proximal duplex to duplex set. Tarsi III and IV each with solenidion proximal, extending almost to mediodistal tactile setae. Each empodium composed of 3 pairs of hairs and 1 pair of somewhat shorter, proximoventral filaments, with the strong mediodorsal spur.

Male:

- Body, including rostrum, 434μm long, 213μm wide.
- Aedeagus upturned distally, terminal knob 3.5μm long, much longer than the width of neck, approximately one half as long as dorsal margin of shaft, the axis of knob sub parallel with dorsal margin of shaft, anterior projection of knob broadly rounded, the posterior projection very narrow, acute.
- Palpus with spinneret about twice as long as broad, dorsal sensillum slender, fusiform.
- The number of setae on leg segments: Tarsus I with 3 tactile setae and 2 solenidia proximal to proximal set of duplex setae with 1 tactile seta and 1 solenidion at or near the level of proximal to proximal duplex set, tarsus II with 3 tactile setae and 1 solenidia proximal to proximal set of duplex setae.
- Empodium I with 1 pair of claw-like divisions and 1 pair of somewhat shorter, proximoventral filaments, and with the strong mediodorsal spur, empodia II-IV each consisting of 3 pairs of hair and 1 pair of proximoventral filaments, with the strong mediodorsal spur.

Table 1: Description of the samples collected

Sl. No.	Sample ID	Description
1	AC1	Alathur Cowpea replication 1
2	AC2	Alathur Cowpea replication 2
3	AC3	Alathur Cowpea replication 3
4	VA1.2	Vellanikkara Amaranthus replication 1.2
5	VA2.2	Vellanikkara Amaranthus replication 2.2
6	VA3.2	Vellanikkara Amaranthus replication 3.2
7	VCu1	Vellanikkara Cucumber replication 1
8	VR3	Vellanikkara Ridge gourd relplication 3
9	AnCuR	Anthikad Cucumber Red mite
10	KB1	KVK Brinjal replication 1
11	KC1	KVK Cowpea replication 1
12	KON	KVK Okra N

13	EC1	Elenad Cowpea replication 1
14	VD3	Vellanikkara Dolichos bean replication 3
15	VD4	Vellanikkara Dolichos bean replication 4
16	AnGA1	Anthikad Green Amaranthus replication 1
17	AnRA2	Anthikad Red Amaranthus replication 2
18	AnRA2.11	Anthikad Red Amaranthus replication 2.11
19	VR2	Vellanikkara Ridge gourd replication 2
20	AnCu3	Anthikad Cucumber replication 3
21	AnCu4	Anthikad Cucumber replication 4

3.4 Molecular analysis

Molecular analyses of spider mite species were carried out with two candidate loci *COI* and *ITS2*. Details of primers used for DNA barcoding are listed in Table 2.

3.4.1 DNA isolation

Modified CTAB method detailed by Rogers and Bendich (1994) was used for the extraction of total genomic DNA. The reagents used for DNA isolation are presented in Annexure II.

Procedure

➢ Five to ten female mites from isolines were taken in autoclaved 1.5ml eppendorf tube containing 20µl CTAB buffer (2X) and crushed using micropestle 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 vortexing 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 supernatent.

➢ Five hundred microlitre 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.

3.4.2 Assessing the quality of DNA using spectrophotometer (NanoDrop ND-1000)

The purity of DNA was checked using NanoDrop ND-1000 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 OD_{260}/OD_{280}. A value 1.8 indicated that the DNA is pure and free from proteins and RNA. When the ratio was <1.8 it meant that the sample is protein contaminated and >1.8 had shown that the sample is RNA contaminated. The quantity of DNA in the pure sample was calculated using the relation.

1 OD at 260 nm = 50 µg DNA/µl

Therefore OD_{260} X 50 gives the quantity of DNA in µg/µl.

Procedure

➢ The Nanodrop spectrophotometer was connected to the computer installed with ND-1000 software.

➢ The option 'Nucleic acid' was selected in the software.

➢ With the sampling arm open, pipetted 1µl distilled water onto the lower measurement pedestal.

➢ The sampling arm was closed and spectral measurement was initiated using the operating software. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made.

➢ The reading was set to zero with sample blank.

➢ Subsequently 1µl of sample was pipette out onto the measurement pedestal and selected the 'Measure' option.

➢ When the measurement has been completed, the sampling arm was opened and the sample was wiped from both upper and lower pedestals using a soft laboratory tissue paper.

3.4.3 DNA amplification conditions

The PCR conditions required for the effective amplification include appropriate proportions of the components of the reaction mixture. Including template DNA, assay buffer A, Taq DNA polymerase, dNTPs and primers. The aliquot of this master mix were dispensed into 0.2 ml PCR tubes and subjected to thermal cycling.

Thermal cycling - Composition and thermal profile:

PCR amplification of *ITS2* locus was performed in a 50µl reaction mixture and the composition of the reaction mixture consisted of,

a) Genomic DNA	- 4 µl
b) 10X Taq assay buffer A	- 5 µl
c) dNTP mix (10mM each)	- 3 µl
d) Taq DNA polymerase (3U)	- 0.5 µl
e) Primer forward	- 0.5 µl
f) Primer reverse	- 0.5 µl
g) Autoclaved distilled water	- 36.5 µl
Total volume	- 50.0 µl

The PCR amplification was carried out for *ITS2* locus with the following thermal profile.

Initial denaturation - 94^0 C for 3 minutes

Denaturation - 94^0 C for 1 minute
ITS2 primer annealing - 52^0 C for 1 minute } 35 cycles
Primer extension - 72^0 C for 2 minutes

Final extension - 72^0 C for 10 minutes

4^0 C for infinity to hold the sample

PCR amplification of *COI* locus was performed in a 50µl reaction mixture and the composition of the reaction mixture consisted of,

a) Genomic DNA - 5 µl
b) 10X Taq assay buffer A - 6 µl
c) dNTP mix (10mM each) - 3 µl
d) Taq DNA polymerase (3U) - 0.5 µl
e) Primer forward - 1 µl
f) Primer reverse - 1 µl
g) Autoclaved distilled water - 33.5 µl

Total volume - 50.0 µl

The PCR amplification for *COI* locus was carried out with the following thermal profile.

Initial denaturation - 94^0 C for 3 minutes

Denaturation - 94^0 C for 1 minute
COI primer annealing - 59^0 C for 1 minute 30 seconds } 35 cycles
Primer extension - 72^0 C for 1 minute 30 seconds

Final extension - 72^0 C for 10 minutes

4^0 C for infinity to hold the sample

Table 2. Details of primers used for DNA barcoding

Sl. No.	Locus	Sequence	Registered name of the primer in BOLD	Annealing temperature ^{0}C	Product length (bp)	Reference
1	*ITS2 F*	ATATGCTTAAA TTCAGCGGG	ITS2 KAU	52	620	Navajas *et al.*, 1998
	ITS2 R	GGGTCGATGA AGAACGCAGC	R ITS2 KAU			
2	COI F	GGAGGATTTG GAAATTGATTA GTTCC	UBC6 F	59	868	Simon *et al.*, 1994
	COI R	GATAAAACGT AATGAAAATG AGCTAC	R COI			

3.4.4 Assessing the PCR products

The amplification of the candidate locus was evaluated through agarose gel electrophoresis on 2% agarose gel.

Reagents and equipments

Composition of buffers and dyes used for gel electrophoresis are presented in Annexure III.

Procedure for Agarose gel electrophoressis

- 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 TAE buffer (1.2ml TAE from stock which was made upto 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 10µl/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 molecular weight marker was loaded (λDNA *Eco*RI/*Hind*III double digest) in first lane.

- 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 fluroscent bands and the degraded one appears as a smear, because of the presence of a large number of bands, which differed in few base length. 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.

3.4.5 Gel documentation

Gel documentation was done with BioRad Gel Documentation System using 'Quantity one^{R}' software. Quantity one^{R} 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 Quantity one^{R} controls in the imaging device window and band size was confirmed. List of laboratory equipments used for the study are presented in annexure IV.

3.4.6 PCR product sequencing

A total of 27 sequences of spider mites were amplified with two selected primers. The PCR products were used for sequencing. If multiple bands were obtained after amplification, only the band having molecular weight nearest to that of the targeted region was eluted and purified. Sequencing was done by outsourcing (SciGenom Lab. Pvt. Ltd., Cochin).

3.5 Data analysis

3.5.1 Analysis of sequence homology

Basic Local Alignment Search Tool (BLAST) was carried out to check homology of the sequence using BLASTn tool of NCBI. Query sequence was compared with a library or database of sequences and the library sequences that resemble the query sequence were identified.

3.5.2 Sequence annotation

Using BLASTn, sequence was confirmed whether it is in 5'-3' forward direction. Sequences which were in reverse direction, the reverse complimentary sequence was generated using BioEdit software. A file was opened *.txt file and all the sequences were pasted in FASTA format. Entry was saved and *.txt file was closed. BioEdit software was opened-File-Open-All files-*.txt file was chosen and Ctrl+Shift+R was pressed.

COI sequences were checked for the presence stop codons using MEGA6. Sequences containing stop codons were trimmed using BioEdit software to get the *COI* sequence free from stop codons. Since *ITS2* is an intron, stop codons were not checked and were directly used for further analysis.

3.5.3 Barcode gap analysis

Barcode gaps are the species specific variations from the base sequence of the genus for the sequence of the barcode locus. The distributions of intra versus inter -specific variability were compared using DNA barcode gap. This analysis was carried out separately for *ITS2* and *COI* sequences. *Tetranychus okinawanus* is a rare species and there was only a single sequence which is not sufficient to perform the analysis. Hence two *T. okinawanus, ITS2* sequences from GenBank and two *COI* sequences from BOLD databases were retrieved to perform barcode gap analysis only. Sequences were aligned using clustalW tool of MEGA6 software and with the mark like "*" was studied. Each gaps were selected which were unique in each species.

3.5.4 Submission to Barcode of Life Data system

Specimen data, specimen images, sequence information and trace files were deposited into Barcode of Life Data (BOLD) online (http://www.boldsystems.org).

3.5.5 Measurement of nucleotide/genetic divergence

It is desirable for barcodes to show very low sequence divergence within a species, with significantly higher sequence divergence at higher taxonomic levels. Distance summary is the tool to measure nucleotide/genetic divergence between sequences which gives a report of sequence divergence between barcode sequences at the conspecific and congeneric levels.

The inter specific distances were calculated using K2P (Kimura 2 parameter) model as recommended by the Consortium of Barcode of Life (CBOL, http:// www.barcoding.si.edu/protocols.html) using Distance summary tool of BOLD (Barcode of Life Data). This measurement was done separately for *ITS2* and *COI* sequences.

3.5.6 Phylogeny analysis

Phylogeny analysis was carried out separately for *ITS2* and *COI* sequences of different spider mite species. The sequences were aligned using clustalW tool of MEGA6 software and were used to construct the phylogenetic tree using 'phylogeny' tool (maximum parsimony model) of MEGA6 software.

3.5.7 Pair wise distance calculation

The inter specific distances were calculated using K2P (Kimura 2 parameter) using MEGA6 software. The "Distance|Compute Pair-wise" command generates the pair wise distances.

3.5.8 Submission to GenBank (NCBI)

A total of 27 DNA sequences from spider mites species were deposited in the NCBI GenBank. Deposition was carried out with the submission tool BankIt. The

procedure involved was:

(i) Logged in to the password account in MyNCBI at http://www.ncbi.nlm.nih.gov/guide/howto/submit-sequence-data/ website.

(ii) Sequence with simple annotation was submitted through BankIt.

(iii) Additional information like date for public release (immediate or at a specified future date), basic information (authors and a working title) for a corresponding reference paper, name of the organism or plant from which the sequence data were isolated and any other related descriptive data were also be provided.

(iv) The accession numbers to the submitted sequences were obtained from NCBI.

Results

4. RESULTS

The study on 'DNA barcoding of spider mites (Prostigmata : Tetranychidae) in vegetables' has been carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Agricultural Entomology, College of Horticulture, Kerala Agricultural University. The objective of the study was to generate DNA barcodes for different species of spider mites in vegetable crops and to study the intra and inter species genetic relationship. The results of various aspects of the investigation are detailed in this chapter.

4.1 Purposive survey

Spider mite infected leaf samples were collected from various locations *viz*; Alathur, Anthikad, Elenad, KVK (Krishi Vigyana Kendra) Thrissur and Vellanikkara. Spider mite infection was observed on vegetable crops *viz*; Amaranthus, brinjal, cowpea, cucumber, dolichos bean, okra and ridge gourd during the study.

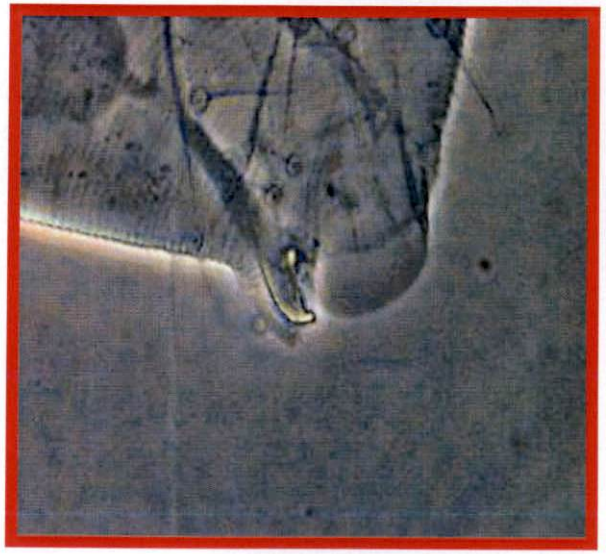
4.2 Maintenance of an isoline

Single gravid female of spider mite from the collection was used for rearing an isoline. Isolines were obtained within 12-15 days.

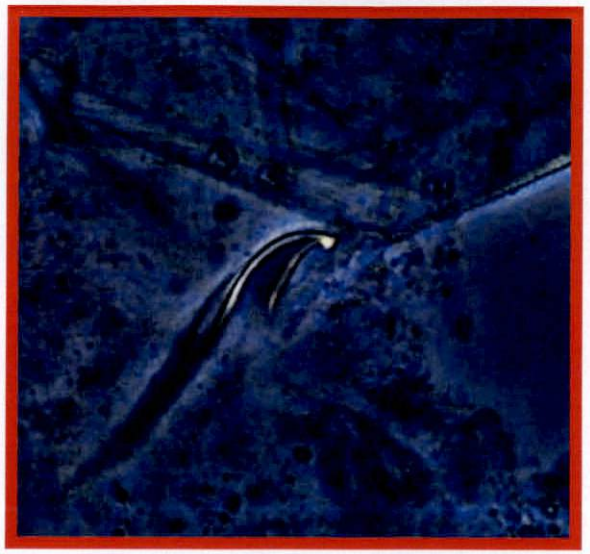
4.3 Preparation of permanent microscopic slides and identification of mite specimens

Morphological characters were recorded from the fully grown up adult males and females of Tetranychid species (spider mites), mounted on microscopic slides. A total of three spider mite species were found in Thrissur district during the study- *Tetranychus truncatus, T. macfarlanei and T. okinawanus*.

