DNA BARCODING OF SPIDER MITES (PROSTIGMATA: TETRANYCHIDAE) ON MAJOR CROP PLANTS OF KERALA

By

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(2015-11-003)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"DNA barcoding of spider mites (Prostigmata: Tetranychidae) on major crop plants of Kerala"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that the thesis entitled **"DNA barcoding of spider mites (Prostigmata: Tetranychidae) on major crop plants of Kerala"** is a record of research work done independently by **Ms. Arunima V** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the advisory committee of **Ms. Arunima V. (2015-11-003)**, a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology**, agree that the thesis entitled **"DNA barcoding of spider mites (Prostigmata: Tetranychidae) on major crop plants of Kerala"** may be submitted by **Ms. Arunima V,** in partial fulfillment of the requirement for the degree.

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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	Deoxyribonucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
g	Gram
L	Litre
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
NCBI	National Centre for Biotechnology Information
ng	Nanogram
^{o}C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion Concentration

ITS2	Second Internal Transcribed Spacer
COI	Mitochondrial Cytochrome Oxidase subunit I
RNA	Ribonucleic Acid
rpm	Revolutions per minute
TAE	Tris Acetate EDTA
TE	Tris EDTA
V	Volts
BOLD	Barcode of Life Database System

Introduction

1. INTRODUCTION

Members of the phytophagous mite family, Tetranychidae, commonly known as spider mites are economically important as pests of large number of agricultural and horticultural crops. This family erected by Donnadieu (1875) comprises of over 1200 described species under six tribes and 71 genera (Bolland *et al*., 1998). The damage caused by them has increased manifolds in the past sixty years (Li *et al*., 2010). Apart from its polyphagous nature, high reproductive potential and short life cycle, factors such as change in climatic conditions and over-use of plant protection chemicals also have helped to compound the mite problem in crops. Recently, spider mites have been reported as serious pests of banana, vegetables and rice from Kerala.

The spider mites colonise the plants mostly on the lower surface of leaves and suck by piercing and sucking the cell, which results in mottling followed by yellowing, browning and dropping of the leaves. In case of severe infestation, mites make extensive webbing on the leaves thus reducing photosynthetic capacity. They have well-developed dispersal mechanisms that enable their populations to spread and fully exploit individual host plants, and colonise widely separated plants over large areas.

The identification of spider mites is mostly based on visible morphology utilizing the taxonomic keys. However the identification is problematic due to their minute size, the limited number of available diagnostic characters and the intraspecific variation. Both sexes of species are often needed in order to arrive at precise determination. Expert taxonomists, microscopic slide preparations and comprehensive keys are essential for the task. As a result, molecular methods are increasingly being applied for taxonomic purposes.

The diversity among DNA sequences can be exploited for the genome based taxon identification. The concept of a DNA barcode has been proposed as a method of identification of species, which uses short sequences consisting of unique combinations of bases occurring in conserved regions of genes that are easily amplified by PCR and direct sequencing (Hebert *et al*., 2003). A DNA

barcode is a short sequence from standardized portions of the genome (like 648 bp of mtCOI). DNA barcoding is technically a simple and rapid approach, in which a small DNA fragment is amplified by PCR from total genomic DNA and PCR product is directly sequenced. The species identification is done by comparing the query sequence with the reference database of DNA barcode library.

The mitochondrial genome in particular has turned out to be exceedingly useful in tracing evolutionary history, as it is present in all eukaryotic organisms, evolves rapidly as compared to nuclear DNA, and does not undergo meiosis and recombination, processes that scramble the evolutionary lineages of nuclear genes. The generation of molecular data from the CO1 region is based on accepted DNA bar-coding principles *.i.e.* barcoding protocols developed by the Barcoding of Life (iBoL) Initiative. Recently, DNA barcoding method is being increasingly applied for species determination in spider mites. Developement of DNA barcodes and species specific markers enable even a non-specialist to identify spider mites up to species level where existence of cryptic species make the identification of spider mites a tedious task. Hence the contribution of molecular taxonomy is undoubtedly necessary to augment the species identification amomg spider mites.

In view of the above facts, the present study entitled "DNA barcoding of spider mites (Prostigmata: Tetranychidae) on major crop plants of Kerala" was undertaken with the objectives to generate DNA barcode for different species of spider mites infesting major crops of Kerala and to understand the variability among them.

Review of literature

2. REVIEW OF LITERATURE

Morphological identification of spider mites is difficult even for well versed taxonomist, as there exist many cryptic species having similar morphologies. Necessity for adult stages and precisely slide mounted specimens make it more troublesome. Molecular taxonomy can act as an augmentation to the existing system and resolve the perplexity in species identification. In this study "DNA barcoding of spider mites (Prostigmata: Tetranychidae) on major crops of Kerala", the two candidate loci *ITS2* and *COI* were chosen for species level identification and generation of DNA barcodes. The relevant literature related to the topic is summarized under different heads in this chapter

2.1. Economic importance of spider mites

Spider mites are one of the most important phytophagous pests with nearly 1200 species worldwide (Bolland *et al.*, 1998) and have many destructive species (Borror *et al.*, 1989). Apart from the worldwide distribution, the infestation can extent to large orchards and greenhouse crops, making spider mites one of the most agriculturally important pests (Cullen and Schramm, 2009).

Spider mites were considered to be a minor pest before the World War II (Hueck, 1953; Lord *et al.*, 1956; Chant, 1963). The arrhenotokous reproduction in spider mites enabled them with an exceptional possibility to develop resistance to pesticides (Helle, 1965). The advancement in science and technology, subsequent improvement in cultural practices and shift to indiscriminate use of modern pesticides followed by World War II led to the sudden upsurge of mite populations. (Channabasavanna, 1969; Huffaker *et al.*,1969).

Utilizing the needle like mouth parts, spider mites feed by sucking on cell sap mostly from abaxial side of the leaf, disrupting both palisade and spongy tissues and as a result removing vital chlorophyll. This causes the mottled or stippled appearance of the leaves followed by yellowing, browning and drying of the leaves. In severe infestation the formation of webbing causes the leaf to be tied together affecting the photosynthetic capacity of the plant (Baker and Connell, 1963; Berry, 1998). The reduction in yield, fiber, fruit and seeds may also result in

cases of severe infestations (Huffaker *et al.*, 1969). Spider mites were reported to cause significant reduction in quality and quantity of the yield in crops (Butani and Mittal, 1992).

In India, Peal (1868) discovered and described tea mites in Assam as red spider and later in the year 1884, Wood Mason described these mites as *Tetranychus bioculatus* and is the first published record on agriculturally important spider mite in India. In the year 1888 the damage done by *Oligonychus coffeae* in one of the tea estates of Sikkim was as high as Rs. 20,000 (Cotes, 1889).

Oligonychus indicus was found as a sporadic pest of sorghum in India (Rahman and Sapra, 1940). They were found to cause considerable damage to sorghum in Mysore and Madras states also (Cherian, 1931; Puttarudriah, 1951). The citrus red mite *Panonychus citri* was also known to exist in India as early as the 1940's (Pruthi and Manl, 1945). The attack of *Tetranychus telarius* and *T. equatorius* was reported from vegetables and ornamentals like tomato, brinjal, gourds, jasmine and rose. (Cherian, 1931; Januja, 1942).

During the period 1951- 1960, there were reports of yield reduction to the tune of 7 per cent in citrus and apples and 6 per cent and 10 per cent reduction for hops and lima beans respectively from United States of America due to the spider mite injury (Le Clerg, 1965). According to Ahmed (1975) as cited by Ismail *et al.* (2007), *T. urticae* infestations were responsible for significant yield losses in vegetable, fruits and other economic crops in Egypt.

According to Basu and Pramanik (1968) spider mite was the next major pest in brinjal after shoot borer. An estimated 13.64 per cent and 31.09 per cent yield reduction were recorded in brinjal from Bangalore and Varanasi, respectively due to infestation of *T. urticae* (Anon, 1998). Even being a rare species, moderate to severe infestation of *T. macfarlanei* was observed in Bangalore in the year 2000 (Anon, 2000). In India *T. ludeni* was also found to infest vegetable crops especially okra and brinjal (Reddy, 2001).

Patil and Nandihalli (2008) estimated the yield loss in brinjal caused by *T. macfarlanei* in summer of 2004-2005. The early infested plants (30 DAT) showed

maximum yield loss and less number of the leaves than the plants infested after 60 DAT and 90 DAT. The yield was found to be maximum for the protected plant (556.72g) and minimum for plant infested after 30 DAT (434.6g).

In another study on relationship between yield loss and mite infestation at various stages of crop growth in tomato (*Lycopersicon esculentum*) conducted by Jayasinghe and Mallik (2010), tomato plants were subjected to *T. urticae* infestation and it was found that infestation at the middle aged crop could contribute up to 50 per cent yield loss due to defoliation and chlorophyll reduction.

Spider mites have been reported as serious pests of banana, vegetables and rice from Kerala (Bhaskar and Thomas, 2011, Bhaskar *et al.*, 2012, Binisha and Bhaskar, 2013). In a study conducted by Bennur *et al.* (2015) three species of mites were identified from various vegetable crops of Thrissur district in Kerala.

Apart from reducing plant vigour, at times spider mites can act as vector to diseases caused by virus and fungus. Schulz (1963) reported that *T. urticae* was known to spread potato virus-Y. Rajagoplan (1974) and Jeppson *et al.* (1975) also reported the role of mites in spreading the Dolichos Enation Mosaic Virus, and Bean Mosaic Virus. The resting spores of Entomophthorales were found in family Tetranychidae (Sarwar, 2015) and *Petrobia latens* was found to be the vector of Barley yellow streak mosaic virus (BaYSMV) in Alberta and Alaska (USA) in *Hordeum vulgare* (Dhooria, 2016).

2.2. Biology and seasonal incidence of spider mites

Many intrinsic factors like level of inbreeding, population density, age and reproductive potential of female and extrinsic factors like temperature, humidity, inter and intraspecific competition and predators can influence the reproduction and life cycle of spider mites (Wrensch, 1985).

The life cycle of spider mite includes egg, larva, protonymph, duetonymph and adult. Even though the fecundity of spider mites is not on par with the other arthropods, since they have short life cycle and number of overlapping generations in a year, they can build into relatively large population and can manifest severe

infestations. Continuous rearing of *T. truncatus* in laboratory by Chao and Lo (1974) for studying the biology of the mite resulted in the observation that thirty one generations were formed in one year. Sex determination in Tetranychidae is primarly haplo-diploid arrhenotoky, however, rarely the sudden shift from arrhenotoky to thelytotoky was also observed in *T. urticae* and *T. pacifus* (Helle and Overmeer, 1973). The spider mite colonies also exhibit a female biased population (Roy *et al.*, 2003). In mites as like in many insects, diapuase is an adaptive strategy for synchronizing the life cycle with that of food availability, which is mostly controlled by photoperiod; as in case of *T. urticae*, diapause is accelerated during long day period (Tehri, 2014). Most spider mites in temperate region have diapause or overwintering as adult or egg (Huffaker *et al.*, 1969). *T. urticae* females can lay upto 20 eggs per day and an average of 90- 114 eggs in life time. The eggs are generally transluscent which hatch in four to six days and complete life cycle may take one to three weeks (Berry, 1998; Cullen and Schramm, 2009).

The infestations are found to be highest during the summer times and at constant temperature of 27° C, a single female mite can result in population build up to 13 million mites (Channabasavanna, 1969). The influence of meteorological parameters on the pest incidence was worked out through simple correlation studies by Mandal *et al.*, (2006), results showed non-significant negative correlation with maximum temperature. Morning and afternoon humidity showed significant negative correlation with activity of mites. Patil (2009) also conducted a study on the seasonal incidence of mites in brinjal and concluded that rainfall is highly detrimental to mite population and there was a significant negative correlation between the relative humidity of morning and evening in mite population build up. Spider mites grow rapidly in hot and dry weather conditions and on plants under water stress (Tehri, 2014) Apart from the weather parameters, the quality of host plants like presence of toxins, digestibility reducers, nutrient balance and physical traits like presence of trichomes or toughness of tissues, also affect the development and survival of plant feeding arthropods (Adango *et al.*, 2006).

2.3. Identification of spider mite by morphological characterization

Though Donnadieu (1875) established the family Tetranychidae, it was Murray (1887) who gave a more comprehensive description of this family. Berlese (1914) established the importance of empodium and tarsus as a taxonomic character for the family. Ewing (1913) stated that the well chitinised male genitalia (aedeagus) are well suited for the systematic studies, as they exhibit visible variation among species. In 1926, Hirst gave a description on the morphology of four different spider mite species, *viz., Tetranychus fici, T. andropogoni*, *Paratetranychus oryzae* and *P. punicae.* The major morphological characters taken for the identification and description of mites were whole body length, chaetotaxy, body segmentation, shape of aedeagus, claw, empodium and presence or absence of tenant hairs. The use of the diamond shaped dorsal integumentary lobe between the third and fourth dorso-central setae on female opistosthoma for species differentiation was proposed by Boudreaux (1956). Ehara (1956) also provided a detailed description of five genera under the family Tetranychidae on mulberry. The description on genus *Tetranychus* was based on the shape of aedeagus, cheatotaxy, folds on the dorsal integumentary in female. Smith Meyer (1987) accounted the presence of single pair of anal setae, empodium split into two to three pairs of proximoventral hairs, presence (or absence) of mediodorsal spur shorter than proximoventral hairs, presence of tridigit spur on male empodium I and dorsally bend aedeagus as some of the characters for recognizing the members of *Tetranychus*. Gupta and Gupta (1994) gave general morphological characters for the identification of spider mite belonging to Tetranychidae, some of which are presence of duplex setae on female tarsus I which is distal to tactile setae, empodium I of male with strong mediodorsal spur and empodium II of male with presence of proximoventral tridigitate spur, aedeagus with tiny knob, bent dorsally and absence of posterior angulation, female hysterosoma with longitudinal striae between third pair of dorsocentral setae.

The taxonomic characters for the identification of tetranychid mites are three to four pairs of setae on propodosoma and eight to thirteen pairs on hysterosoma. The chelicera is fused to stylophore, the claws and empodium with

tenant hairs. Pedipalp have well developed thumb and claw process (Bhaskar, 2016).