Plate 8: Taxonomic key - male aedeagus of *Tetranychus truncatus, Tetranychus macfarlanei* and *Tetranychus okinawanus*



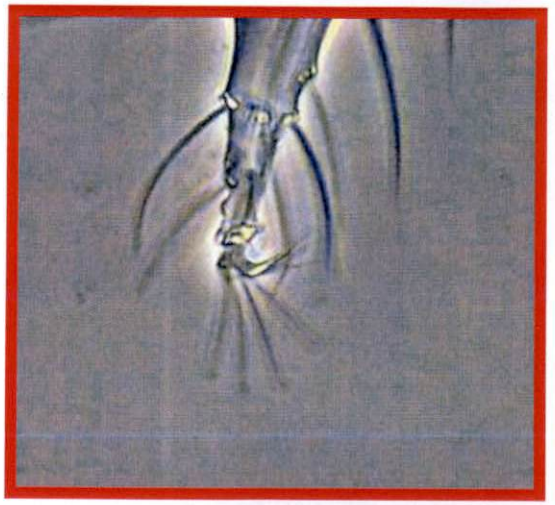
Tetranychus truncatus



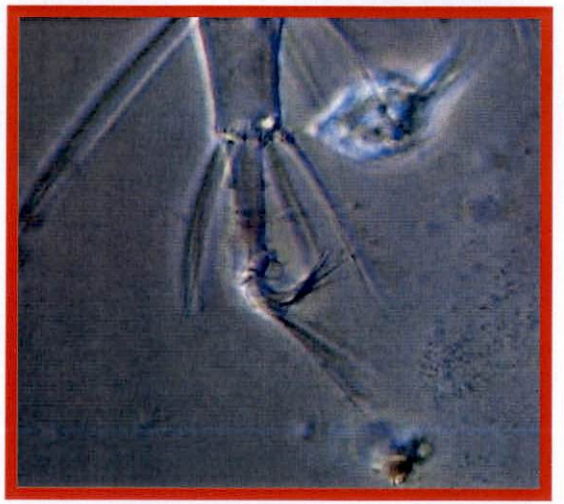
Tetranychus macfarlanei

Tetranychus okinawanus

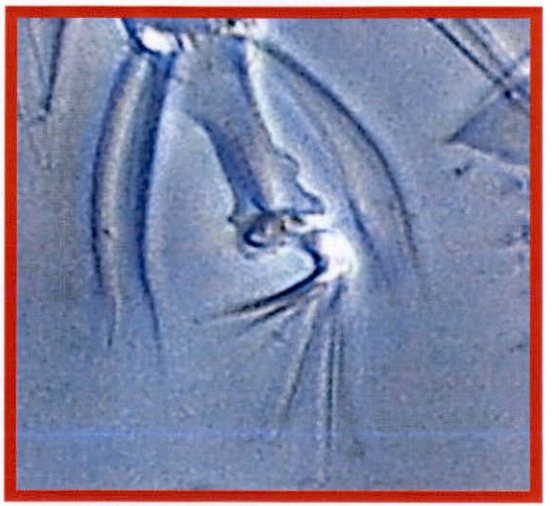
Plate 9: Taxonomic key- Empodium of *Tetranychus truncatus, Tetranychus macfarlanei* and *Tetranychus okinawanus*



Tetranychus truncatus

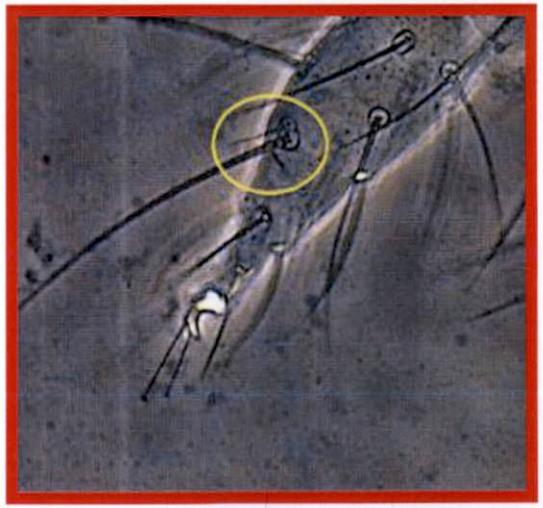


Tetranychus macfarlanei



Tetranychus okinawanus

Plate 10: Taxonomic key- Tarsus I with duplex setae of *Tetranychus truncatus, Tetranychus macfarlanei* and *Tetranychus okinawanus*



Tetranychus truncatus

Tetranychus macfarlanei

Tetranychus okinawanus

Morphological characters of different spider mites species were identified by experts in Tetranychid mite taxonomy using keys given by Gupta and Gupta (1994). List of spider mite species identified from different vegetable crops and from different locations of Thrissur district of Kerala is given in table 6 and list of major taxonomic keys used for differentiating spider mite species is given in table 7. Taxonomic key male aedeagus of *Tetranychus truncatus, Tetranychus macfarlanei* and *Tetranychus okinawanus* is given in plate 8. Taxonomic key Empodium of *Tetranychus truncatus, Tetranychus macfarlanei* and *Tetranychus okinawanus* is given in plate 9. Taxonomic key Tarsus I with duplex setae of *Tetranychus truncatus, Tetranychus macfarlanei* and *Tetranychus okinawanus* is given in plate 10.

4.3.1 Taxonomic keys to identify *Tetranychus truncatus* Ehara (1956).

Female:

- Empodia with 6 proximoventral hairs.
- Tarsus I with sockets of 4 tactile setae proximal to proximal pair of duplex setae.

Tarsus III with 1 proximal tactile seta, peritreme hook 22-25μm long, ventral stria without lobes, pregenital stria entire.

Male:

- Empodia I-II each with an obvious dorsal spur, I-II 3-4 μm long, III-IV 2 μm long.
- Empodium I claw like (uncinate), empodium II-IV with proximoventral hairs free and long.
- Aedeagus with small knob, anterior projection rounded, short, posterior projection pointed, short, dorsal surface flat to slightly, convex, with medial indentation.

4.3.2 Taxonomic keys to identify *Tetranychus macfarlanei* (Baker and Pritchard, 1960).

Female:

- Empodia with 6 proximoventral hairs, empodia I-IV each with dorsal spur 2-3 μm long.
- Tarsus I with the sockets of 3 tactile setae overlapping with the socket of the proximal duplex setae, socket of 1 tactile seta proximal to socket of proximal duplex setae.
- Tarsus III with 1 proximal tactile setae, dorsal stria with lobes, ventral stria between genital region and setae with lobes.
- Pregenital stria almost entire but weak and/or broken medially.

Male:

- Empodia I claw-like (uncinate), empodia II-IV each with proximoventral hairs long and free.
- Aedeagus with small anvil-shaped knob, anterior and posterior projections tiny, dorsal surface flat to slightly convex

4.3.3 Taxonomic keys to identify *Tetranychus okinawanus* (Ehara, 1995)

Female:

- Body, including rostrum, 476μm long , 266"μm wide, red in color.
- Dorsal setae on idiosoma slender, pubescent, much longer than distance between consecutive setae.
- Hysterosoma with longitudinal stria between pair of setae C3 and between pair of setae C4, forming a daimond shaped figure between these setae, lobes on dorsal striae very variable in shape, mostly rounded.
- Peritremes strongly hooked distally.
- Pregenetal area with longitudinal striae, the striae broken medially, solid laterally,

genital flap with longitudinal striae on anterior part, with transverse striae on posterior part.

- Palpus with spinneret slightly longer than broad, dorsal sensilum fusiform, approximately as long as spinneret.
- The number of setae on leg segments: Tarsus I with 3 tactile setae proximal to proximal set of duplex setae, with 1 tactile setae and 1 solenidion at or near the level of proximal duplex to duplex set. Tarsi III and IV each with solenidion proximal, extending almost to mediodistal tactile setae. Each empodium composed of 3 pairs of hairs and 1 pair of somewhat shorter, proximoventral filaments, with the strong mediodorsal spur.

Male:

- Body, including rostrum, 434μm long, 213μm wide.
- Aedeagus upturned distally, terminal knob 3.5μm long, much longer than the width of neck, approximately one half as long as dorsal margin of shaft, the axis of knob sub parallel with dorsal margin of shaft, anterior projection of knob broadly rounded, the posterior projection very narrow, acute.
- Palpus with spinneret about twice as long as broad, dorsal sensillum slender, fusiform.
- The number of setae on leg segments: Tarsus I with 3 tactile setae and 2 solenidia proximal to proximal set of duplex setae with 1 tactile seta and 1 solenidion at or near the level of proximal to proximal duplex set, tarsus II with 3 tactile setae and 1 solenidia proximal to proximal set of duplex setae.
- Empodium I with 1 pair of claw-like divisions and 1 pair of somewhat shorter, proximoventral filaments, and with the strong mediodorsal spur, empodia II-IV each consisting of 3 pairs of hair and 1 pair of proximoventral filaments, with the strong mediodorsal spur.

4.4 Molecular analysis

4.4.1 DNA isolation

Total genomic DNA was isolated from five to ten female mites taken from an isoline using modified CTAB method (Rogers and Benedich, 1994). The subsequent agarose gel electrophoresis has indicated clear bands without contamination and spectrophotometric analysis gave ratio of UV absorbance ratio ($A_{260/280}$) around 1.8.

4.4.2 Assessing the quality of DNA using spectrophotometer (NanoDrop ND-1000)

The purity of DNA was checked using NanoDrop ND-1000 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 OD_{260}/OD_{280}. A value 1.8 indicated that the DNA is pure and free from proteins and RNA. Spectrophotometric analysis gave ratio of UV absorbance ratio ($A_{260/280}$) around 1.8. Quality and quantity of DNA isolated from *Tetranychus* species assessed by NanoDrop spectrophotometer method are presented in Table 3.

Table 3: Quality and quantity of DNA isolated from *Tetranychus* species assessed by NanoDrop spectrophotometer method

Sample ID	UV absorbance at 260 nm (A260)	UV absorbance at 280 nm (A280)	A260/A280	Quantity (µg/µl)
AC1	2.072	1.129	1.84	103.6
AC2	2.586	1.348	1.92	129.3

AC3	1.567	0.789	1.96	78.35
VA1.2	4.343	2.308	1.88	217.15
VA2.2	5.529	2.833	1.95	276.45
VA3.2	4.956	2.588	1.82	247.8
VCu1	0.889	0.478	1.86	44.45
VR3	10.294	5.000	2.06	514.7
AnCuR	2.220	1.220	1.80	111
KB1	0.640	0.354	1.81	32
KC1	1.567	0.789	1.96	78.35
KON	4.956	2.588	1.82	247.8
VD3	0.889	0.478	1.86	44.45
VD4	2.056	1.145	1.79	102.8
AnGA1	2.220	1.220	1.80	111
AnRA2	2.345	1.289	1.81	117.25
AnRA2.11	4.390	2.008	2.18	219.5
VR2	10.294	5.000	2.06	514.7
AnCu3	2.220	1.220	1.80	111
AnCu4	2.220	1.220	1.80	111

4.4.3 Amplification of barcode loci with selected primers

Good quality total genomic DNA was used for the amplification of the barcode loci, using the previously reported primers. Total genomic DNA was used since it had both nuclear and mitochondrial DNAs.

ITS2 and *COI* markers from nuclear second internal transcribed spacer and mitochondrial Cytochrome c Oxidase subunit I gene, respectively, were used for

generating species specific signature sequences from nuclear and mitochondrial genome. The PCR assay was standardized and gave good results.

4.4.4 Assessing the PCR products

The amplification of the candidate locus was evaluated through 2 per cent agarose gel electrophoresis. The barcoding primers used in this study has mostly amplified a clear distinct band of size range 620bp for *ITS2* and 868bp for *COI* and has indicated clear bands without contamination (Figure 1 to 6).

4.4.5 PCR product sequencing

The PCR products amplified using *ITS2* and *COI* primers were run on 2 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 SciGenom Lab. Pvt. Ltd., Cochin. List of *ITS2* and *COI* sequences obtained from spider mite species are given in annexure V.

4.5 Data analysis

4.5.1 Analysis of sequence homology using BLASTn

All the 27 sequences were annotated with the database using BLASTn tool. Analysis of sequences by BLASTn are given in Table 4 and BLASTn results for selected sequences has been presented in figure 14, 15 and 16.

Amplification of *ITS2* (620bp) and *COI* (868bp) regions of spider mites using the barcoding primers

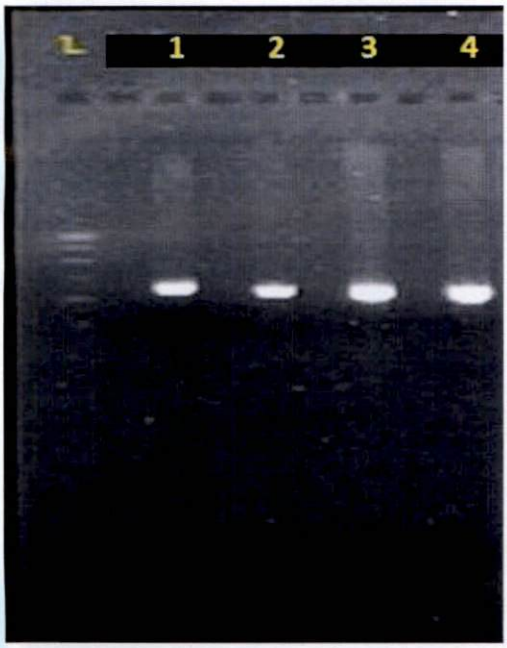


Fig 1: *ITS2* locus, L- Ladder, 1- AC1, 2- AC2, 3- AC3, 4- VA1.2,

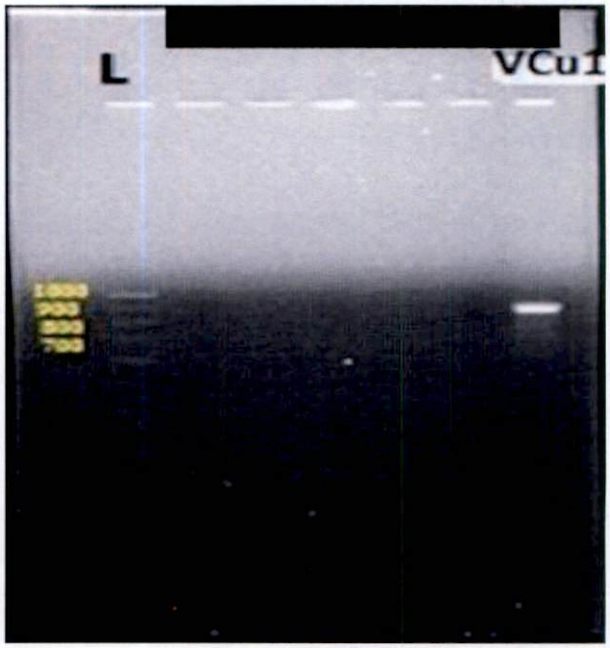


Fig 2: *COI* locus, L-Ladder, VCu1

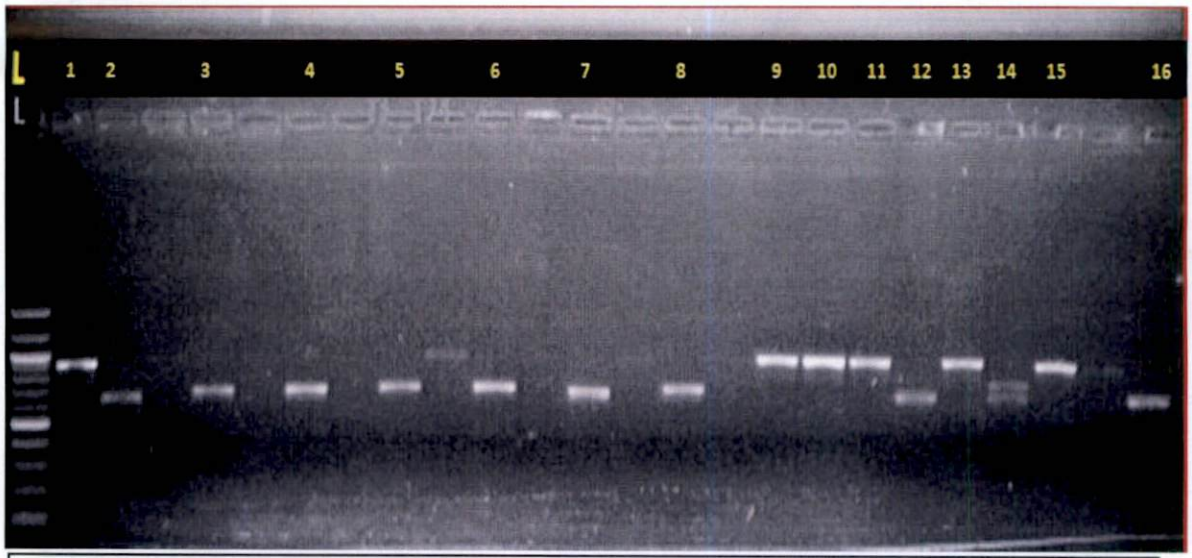


Fig 3: L- Ladder, 1.EC1(*COI*) 2.EC1(*ITS2*) 3. KB1(*ITS2*) 4.KC1(*ITS2*) 5.KON(*ITS2*) 6.VD3(*ITS2*) 7.VD4(*ITS2*) 8- VD6(*ITS2*), 9- AC1(*COI*), 10- AC3(*COI*), 11- VA1.2(*COI*), 12- VR3(*ITS2*), 13- AnCuR(*COI*), 14- AnCuR(*ITS2*), 15- EC1.1(*COI*), 16- VCu1(*ITS2*)