2.4. Molecular systematics in spider mite

Since only 15 per cent of the estimated 10 million existing species biodiversity has been described (Kamien and Kaul, 2005), there exists a taxonomic deficit (*i.e.* is the ratio of expected taxa to named taxa) in many taxa and which is more for the organisms with smaller body size (Blaxter, 2004). This taxonomic deficit needs to be addressed and molecular taxonomy can bridge this gap by accelerating taxonomic identification by assisting classical taxonomical approaches.

Despite having an arrhenotokous mode of reproduction, spider mites exhibit potential for rapid adaptations. A possible reason for this may be that, the new mutations despite of being dominant or recessive gets expressed in males due to their haploidy and these mutations gets accumulated and fixated due to the continuous inbreeding (Helle and Overmeer, 1973). DNA sequences evolve at constant rate over time, and rate of evolution varies with the nature of DNA sequence, for e.g. the pseudogenes and introns evolve faster than mitochondrial DNA (Futuyama, 1997). Due to this constant evolution in DNA sequences and capacity of the spider mites to accumulate these variations, there is a wide variability in spider mite population.

Distinguishing spider mites only based on morphology is difficult due to the very limited potential diagnostic characters and also due to the need of both sexes for identification (Ros and Breeuwer, 2007; Ben-David *et al.*, 2007). The existence of cryptic species is another issue of concern in morphological identification, for e.g. considering red *T. urticae* as separate species from that of the green *T. urticae* is an issue of debate and moreover it is also supposed that *T. urticae* is a species complex with forty four synonyms (Navajas, 1998). Molecular systematics can step in to reinforce the morphological identification so as to reduce the perplexity existing in spider mite identification. It has additional advantages such as being rapid, versatile and can be carried out with any stage of the life cycle or fragmented

body parts (Marrelli *et al.*, 2005; Leigh *et al.*, 2008). However, construction of phylogeny with DNA data cannot always guarantee accurate results, since the correlation between genetic divergence and speciation is not universal; moreover retention of ancestral polymorphisms and masking of information caused by multiple substitutions may also cause trouble (Simon *et al.*, 1994)

2.4.1. Preservation of specimens for molecular studies

Preservation of specimens for future use is a common practice. However the specimen stored for molecular studies need to be wet preserved with special care for preventing the degradation of DNA.

In a study conducted by Post *et al.* (1993) on *Simulium damnosum* (Black fly) it was found that storing specimens in 100 per cent ethanol at 4° C yielded maximum quantity of DNA. Ethanol proved better than methanol and propanol and ANOVA studies on storage temperatures indicated that storing in 4° Celsius was optimum and in 100 per cent ethanol, specimens could be even stored at room temperature up to five years for molecular studies. The study also proposed that ethanol preservation can be a feasible alternative for liquid nitrogen storage, for later DNA analysis.

According to Reiss *et al.* (1995), preservation in 95 per cent ethanol, DNA isolation buffer, and cryopreservation all yielded intact DNA from *Amara glacialis* (Coleoptera: Carabidae) and for the field conditions as well as short term storage ethanol was most convenient, as it required no special equipment. Moreover the specimens could be maintained in an undamaged and manipulated condition for later identification.

A comparison of various preservation techniques for DNA extraction was studied in two species of parasitic wasps, *Venturia canescens* and *Leptomastix dactylopii* by Dillon *et al.* (1996). It was found that specimen frozen and stored at -80° C and 100 per cent ethanol yielded same quantity of total DNA as that from fresh specimens.

Craemer *et al.* (2000) also recommended wet preservation in 70-95 per cent alcohol for arachnids and soft bodied insects. According to Cruickshank

(2002) effective method of preserving specimen for molecular work was ultra-cold freezing (-80) of live specimens and alternatively the storage was in 100 per cent ethanol.

King and porter (2004) killed and stored three ant species *Solenopsis invicta*, *Camponotous floridanus* and *Dorymyrmex bureni* in varying concentration of ethanol and isopropanol, over three time periods. The study revealed that specimens stored in either 100 per cent or 95 per cent alcohol were best suited for processing and mounting with minimal shrinkage and swelling of body part. This also resulted in removal of water and oxygen and sterilization of tissue to prevent damage from autolytic process and prevented DNA decay.

2.4.2. DNA isolation

DNA isolation is the first step in any molecular study. Murray and Thompson (1980) proposed a technique for rapid isolation of high molecular weight DNA from different plant species. One per cent CTAB (Cetyl trimethyl bromide) extraction buffer was used for isolation. Even with a small capacity ultra-centifuge (Dupont- sorval TV-865) 20-70 ug of DNA per 100 mg dry weight was obtained. The spectrophotometer readings after CsCl centrifugation were A260/230 between 2.2-2.5 and A260/280 between 1.8-1.9 indicating the purity of DNA.

Phillips and Simon (1995) put forward a simple and nondestructive method for DNA isolation from museum specimens of arthropods. In the proposed technique, perforated exoskeleton of various specimens was placed in Eppendorf tubes containing 8 per cent DTAB (dodecyl trimethylammonium bromide) as extraction buffer. The technique originally intended for DNA isolation from blood sample gave satisfactory results for DNA isolation from various arthropod taxa like Acarina, Collembola, Neuroptera, Orthoptera, Odonata and Lepidoptera. The DNA extracted when subjected to PCR, yielded 700bp region corresponding to the cytochrome oxidase II gene. The whole organism or any part of it can be utilized for the extraction of DNA. When Klompen (2000) found that for DNA isolation from mites at least 2-5 individuals should be taken, Anderson and Trueman (2000)

suggested a method which stated that DNA could be isolated even from dissected leg tissue of individual mites.

On comparison of five DNA isolation techniques with corn rootworm beetle (*Diabrotica virgifera*) *viz.,* SDS method, CTAB method, DNAzol reagent, purgene solutions and DNeasy column by Chen *et al.* (2010), it was found that SDS method and CTAB method yielded good quality DNA at lower expenses and yield was also found to be higher than DNAzol, Purgene and DNeasy column. The gel electrophoresis also showed presence of good bands with fewer smears, affirming lower degradation of DNA and the utility of the methods. The PCR of extracted DNA with microsatellites also proved the suitability of the methods.

Wang and Wang (2012) compared three different DNA isolation methods for DNA extraction from single chironomid (Diptera: Chironomidae) for PCR analysis. The methods used were Protinase k, CTAB method, and Chelex-100. It was found even though all the three methods yielded DNA, the quality of DNA obtained from CTAB method was better than the other two, with an OD value of 1.8 ± 0.3. The PCR was carried out using two primers COXII and 28SrDNA D3-D5, the results were obtained as clear bands of 778bp and 480bp, respectively.

DNA degradation from older samples or preserved samples lead to lower yields on later extraction. An *in-situ* DNA extraction method was put forth by Almakarem *et al.* (2012) for plant and fungi tissues. The samples were homogenized in warm 2X CTAB buffer in the field with portable equipment like battery operated centrifuges and on precipitation of DNA as CTAB salt, samples were taken to the laboratory for further steps in isolation. Depending on the tissue, the yields were 3ng-200ng/mg of the sample. The PCR amplification obtained with SSU rRNA, ITS1, *ITS2*, and LSU rRNA primers were successful and comparable to that of fresh samples.

Three methods of DNA extraction were compared in *Parachipteria willmanni* (Acari: Oribatida) in Turkey by Per and Ercan (2015). A single mite was taken and DNA isolated using each of the techniques *viz.,* Chelex resin (C100), Qiagen DNA extraction kit and CTAB method. It was found that, the CTAB

method provided more quantity of DNA than Qiagen DNA extraction kit and purity of DNA was higher than that of the C-100 method. RAPD-PCR was carried out by Opc2 random primer and clear bands were obtained after Agarose Gel Electrophoresis (AGE).

2.4.3. Molecular phylogeny in spider mites

Beyond 1965, experiments were initiated to discover markers in spider mites which resulted in discovery of distinct markers. Most of the earlier markers were biochemical and were profoundly discovered in haemolymph and for eye pigmentation. Several mutations were observed in mites for pigmentation, which was even found to be high for some strains. Resistance to pesticides was developed into markers in *Tetranychus* species and it was found that the inheritance of these markers was normal. Due to the male haploidy and due to high rate of inbreeding, the fixation of the structural mutations in a population was prominent (Helle and Overmeer, 1973). The utilization of the differences in single gene to investigate the evolutionary relationship among the prokaryotic domains was done by Woose and Fox in 1977, which can be regarded as one of the earliest attempts in utilizing DNA based phylogeny. The phylogenetic analysis of the prokaryotic domain, based on the ribosomal RNA sequence revealed that the living systems represent three primordial lines of descents which are; Eubacteria, Archaebacteria and Ukaryotes (Woose and Fox, 1977)

Even though the ideal marker for phylogenetics is the one which should be present as a single copy but due to the difficulties in amplification, sequences with multiple copies yet same sequences can also be utilized. Moreover the ideal markers should be easy to align, with substitution only high enough to provide required informative sites and an unbiased base composition with straight forward mode of genetic transmission. However a single marker is unable to suit every criterion and the nuclear ribosomal genes and the mitochondrial genes are the two groups of genes which can be utilized in this context which is currently the most commonly used in molecular phylogenetics (Avise *et al.*, 1987; Cruickshank, 2002).

ITS2 is the second internal transcribed spacer present in rDNA construct and are present in thousands of copies, organized in nucleolar organizer region. Despite being present in thousands of copies, all units evolve in concerted manner thus the homogeneity of sequences within a species remains the same. In addition to the above *ITS2*, markers follow bi-parental inheritance and harbor more variability due to low selection pressure (Cruickshank, 2002; Vijayan and Tsou, 2010)

In the year 1992, the phylogenetic relationship between six species (*Eotetranychus carpini*, *E. pruni*, *Tetranychus pacificus*, *T. turkestani*, *T. urticae*, *T. mcdaneili*) of economically important tetranychid mites were constructed from the sequence information from 300bp region of *ITS2*. Sequences were obtained by designing the primers for the flanking region. Intra-generic phylogenetic tree constructed for the genus *Tetranychus* was found similar to that constructed with morphological data, indicating the utility of the maker in assessment of systematics and evolution of the group (Navajas *et al*., 1992). The intraspecific diversity in Cassava Green Mite (*Mononychellus progresivus*; Acari: Tetranychidae) collected from Congo, Benin, Brazil and Colombia were examined by Navajas *et al*. (1994) utilizing *COI* and *ITS2* markers. The variation in length of dorsal setae from one strain to another of these mites, led to the belief that they belonged to distinct taxa. However the study elucidated that the sequence divergence is low for both *COI* (0-2.1 %) and *ITS2* (0-0.4%) and on the inter-strain comparison; the two African population appeared identical, which were not in consensus with the morphological data. The data obtained thus supported the hypothesis of single introduction of the species in two African populations and established the utility of molecular marker in delineation of taxonomic status and also the dispersion paths.

The variability in ITS sequences among *Ixodes scapularis* (Acari: Ixodidae) were assessed on a Macro-geographic scale by McLain *et al.* (1995). Nearly forty sequences were generated from the specimens collected from Georgia, Florida, North Carolina, Maryland, Massachusetts, New Jersey and New York. The sequence variation between locations was found only to be 23 per cent which indicated that *I. scalpularis* constituted only one species.

A comparison between molecular phylogeny and changes of morphological traits were conducted by Navajas *et al.* (1996). A part of *COI* was sequenced in 20 species of phytophagous mites. In accordance to the earlier reports, these regions were found to be AT rich (75% - 95%) with an exception of one of the genera (68%) indicating that the base composition can substantially vary in even short periods of time. Most of the variations were assessed to be transition substitution, but transversions were also shown to accumulate at a steady rate, as a function of non-synonymous differences which pointed out the utility of the gene in conducting phylogenetic analysis at various taxonomic level. The species *Tetranychus viennensis* was placed in another group on phylogenetic tree and on close examination of empodial shape it led to the discovery that the mediodorsal spur was absent, which is one of the most distinguishing feature of genus *Tetranychus*, moreover it retained the ancestral shape of peritreme thus pointing out the necessity for taxonomic revision. In the study they also suggested that many morphological characters in Tetranychidae may have had convergent evolution.

T. urticae and *T. puerariсola* are considered sibling species and the similarity in the shape of aedeagal knobs makes morphological differentiation difficult between these two species. Gotoh *et al.* (1998) conducted a molecular comparison between these two species using the marker *ITS2*. Even though the sequences showed similarity as high as 93.8 per cent, the restriction of these *ITS2* sequences with *RsaI* and *DraI* resulted in species specific restriction site, thus the utility of the molecular marker in species delineation was established.

The intraspecific divergence of *ITS2* and *COI* were investigated for *T. urticae* collected from wide geographic regions around Mediterranean Basin. *COI* sequences formed 15 haplotypes with an average nucleotide divergence of 5 per cent, but *ITS2* sequences exhibited no intraspecific polymorphism but were shown to evolve 2.5 times faster than *COI* at generic level. The concerted evolution of *ITS2* has its utility as a good indicator of long term isolation between species and on the other hand *COI* can shed light on the recent geographic colonization patterns of the species. (Navajas, 1998; Navajas *et al.*, 1998). Lee *et al.* (1999) constructed

a phylogenetic tree from *COI* sequences of six species of genus *Tetranychus* in Korea. The nucleotide variation from species to species was in a range of 6.7-14.9 per cent and on pair wise comparison transversion were found to be as high as 55 per cent. *T. urticae*, *T. cinnabarinus* and *T. kanzawai* formed an entire different group from *T. truncatus*, *T. viennensis* and *T. piercei* and were found to be sister species, while the maximum genetic distance was found between *T.viennensis* and *T. piercei*.

The *ITS2* region was amplified and sequenced followed by a PCR-RFLP to discriminate *T. evansi* and *T.urticae* which were commonly confused in South Africa on tomato in Zimbabwe. The restriction profile of the amplified site with enzymes *RsaI*, *AluI* and *TruI* showed the presence of two bands in *T. urticae,* while only one in *T. evansi* indicating that a point mutation must have resulted and it was also established that the above enzymes can be utilized for the discrimination between the two species effectively. The validity of the restriction profile was confirmed by analyzing the sequence data of *ITS2* (Knapp *et al.*, 2003).

After the analysis of the single individual from 200 closely associated species of Lepidoptera, a model *COI* profile was generated by Herbert *et al.* (2003). Apart from the 100 per cent success of identification of the subsequent specimens, it was found that these *COI* profiles can be utilized for species level assignments as well. The authors also suggest that when fully developed the *COI* system can be cost effective, reliable and accessible solution for species identification and develop as the core global bio-identification system for animals. In an another study conducted by Herbert *et al.* (2003) it was found that with an exception of the Cnidaria, sequence divergences at *COI* can enable discrimination of closely associated species of all animal phyla. The diversity in the amino acid sequences at the 5' end of the *COI* sequence can be used to place species onto higher category reliably, for e.g. The divergence in the *COI* sequences in 13320 congeneric species pairs indicated a range from zero per cent to as high as fifty three per cent.

Ben-David (2008) conducted molecular characterization of 21 species of spider mites in Israel, collected from twenty different sites. *ITS2* sequences from 16 species were obtained and it was found that the sequence divergence were higher between species than within species. Nine different *ITS2* sequences were obtained from *Eutetranychus orientalis* making it the most polymorphic species and also suggesting the possible existence of cryptic species. As a conclusion to the study, it has been suggested that *ITS2* sequences can be used to generate barcode for spider mite

Alasaad *et al.* (2009) investigated the utility of *ITS2* markers in the molecular characterization of Sarcoptes mites from various locations in Europe, during which they confirmed that, even though *ITS2* sequences are not suitable for distinguishing among closely related species, as the intraspecific variation is not observed in these sequences, they are suitable genetic markers for examining phylogenetic relationships within a genera or between genera.

A microsatellite (TUI, TUII, and TU35b) based study to understand the population genetic structure between sibling species *T. urticae* and *T. cinnabarinus* was conducted by Li *et al.* (2009) in China. The genetic differentiation between the two species was immense even for the species collected from adjacent and sympatric areas indicating that there is a lack of gene flow between the species. However *T. cinnabarinus* exhibited far more overall genetic diversity than *T. urticae* but less geographical distribution.

On studying the sequence divergence of mitochondrial genes, ribosomal RNA genes and tRNAs, Jeyprakash and Hoy (2009) established that all chelicerates are monophyletic (*i.e.* having common ancestor) and diversification into spiders, scorpions, mites and ticks must have occurred during the late Paleozoic era.

Even though *ITS2* is insufficient to resolve the relationships within closely related species and thus is less reliable in barcoding, the aligning of the secondary structure of the *ITS2* sequences can result in an informative phylogenetic tree (Agnarsson, 2010)

Li *et al.* (2010) conducted a molecular phylogeny study in nine agriculturally important species of tetranychid in China. It was found that both nuclear and the mitochondrial markers have similar phylogeny and the phylogenetic tree constructed using the *COI*, *ITS1* and *ITS2* sequences showed significant geographical differences within *T. cinnabarinus*, suggesting the existence of cryptic species. The study also validated that all the three markers are suited for the phylogenetic analysis.

Genus *Oligonychus* include some morphologically similar species which are difficult to distinguish and are generally categorized into two groups based on the direction of the curvature of aedeagus. Matsuda *et al.* (2012) utilized *COI* sequences as a DNA based identification technique to distinguish between species in *Oligonychus.* Seventeen species were accurately distinguished and intraspecific divergences were found to be less than the interspecific divergences. The species with ventrally curved aedeagus and dorsally curved aedeagus formed two distinct clades, when phylogenetic trees were constructed indicating that, *Oligonychus* species inhabiting the gramineous crops formed clearly defined sub clades.

In another study, Matsuda *et al.* (2013) conducted a DNA based study to establish molecular evidence for the existence of cryptic species in genus *Tetranychus*. *COI* and *ITS2* sequence information were studied for 13 known species in Japan and *Tetranychus kanzawai* and *T. Parakanzawai* separated into two monophyletic clades indicating the existence of cryptic species within each species.

Arimoto *et al.* (2013) developed a PCR-RFLP based technique for the rapid identification of *Tetranychus* species. The technique targeted the ITS sequences and could distinguish up to 14 species within eight hours. Currently this technique is being used under plant quarantine division of Japan

Upon investigation of phylogenetic tree constructed for the subfamily Tetranychinae from18S and 28S sequences, it was found that the tribes Petrobini, Bryobini, Eutetranychini formed monophyletic group while Tetranychini branched

out into a polyphyletic group indicating that Tetranychini was diversified from the rest in the course of evolution. At generic level *Oligonychus*, *Tetranychus*, *Schizotetranychus*, *Eotetranychus* were also shown to be polyphyletic (Matsuda *et al.*, 2014).

ITS2 sequences were utilized to distinguish between seventeen thelytokous lines of native/ introduced *Trichogramma* species collected from Peru, Colombia and USA. For the first time, *Wolbachia* induced parthenogenesis were found in the eleven *T. pretiosum* lines, moreover based on the size of restricted *ITS2*-PCR products, a dichotomous key for species identification was built (Almeida and Stouthamer, 2015)

Li *et al.* (2015) proposed a RT-PCR (Real Time PCR) based technique for the rapid identification of *T.urticae* in New Zealand, by amplifying the ITS1 sequence. The assay was validated for *T.urticae* specimens from various countries and hosts. After standardization, the process takes three hour for completion and is efficient to distinguish between closely related species in *T.urticae* from any life stage of the mite. Bennur *et al.* (2015) conducted a study on the vegetable mites in Thrissur district of Kerala and DNA barcodes were generated and intraspecific divergence within the genus was found to be 9.14 to 9. 89 per cent for *COI* and 7.7-7.92 per cent for *ITS2* sequences. The study established the utility of both *ITS2* and *COI* as markers to differentiate various *Tetranychus* species.

2.5. DNA barcoding

DNA barcoding is a well- established technique for species identification in animals (Vijayan and Tsou, 2010). The diversity among DNA sequences can be exploited for the genome based taxon identification. Herbert and co- workers came up with idea of 'DNA barcode' in the year 2003. The concept was based on Universal Product Codes, which utilizes the 10 digit numeral system for product identification. As DNA molecule is made of four different nucleotides, it was conceptualized that genomic barcodes can be generated utilising these four alternate nucleotides at each position. As the string of sites available for inspection is huge, the possible combination of codes that can be generated is 100 times the

number that would be required to discriminate life in each taxon uniquely. Thus certain key sequences can be rendered as pattern 'barcode' embedded in every cell which can be utilized as tool for the molecular identification. The features of these sequences are minimum intraspecific variation yet mutation high enough to generate inter specific variability, easy to obtain, fewer deletions and insertions which may result in low alignment (Waugh, 2007). In the above context, mitochondrial DNA has more utility than nuclear DNA and *COI* is a well-established marker for animal to generate a standard barcode. In a study conducted by Herbert *et al.* (2003), it was found that the 648bp region of the mitochondrial gene *COI*, could serve as a reliable barcode for the identification of animal species.

According to Schindel and Miller (2005), DNA barcoding could aid taxonomists in three different ways, firstly barcodes can be utilized to organize new collection into known species and into ones new to science, secondly in absence of morphological identification (partial or damaged specimen) barcode based identification can assign specimen to species on preliminary basis and finally DNA barcodes can augment taxonomic datasets in the process of delimiting species boundaries. Faster extinction rates of species and rapid introduction of invasive species, on the grounds of globalization and increased international commerce demands a prompt species identification service. Moreover in tropical developing countries with relatively high species diversity yet fewer taxonomic resources is also in demand of rapid and cost effective technique for species delineation. DNA barcoding can be resorted for both the above scenario with reliability (Miller, 2007). When DNA barcoding is assorted as a method of identification, then both the DNA and specimen from which it was extracted should be vouchered for more reliability and future reference (Rowley *et al.*, 2007).

According to Ros and Breeuwer (2007) the use of single gene sequence DNA barcoding technique is however unreasonable and a centralizing approach of utilizing nuclear and mitochondrial gene, morphological data and ecological information should be employed for more comprehensive DNA barcoding. The concept of DNA barcoding has gained considerable acceptance and apart from arthropods is being used in identifying wide range of organisms like birds (Herbert

et al., 2004), Antaratic marine larvae (Webb *et al.*, 2006), marine fishes (Ward *et al.*, 2008; Zemlak *et al.*, 2009) viruses, bacteria, protists and Rhodophyta (Waugh, 2007) and fungi (Seifret, 2009).

It was found that, the neotropical skipper butterfly (*Astraptes fulgerator*) adults have subtle differences in morphology with almost no genitalic divergence, however the larval stages were distinct in appearance, host plant and ecosystem preferences. The DNA barcode analysis along with morphological study indicated the existence of cryptic species and found that *A. fulgerator* is a complex of at least 10 species (Herbert *et al.*, 2004). Barcoding performance was analysed in a diverse group of marine gastropods and cowries by Meyer and Pauly (2005). They concluded that DNA barcoding could show high error rates (4 -7%) in poorly sampled and taxonomically understudied groups and holds promise only for well understood and well-studied clades.

Moulting into adults of considerable size difference and genitalic polymorphisms recently discovered in some spiders complicated the identification of spiders. Barrett and Herbert (2005) conducted a study on the utility of *COI* sequences in resolving the problem and generating the barcodes in 203 arachnid species. By including one representative sample from every species, 327 *COI* sequences were obtained and the identification success for the tested individuals were 100 per cent, without any overlap between mean nucleotide divergence at the inter or intra specific levels. The study also pointed out the utility of *COI* marker in the identification of arachnid species. Hajibabaei *et al.* (2006) utilized the *COI* sequences to generate barcodes for the tropical Lepidoptera near Costa Rica (Central America). *COI* sequences were obtained from 4,260 adults and showed that the barcode data generated for 521 species were 97.9 per cent in consensus with the prior reported taxonomic data. However in five species, higher divergence in sequences were found within themselves indicating the existences of cryptic species and in other 11 species overlapping regions at barcodes were observed indicating mixed species cluster and suggesting the very recent speciation or hybridization. The utility of the *COI* sequences in barcoding of Lepidoptera was thus established.

Karytotypically different species of genus *Agrodiaetus* belonging to the butterfly family Lycaenidae were studied for their inter and intra specific variation and for generating barcodes. It was found that due to low interspecific variation, 18 per cent overlapping sequences were obtained and 16 per cent specimens with conspecific sequences were mis-identified. They conclude that although DNA barcodes can be utilized in distinguishing species, they should be utilized in combination with other relevant morphological data.

Hernandez-Triana *et al.* (2012) employed *COI* DNA barcoding region for investigating the unexposed diversity existing in subgenus of Black fly, *Trichodagmia enderlein*. The sequences provided good resolution to species identification and in association with morpho-taxonomic framework, the technique proved to be of great value in unraveling the unexposed diversity in the subgenus *Trigchodagmia*. Abd-Rabou *et al.* (2012) investigated the mealy bug population in Egypt by DNA barcoding approach. DNA barcoding was utilized to identify Molecular Operational Taxonomic Units (MOTU) in sub artic Hymenoptera and it was found that there existed a great deal of diversity in Hymenoptera in this region and also pointed out their dominance as parasitoids (Stahlhut *et al.*, 2013). Utilising the *COI* sequence variability, Asfaq *et al.* (2014) determined the mosquito diversity in Pakistan and conspecific divergence was found to be 0-2.4 per cent, while congeneric divergence was found to be between 2.3-17.8 per cent. This indicated the efficacy of the sequence to distinguish at both specific and generic level.

Heindrich *et al.* (2015) conducted the first extensive test for efficacy of DNA barcodes for the European beetles on regional basis and generated a comprehensive DNA barcode database with a focus on Germany. The study added more than 3500 identified species to BOLD and now is regarded as the largest reference database for Coleoptera globally, with a reference available for up to 15948 individuals belonging to 3514 well identified species.

Despite the few drawbacks like lack of clearly stated objective hypothesis, presence of overlapping regions found between intra and inter specific divergence values, absence of standard threshold value for species delimitation, possibility for

human error, need for clearly defined and comprehensive samples for arriving at reasonable conclusions, DNA barcoding is found to be successful for more than 95 per cent of studied species and is widely used by biological taxonomists (Collins and Cruickshank, 2012; Muhammed and Aktar, 2015).

Materials and methods

MATERIALS AND METHODS

The study entitled "DNA barcoding of spider mite (Prostigmata: Tetranychidae) on major crops of Kerala was carried out at CPBMB and Acarology Laboratory of AINPAA (All India Network Project on Agricultural Acarology), Department of Agricultural Entomology, College of Horticulture during the period 2016-2017. The materials used and methodology adopted for conducting the study are detailed below.

3.1. Collection and preservation of spider mite

3.1.1. Field survey

Purposive surveys were conducted in selected farmer's fields of Thrissur, Palakkad, Malapuram and Ernakulam districts during the months of February-May and November- December, 2016 and February- May, 2017. Spider mites associated with selected crops namely tapioca, banana, papaya, cowpea, ashgourd, brinjal, okra, pumpkin, *Adenium*, and *Dahlia* were collected during the survey. Leaves showing symptoms of speckling typical to spider mite infestation were observed on the abaxial side using a magnifying glass/hand lens (5X) for the presence of mites. Mite infested leaf samples were collected in polythene bags, and proper labels describing the locality, crop and date of collection were also placed inside each bag and tied with rubber bands. The samples were brought to the laboratory for further observation and maintenance of culture. The different locations from which spider mite were collected are presented in Plate 1 and symptoms of spider mites infestation are presented in Plate 2. Materials used for the collection of mites are presented in the Annexure I.

3.1.2. Maintenance of isoline culture

After observing under the microscope for the presence of spider mites (Prostigmata: Tetranychidae), single gravid female mite was transferred to a fresh mulberry leaf using a fine camel hair brush. The mulberry leaf was then placed on a wet sponge surrounded by water on a tray. The population arising from this single gravid female was maintained as iso-line assigning unique accession number.