Amplification of *ITS2*(620bp) and *COI*(868bp) regions of spider mites using the barcoding primers

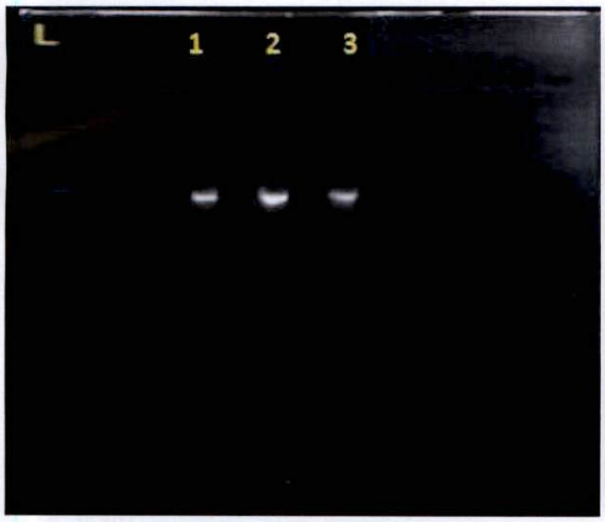


Fig 4: *COI* locus, L-Ladder 1-AnGA1 2- VA2.2 3- VA3.2

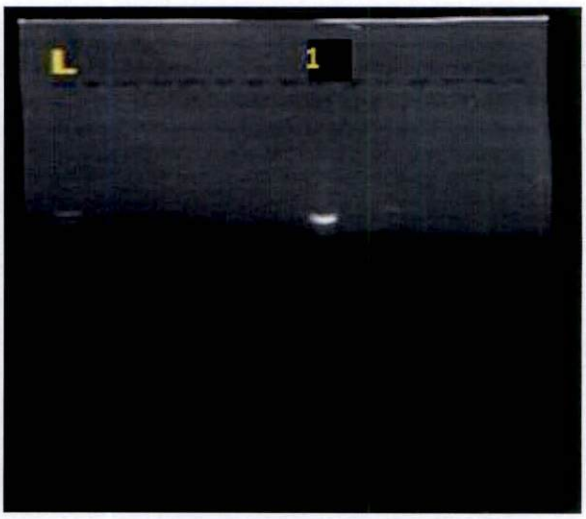


Fig 5: *COI* locus, L-Ladder 1 - AnRA2

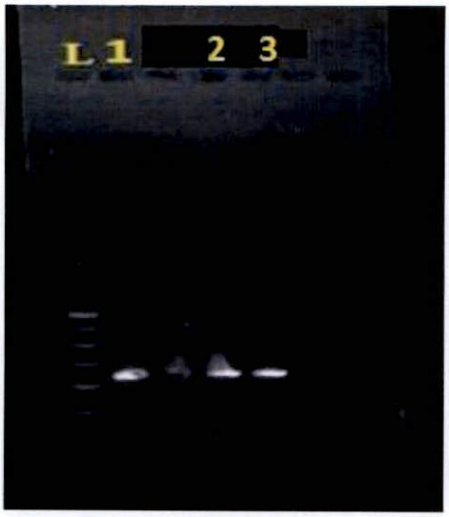


Fig 6: *ITS2* locus, L-Ladder 1-AnGA1, 2-AnRA2, 3-AnRA2.11

Table 4: Analysis of sequences by BLASTn

Sequence ID	Locus	Query coverage in %	Identity in %	Name of the species	E-Value
AC1	*ITS2*	100	100	*Tetranychus truncatus*	0.0
AC2	*ITS2*	100	100	*Tetranychus truncatus*	0.0
AC3	*ITS2*	99	99	*Tetranychus truncatus*	0.0
VA1.2	*COI*	99	100	*Tetranychus truncatus*	0.0
VA2.2	*COI*	99	99	*Tetranychus truncatus*	0.0
VA3.2	*COI*	100	100	*Tetranychus truncatus*	0.0
VCu1	*COI*	100	99	*Tetranychus truncatus*	0.0
VA1.2	*ITS2*	99	100	*Tetranychus truncatus*	0.0
VR3	*ITS2*	99	99	*Tetranychus macfarlanei*	0.0
VCu1	*ITS2*	99	100	*Tetranychus truncatus*	0.0
AC1 Nov	*COI*	100	99	*Tetranychus truncatus*	0.0
AC3 Nov	*COI*	100	99	*Tetranychus truncatus*	0.0
AnCuR	*COI*	99	89	*Tetranychus okinawanus*	0.0
AnCuR	*ITS2*	100	99	*Tetranychus* species	0.0
EC1	*COI*	100	99	*Tetranychus truncatus*	0.0
EC1	*ITS2*	97	99	*Tetranychus truncatus*	0.0
EC1.1	*COI*	100	99	*Tetranychus truncatus*	0.0
KB1	*ITS2*	99	99	*Tetranychus macfarlanei*	0.0
KC1	*ITS2*	95	98	*Tetranychus*	0.0

				macfarlanei	
KON	*ITS2*	97	99	*Tetranychus macfarlanei*	0.0
VD3	*ITS2*	98	99	*Tetranychus macfarlanei*	0.0
VD4	*ITS2*	75	98	*Tetranychus macfarlanei*	0.0
AnGA1	*COI*	100	99	*Tetranychus truncatus*	0.0
AnGA1	*ITS2*	100	100	*Tetranychus truncatus*	0.0
AnRA2	*COI*	100	97	*Tetranychus truncatus*	0.0
AnRA2	*ITS2*	99	100	*Tetranychus truncatus*	0.0
AnRA2.11	*ITS2*	99	99	*Tetranychus truncatus*	0.0

4.5.2 Sequence annotation

Sequence annotation is the process through which the coding and non coding regions and other specific locations that are of importance in a DNA sequence are identified. Using BLASTn, sequence was confirmed whether it is in 5'-3' forward direction. Sequences which were in reverse direction, the reverse complimentary sequence was generated using BioEdit software. *COI* sequences were checked for the presence stop codons using MEGA6. Sequences containing stop codons were trimmed using BioEdit software to get the *COI* sequence free from stop codons. Since *ITS2* is an intron, stop codons were not checked and were directly used for further analysis.

4.5.3 Barcode gap analysis

The distributions of intra versus inter -specific variability were compared using DNA barcode gap. This analysis was carried out separately for *ITS2* and *COI* sequences. Sequences were aligned using clustalW tool of MEGA6 software and with

the mark like "*" was studied. Each gaps were selected which were unique in each species. Barcode gap analysis of *ITS2* sequences showed the presence of adenine nucleotide in *T. truncatus* where as there was thymine in case of *T. macfarlanei* and cytosine in case of *T. okinawanus*. Barcode gap analysis of *COI* sequences showed the presence of thymine in case of *T. truncatus* but cytosine in *T. okinawanus*. The results of barcode gap analysis of *ITS2* and *COI* sequences are presented in figure 7 and 8.

4.5.4 Submission to Barcode of Life Date (BOLD)

Specimen data, specimen images, sequence information and trace files were deposited into Barcode of Life Data (BOLD). Process IDs were generated for the submitted data for which barcodes were generated subsequently. List of process IDs generated by BOLD are given in table 5 and a copy of details of BOLD submission of three species has been attached. Details of BOLD (Barcodes of Life Data) submission is presented in plate 11.

4.5.5 Measurement of nucleotide/genetic divergence

The inter specific distances were calculated using K2P (Kimura 2 parameter) model as recommended by the Consortium of Barcode of Life (CBOL, http:// www.barcoding.si.edu/protocols.html) using Distance summary tool of BOLD (Barcode of Life Data). This measurement was done separately for *ITS2* and *COI* sequences and the results are presented in figure 9 and 10.

4.5.6 Phylogeny analysis

Phylogenetic tree drawn using MEGA6 software (Maximum parsimony model) for *ITS2* sequences of spider mite species are shown in figure 11. Phylogenetic tree drawn using MEGA6 software (Maximum parsimony model) for *COI* sequences of spider mite species are shown in fig 12. These results have revealed that *T. truncatus*