Plate 1 Survey conducted at various locations

A. KVK Vellanikkara **B.** Potta **C**. Kodali

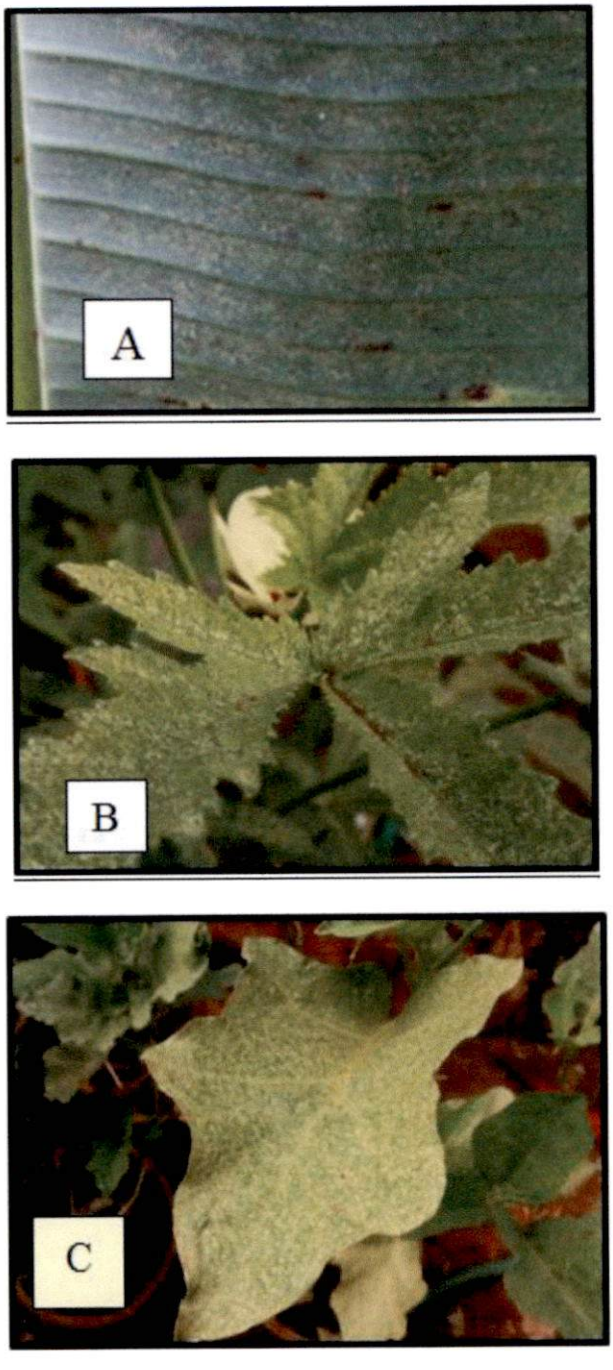
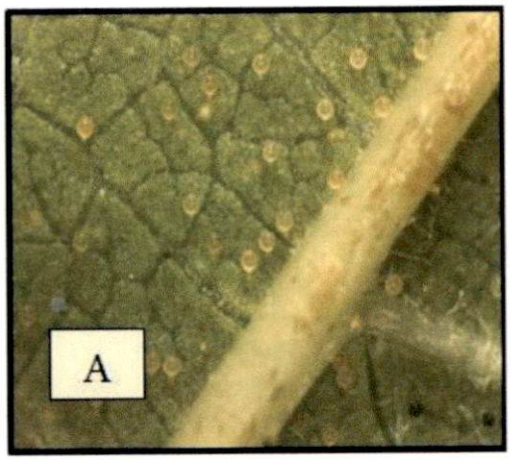


Plate 2 Symptoms of spider mite infestation. **A**.Banana (Potta) **B**. Okra (Kaloor) **C.** Brinjal (Tavannur)

Plate 3. Isoline culture of spider mites maintained at Acarology Laboratory



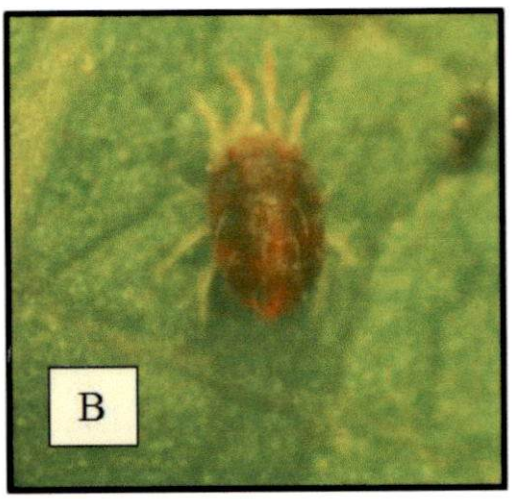


Plate 4 Different stages of spider mites **A.** Eggs **B.** Adult female **C.** Adult Male

Isolines were maintained separately for mites collected from different crops and from different localities surveyed. The technique of maintaining isoline culture is presented in Plate 3 and different stages of spider mites are represented Plate 4.

3.1.3. Wet preservation

Once the isoline was established, the adult mites, both males and females were picked and placed in 0.5mL eppendorf tubes labeled with accession numbers containing 100 per cent alcohol and stored at -4° C for molecular studies (Post *et al.*, 1993)

3.1.4. Preparation of permanent microscopic slides of mite specimens

Permanent slides of mite specimens from different isoline cultures were prepared by mounting separately adult female and male mites dorsally in a drop of Hoyer's medium placed on the middle on the glass slide. Mites were made to settle to the bottom of the drop by gentle tapping with a micro-needle. After proper positioning, cover slip was placed carefully over it, avoiding the chances of entrapping air inside. For species level identification, male mites were also mounted in lateral position according to technique suggested by Henderson (2001) for observing the shape of the aedeagus, a key character for species level identification.

The slides were then labeled with details *viz.* host, locality, date of collection, and collector's name. The slides thus prepared were dried at 40-45° C for 5-6 days in hot air oven. After observing under phase contrast microscope to ensure proper drying, slides were sealed well using transparent nail polish and later utilized for morphological characterization and identification of species using taxonomic keys. The figure of Permenant slides prepared is presented in Plate 5. Materials and equipment required for preparation of permanent microscopic slides are listed in Annexure I.

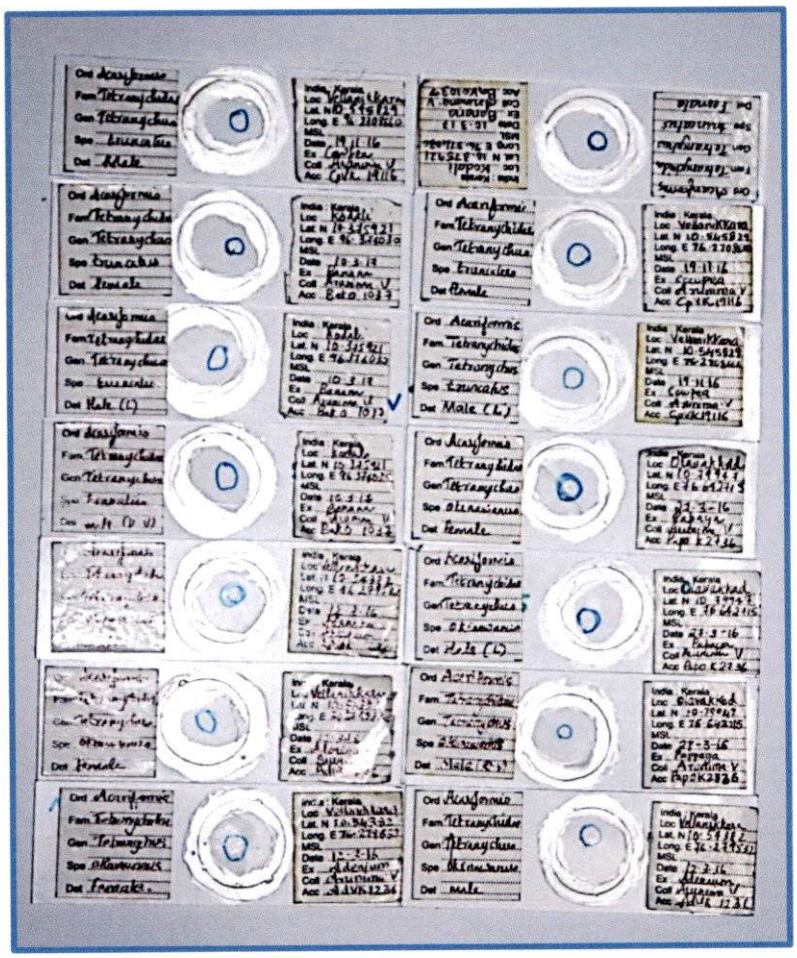


Plate 5 Permanent slides for morphological identification

3.2. Identification of mite specimen

3.2.1. Morphology based identification

The slide mounted specimens were observed under phase contrast microscope to study the following morphological features:

1. Chaetotaxy of hysterosoma and leg
2. Empodium
3. Aedeagus (male genitalia).

Based on the characters studied, the mite specimens were identified up to species level using standard taxonomic keys (Gupta and Gupta, 1994; Ehara, 1995; Srinivasa *et al*., 2012)

3.2.2 Molecular based identification

3.2.2.1. DNA isolation

The procedure adopted for isolation of total genomic DNA of mite specimen from different iso-lines is detailed below. The total genomic DNA isolation was done using the modified CTAB method detailed by Roger's and Benedict (1994). The required reagents are listed in the Annexure II. Ten to fifteen adult female mites were picked and placed in autoclaved and labeled (accession number) 1.5 ml Eppendorf tube containing 20µL CTAB buffer (2X) and crushed thoroughly with clean micro pestle. Eighty micro litre of CTAB pre-warmed at 65° C was added to make up the volume as 100µL. The eppendorf tubes were incubated at 65° C for one hour with intermittent vortexing of two minutes each. Equal volume of Chloroform: Isoamyl alcohol (24:1) was added and gently mixed by inversion. The mixture was centrifuged at 10000 rpm for fifteen minutes at 4° C. Topmost aqueous layer was carefully pipetted out from the three distinct phases which appear after centrifugation and transferred to new eppendorf tubes. To the pipetted aqueous layer (70-80µL) 200ml ninety six per cent alcohol and 30ml sodium acetate were added and kept at incubation for 1-3 hours at -20° C. Later the mixture was centrifuged at 13000 rpm for ten minutes at 4° C and the supernatant was decanted. Two alcohol washes, one with 70 per cent and another with 100 per

cent alcohol were done at centrifugation speed of 13000 rpm for 10 minutes. The supernatant was discarded and pellets were air dried in Laminar Air Flow cabinet (LAF) to remove all traces of alcohol. The extracted DNA was dissolved in 50µL of double distilled water for storage and future use.

3.2.2.2 Assessing the quality of the DNA using Nano drop spectrophotometer

The quality of the DNA samples was analyzed using Nano drop spectrophotometer. Absorbance at the 260nm and 280nm wavelength were recorded and purity was indicated by the ratio A260/A280. The assessment was done using computer installed ND-1000 software. The procedure for analysis is as follows.

a. Entering the software ND-1000, option '**NUCLEIC ACID**' was selected
b. Spectral measurement was initiated by placing one micro-litre distilled water on to the lower measurement pedestal and then after lowering the sampling arm, the option '**OK**' was clicked.
c. For setting blank, one micro-litre of distilled water was again placed in lower measuring pedestal and the option '**Blank'** was selected
d. For measuring the absorbance of DNA samples; one micro-litre of the sample was placed in the measurement pedestal and the option '**Measure**' was selected.
e. After the measurement, the sample was wiped from both measurement pedestal and sampling arm using a soft tissue paper. Subsequent samples were loaded for measurement and above procedure repeated.
f. The readings were noted down.

3.2.2.3. DNA amplification with Polymerase Chain reaction (PCR)

By providing the ample conditions like intact template DNA, Assay buffer A, Taq DNA polymerase, dNTP's, $MgCl_2$ and primers the amplification of the conserved sequences could be ensured in Polymerase Chain Reaction (PCR). The reaction mixture prepared was dispensed into 0.2 mL PCR tubes and subjected to thermal cycling. The reaction mixture put forth by Li *et al.* (2010) for the

amplification of the genus *Tetranychus* was utilized for the amplification of both the loci in this study. Two candidate loci selected for PCR analysis were *ITS2* and *COI*.

For the PCR amplification of *ITS2* locus, a 25µL reaction mixture with the following composition was used.

a) Genomic DNA	- 2µL
b) 10X Taq assay buffer A	- 2µL
c) dNTP mix (10mM)	- 2µL
d) $MgCl_2$	- 0.75µL
e) Taq DNA polymerase (3U)	- 0.5µL
f) Primer forward	- 1µL
g) Primer reverse	- 1µL
h) Autoclaved distilled water	-15.75µL
Total volume	- 25.0µL

Thermal profiling for the *ITS2* locus was carried out with the following programme. The annealing temperature was standardized by setting up a gradient PCR and was documented.