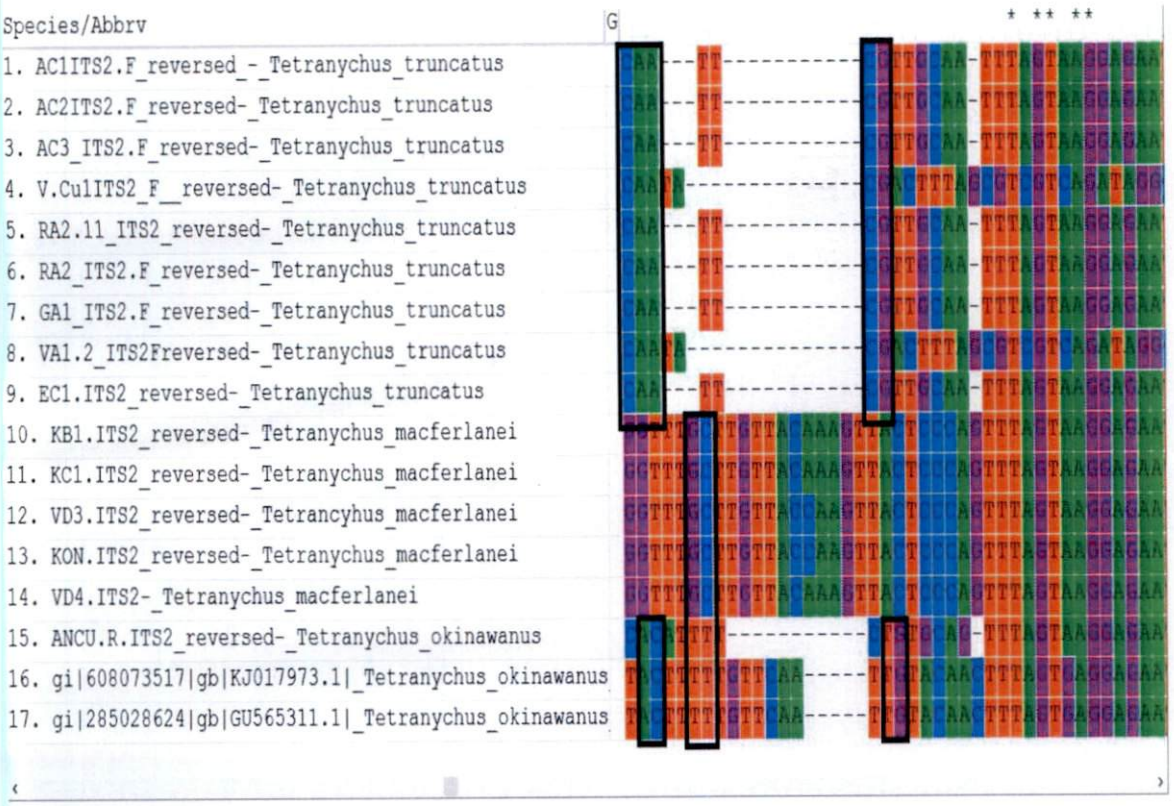


Fig 7: Barcode gap analysis of *ITS2* sequences of spider mite species

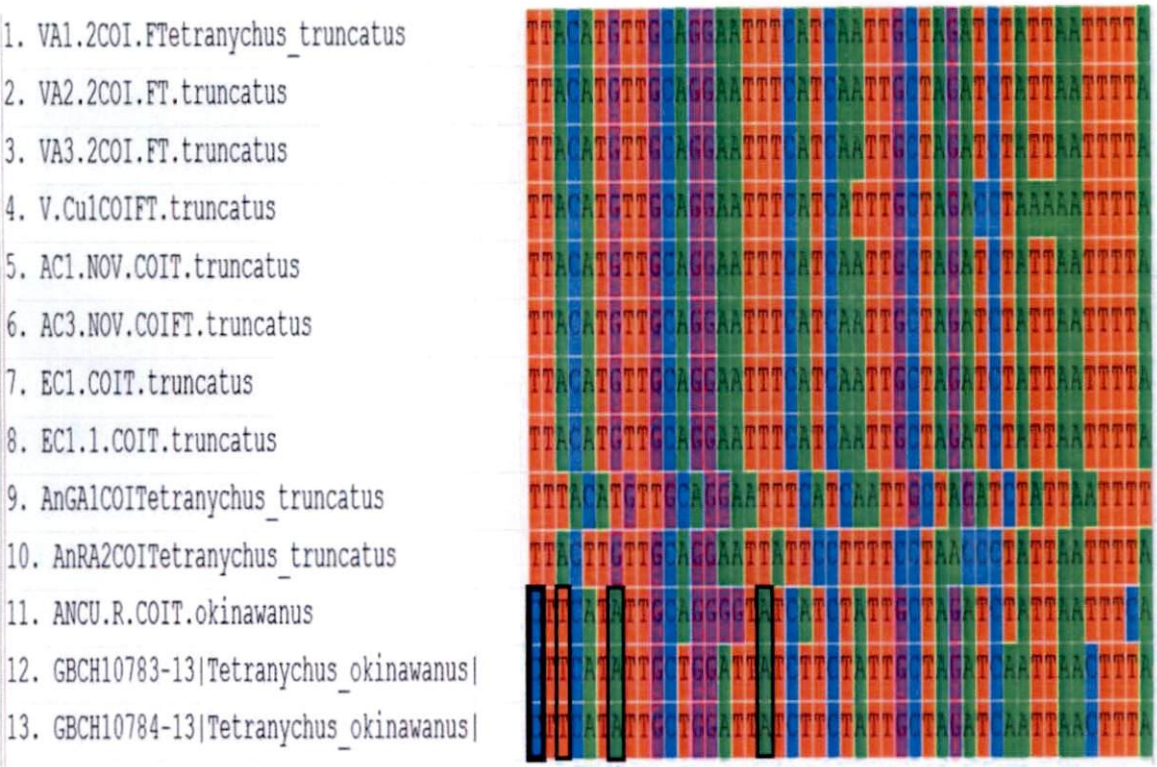


Fig 8: Barcode gap analysis of *COI* sequences of spider mite species

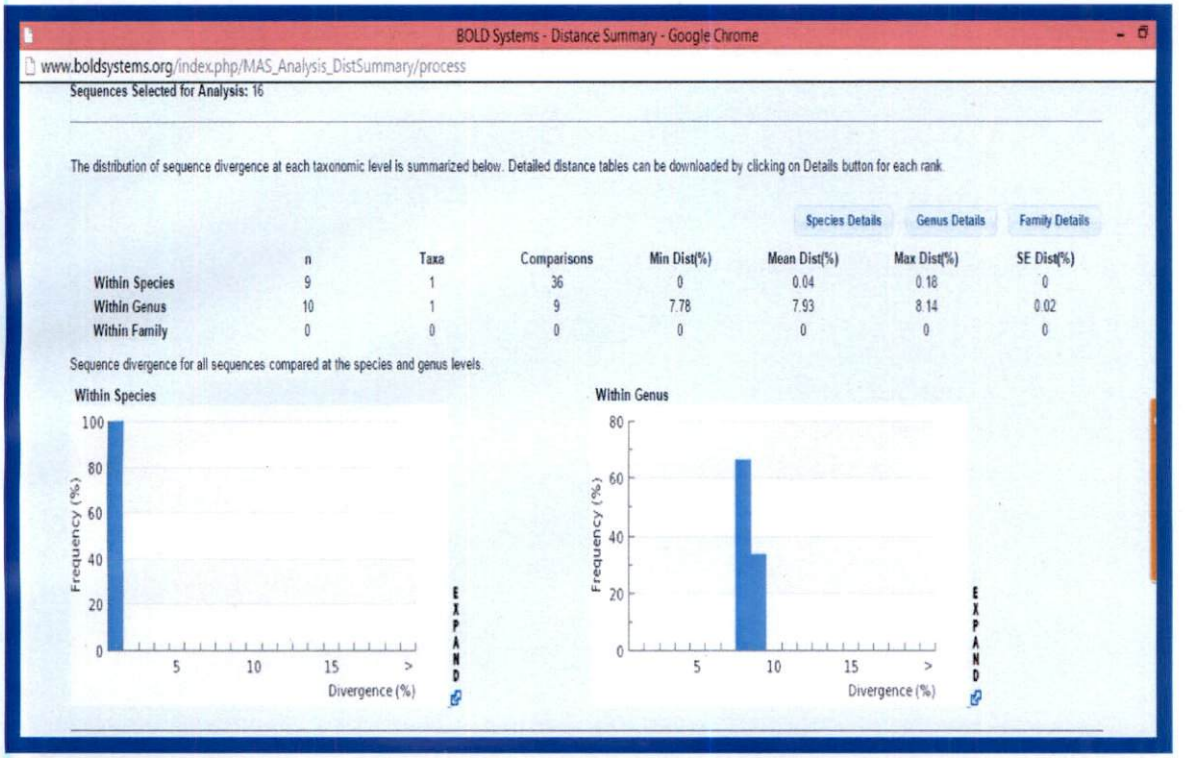


Fig 9: Distance summary results of *ITS*2 sequences spider mites species

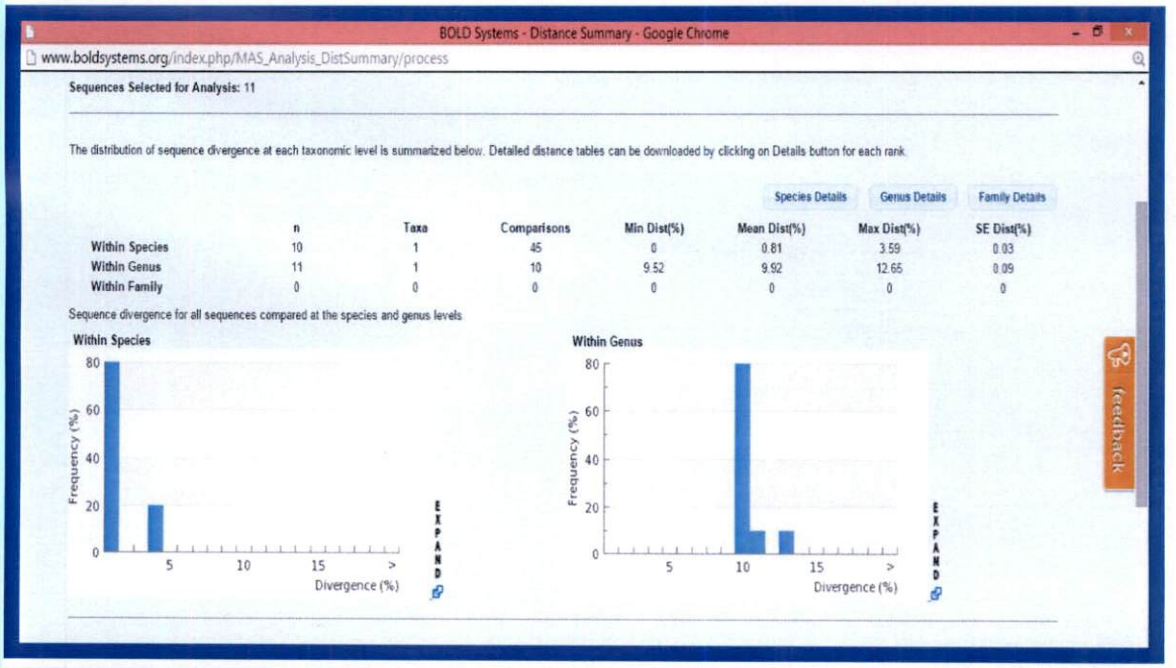


Fig 10: Distance summary results of *COI* sequences of spider mites species

Fig 11: Phylogenetic tree drawn using MEGA6 software for *ITS2* sequences of spider mite species.

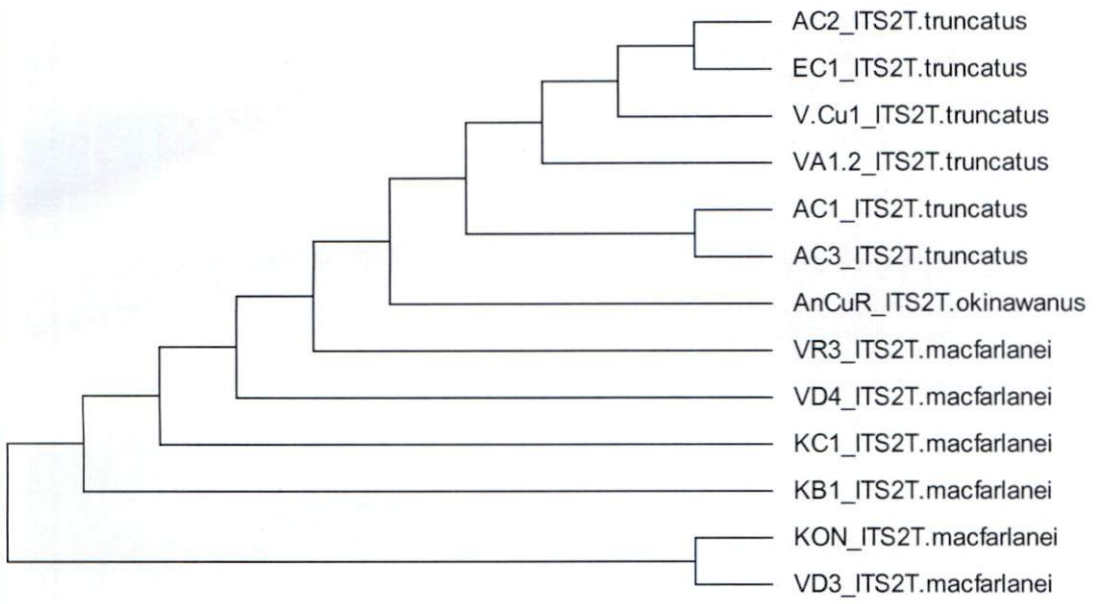
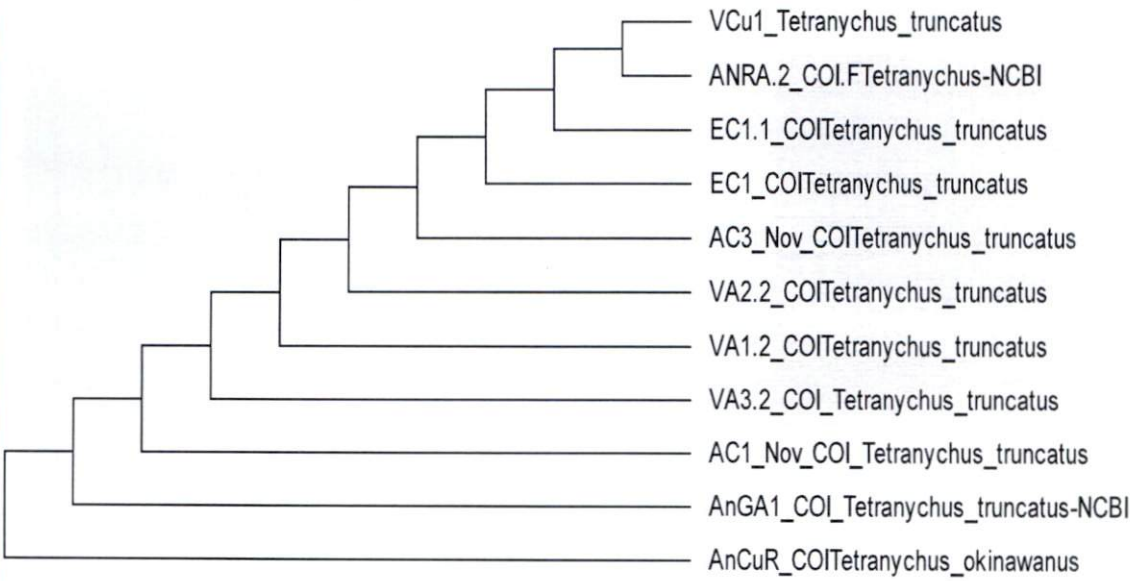
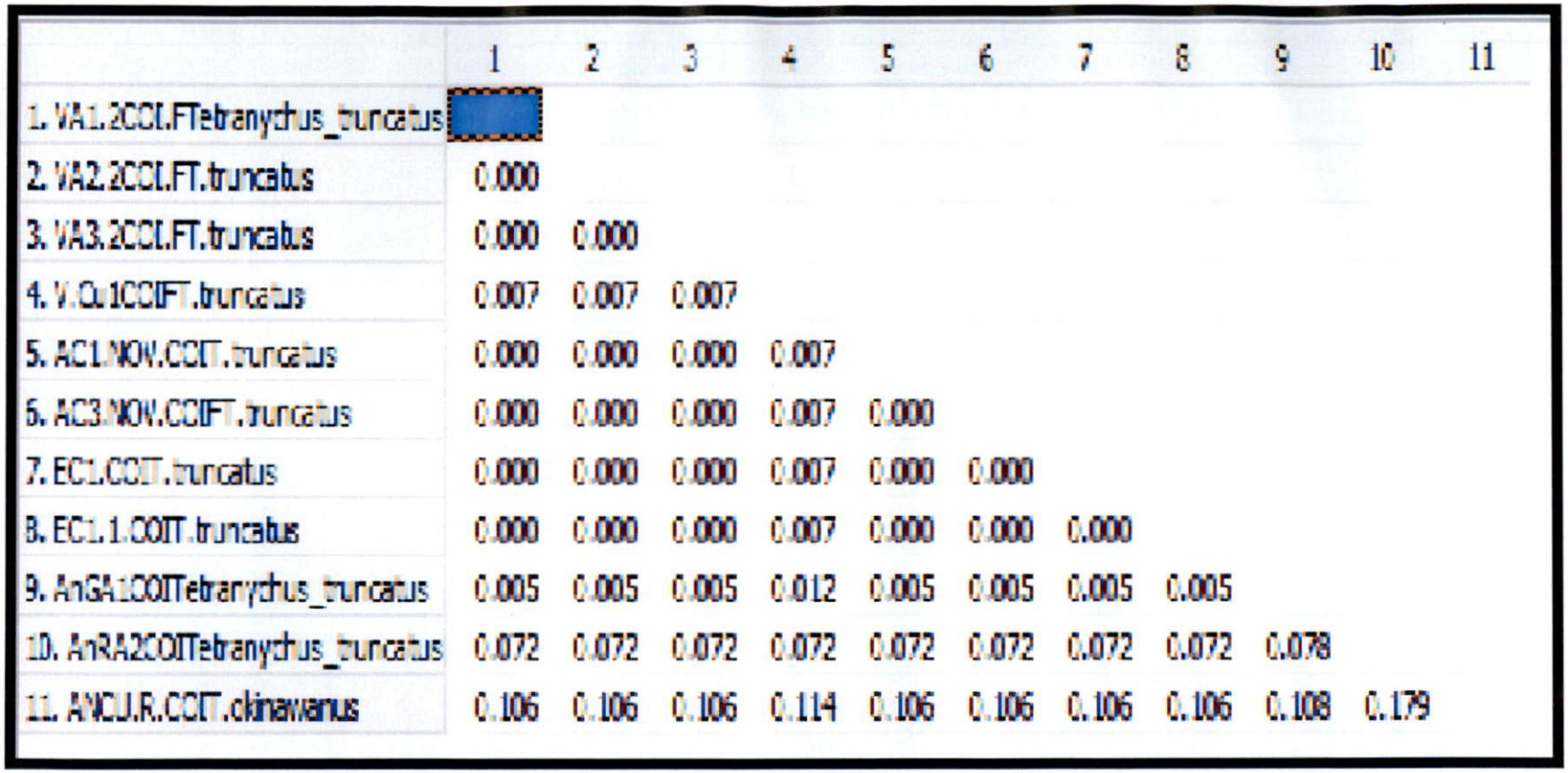


Fig 12: Phylogenetic tree drawn using MEGA6 software for *COI* sequences of spider mite species.



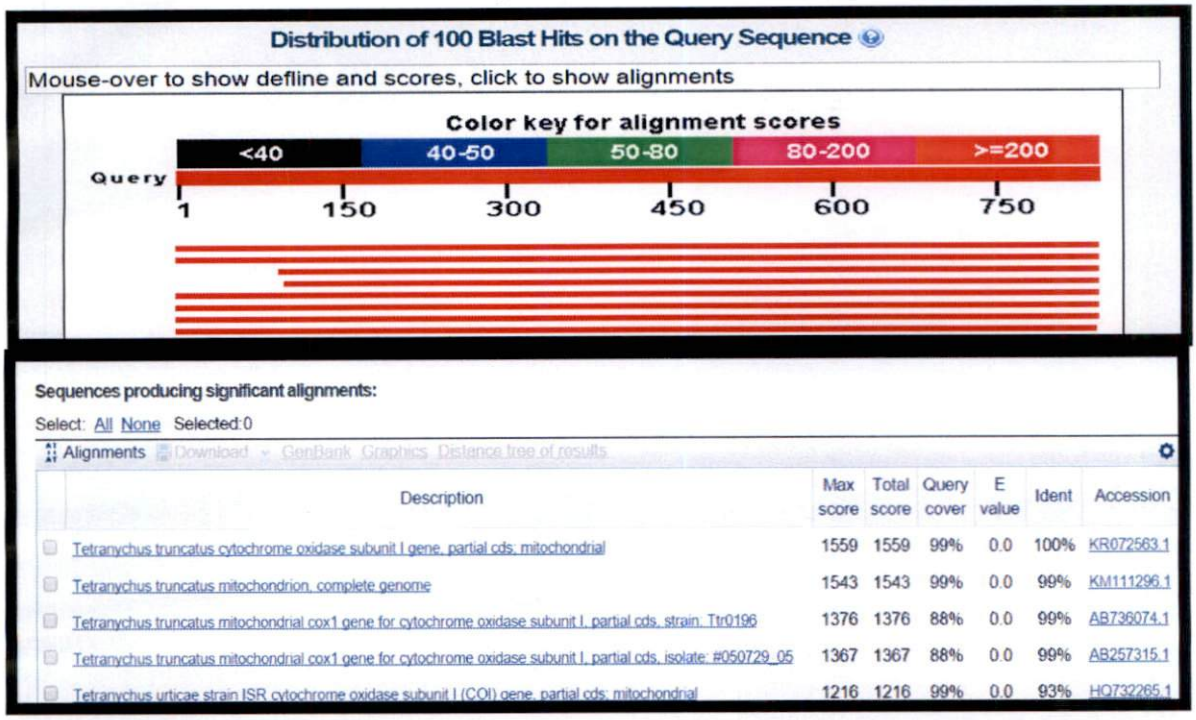


	1	2	3	4	5	6	7	8	9	10	11
1. VA1.2COI.FTetranychus_truncatus											
2. VA2.2COI.FT.truncatus	0.000										
3. VA3.2COI.FT.truncatus	0.000	0.000									
4. V.Ou1COIFT.truncatus	0.007	0.007	0.007								
5. AC1.NOV.COIT.truncatus	0.000	0.000	0.000	0.007							
6. AC3.NOV.COIFT.truncatus	0.000	0.000	0.000	0.007	0.000						
7. EC1.COIT.truncatus	0.000	0.000	0.000	0.007	0.000	0.000					
8. EC1.1.COIT.truncatus	0.000	0.000	0.000	0.007	0.000	0.000	0.000				
9. AnGA1COITetranychus_truncatus	0.005	0.005	0.005	0.012	0.005	0.005	0.005	0.005			
10. AnRA2COITetranychus_truncatus	0.072	0.072	0.072	0.072	0.072	0.072	0.072	0.072	0.078		
11. ANCU.R.COIT.okinawanus	0.106	0.106	0.106	0.114	0.106	0.106	0.106	0.106	0.108	0.179	