Initial denaturation	-94 ° C for 3 minute	
Denaturation	-94° C for 1 minute	35 cycles
ITS2 primer annealing	- 55° C for 1minute	
Primer extension	-72° C for 1 minute	
Final extension	-72° C for 10 minutes	
Storage	- 4° C for infinity	

For PCR amplification of *COI* locus, 25µL reaction mixture of the following composition was made.

a) Genomic DNA - 4µL

b) 10X Taq assay buffer A - 2µL

c) dNTP mix (10mM each) -2µL

d) $MgCl_2$ -0.75µL

e) Taq DNA polymerase (3U) -0.5µL

f) Primer forward -1µL

g) Primer reverse -1µL

h) Autoclaved distilled water -13.75µL

Total volume - 25.0µL

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

Initial denaturation	-94° C for 3 minute	
Denaturation	-94° C for 1 minute	35 cycles
COI primer annealing	-59 ° C for 1minute 30 seconds	
Primer extension	-72 ° C for 1 minutes 30 seconds	
Final extension	-72° C for 10 minutes	
Storage	- 4 ° C for infinity	

Table No. 1 Details of Primers used in the study

SI No.	Locus	Sequence	Registered name of Primer in BOLD	Reference
1	ITS2 F	ATATGCTTAAATTCAGCGGGG	ITS2 KAU	Navajas *et al.*, 1998
	ITS2 R	GGGTCGATGAAGAACGCAGC	R ITS2 KAU	
2	COI F	GGAGGATTTGGAAATTGATTAGTTCC	UBC6 F	Simon *et al.*, 1994
	COI R	GATAAAAACGTAATGAAAATGAGCTAC	R COI	Gotoh *et al.*, 2005

3.2.2.4. Assessing the PCR Products

The assessment of proper amplification of the candidate loci was done by Agarose Gel Electrophoresis (AGE) on 2 per cent agarose gel. The procedure is as follows:

1. Agarose gel (2%) was prepared by dissolving agaorse at the rate of 2g per 100ml of the 1X TAE buffer. The solution was then heated in an oven to properly dissolve the agarose and form a uniform gel.
2. The solution was allowed to cool down and 4µL of ethidium bromide (intercalating agent) was added to the solution and mixed well for uniform distribution.
3. The solution was then carefully poured into the casting tray avoiding the formation of bubbles and with a comb properly placed for the formation of wells. It was allowed to solidify at room temperature.
4. The combs were carefully removed exposing the wells and the tray was placed in the electrophoresis unit by positioning the wells near the negative terminal. The unit was filled with running buffer (1X TAE) until the wells were submerged sufficiently.
5. The samples for loading were prepared by mixing 4µL of 6X loading dye for every 10µL of PCR product and loading 14µL in each well. The first well was loaded with 100 bp ladder and in second well blank sample was loaded.
6. The terminals of the electrophoresis unit was carefully connected and electrophoresis was carried out at 85 volts until the dye migrated to two third length of the gel

3.2.2.5. Gel documentation

After electrophoresis, gel documentation was done with 'BioRad gel documentation system' and software 'Quantity one". In the system, the gel was auto exposed to UV radiations and the image which appeared on the computer screen was 'freezed' at the right moment to obtain the clear image of the ladder and the bands. The bands appeared as bright orange colour due to the intercalating

ethidium bromide. The image was later saved in the JPEG format. The composition of buffers and dye for gel electrophoresis is listed in Annexure III.

3.2.2.6. Sequencing of PCR product

After the PCR, single and distinct bands were observed and the PCR products were used for sequencing. Seven sequences amplified with *ITS2* and nine sequences amplified with *COI* were sequenced by outsourcing at AgriGenome Labs, Pvt. Ltd., Cochin.

3.2.3 Data analysis using *In-silico* tools

3.2.3.1. Sequence analysis and annotation

The forward and reverse sequences obtained for each accession were merged to form contigs using CAP3 sequence assembly programme (http://doua.prabi.fr/software/cap3). The *COI* sequences which are protein coding sequences were analysed for the presence of stop codons. For this, the merged *COI* sequences were aligned using "align by muscle" option in MEGA 7. These aligned sequences were translated and were compared to the invertebrate genetic code table to assess the presence of stop codons. The stop codons if present were removed using Bio-Edit software.

3.2.3.2. Analysis of sequence homology

Basic Local Alignment Search Tool (BLAST), a sequence similarity search tool provided by NCBI was utilized to assess the homology of sixteen sequences to the sequences present in NCBI database. For this the individual accessions were subjected to nucleotide BLAST (BLASTn). The sequences from the database showing maximum identity, query coverage and with least expected value (E value) to the query sequence were identified.

3.2.3.3. Barcode gap analysis

The sequences were aligned using the Clustal W tool provided by MEGA 7 software and the barcode gaps were identified by analysing the regions with absence of '*' symbol. The regions which showed variation in nucleotide were marked.

3.2.3.4. Calculation of pairwise distance

For the calculation of pairwise distance the sequences were initially aligned using the "align by muscle" option and the clustering method used was neighbor joining method. By clicking on the "Distance/Compute pairwise" command on the task bar of MEGA 7 main window, the pairwise distances between the sequences were calculated using the Kimura 2 Parameter.

3.2.3.5. Distance summary analysis

The genetic distances between sequences were analysed seperately for nine *COI* and four *ITS2* sequences using the sequence analysis tools provided by BOLD (Barcode of Life Data Systems). The sequences were aligned using the "Muscle" algorithm and were analysed based on Kimura 2 Parameter model (K2P).

3.2.3.6. Construction of the phylogenetic tree

The sequences were initially aligned by "Clustal W" tool and the phylogenetic tree was constructed using the phylogeny tool in MEGA 7 (based on the neighbor joining method). Separate phylogenetic trees were constructed for *COI* and *ITS2* sequences.

3.2.3.7. Submission of sequences to NCBI GenBank

An account was created in NCBI BankIt (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) and by logging into the account, fourteen sequences were submitted. In step wise procedure the details regarding authors, title of the work, address for correspondence, sequence information, name of organism, source modifier details like country, date of

collection of sample, sample description were also provided while submitting the data.

3.2.3.8. Submssion to Barcode of Life Data Systems (BOLD)

For the generation of species specific barcodes, the fourteen sequences were submitted to Barcode of Life Data Systems online (http://www.boldsystems.org/). Logging into the workbench the data submission proceeds step by step submission of specimen data, sequence data, traces, images and primer information.

Results

4. RESULTS

The results of the study entitled "DNA barcoding of spider mite (Prostigmata: Tetranychidae), carried out at CPBMB and AINP on Agricultural Acarlogy, College of Horticulture during the period 2016-2017 are presented below.

4.1. Morphology based identification

A total of sixteen accessions of spider mites (Tetranychidae) representing five vegetable crops, two fruit crops, two ornamental plants and one tuber crop from eleven different localities of Thrissur, Malappuram, Palakkad and Ernakulam districts were maintained in Acarology Laboratory as iso-lines. Details of the iso-line cultures are furnished along with the GPS co-ordinates of different localities are presented in Table 2.

The study revealed that the spider mites collected from different localities from different crops belonged to a single genus, *Tetranychus*. Detailed morphological characterization of slide mounted specimens representing different accessions revealed that three species of *Tetranychus viz., Tetranychus okinawanus* Ehara, *Tetranychus truncatus* Ehara and *Tetranychus udaipurensis* Gupta and Gupta were found associated with the crops surveyed. *Tetranychus okinawanus* was recorded on papaya, cowpea, ashgourd, brinjal and adenium; *T. truncatus* was recorded on tapioca, cowpea, pumpkin, banana and dahlia and *T. udaipurensis* was recorded on tapioca, okra and banana. (Table 3)

The morphological characters of the genus and species are detailed below

Genus *Tetranychus* Dufour, 1832

Hysterosoma with fourth pair of dorso-central setae in normal dorsal position, with one pair of para anal setae; empodium split distally, with three pairs of proximoventral hairs and mediodorsal spur much shorter than hairs; peritreme recurved distally; tarsus I with two pairs of duplex setae usually widely separated; aedeagus of male always bent dorsad (Plate 6).

Table 2. Isoline cultures of spider mites

SI No.	Accessions	Crop	Location	Districts	GPS co-ordiantes (Decimal degrees)		Date of collection
					Longitude	Latitude	
1	AdVk1236	Adenium	Vellanikkara	Thrissur	10.54382	76.279532	12-03-16
2	PapOk2736	Papaya	Olavakkod	Palakkad	10.79947	76.642715	27-03-16
3	BrTv0356	Brinjal	Tavannur	Malappuram	10.85260	75.982938	3-05-16
4	DaId30316	Dahlia	Idukki	Idukki	9.918897	77.102490	30-03-16
5	PapKa1256	Papaya	BRS kannara	Thrissur	10.53733	76.320060	12-05-16
6	CpVk19116	Cowpea	Vellanilkkara	Thrissur	10.545829	76.2708660	19-11-16
7	AgOl21116	Ashgourd	Vellanikkara	Thrissur	10.548015	76.2830295	21-11-16
8	TpMk29116	Tapioca	Manjakunnu	Thrissur	10.549178	76.3463116	29-11-16
9	TpOk12126	Tapioca	Olavakkod	Palakkad	10.799477	76.642715	10-12-16
10	OkEr12126	Okra	Kalur/Ernakulam	Ernakulam	9.992436	76.289916	12-12-16
11	TpKK0227	Tapioca	Kakkani	Palakkad	10.818490	76.635218	02-02-17
12	PmMK0927	Pumpkin	Manjakunnu	Thrissur	10.549178	76.3463116	09-02-17
13	TpCm1827	Tapioca	Chuvannamannnu	Thrissur	10.562853	76.357942	18-02-17
14	BaPo2327	Banana	Potta	Thrissur	10.843466	76.643865	23-02-17
15	CpVl2827	Cowpea	Vellayani	Trivandrum	8.429437	76.988012	28-02-17
16	BaKo1037	Banana	Kodali	Thrissur	10.375921	76.374030	10-03-17

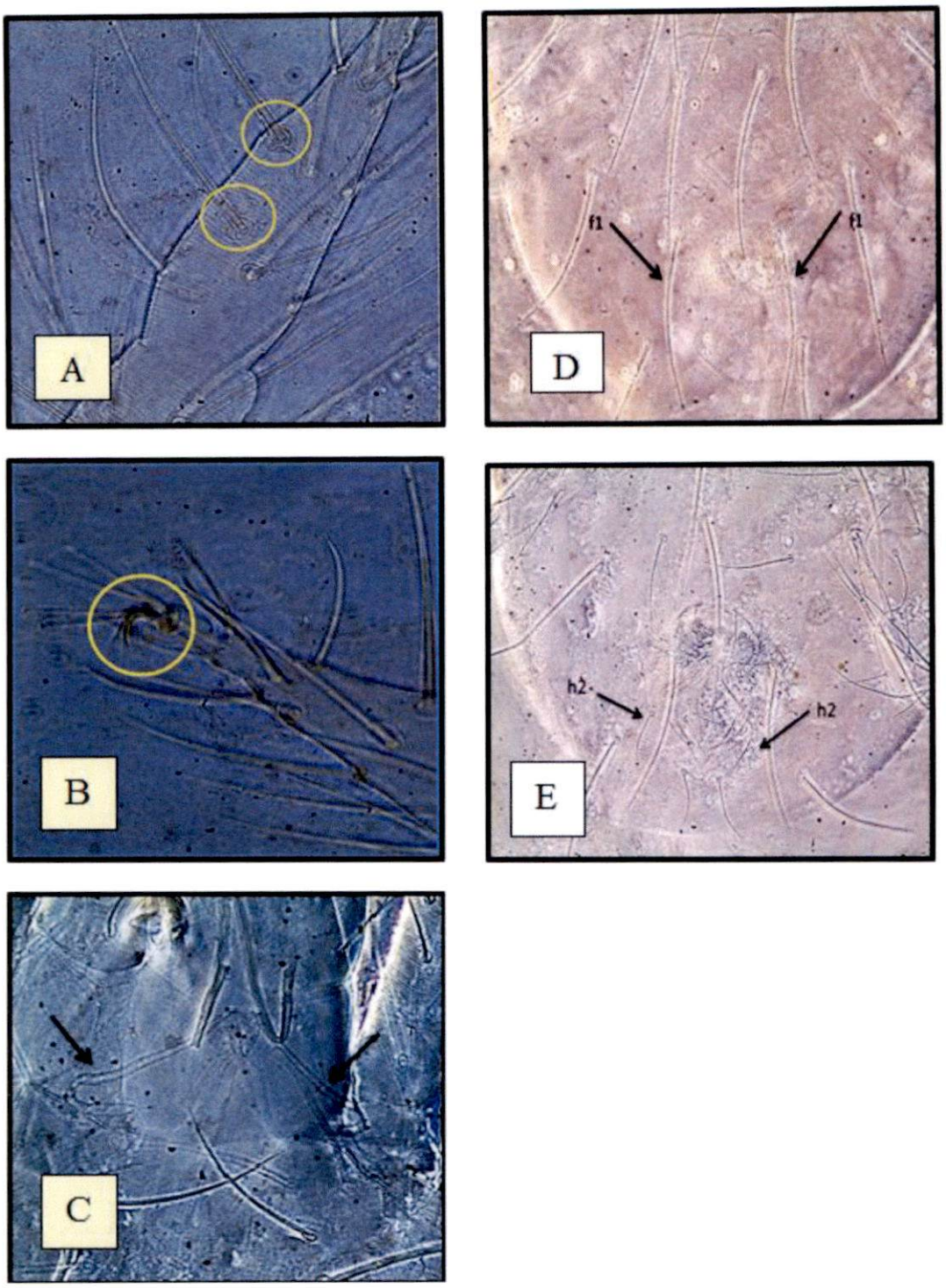


Plate 6 Taxonomic character of genus *Tetranychus*

A Duplex setae (widely separated) **B** Empodium (distally split) **C** Peritreme (recurved distally) **D** Dorsocentral setae (present on normal position) **E** Para anal setae (one pair)

***Tetranychus truncatus* Ehara**

Female: Empodia with six proximoventral hairs; empodia I-IV each with a minute spur; tarsus I with at least four tactile setae proximal to proximal duplex setae; tarsus III with one proximal tactile seta; peritreme hooked; dorsal striae between e1-e1 longitudinal; dorsal striae between e1 and f1 transverse forming a diamond shape medially.

Male: Empodia I-II each with an obvious dorsal spur; empodium I claw like; empodia II-IV with six long proximoventral hairs; aedeagus with small knob, with rounded short anterior projection and pointed short posterior projection, dorsal surface of the knob flat to slightly convex with an indentation in the posterior half; axis of the knob forms almost right angle with shaft (Plate 7).

***Tetranychus okinawanus* Ehara**

Female: Empodium with three pairs of hairs and one pair somewhat shorter, proximoventral filaments, with a strong mediodorsal spur; tarsus I with three tactile setae proximal to duplex setae; Tarsus II with three tactile setae and one solenidion proximal to duplex setae; striae between setae e1- e1 and between pair of f1- f1 forming a diamond shape; peritreme strongly hooked distally

Male: Empodium I with one pair of claw like divisions and one pair of somewhat shorter proximoventral filaments and with a strong mediodorsal spur; empodia II – IV each consisting of three pairs of hairs and one pair poximoventral filaments, with a strong mediosdorsal spur; tarsus I with three tactile setae and two solenidia proximal to proximal set of duplex setae; tarsus II with three tactile setae and one solenidion proximal to duplex setae; aedeagus upturned distally; terminal knob much longer than the width of neck; anterior projection broadly rounded, the posterior projection very narrow acute (Plate 8).