Fig 13: Pairwise distance of *COI* sequences of spider mite species

Fig 14- BLASTn results of selected sequences

Sample ID- VA1.2



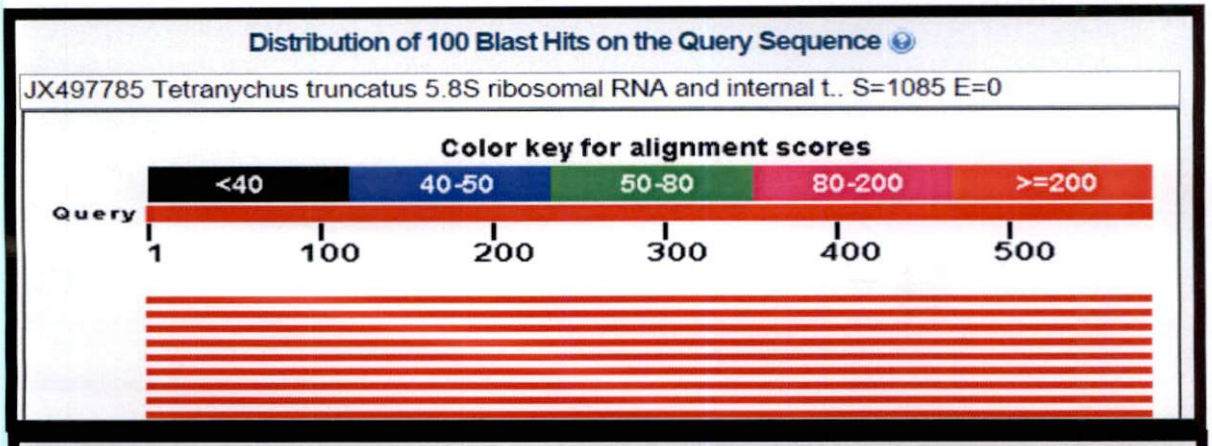
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus truncatus cytochrome oxidase subunit I gene, partial cds; mitochondrial	1559	1559	99%	0.0	100%	KR072563.1
Tetranychus truncatus mitochondrion, complete genome	1543	1543	99%	0.0	99%	KM111296.1
Tetranychus truncatus mitochondrial cox1 gene for cytochrome oxidase subunit I, partial cds, strain: Ttr0196	1376	1376	88%	0.0	99%	AB736074.1
Tetranychus truncatus mitochondrial cox1 gene for cytochrome oxidase subunit I, partial cds, isolate: #050729_05	1367	1367	88%	0.0	99%	AB257315.1
Tetranychus urticae strain ISR cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1216	1216	99%	0.0	93%	HQ732265.1

Sample ID-AC2



Sequences producing significant alignments:

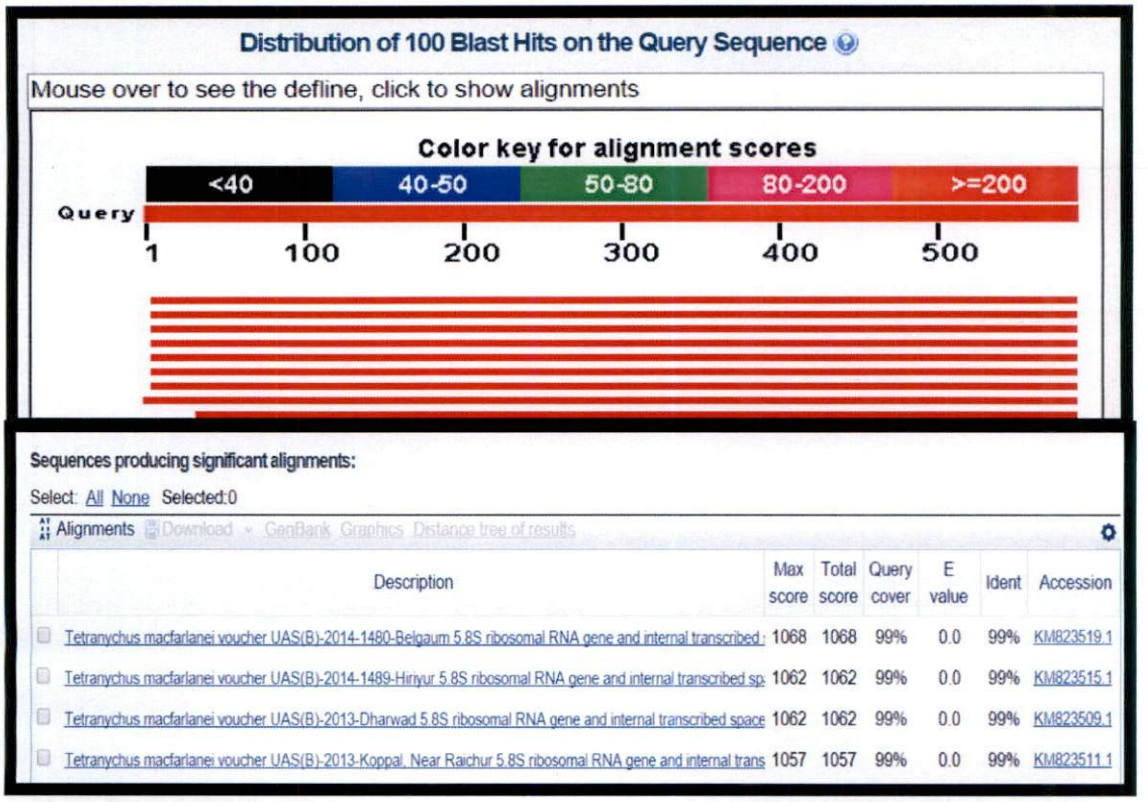
Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus truncatus 5.8S ribosomal RNA and internal transcribed spacer 2, partial sequence	1085	1085	100%	0.0	100%	JX497785.1
Tetranychus cinnabarinus from China internal transcribed spacer 2, partial sequence	1085	1085	100%	0.0	100%	DQ515786.1
Tetranychus truncatus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, isol	1079	1079	100%	0.0	99%	AB257729.1
Tetranychus cinnabarinus isolate Anqing population internal transcribed spacer 2, partial sequence	1079	1079	100%	0.0	99%	DQ512869.1

Fig 15- BLASTn results of selected sequences

Sample ID- KB1



Sample ID- AC3

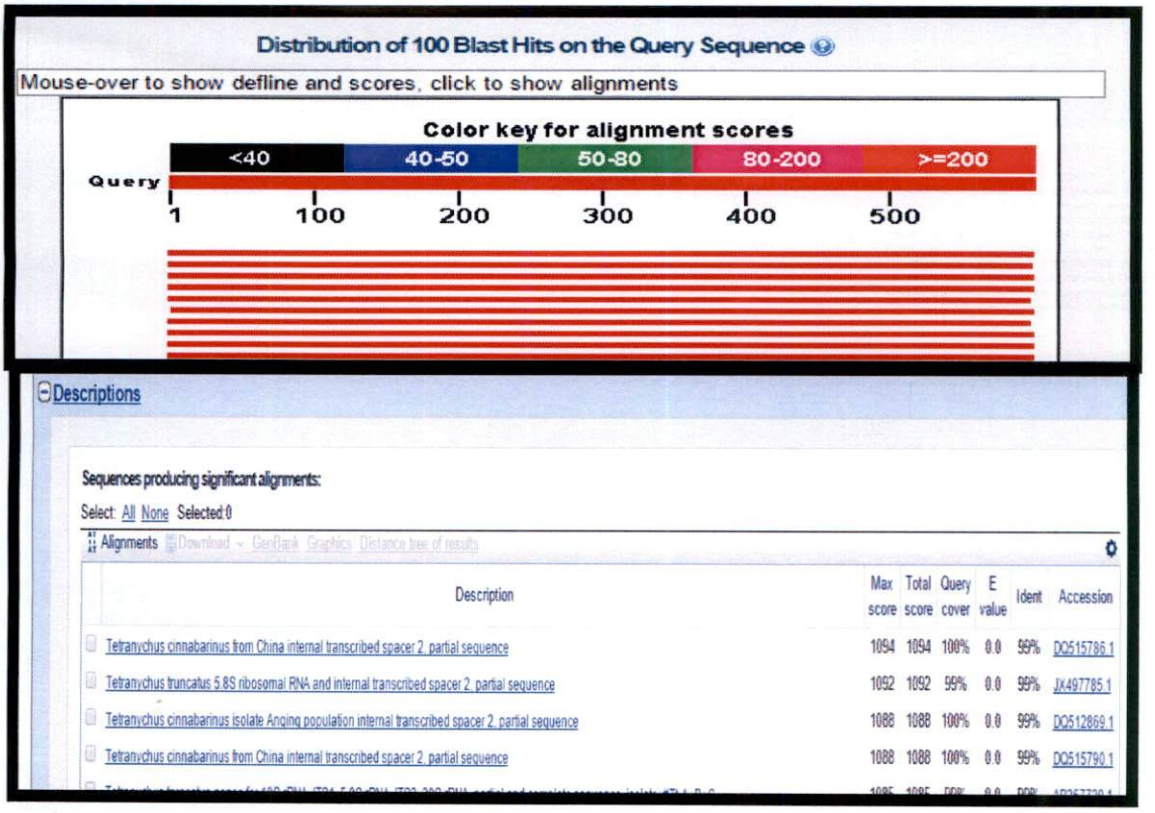
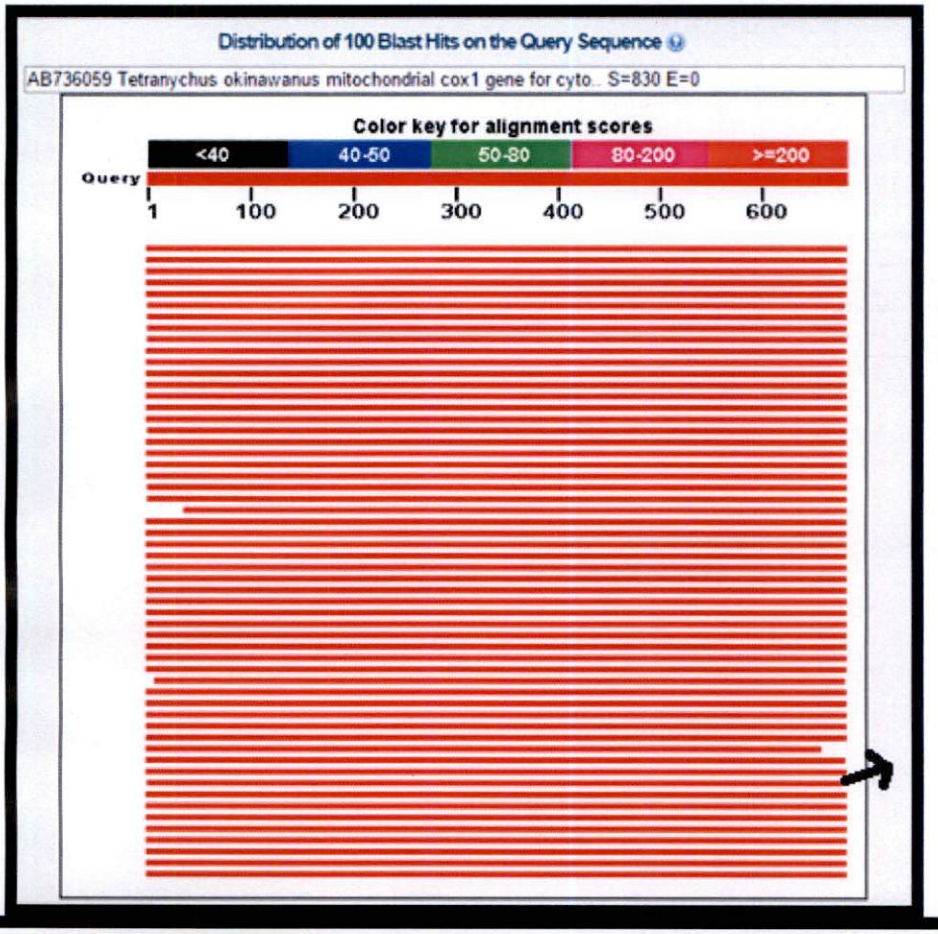


Fig 16- BLASTn results of selected sequences

Sample ID- AnCuR



Tetranychus kanzawai cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	835	835	96%	0.0	90%	AY044642.1
Tetranychus urticae mitochondrial cox1 gene for cytochrome oxidase subunit I, partial cds, strain: TuR0173	833	833	99%	0.0	89%	AB736080.1
Tetranychus evansi strain Algarrobo1 cytochrome oxidase subunit I gene, partial cds; mitochondrial	830	830	100%	0.0	89%	KF447576.1
Tetranychus okinawanus mitochondrial cox1 gene for cytochrome oxidase subunit I, partial cds, strain: Tok0373	830	830	99%	0.0	89%	AB736059.1
Tetranychus evansi mitochondrial cox1 gene for cytochrome oxidase subunit I, partial cds, strain: Tev0211	830	830	100%	0.0	89%	AB736040.1
Tetranychus evansi mitochondrial cox1 gene for cytochrome oxidase subunit I, partial cds, strain: Tev0210	830	830	100%	0.0	89%	AB736039.1
Tetranychus evansi strain Valencia cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	830	830	100%	0.0	89%	GU145108.1
Tetranychus evansi strain KM cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	830	830	100%	0.0	89%	FJ440678.1
Tetranychus evansi strain TW cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	830	830	100%	0.0	89%	FJ440677.1

Specimen - AnGA1 COI- Tetranychus truncatus [ANGAO]

Print

Edit Specimen

IDENTIFIERS

Sample ID:	AnGA1COI
Process ID:	ANGAO001-15
Institution Storing:	Kerala Agricultural University
Field ID:	Andhikad
Museum ID:	CPBMB
Collection Code:	

TAXONOMY

Identification:	Tetranychus truncatus (Ehara, 1956, 1956)	
Rank:	Species	
Identifier		
Identification Method:		
Identifier Institution:		
Identifier Email:		
Taxonomy Note:		
Rank	Current Record (AnGA1COI)	Others in BIN (BOLD:ACI1028)
Phylum:	Arthropoda	Arthropoda [4]
Class:	Arachnida	Arachnida [4]
Order:	Trombidiformes	Trombidiformes [4]
Family:	Tetranychidae	Tetranychidae [4]
Subfamily:		
Genus:	Tetranychus	Tetranychus [4]
Species:	Tetranychus truncatus	Tetranychus truncatus [4]

SPECIMEN DETAILS

Voucher Status:	
Tissue Descriptor:	
Sex:	Female
Reproduction:	
Life Stage:	Adult
Extra Info:	
Note:	
Associated Taxa:	
Associated Specimens:	
Reference Link:	

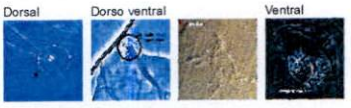
ANNOTATION

Add Tags & Comments Comments: 0 Associated Tags: No Tags

PHOTOGRAPHS

License: Nrr (2015)
License Holder: Shruti Bennur, Kerala Agricultural University

Add Tags & Comments Comments: 0 Associated Tags: No Tags



GEOGRAPHY

Country:	India
Province/State:	Kerala
Region/County:	
Sector:	
Exact Site:	
Lat/Lon:	

COLLECTION DETAILS

Collectors:	Shruti Bennur
Collection Event ID:	
Date Collected:	
Date Accuracy:	
Time Collected:	
Site Code:	
Habitat:	
Sampling Protocol:	
Coord. Source:	
Coord. Accuracy:	
Elevation:	
Elevation Accuracy:	
Depth:	
Depth Accuracy:	
Collection Notes:	

Sequence - AnGA1 COI- Tetranychus truncatus [ANGAO]

Print

IDENTIFIERS

Sample ID:	AnGA1COI
Process ID:	ANGAO001-15
Identification:	Tetranychus truncatus
BIN:	BOLD:ACI1028

COI-5P

SEQUENCE DATA

Genbank Accession:	KT208292
Translation Matrix:	Invertebrate Mitochondrial
Last Updated:	2015-06-09

Clear Sequence Edit Sequence

NUCLEOTIDE SEQUENCE

Sequence: *834 bp*

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GTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATATCTTCTTCTATT
GAAATAATAATTTTTTCTTTACATGTTGCAGGAATTTCATCAATTGCTAGATCTATTAAT
TTTATTTCAACTATTTTATTAATAAAAAATAAAAACTATTATTTAAGTAATTTAACATTG
TTTTCTTTATCAATTTTAATTACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCA
GGTGCTATTACAATAATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGA
GGAGGAGGAGACCCAATTTTATATCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTT
TATATTTTAATTTTACCAGGTGTTGGAATGATTTCACACATTATTAGATATAATTTAGGT
AAAAAAGAAGTTGTTGGAAAAATTGGAATAATATTTGCTATAATATCGATTGGTTTATTA
GGTTTTATTGTATGAGCTCACCACATATTTACAGTAGGAATAGATGTTGATACACGAGCT
TACTTTACTGCTGCTACAATAATTATTGCTATTCCTACTGGAATTAAAATTTTTAGTTGA
TTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATATTGATCTATAGGA
TTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCAAATTCA
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Composition: A (278), G (99), C (101), T (356)

Ambiguous Characters: 0

Identify Sequence Using:

Full DB Species DB Published DB Full Length DB

AMINO ACID SEQUENCE

Sequence: *300 residues*

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PLMVNTVDLCFPRINNMSFWLLIPSLILMISSSMKSVMNGVGWTMYPPLTSIQYFMSSSI
EMMIFSLHVAGISSIASSINFISTILLMKNKNYYLSNLTLFSLSILITTLLLLLALPVLA
GAITMILMDRNFNTSFFDPSGGGDPILYQHLFWFFGHPEVYILILPGVGMISHIISYNLG
KKEVVGKIGMMFAMMSIGLLGFIVWAHHMFTVGMDVDTRAYFTAATMIIAIPTGIKIFSW
FTTILNSHINFNISMYWSMGFLIMFSIGGFTGIVASNS
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ILLUSTRATIVE BARCODE

SEQUENCING RUNS: Kerala Agricultural University

Run Date	Direction	Trace File	Seq Primer	Quality
PCR Primers: UBC6 F/R COI				
2015-05-20	r	ANGA1COIR.ab1	R COI	high
2015-05-20	f	ANGA1COIF.ab1	UBC6 F	fail

Sequence Editor View Trace Files Download

ANNOTATION

Add Tags & Comments Comments: 0 Associated Tags: No Tags

SAMPLE REPORT FROM LIMS

BOLDLIMS

No Report Found on LIMS.