***Tetranychus udaipurensis* Gupta and Gupta**

Female: Empodium I to IV with short proximoventral hairs; dorsal idiosomal setae one and a half times longer than the interval between their longitudinal bases; tibia I with one sensory and nine tactile setae, tarsus I with one sensory and two tactile

setae proximal to duplex setae; tibia II with seven tactile setae, tarsus II with one sensory and two tactile setae proximal to duplex setae; peritreme hooked distally with more bend.

Male: Dorsal idiosomal setae simple, tapering gradually and one and a half times longer than the interval between their longitudinal bases; tibia I with three sensory and eight tactile setae, tarsus I with four sensory and one tactile setae proximal to tactile setae; peritreme hooked distally; aedeagal knob tiny with anterior lobe angulate and posterior lobe rounded (Plate 9)

4.2. Molecular identification

4.2.1 Quality of isolated DNA

Total genomic DNA was isolated from 16 spider mite accessions collected from various locations in Thrissur, Palakkad, Ernakulam, and Malappuram districts using the modified CTAB method

The purity of DNA was checked using nanodrop ND-1000 spectrophotometer. Nucleic acid showed absorption maximum at 260 nm whereas protein showed peak absorbance at 280nm. The DNA concentration was obtained in the range of 120-350 ng/µL. The readings of spectrophotometer are presented in the Table 4 and Fig. 1.

4.2.2. DNA amplification with Polymerase Chain reaction (PCR)

The amplification of DNA was carried out according to the PCR protocol suggested by Li *et al.* (2010) for the genus *Tetranychus*. The annealing temperature for the locus *ITS2* was standardized by setting up a gradient PCR (Sure cycler 8800, Agilent technologies). The optimum annealing temperature was found to be 55.3° C.

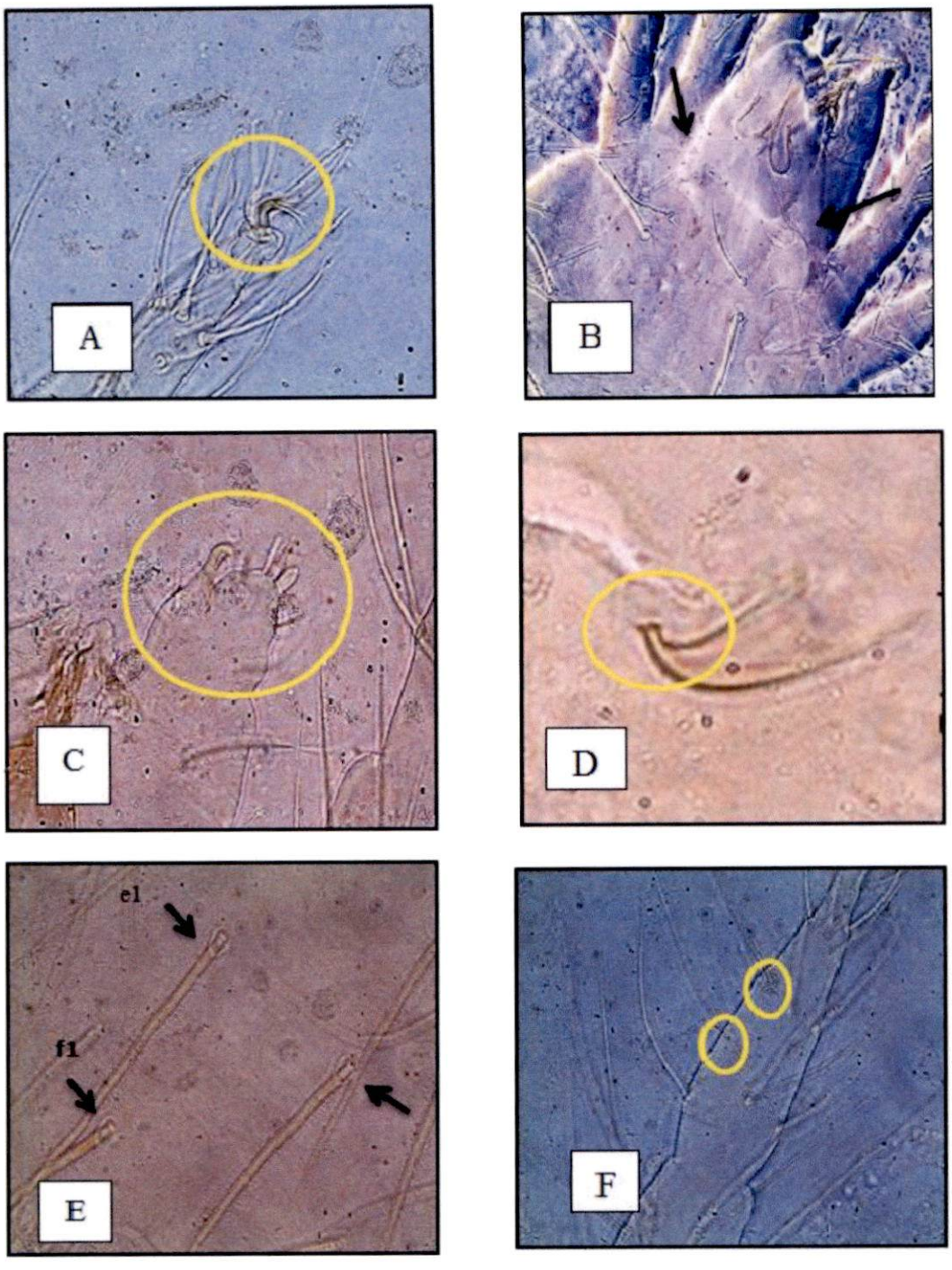


Plate 7 Taxonomic characters of *Tetranychus truncatus* **A**. Empodium **B.** Peritreme (distally hooked) **C.** Pedipalp **D**. Aedeagus **E.** Female Striae between e1 and f1 **F.** Female tarsus I (duplex setae)

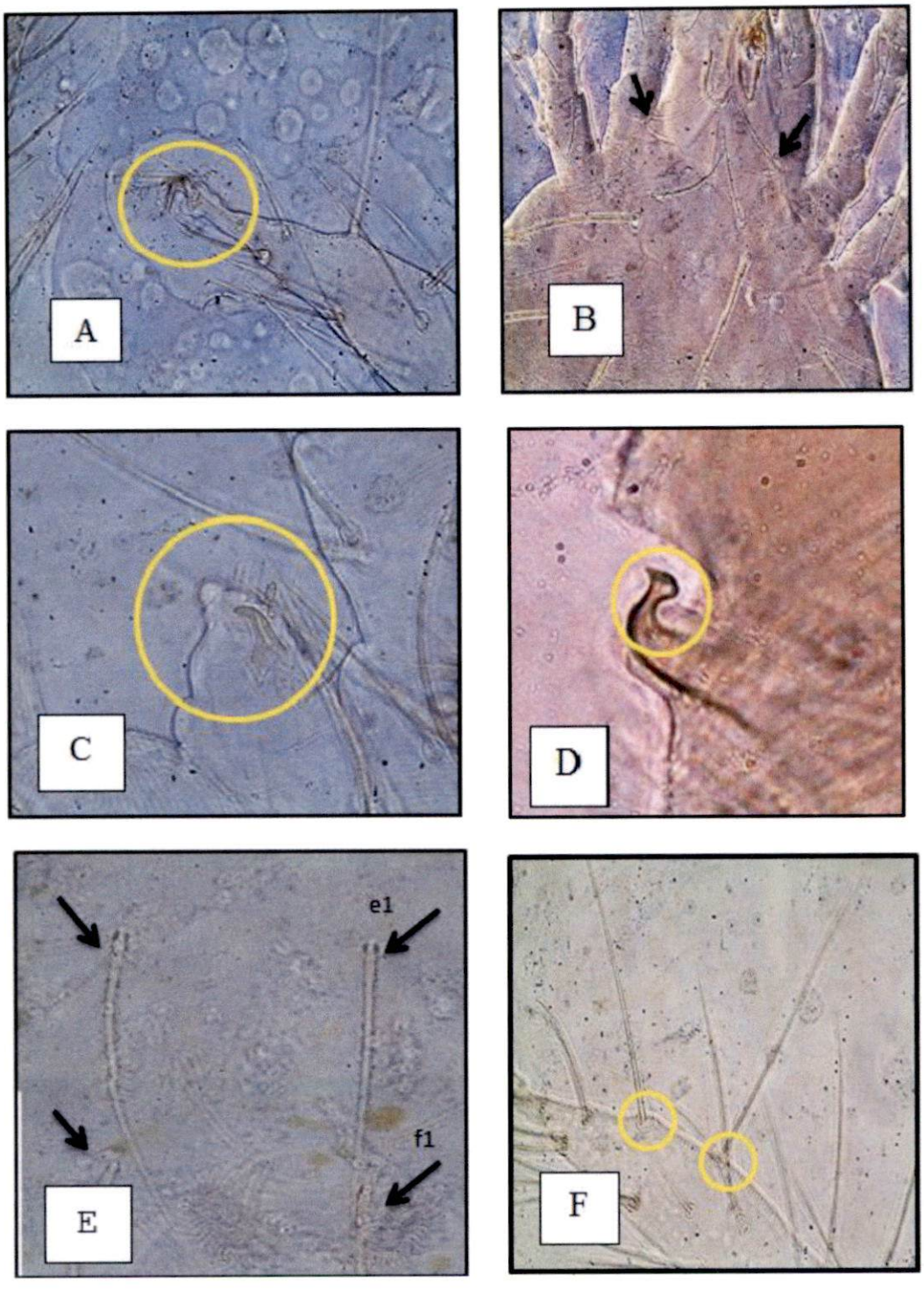


Plate 8 Taxonomic characters of*Tetranychus okinawanus* **A.** Empodium (female) **B**. Peritreme **C.** Pedipalp **D.** Aedeagus (male) **E.** Striae between e1 and f1 setae (female) **F.** Duplex setae

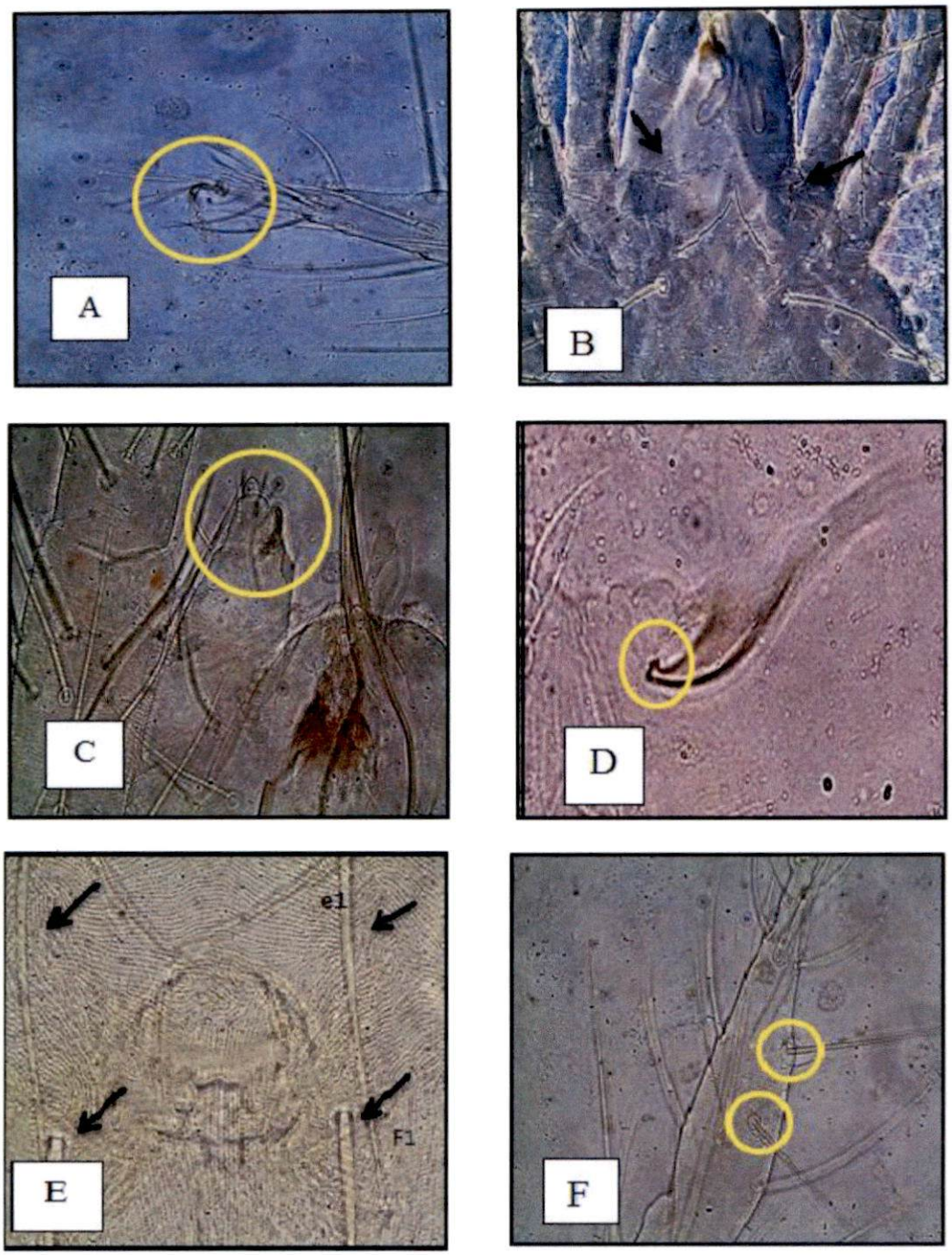


Plate 9 Taxonomic characters of *Tetranychus udaipurensis* **A** . Empodium **B.** peritreme **C.** pedipalp **D**. aedeagus **E** Female striae between e1 and f1 **F** Duplex setae

Table 3 Spider mites associated with some economically important crops

Sl. No.	Crop	Species	Location	District
1	Cowpea	*Tetranychus okinawanus* *Tetranychus truncatus*	Vellayani Vellanikkara	Trivandrum Thrissur
2	Brinjal	*Tetranychus okinawanus*	Tavannur	Malappuram
3	Okra	*Tetranychus udaipurensis*	Kaloor	Ernakulam
4	Ashgourd	*Tetranychus okinawanus*	Vellanikkara	Thrissur
5	Pumpkin	*Tetranychus truncatus*	Manjakunnu	Thrissur
6	Tapioca	*Tetranychus truncatus* *Tetranychus udaipurensis*	Olavakkod, Manjakunnu Chuvannamannu, Kakkani,	Palakkad Thrissur Thrissur Palakkad
7	Banana	*Tetranychus truncatus* *Tetranychus udaipurensis*	Kodali Potta	Thrissur Thrissur
8	Papaya	*Tetranychus okinawanus*	Olavakkod, Kannara	Palakkad Thrissur
10	Dahlia	*Tetranychus truncatus*	Thodupuzha	Idukki

Table 4 Quality of spider mite DNA assessed using spectrophotometer (NanoDrop ND - 1000)

SL NO.	Accession number	A260/A280	A260/A230	Concentration (ng/µL)
1	PapOk2736	1.89	2.10	172.3
2	BrTv0356	1.97	2.06	257.7
3	DaId30316	1.89	1.84	175.3
4	PapKa1256	1.82	2.00	216.5
5	CpVk19116	1.84	1.57	261.9
6	AgOl21116	1.96	1.77	359.2
7	TpMk29116	2.02	2.10	191.6
8	TpOk12126	2.01	2.21	284.7
9	OkEr12126	1.99	1.97	321.5
10	TpKK0227	1.97	2.18	106.4
11	PmMK0927	1.87	2.24	228.6
12	TpCm1827	1.95	2.13	264.8
13	BaPo2327	1.78	1.43	166.0
14	CpVl2827	1.85	1.59	134.3
15	BaKo1037	1.80	1.81	193.9
16	AdVK1236	1.88	2.10	213.3

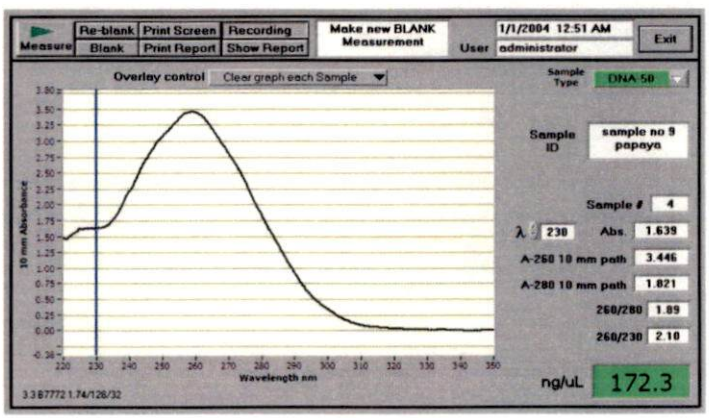


Fig. 1. Absorbance peak of DNA at 260 nm for the accession PapOk2736

4.2.3. Assessing quality of PCR products

After the completion of thermo cycling, the PCR products were assessed with 2 per cent agarose gel for the presence of amplicons. Single and distinct bands in range of 700-800 bp and 800-900 bp were obtained for *ITS2* and *COI* respectively. Gel pictures for gradient PCR as well as the amplification of *COI* and *ITS2* loci are displayed in Plate 10 , 11, 12, and 13.

4.2.4. Sequencing of PCR products

Sixteen sequences were obtained after the sequencing by Sanger dideoxy method carried out at AgriGenom Labs. Pvt. Ltd., Cochin. List of *ITS2* sequences and *COI* sequences obtained from each accession is furnished in Annexure V

4.3 Data analysis using *In-silico* tools

4.3.1. Sequence analysis and annotation

On merging of the forward and reverse sequences with CAP3 sequence assembler, proper contigs were formed for 14 accessions. The accessions AdVk1236 and CpVl2827 did not form contigs and were merged using Emboss GUI tool for the BLASTn analysis of the sequences. The contigs formed are presented in the Annexure VI. Length of forward, reverse and contig sequences (in bp) obtained for the accessions are presented in Table No. 5.

Standardisation of annealing temperature – *ITS2* loci

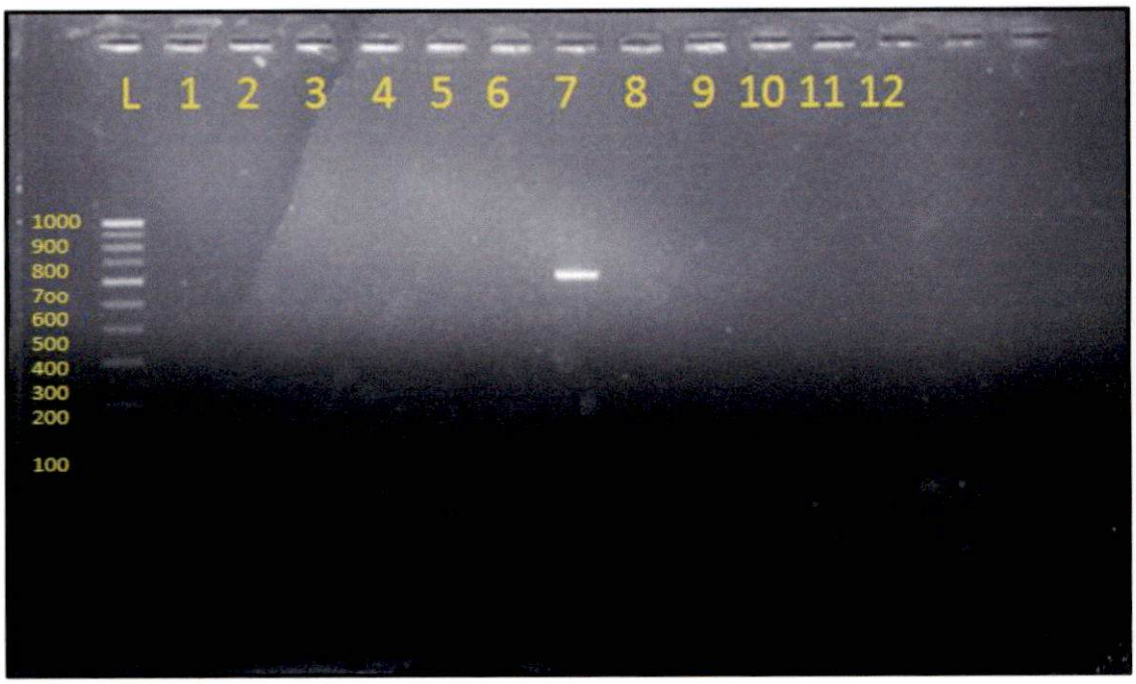


Plate 10 (Gradient PCR temperature in degree Celsius) L- Ladder **1.** 52.1 **2.** 52.3 **3**. 52.8 **4**.53.5 **5.** 54.1 **6.** 54. 7 **7.** 55.3 **8.** 55.8 **9.** 56.4 **10.** 57.1 **11.** 57.8 **12.** 58.0

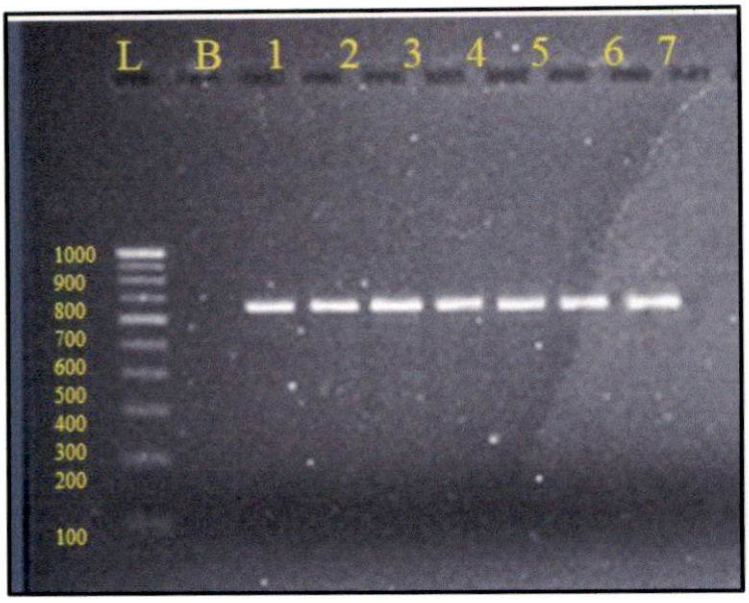


Plate 11 Amplification of *ITS2* locus (I) L. Ladder B. blank **1**. CpVl2827 **2**.AgOl21116 **3**.BaItp1847 **4.** BrTv0356 **5**.PapK91256 **6**. DaId30316 **7.** PapOK2736

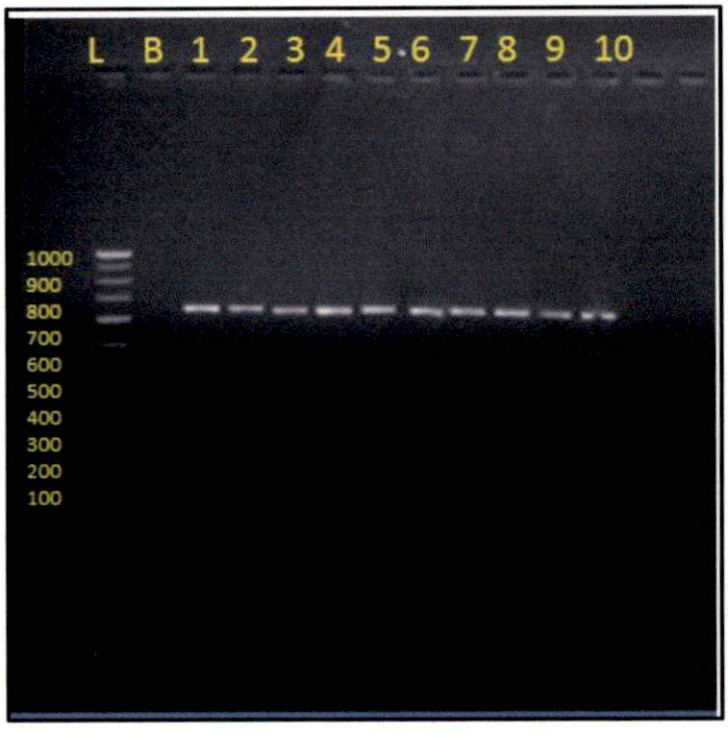


Plate 12 Amplification of *ITS2* locus (II) **L**. Ladder **B**. blank **1**. TpOk12126 **2.**TpMk29116 **3**. BaKo1037 **4.** CpVK19116 **5**. BaPo2327 **6**. OkEr12126 **7**.TpKk0227 **8.** TpCm1827 **9.** PmMk0927

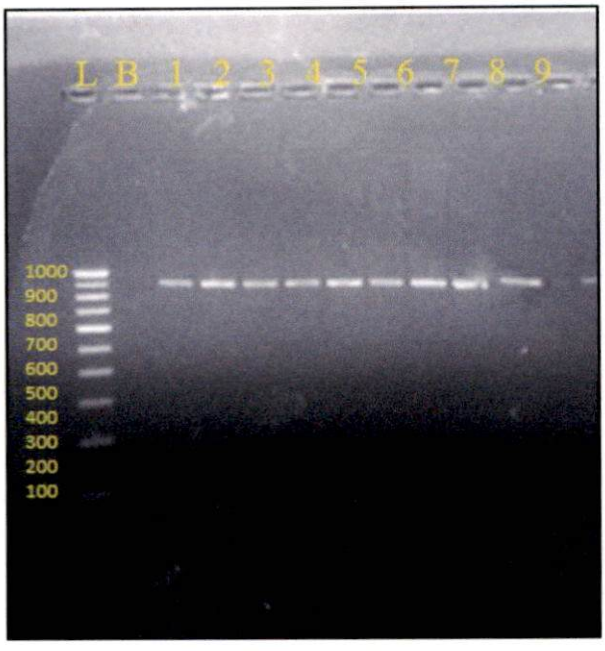


Plate 13 Amplification of *COI* locus **L**. Ladder **B**. blank **1**. TpOk12126 **2.**TpMk29116 **3**. BaKo1037 **4.** CpVK19116 **5**. BaPo2327 **6**. OkEr12126 **7**.TpKk0227 **8.** TpCm1827 **9.** PmMk0927

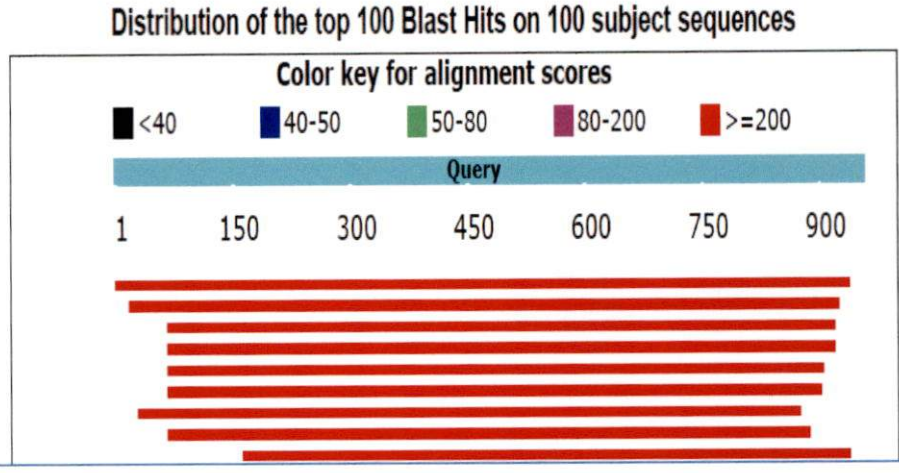
Table 5 Details of forward, reverse and contig sequences