Specimen - Tetranychus macfarlanei - KB1 ITS2 [KBONE]

Print

Edit Specimen

IDENTIFIERS

Sample ID:	KB1 ITS2
Process ID:	KBONE001-15
Institution Storing:	Kerala Agricultural University
Field ID:	KVK
Museum ID:	CPBMB
Collection Code:	

TAXONOMY

Identification:	Arthropoda
Rank:	Phylum
Identifier	
Identification Method:	Taxanomic keys
Identifier Institution:	
Identifier Email:	
Taxonomy Note:	
Rank	Current Record (KB1 ITS2)
Phylum:	Arthropoda
Class:	
Order:	
Family:	
Subfamily:	
Genus:	
Species:	

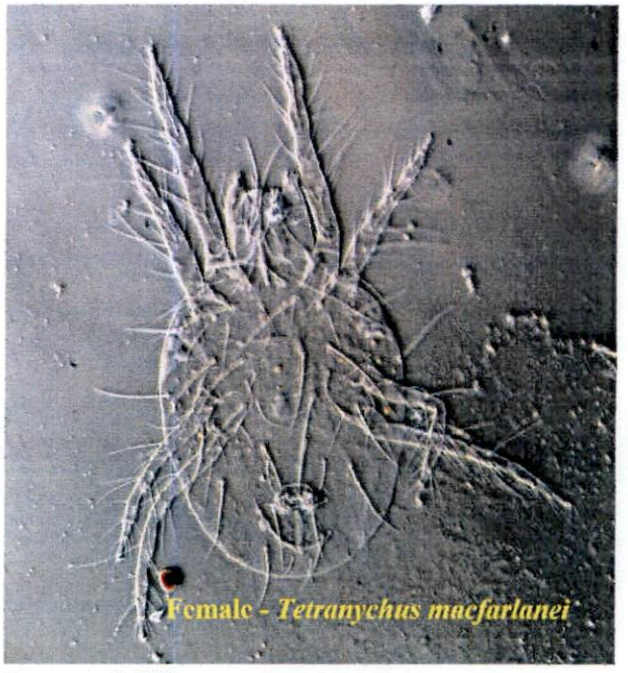
SPECIMEN DETAILS

Voucher Status:	
Tissue Descriptor:	
Sex:	
Reproduction:	
Life Stage:	
Extra Info:	
Note:	
Associated Taxa:	
Associated Specimens:	
Reference Link:	

ANNOTATION

Add Tags & Comments Comments: 0 Associated Tags: No Tags

PHOTOGRAPHS



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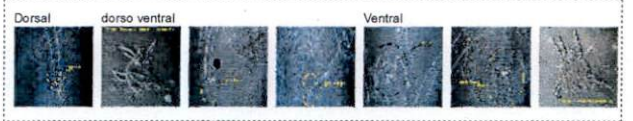
Add Tags & Comments Comments: 0 Associated Tags: No Tags



GEOGRAPHY

Country:	India
Province/State:	Kerala
Region/County:	
Sector:	
Exact Site:	
Lat/Lon:	

COLLECTION DETAILS

Collectors:	Shruti Bennur
Collection Event ID:	
Date Collected:	
Date Accuracy:	
Time Collected:	
Site Code:	
Habitat:	
Sampling Protocol:	
Coord. Source:	
Coord. Accuracy:	
Elevation:	
Elevation Accuracy:	
Depth:	
Depth Accuracy:	
Collection Notes:	

Sequence - Tetranychus macfarlanei - KB1 ITS2 [KBONE]

Print

IDENTIFIERS

Sample ID:	KB1 ITS2
Process ID:	KBONE001-15
Identification:	Arthropoda

ITS2

SEQUENCE DATA

Genbank Accession:	
Translation Matrix:	N/A - NON-CODING MARKER
Last Updated:	2015-04-24

Clear Sequence Edit Sequence

NUCLEOTIDE SEQUENCE

Sequence: *589 bp*

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AGGTATTTGCAATATCTCTTTTTTAAACTCTGCTTCAAGACAGAATGAATTAGTTACCAT
TTTGTAAGTATATAGCATACAAGTGCATAGAGAATTCTGTTTTGAACAGGTTAGCTTATC
ACCATGGTTATCTGTAATACGACTTTGACGTCGTCAGATAGGCGACTAACCTAAGAATCT
CATGCTAGTATCTAATCATATATACTGCTTGCAGAGATGAAATATGCATTGTACACAAAA
TTTATTAGTGTGTAACAAGTAGATTTGAGATTCTCCTTACTAAACTGGGAGTAACTTTGT
AACAAGCAAACCCAGTTAAGAAAAGGAATAAAACATCAAGATTCATACGAATCCCAAAAT
ACTGCAACTACTGGTTAGAGTAGTATACAAGTATTTTGTTGATTATTTTACATCTCAACT
CTCAGACAGATGTGACCTCGGAAAAGACCCGAAAGCCGCAATGTGCGTTGGAAGCTCAAG
TGCTCGGCGTGTCCTGCAATTCGCACCGATTATCGTAGCTAGCTGCGTT
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Composition:	A (191), G (110), C (106), T (182)
Ambiguous Characters:	0

ILLUSTRATIVE BARCODE

SEQUENCING RUNS: Kerala Agricultural University

Run Date	Direction	Trace File	Seq Primer	Quality
PCR Primers: ITS2 KAU/R ITS2 KAU				
2015-04-13	r	KB1ITS2R.ab1	R ITS2 KAU	high
2015-04-13	f	KB1ITS2F.ab1	ITS2 KAU	high

Sequence Editor View Trace Files Download

ANNOTATION

Add Tags & Comments Comments: 0 Associated Tags: No Tags

SAMPLE REPORT FROM LIMS

BOLDLIMS

No Report Found on LIMS.

Specimen - Tetranychus okinawanus - AnCuR COI [ANCRI]

Print

Edit Specimen

IDENTIFIERS

Sample ID:	ANCURCOI
Process ID:	ANCRI001-15
Institution Storing:	Kerala Agricultural University
Field ID:	Andhikad
Museum ID:	CPBMB
Collection Code:	

TAXONOMY

Identification:	Tetranychus okinawanus
Rank:	Species
Identifier	
Identification Method:	Taxanomic keys
Identifier Institution:	
Identifier Email:	
Taxonomy Note:	
Rank	Current Record (ANCURCOI)
Phylum:	Arthropoda
Class:	Arachnida
Order:	Trombidiformes
Family:	Tetranychidae
Subfamily:	
Genus:	Tetranychus
Species:	Tetranychus okinawanus

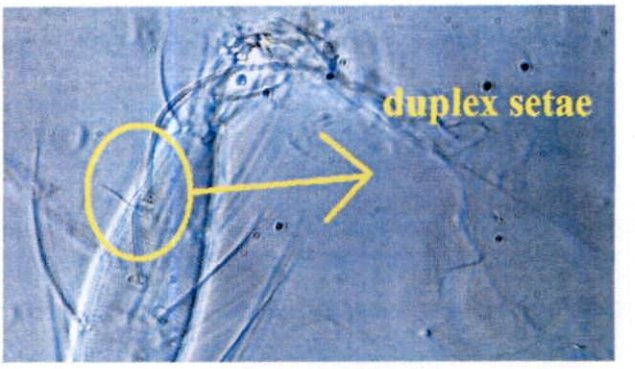
SPECIMEN DETAILS

Voucher Status:	
Tissue Descriptor:	
Sex:	
Reproduction:	
Life Stage:	adult
Extra Info:	
Note:	
Associated Taxa:	
Associated Specimens:	
Reference Link:	

ANNOTATION

Add Tags & Comments Comments: 0 Associated Tags: No Tags

PHOTOGRAPHS



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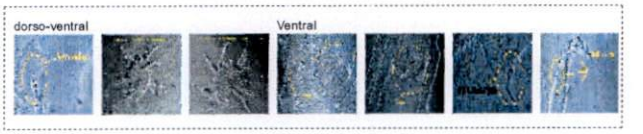

GEOGRAPHY

Country:	India
Province/State:	Kerala
Region/County:	
Sector:	
Exact Site:	
Lat/Lon:	

COLLECTION DETAILS

Collectors:	Shruti Bennur
Collection Event ID:	
Date Collected:	
Date Accuracy:	
Time Collected:	
Site Code:	
Habitat:	
Sampling Protocol:	
Coord. Source:	
Coord. Accuracy:	
Elevation:	
Elevation Accuracy:	
Depth:	
Depth Accuracy:	
Collection Notes:	

Sequence - Tetranychus okinawanus - AnCuR COI [ANCRI]

Print

IDENTIFIERS

Sample ID:	ANCURCOI
Process ID:	ANCRI001-15
Identification:	Tetranychus okinawanus

COI-5P

SEQUENCE DATA

Genbank Accession:	KT186807
Translation Matrix:	Invertebrate Mitochondrial
Last Updated:	2015-04-29

Clear Sequence Edit Sequence

NUCLEOTIDE SEQUENCE

Sequence: *607 bp*

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TTGCAGGGGTATCATCTATTGCTAGATCTATTAATTTCATTTCAACTATTTTACTAATAA
AAAATAAAAATTATATTATAAGAAACTTAACTTTATTTACTTTATCAATTTTAATTACTA
CCTTTTTACTTTTACTAGCTTTACCAGTATTAGCAGGTGCTATTACAATAGTTTTAATAG
ATCGAAATTTTAACACGTCTTTTTTTGATCCTACAGGAGGGGGAGATCCTATTTTGTATC
AACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTATATTTTAATCTTACCAGGTTTTG
GAATAATTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGTTTGGAAAAGTTG
GAATATTATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGAGCTCATCATA
TATTTACAGTAGGAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTA
TTGCAATTCCTACTGGAATTAAAATTTTTAGATGATTTACAACTATTATTAATTCACATA
TTATTTT
```

Composition:	A (202), G (73), C (74), T (258)
Ambiguous Characters:	0

Identify Sequence Using:

Full DB Species DB Published DB Full Length DB

AMINO ACID SEQUENCE

Sequence: *220 residues*

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LQFNIYMSSSIEMMIFSLHIAGVSSIASSINFISTILLMKNKNYIMSNLTLFTLSILITT
FLLLLALPVLAGAITMVLMDRNFNTSFFDPTGGGDPILYQHLFWFFGHPEVYILILPGFG
MISHIISYNLGKKEVFGKVGMLFAMMSIGLLGFIVWAHHMFTVGMDVDTRAYFTAATMII
AIPTGIKIFSWFTTIINSHIIX
```

ILLUSTRATIVE BARCODE

SEQUENCING RUNS: Kerala Agricultural University

Run Date	Direction	Trace File	Seq Primer	Quality
PCR Primers: UBC6 F/R COI				
2015-04-13	r	ANCURCOIR.ab1	R COI	fail
2015-04-13	f	ANCURCOIF.ab1	UBC6 F	med

Sequence Editor View Trace Files Download

ANNOTATION

Add Tags & Comments Comments: 0 Associated Tags: No Tags

SAMPLE REPORT FROM LIMS

BOLDLIMS No Report Found on LIMS.

and *T. okinawanus* are closely related species.

4.5.7 Pairwise distance calculation

The inter specific distances were calculated using K2P (Kimura 2 parameter) using MEGA6 software. The "Distance|Compute Pair-wise" command generates the pair wise distances. The results of pairwise distance of *COI* sequences of spider mite species is presented in figure 13.

4.5.8 Submission to NCBI

A total of 27 DNA sequences from spider mites species were deposited in the NCBI GenBank and the list of accession numbers generated for the sequences deposited in NCBI are given in table 5.

Table 5: List of accession numbers generated for the sequences deposited in NCBI and process IDs generated by BOLD

Sl. No.	Sample ID	Locus	Accession no. (NCBI)	Process ID (BOLD)
1	AC1	*ITS2*	KR052246	ACONE001-15
2	AC2	*ITS2*	KR052247	ACTWO001-15
3	AC3	*ITS2*	KR063238	ACTRE001-15
4	VA1.2	*COI*	KR052245	VACOI001-15
5	VA2.2	*COI*	KR063239	VATPT001-15
6	VA3.2	*COI*	KR072563	VATRE001-15
7	VCu1	*COI*	KR260209	VCUMT001-15
8	VA1.2	*ITS2*	KR007962	KAU002-15

9	VR3	*ITS2*	KR095582	VRTRE001-15
10	VCu1	*ITS2*	KT070711	VCUIT001-15
11	AC1 Nov	*COI*	KR271023	ACCOI001-15
12	AC3 Nov	*COI*	KR271024	ACTNC001-15
13	AnCuR	*COI*	KT186807	ANCRI001-15
14	AnCuR	*ITS2*	KR271022	ANCUR001-15
15	KB1	*ITS2*	KR297220	KBONE001-15
16	KC1	*ITS2*	KR297221	KCONE001-15
17	KON	*ITS2*	KR297222	KONIT001-15
18	EC1	*COI*	KR297226	ECONE001-15
19	EC1	*ITS2*	KR297223	ECITS001-15
20	VD3	*ITS2*	KR297224	VDTRE001-15
21	VD4	*ITS2*	KR297225	VDFOU001-15
22	AnGA1	*COI*	KT208292	AnGAO001-15
23	AnGA1	*ITS2*	KT070708	ANGAI001-15
24	AnRA2	*COI*	KT208293	RACOI001-15
25	AnRA2	*ITS2*	KT070709	ARAIT001-15
26	AnRA2.11	*ITS2*	KT070710	ANRAT001-15
27	EC1.1	*COI*	KT070711	ECOPO001-15

Table 6: List of spider mite species identified from different vegetable crops from different locations of Thrissur district of Kerala

Sl. No	Locations	Crops	Species found
1	Alathur	Cowpea	*Tetranychus truncatus*
2	Anthikad	Red amarathus Green amaranthus Cucumber	*Tetranychus truncatus* *Tetranychus truncatus* *Tetranychus okinawanus*
3	Elenad	Cowpea	*Tetranychus truncatus*
4	KVK, Thrissur	Brinjal Cowpea Okra	*Tetranychus macfarlanei* *Tetranychus macfarlanei* *Tetranychus macfarlanei*
5	Vellanikkara	Amaranthus Cucumber Ridgegourd Dolichos bean	*Tetranychus truncatus* *Tetranychus truncatus* *Tetranychus macfarlanei* *Tetranychus macfarlanei*

Table 7: List of major taxonomic keys used for differentiating spider mite species

Taxonomic keys	*T. truncatus*	*T. macfarlanei*	*T. okinawanus*
Male aedeagus	Aedeagus with small knob, anterior projection rounded, posterior projection pointed, short, dorsal surface flat to slightly with medial intendation	Aedeagus with small anvil-shaped knob, anterior and posterior projections tiny, dorsal surface flat to slightly convex	Aedeagus upturned distally, terminal knob 3.5µm long, much longer than the width of neck, anterior projection of knob broadly rounded, the posterior projection very narrow, acute
Empodium	Empodium I uncinate, empodium II-IV with proximoventral hair free and long	Empodia I-IV each with obvious dorsal spur 4µm long	Empodia composed of 3 pairs of hairs and 1 pair of somewhat shorter, proximoventral filaments, with strong mediodorsal spur
Tarsus I	Tarsus I with at least 4 tactile setae proximal to proximal duplex setae	Base of most proximal tactile seta on tarsus I just proximal to the proximal duplex seta), other 3 proximal tactile setae clearly overlapping with duplex seta.	Tarsus I with 3 tactile setae and 2 solenidia proximal to proximal set of duplex setae, with 1 tactile seta and 1 selenidion at or near the level of the proximal duplex setae.

Discussion

5. DISCUSSION

The family Tetranychidae, or spider mites comprises of 19 genera, 6 tribes and 2 subfamilies in the Tetranychidae family. The subfamilies are Bryobiinae and Tetranychinae. Gupta (1985) in his Handbook Plant mites of India, has included 82 species under 18 genera. The family Tetranychidae comprises 1200 species worldwide (Bolland *et al.*, 1998). It includes the most injurious plant-feeding mites (Helle and Sabelis 1985; Meyer 1987; Zhang 2003). Some infest a wide range of host plants, whereas others are highly specific (Bolland *et al.,* 1998). Spider mites can rapidly disperse to exploit new feeding sites, damage agricultural and horticultural crops, causing severe economic losses.

The precise identification of tetranychid taxa is problematic due to the limited number of available morphological characters and to the similarity between species. In addition, both sexes of many species are often needed in order to arrive to precise determinations (Jeppson *et al.*, 1975; Helle and Sabelis, 1985). For these reasons molecular methods are increasingly being applied for taxonomic purposes.

The DNA barcoding was used for precise identification of unknown species, with no taxonomic expertise (Hebert *et al.,* 2003). This study was carried out to to generate DNA barcode for different species of spider mites in vegetable crops and to study the intra and inter species genetic relationship.

5.1 Purposive survey

Spider mite infected leaf samples were collected from different locations of Thrissur district, Kerala and spider mite infection was observed on various vegetable crops *viz*; Amaranthus, brinjal, cowpea, cucumber, dolichos bean and ridge gourd. A total of three different *Tetranychus* species *viz*; *Tetranychus truncatus, T. macfarlanei*

and *T. okinawanus* belonging to the family Tetranyhcidae were recorded during the study. Similar survey, conducted by Binisha and Bhaskar (2013) reported *T. urticae* as a dominant species on vegetables in Thrissur district, Kerala.

5.2 Maintenance of an isoline

Spider mites are very minute in their size and to obtain good quality and quantity of DNA for molecular studies, five to ten female spider mites are needed. Under field conditions, there is a chance that, more than one Tetranychid species may reside together on a single leaf. Since all the members belonging to Tetranychidae morphologically look similar, it is difficult to select females for molecular studies. To avoid such confusions, there is a need for maintaining an isoline in order to avoid DNA contamination of different species and from the same isoline, individuals are to be used for morphological analysis.

In this study, single gravid female of spider mite from the collection was used for rearing an isoline. Isolines were obtained within 12-15 days which were later used for morphological and molecular analysis. Maintainance of isoline was also done by Srinivasa *et al.* (2012).

5.3 Preparation of permanent microscopic slides and identification of mite specimens

Morphological characters were recorded from the fully grown up adult males and females of *Tetranychus* species (spider mites), mounted on microscopic slides. Taxonomic keys such as, the presence of well developed duplex setae on tarsus I, one pair of para-anal setae, tarsus I with duplex setae, well separated, dividing segment into three equal parts and empodium splits distally into three pairs have already been described as the key characters for confirming *Tetranychus* genus by Gupta and Gupta

(1994). Taxonomic keys referred to identify *Tetranychus okinawanus* was given by Ehara (1995). Same keys were used in this study also.

Morphological characters of *Tetranychus* species collected from different vegetables of Thrissur district during the study revealed the presence of three species belonging to family Tetranychidae *viz; Tetranychus truncatus, T. macfarlanei and T. okinawnaus*. The species were distinguished based on shape of the male aedeagous, structure of distal segment of pedipalp thumb claw. These key characters are described for *Tetranychus truncatus* by Ehara (1956), *Tetranychus macfarlanei* by Baker and Pritchard (1960) and *Tetranychus okinawanus* by Ehara (1995).

Among spider mites infesting vegetable crops in India, *T. urticae* was considered as predominant species which infest wide variety of crops. In Kerala, a survey conducted to explore phytophagous and predatory mites associated with major vegetable crops by Binisha and Bhaskar during 2011- 2012. Their study revealed *Tetranychus urticae* to be the dominant phytophagous mite genus on Brinjal, Bhindi, Amaranthus and Cowpea. However in the present study, *T. urticae* was not recorded from any host plants surveyed where as *T. truncatus* was found to be the dominant. Result in present study suggest chance for species displacement in a short period of two years. Exhaustive survey has to be carried out to arrive at the correct conclusion.

5.4 Molecular analysis

Three different spider mite species were characterized using *ITS2* and *COI* primers in this study. Accumulating large number of spider mites sequences, especially from genes such as the mitochondrial cytochrome oxidase I (*COI)* and the transcribed spacer regions (*ITS1* and *ITS2)* of nuclear ribosomal DNA (Navajas *et al.*, 1996, 1998), has served as the scaffold of a molecular method that could simplify spider mites identification. At present mitochondrial *COI* gene is used for species

identification in the animal kingdom (Hebert *et al.*, 2003).

5.4.1 DNA isolation

Candidate loci used in the study are *ITS2* and *COI* which lies in nuclear DNA and mitochondrial DNA respectively. Since total genomic DNA contains both nuclear DNA and mitochondrial DNA, isolation of total genomic DNA using the modified CTAB method (Rogers and Benedich, 1994) was followed in this study. To increase the concentration of DNA, spider mites were kept in CTAB buffer and incubated for five minutes at 60 degree celcius before crushing. This modification in DNA isolation protocol helped in getting more quantity of DNA. This pre-incubation was one of the finding in this study.

Spider mites are very minute in size and DNA isolation from single mite is not enough for further analysis. Female spider mites are bold and bigger in size than males. Hence five to ten adult female mites taken from an isoline gave good quality DNA in sufficient quantity. Since spider mites lack phenols, browning was not a problem and the use of antioxidant such as PVP was not necessary. Srinivasa *et al.* (2012) had also used females from isolines for DNA isolation.

Klompen (2000) found it necessary to use 2-5 individuals in each extraction whereas Anderson and Trueman (2000) used only leg tissue dissected from individual mites. If possible it is preferable to extract DNA from single individuals to prevent mixing of distinct genotypes, particularly if there is any question about the identity of the mites.

5.4.2 Quality assessment of DNA using spectrophotometer (NanoDrop ND-1000)

The quality of DNA was tested by subjecting it to spectrophometric method. A DNA sample was reported as high quality if it has a low amount of RNA

(Wettasingf and Peffley, 1998). DNA obtained in the study also showed less amount of RNA.

In spectrophotometer method, the ratio of optical density at 260 and 280nm was worked out to test the quality. The absorbance ratio was calculated as OD at 260/280, for various samples. Those samples with the ratio between 1.8 and 2.0 were considered to be of high quality. Majority of the samples recorded a ration between 1.8 to 2.0.

5.4.3 PCR product sequencing

A total of 27 sequences of spider mites were amplified with two selected primers and sequenced by outsourcing (SciGenom Lab. Pvt. Ltd., Cochin). 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.

5.5 Data analysis

5.5.1 Analysis of sequence homology using BLASTn

BLAST, or the Basic Local Alignment Search Tool, was specifically designed to search nucleotide and protein databases. 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).

In this study, all the 27 sequences were annotated using BLASTn tool. *ITS2* sequences of sample ID AC1, AC2, AC3, VA1.2, VR3, VCu1, EC1, KB1, KON, AnGA1 and AnRA2 have shown almost 99-100% identity with *Tetranychus truncatus.* But in the sequence of sample ID KC1, the query coverage was 95% and identity was

98%. In VD4 also query coverage was less i.e 75% and identity was 98%. AnGA1 has shown 100% identity with 100% query coverage. Sample ID AnCuR has shown 100% query coverage with 99% identity with *Tetranychus sp.* Its has not shown identity with *T. okinawnaus* clearly with respect to species. In case of *COI* sequences also, almost all samples have shown 99-100% identity with the respective species but sample ID AnCuR 89% identity with *T. okinawanus*. However, BLASTn results have revealed that both *ITS2* and *COI* loci are powerful to yield up to an average of 99.25% & 98.45% efficiency respectively to differentiate the species and at genus level both loci have shown 100% efficacy. BLAST analysis was also carried out by Liu *et al.* (2011) to find homology.

One pitfall of BLAST analysis is that there is chance of a sequence showing homology with other related species. John *et al.* (2012) have also mentioned about the problems in GenBank BLAST analysis during their study on practical evaluation of DNA barcode efficacy. In the present study also the problem of accuracy was encountered. For example, in the sample ID AC3 (Fig 16) which is identified as *T. truncatus* based on morphological analysis, showed 99% identity with *T. truncatus* (Query coverage- 99%) and also with *T. cinnabarinus* (Query coverage- 100% and Identity- 99%). This is a situation where it again leads to the confusion about identity and this also strengthens the need for DNA barcoding for precise species identification.

5.5.2 Sequence annotation

Sequence annotation is the process through which the coding and non coding regions and other specific locations that are of importance in a DNA sequence are identified. There is a chance that obtained sequences may be in reverse direction. Checking sequence alignment using BLASTn makes the sequence suitable for further analysis.

5.5.3 Barcode gap analysis

A robust DNA barcode should have separate and non-overlapping genetic variations among species. If all species share the identical sequences, as straight forward failures, but when all individuals of a species show change in some sequence, it is treated as successful discrimination (Mathew, 2014). Wiemers and Fiedler (2007) have also carried out barcode gap analysis in case study in blue butterflies (Lepidoptera: Lycaenidae).

A total of 27 sequences were aligned and the position of nucleotide change has been observed. The distributions of intra- versus inter- specific variation in *ITS2* and *COI* barcode was examined separately. In Fig. 7 and 8, the results demonstrated the characteristic variation among the sequences of different species. For example in figure 7, among *ITS2* sequences of different species, there is a nucleotide A (adenine) in all the sequences of *T. truncatus,* T (thymine) in all the sequences of *T. macfarlanei* and C (cytosine) in all the sequences of *T. okinawanus.* This is regarded as barcode gap. Both *ITS2* and *COI* were successful in showing the variation. *COI* locus is already regarded as a potential locus for barcoding animal kingdom (Hebert *et al.*, 2003). David *et al.* (2007) have tentatively concluded that rDNA *ITS2* sequence barcodes may serve as an effective tool for the identification of spider mite species.

5.5.4 Measurement of nucleotide/genetic divergence

The inter specific distances were calculated using K2P (Kimura 2 parameter) model as recommended by the Consortium of Barcode of Life (CBOL, http:// www.barcoding.si.edu/protocols.html) using Distance summary tool of BOLD (Barcode of Life Data).

It is desirable for barcodes to show very low sequence divergence within a species, with significantly higher sequence divergence at higher taxonomic levels.

The Distance Summary tool gives a report of sequence divergence between barcode sequences at the conspecific and congeneric levels. *ITS2* sequences have given mean distance within species as 0.04% and mean distance within genus as 7.93% whereas *COI* sequences have given mean distance within species as 0.81% and mean distance within genus 9.92%. Within species distance is very less for *ITS2* sequences when compared with *COI* sequences. Similar distance summary calculation was done by Wiemers and Fiedler (2007) in case study in blue butterflies (Lepidoptera: Lycaenidae).

5.5.5 Phylogeny analysis

The genetic similarity was determined for the three *Tetranychus* species based on *ITS2* and *COI* sequences separately. Phylogeny analysis done in this study have revealed that *Tetranychus truncatus* and *T. okinawanus* are closely related species. Same results were obtained by Matsuda *et al.* (2014) in their phylogenetic study of spider mites based on *COI* and rRNA genes.

5.5.6 Pairwise distance calculation

The inter specific distances were calculated using K2P (Kimura 2 parameter). Navajas *et al.* (1998) have also performed pairwise distance calculation using *COI* and *ITS2* sequences of *T. urticae* where in the 15 *COI* haplotypes found fell into two major phylogenetic lineages differing by an average of 5% nucleotide divergence. In contrast, no variation at all was found at the ITS2 in this species.

In the present study, range of distance within *T. truncatus* (*ITS2*) was 0% to 0.925%. Range of distance within *T. macfarlanei* (*ITS2*) was 0% to 0.006%. Distance between *T. macfarlanei* and *T. truncatus* was in the range of 0.145% to 0.972%. Distance between *T. macfarlanei* and *T. okinawanus* was in the range of 0.145% to 0.150%. Distance between *T. okinawanus* and *T. truncatus* was in the range of 0.011%

to 0.101%. Range of distance within *T. truncatus* (*COI*) was 0% to 0.078% and range of distance between *T. truncatus* and *T. okinawanus* was 0.106% to 0.179%.

5.6 Utilization of the developed barcodes in spider mites identification

The species specific barcodes generated from this study could be used for the species allocation of *Tetranychus* accession. *COI* or *ITS2* locus may be amplified using the primer sets and sequence could be generated. Based on the BLAST with the basic sequence of 868bp (*COI*) and 620bp (*ITS2*) generated and deposited in NCBI, the barcoding gaps could be determined in the sample and from this, the species interpretation could be easily done.

5.7 Future line of work

At present, BOLD (Barcode of Life Data) accepts only *COI* marker for DNA barcoding. In this study efficiency of *ITS2* as a DNA barcoding marker was studied and found that it is equally good. In future, even *ITS2* can be used as an efficient DNA barcode tool. In future, sequence analysis can also be done using *ITS1* which also has similar properties to *ITS2* (McLain *et al.*, 1995) and its efficiency can be measured for DNA barcoding of *Tetranychus* species. Primer sets used in this study can be used for barcoding other species belonging to family Tetranychidae. Spider mites collected from different vegetable crops and from different locations were identified using taxonomic keys and further by molecular taxonomy. The study can be further extended to know the host mediated and ecology based variations in spider mites species to know the ecotypes using specific SSR molecular markers already identified.

Summary

6. SUMMARY

The study entitled "DNA barcoding of spider mites (Prostigmata: Tetranychidae) in vegetables" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Agricultural Entomology, College of Horticulture, Vellanikkara, during the period 2013-2015. The objective of the study was to generate DNA barcode for different species of spider mites of vegetable crops and to study the intra and inter species genetic relationship. *ITS2* and *COI* candidate loci were selected for the study. In Thrissur district of Kerala, three spider mite species were found on vegetables during this study: *Tetranychus truncatus, T. okinawanus* and *T. macfarlanei.* Spider mites were collected from different location in Thrissur district like Alathur, Anthikad, Elenad, KVK (Thrissur) and Vellanikkara. Crops from which spider mites were collected are Amaranthus, brinjal, cowpea, dolichos bean, okra, ridge gourd and Cucumber.

The salient findings of the study are stated below:

1. The protocol for genomic DNA isolation from spider mites was standardised. The protocol suggested by Rogers and Benedict (1994) with slight modification made before and after crushing the sample was found to be most appropriate for isolation of DNA from spider mites.

2. The quality and quantity of DNA was analyzed by NanoDrop^{R} ND-1000 spectrophotometer. The absorbance ratio ranged from 1.8 - 1.9, which indicated good quality DNA and the recovery was high. This DNA was suitable for PCR amplification analysis.

3. Primers set of common barcoding locus *COI* was studied along with the *ITS2* locus. Both the primers generated the expected molecular weight bands which were suitable for barcoding.

4. *ITS2* was found to be an efficient tool which gave species level resolution. This can be used as supporting marker for *COI* and can also be used for DNA barcoding of spidermites.

5. Both the barcode primers have yielded >500bp bands and the primers were useful in further analysis.

6. The species identification success rate was calculated using BLAST using all *ITS2* and *COI* sequences as query sequences, which indicated both the loci are powerful to yield up to 99.25 per cent and 98.45 per cent respectively, efficiency to differentiate the species and at the genus level with 100 per cent efficiency.

7. A total of 30 sequences of spider mites have been submitted to the NCBI GenBank (Accession numbers: KR052246, KR052247, KR063238, KR052245, KR007962, KR063239, KR072563, KR095582, KR271023, KR271024, KR297220, KR297221, KR260209, KR297222, KR297223, KR297224, KR297225, KR271022, KR297226, KT070708, KT070709, KT070710 and KT070711).

8. All the sequences were used to construct the phylogenetic tree using MEGA 6 (maximum parsimony). The phylogenetic tree generated in the study discriminated all the three species. Results revealed that *T. truncatus* and *T. okinawanus* are closely related species.

9. The genetic distance (*ITS2* and *COI* separately) was computed for all the sequences belonging to 3 species of family Tetranychidae based on K2P model using BOLD Distance summary tool.

10. Results of morphological and molecular analysis were correlated and the results matched.

11. A new species *Tetranychus okinawanus* was found on Cucumber crop from Andhikad, Thrissur district, Kerala which is first time getting reported from India.

12. Purposive survey carried out at different locations has revealed that *T. truncatus* is the dominant spider mite species on vegetables in Thrissur district, Kerala.

Future line of work includes; At present, BOLD (Barcode of Life Data) accepts only *COI* marker for DNA barcoding of the members that belong to animal kingdom. In this study, *ITS2* was also used as a DNA barcoding marker to differentiate *Tetranychus* species and found that it is equally good. In future, even *ITS2* can be used as an efficient DNA barcode tool. In future, sequence analysis can also be done using *ITS1* which also contains conserved regions and its efficiency can be measured for DNA barcoding of Tetranychid species. Primer sets used in this study can be used for barcoding other Tetranychid species also. Spider mites collected from different vegetable crops and from different locations were identified using taxonomic keys and further by molecular taxonomy. The study can be further extended to know the host mediated and ecology based variations in spider mites species to know the ecotypes using specific SSR molecular markers already identified.

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Annexures

ANNEXURE I

Materials used during spider mites collection and rearing

Polythene bags and rubber bands

Petriplates: Tarsons glasswares

Microscope: 10X,30X and 40X lens

Insulin syringe

Paint brush (Zero size)

Cotton pads

Slides

Cover slips

Composition of Hoyer's medium

Sl. No.	Content	Quantity
1	Chloral Hydrate	200g
2	Gum Arabic	30g
3	Glycerol	20ml
4	Distilled water	50ml

ANNEXURE II

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	: 10ml
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 9^0C 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**- BioRad power PAC 1000, gel casting tray, comb.

ANNEXURE IV

List of laboratory equipments used for the study

Refrigerated centrifuge: Kubota, Japan

Horizontal electrophoresis System: BioRad, USA

Thermal cycler: Veriti Thermal Cycler (Applied Biosystem, USA)

Gel documentation system: BioRad, USA

Nanodrop^{R} ND-1000 spectrophotometer: Nanodrop^{R} Technologies Inc. USA

ANNEXURE V

List of *ITS2* and *COI* sequences obtained from spider mite species

>AC1ITS2.Freversed-Tetranychus truncatus
AGAACGCAGCTAGCTACGATAATCGGTGCGAATTGCAGGACACGCCGAGCACTTGAGCTTCCAACGCACATTGCGGCTTTC
GGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCATACTACCATATATGCATT
GTTTTGAGAGGATTGCATATTTATATGCATGAATCTTGATGTTTTATTCCTTTTCTTAATTGCAATTCGTTGCAATTTAGTAAGG
AGAATCTCAAATCTACTTGTTTCACATGATAAATTTTGTGTACAATGCATATTTCATCTCTGCAAGCAGTATATATGAATAGATA
CTAGCATGAGATTCTAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAACTAACCTGC
TAAATAATGAATCTTATTGCACTTGTATAAAGCATACAAATAGTAGCTATTTCATTCTGTTAAAGCAGACCTAAGAAGTAATGC
AAAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTTGCTTGCTTGCAAACAACACAAATATACATTAATCAACTTAATC
AA
>AC2ITS2.Freversed- Tetranychus truncatus
AGAACGCAGCTAGCTACGATAATCGGTGCGAATTGCAGGACACGCCGAGCACTTGAGCTTCCAACGCACATTGCGGCTTTC
GGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCATACTACCATATATGCATT
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AGAATCTCAAATCTACTTGTTTCACATGATAAATTTTGTGTACAATGCATATTTCATCTCTGCAAGCAGTATATATGAATAGATA
CTAGCATGAGATTCTAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAACTAACCTGC
TAAATAATGAATCTTATTGCACTTGTATAAAGCATACAAATAGTAGCTATTTCATTCTGTTAAAGCAGACCTAAGAAGTAATGC
AAAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTTGCTTGCTTGCAAACAACACAAATATACATTAATCAACTTAATC
AAT
>AC3_ITS2.Freversed- Tetranychus truncatus
GGTCGATGGAAGAACGCAGCTAGCTACGATAATCGGTGCGAATTGCAGGACACGCCGAGCACTTGAGCTTCCAACGCACAT
TGCGGCTTTCGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCATACTACC
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TTTAGTAAGGAGAATCTCAAATCTACTTGTTTCACATGATAAATTTTGTGTACAATGCATATTTCATCTCTGCAAGCAGTATATA
TGAATAGATACTAGCATGAGATTCTAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCA
ACTAACCTGCTAAATAATGAATCTTATTGCACTTGTATAAAGCATACAAATAGTAGCTATTTCATTCTGTTAAAGCAGACCTAA
GAAGTAATGCAAAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTTGCTTGCTTGCAAACAACACAAATATACATTAA
TCAACTTAATCAA
>V.Cu1ITS2 Freversed- Tetranychus truncatus
TGATTAAGTTGATTAATGTATATTTGTGTTGTTTGCAAGCAAGCAACGTAAATCTACTTT
AACGTTTGCACAAATTTTGCCTTTGCATTACTTCTTAGGTCTGCTTTAACAGAATGAAAT
AGCTACTATTTGTATGCTTTATACAAGTGCAATAAGATTCATTATTTAGCAGGTTAGTTG
ATCACCATAGTTATCTGCAATACGACTTTAGCGTCGTCAGATAGGCGACTAACCTTAGAA
TCTCATGCTAGTATCTATTCATATATACTGCTTGCAGAGATGAAATATGCATTGTACACA
AAATTTATCATGTGAAACAAGTAGATTTGAGATTCTCCTTACTAAATTGCAACGAATTGC
AATTAAGAAAAGGAATAAAACATCAAGATTCATGCATATAAATATGCAATCCTCTCAAAA
CAATGCATATATGGTAGTATGCAAGTGTTTTGTTGATTATTTTACATCTCAACTCTCAGA
CAGATGTGACCTCGGAAAAGACCCGAAAGCCGCAATGTGCGTTGGAAGCTCAAGTGCTCG
GCGTGTCCTGCAATTCGCACCGATTATCGTAGCTAGCTGCGTTCTC
>RA2.11_ITS2reversed- Tetranychus truncatus
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GCGGCTTTCGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCATACTACCA
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CTAACCTGCTAAATAATGAATCTTATTGCACTTGTATAAAGCATACAAATAGTAGCTATTTCATTCTGTTAAAGCAGACCTAAG
AAGTAATGCAAAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTTGCTTGCTTGCAAACAACACAAATATACATTAAT
CAACTT
>RA2_ITS2.Freversed- Tetranychus truncatus
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TTCGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCATACTACCATATATGC
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GGAGAATCTCAAATCTACTTGTTTCACATGATAAATTTTGTGTACAATGCATATTTCATCTCTGCAAGCAGTATATATGAATAGA
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GCTAAATAATGAATCTTATTGCACTTGTATAAAGCATACAAATAGTAGCTATTTCATTCTGTTAAAGCAGACCTAAGAAGTAAT
GCAAAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTTGCTTGCTTGCAAACAACACAAATATACATTAATCAACTTA
ATC
>GA1_ITS2.Freversed- Tetranychus truncatus
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GGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCATACTACCATATATGCATTG
TTTTGAGAGGATTGCATATTTATATGCATGAATCTTGATGTTTTATTCCTTTTCTTAATTGCAATTCGTTGCAATTTAGTAAGGA
GAATCTCAAATCTACTTGTTTCACATGATAAATTTTGTGTACAATGCATATTTCATCTCTGCAAGCAGTATATATGAATAGATAC
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AAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTTGCTTGCTTGCAAACAACACAAATATACATTAATCAACTTAATCA
A
>VA1.2_ITS2Freversed- Tetranychus truncatus
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TTAGTTGATCACCATAGTTATCTGCAATACGACTTTAGCGTCGTCAGATAGGCGACTAACCTTAGAATCTCATGCTAGTATCTA
TTCATATATACTGCTTGCAGAGATGAAATATGCATTGTACACAAAATTTATCATGTGAAACAAGTAGATTTGAGATTCTCCTTA
CTAAATTGCAACGAATTGCAATTAAGAAAAGGAATAAAACATCAAGATTCATGCATATAAATATGCAATCCTCTCAAAACAAT
GCATATATGGTAGTATGCAAGTGTTTTGTTGATTATTTTACATCTCAACTCTCAGACAGATGTGACCTCGGAAAAGACCCGAA
AGCCGCAATGTGCGTTGGAAGCTCAAGTGCTCGGCGTGTCCTGCAATTCGCACCGATTATCGTAGCTAG
>EC1.ITS2reversed- Tetranychus truncatus
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GGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCATACTACCATATATGCATTG
TTTTGAGAGGATTGCATATTTATATGCATGAATCTTGATGTTTTATTCCTTTTCTTAATTGCAATTCGTTGCAATTTAGTAAGGA
GAATCTCAAATCTACTTGTTTCACATGATAAATTTTGTGTACAATGCATATTTCATCTCTGCAAGCAGTATATATGAATAGATAC
TAGCATGAGATTCTAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAACTAACCTGCT
AAATAATGAATCTTATTGCACTTGTATAAAGCATACAAATAGTAGCTATTTCATTCTGTTAAAGCAGACCTAAGAAGTAATGCA
AAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTTGCTTGCTTGCAAACAACACAAATATACATTAATCAACTA
>VR3ITS2F-Tetranychus macferlanei
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CTA
>KB1.ITS2reversed- Tetranychus macferlanei
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CAACT
>KC1.ITS2reversed- Tetranychus macferlanei
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CAACTTT
>VD3.ITS2reversed- Tetrancyhus macferlanei
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CATATACAATCAAC
>KON.ITS2reversed- Tetranychus macferlanei
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>VD4.ITS2-Tetranychus macferlanei

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TTTAAAAAAGAGATATTGCAAATACCTAAATTTGTGTTAACGCATAAG

>ANCU.R.ITS2reversed- Tetranychus okinawanus

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GCTTTCGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGTATACTACCATATA
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>VA1.2 COI[Tetranychus truncatus]

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TGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATATCTTCTTCTATTGA
AATAATAATTTTTTCTTTACATGTTGCAGGAATTTCATCAATTGCTAGATCTATTAATTT
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TACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATATTGATCTATAGGATT
TTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCAAATTCATGTTTGGA
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>VA2.2 COI[Tetranychus truncatus]

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GTTATAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATA
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TTTGATCCTAGAGGAGGAGGAGACCCAATTTTATATCAACATTTATTCTGATTTTTTGGG
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ATTTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATAT
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>VA3.2 COI [Tetranychus truncatus]

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GTTATAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATA
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ATTTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATAT
TGATCTATAGGATTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCA
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>VCu1 [Tetranychus truncatus]

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>AC1 Nov COI [Tetranychus truncatus]
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>AC3 Nov COI[Tetranychus truncatus]
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T
>AnCuR COI[Tetranychus okinawanus]
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>EC1 COI[Tetranychus truncatus]
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>EC1.1 COI[Tetranychus truncatus]
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>AnGA1 COI [Tetranychus truncatus-NCBI]
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>ANRA.2 COI.F[Tetranychus-NCBI]
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AGC

DNA barcoding of spider mites (Prostigmata: Tetranychidae) in vegetable crops

By

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ABSTRACT OF THE THESIS

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ABSTRACT

DNA barcoding is a novel system designed to provide rapid, accurate, and automatable species identification using short DNA sequences from a standardized region of the genome. It helps in precise identification of species that can be applicable as a diagnostic tool for quarantine and other pest management activities. DNA barcoding is based on the variation on the sequences identified in genomic regions, which can distinguish individuals of a species. Species identification through barcoding is usually achieved by the retrieval of a short DNA sequence i.e. the 'barcode' from a standard region of the genome, a specific gene region either from mitochondria or nuclear genome. The barcode sequence from the unknown specimen could be compared with a library of reference barcode sequences derived from individuals of known identity.

The family Tetranychidae or spider mites include the most injurious plant-feeding mites. Some infest a wide range of host plant whereas some others are highly specific. The family includes about 12,000 species of phytophagous mites which can rapidly disperse to exploit new feeding sites, damage agricultural and horticultural crops causing severe economic losses. Precise identification up to species level is difficult since all species look similar. So molecular methods are increasingly applied in tetranychid mite taxonomy to establish species identity and DNA barcoding is the best option for this.

The study entitled "DNA barcoding of spider mites (Prostigmata : Tetranychidae) in vegetables" was done at Centre for Plant Biotechnology and Molecular Biology and Department of Agricultural Entomology, College of Horticulture, Vellanikkara. The objective of this study was to generate DNA barcodes for different species of spider mites in vegetable crops and to study the intra and inter species genetic relationship. For this, spider mites were collected from different locations *viz*; Anthikad, Alathore, Elenad, KVK, Thrissur and Vellanikkara and from

different vegetable crops *viz*; Amaranthus, brinjal, cowpea, cucumber, dolichos bean, okra and ridge gourd. After collection, spider mites were reared in the laboratory to get an isoline from which few males and females were used for microscopic slide preparation. Acarologists used slides to identify the spider mite species based on morphological taxonomic keys.

Total genomic DNA isolated using modified CTAB method (Rogers and Benedict, 1994) was subjected to PCR assay using markers for two different loci *ITS2* (second internal transcribed spacer) and *COI* (mitochondrial cytochrome c oxidase subunit I) which yielded bands of 620 and 868bp respectively. The obtained bands were eluted and subjected for sequencing. Nucleotide divergence among the sequences was calculated using Barcode of Life Data (BOLD) tool 'Distance summary'. It is desirable for barcodes to show very low sequence divergence within species.

Multiple sequence alignment using clustalW of MEGA6 software was performed for all the sequences and phylogenetic analysis has discriminated three different species of Tetranychidae family: *Tetranychus truncatus, T.macferlanei and T.okinawanus.* Barcoding gap, a position in the sequence at which unique nucleotide is present in all the members of a *Tetranychus* species was also assessed. Morphological and molecular analysis results were correlated with each other and results matched. *Tetranychus okinawanus* found on cucumber during the study is first time getting reported from India.

The study had shown that both *ITS2* (99.25%) and *COI* (98.45%) sequences efficiently classified the spider mite species. However, *ITS2* was found to be an efficient tool which gave species level resolution in spider mites. This can be used as supporting marker for *COI* to barcode spider mites species. In future, these primers can be used to barcode other *Tetranychus* species also and sequence analysis can also be done using *ITS1* locus and its efficiency can be measured for DNA barcoding of *Tetranychus* species.