SI No.	Accession No.	Crop and locality	Loci	Length of sequence obtained after sequencing (bp)		Length of contig formed (bp)
				Forward	Reverse	
1	TpOk12126	Tapioca/ Olavakkod	COI	895	904	932
2	TpCm1827	Tapioca/ Chuvannamannu	COI	896	895	926
3	TpMk29116	Tapioca/ Manjakunnu	COI	889	895	920
4	TpKk0227	Tapioca/ Kakkani	COI	896	895	928
5	CpVk19116	Cowpea/ Vellanikkara	COI	894	898	921
6	BaPo2327	Banana/ potta	COI	896	894	923
7	BaKo1037	Banana/ Kodali	COI	893	891	921
8	PmMk0927	Pumpkin/ Manjakunnu	COI	722	558	864
9	OkEr12126	Okra / Erankulam	COI	891	897	926
10	PapOk2736	Papaya/ Olavakkod	ITS2	625	628	652
11	PapKa1256	Papaya/ Kannara	ITS2	624	626	655
12	BrTv0356	Brinjal/ Tavannur	ITS2	629	595	629
13	AgOl12116	Ashgourd/ Vellanikkara	ITS2	626	627	654
14	DaId3036	Dahlia/ Idukki	ITS2	621	620	655
15	AdVK1236	Adenium/ Vellanikkara	ITS2	716	723	No contigs
16	CpVl2827	Cowpea/ Vellayani	ITS2	723	721	No contigs

4.3.2. Analysis of sequence homology

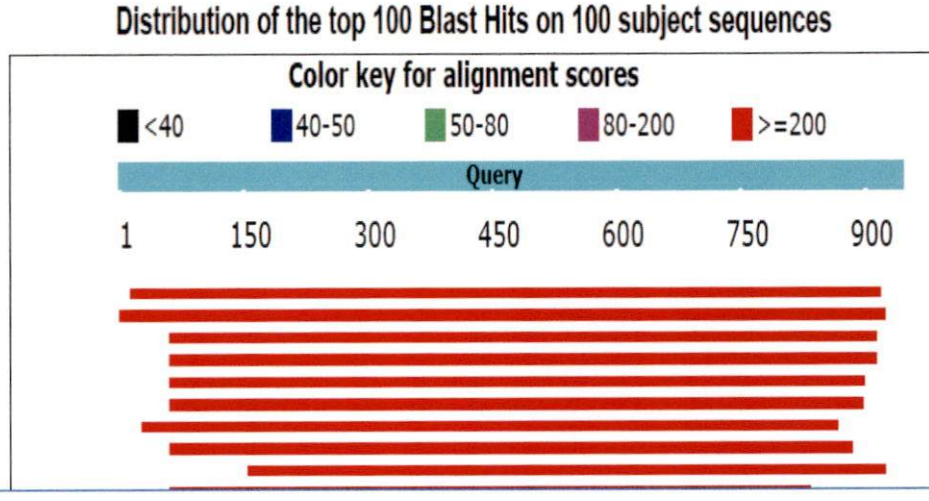
Sequence homology was studied using Mega BLASTn for all the sixteen accessions. Three species were identified with BLAST homology search using the sixteen sequences. The accessions TpOk12126 (tapioca, Olavakkod), TpMK29116 (tapioca, Manjakunnu), BaKo1037 (banana, Kodali), PmMK0927 (pumpkin, Manjakunnu) and DaId30316 (dahlia, Idukki) showed maximum similarity towards *T. truncatus* on BLASTn and the query coverage and identiiy of these sequences were in the range of 96-99 and 97-99 per cent respectively. The accession TpOk12126 (Tapioca, Olavakkod) showed maximum similarity towards NCBI accession KM111296.1 reported by Chen *et al.* (2014), while rest of the accessions showed similarity towards the NCBI accessions KR072563.1 and KRO63238.1 both reported by Bennur *et al.*, (2015). The accessions TpCm1827 (tapioca, Chuvannamannu), TpKk0227 (tapioca, Kakkani), Bapo2327 (banana, Potta) and OkEr12126 (Okra Ernakulam) showed maximum similarity towards *T. udaipurensis* and maximum hit was for the NCBI accession KU738616.1 and query coverage for these sequences were in the range of only 77-79 per cent, while the identity were in the range of 98-99 per cent. The identity towards *T. okinawanus* (*ITS2* locus) was shown by the accessions PapOk2736 (Papaya, olavakkod), AgOl121116 (Ashgourd, Vellanikkara), BrTv0356 (Brinjal, Tavanur) and PapKa1256 (Papaya, kannara) and the identity percentage were in the range of 99 – 100. The BLAST analysed results are presented in Fig. 2-7 and Table 6.

Fig. 2 BLASTn result of accession TpOK12126



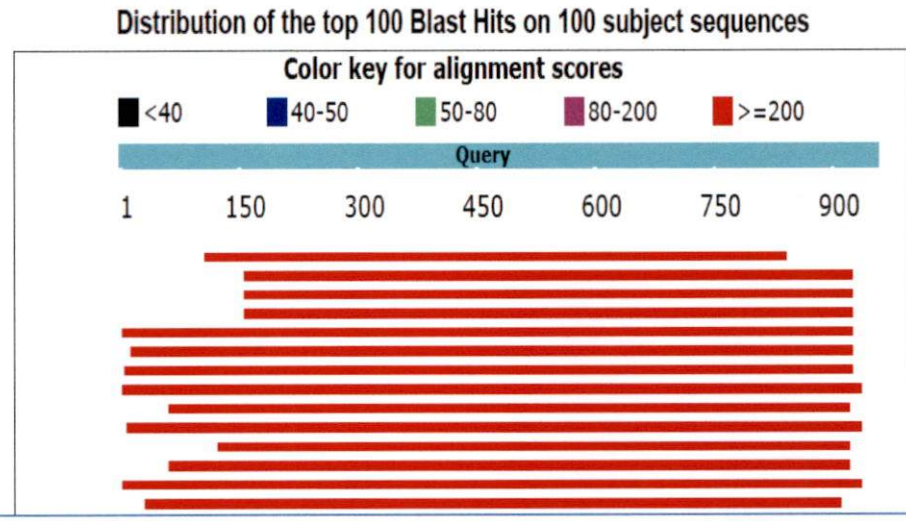
Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus truncatus mitochondrion, complete genome	1557	1557	99%	0.0	97%	KM111296.1
Tetranychus truncatus cytochrome oxidase subunit I gene, partial cds; mitochondrial	1548	1548	96%	0.0	98%	KR072563.1
Tetranychus truncatus isolate VA1.2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1476	1476	90%	0.0	98%	KR052245.1

Fig. 3 BLASTn result for accession TpMk0927



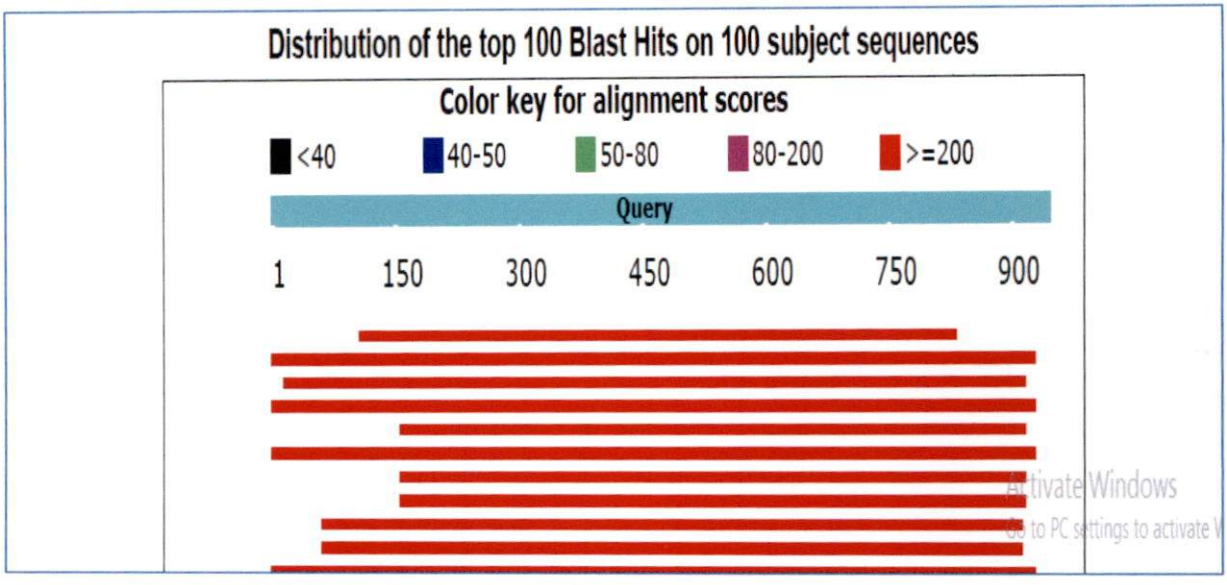
Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus truncatus cytochrome oxidase subunit I gene, partial cds; mitochondrial	1522	1522	97%	0.0	97%	KR072563.1
Tetranychus truncatus mitochondrion, complete genome	1509	1509	99%	0.0	96%	KM111296.1
Tetranychus truncatus isolate VA1.2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1471	1471	91%	0.0	98%	KR052245.1

Fig. 4 BLASTn result of accession TpCm1827



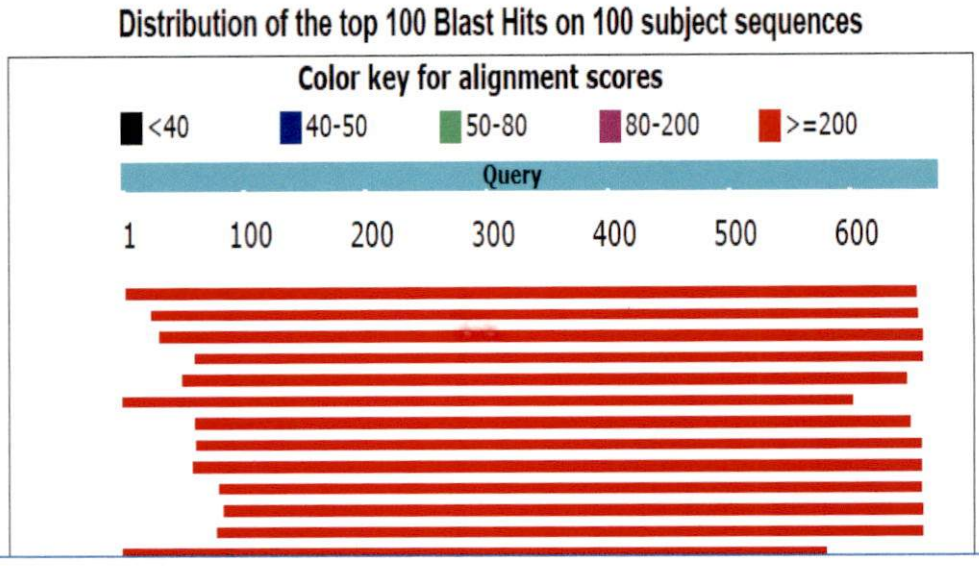
Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus udaipurensis cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1247	1247	77%	0.0	98%	KU738616.1
Tetranychus neocaledonicus mitochondrial cox1 gene for cytochrome oxidase subunit I, partial cds, strain: Tne0003	1081	1081	81%	0.0	92%	AB736056.1

Fig. 5 BLASTn result of accession BaPo2327



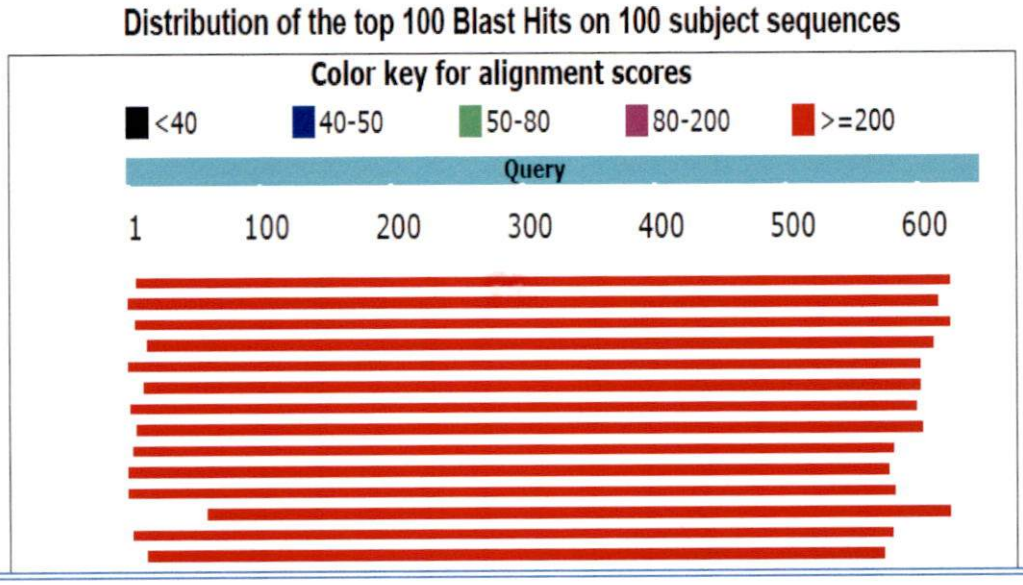
Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus udaipurensis cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	1264	1264	78%	0.0	98%	KU738616.1
Tetranychus truncatus mitochondrion, complete genome	1160	1160	100%	0.0	89%	KM111296.1
Tetranychus truncatus cytochrome oxidase subunit I gene, partial cds; mitochondrial	1153	1153	97%	0.0	90%	KR072563.1

Fig. 6 BLASTn result of accessions AgOl12116



Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus okinawanus isolate AnCuR 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1192	1192	98%	0.0	100%	KR271022.1
Tetranychus neocaledonicus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, isolate: Tn-1	1127	1127	95%	0.0	99%	AB738752.1

Fig 7 BLASTn result of accessions BrTv0356



Description	Max score	Total score	Query cover	E value	Ident	Accession
Tetranychus okinawanus isolate AnCuR 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1116	1116	97%	0.0	99%	KR271022.1
Tetranychus sp. UASB 2014 Trivandrum 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence	1098	1098	96%	0.0	99%	KM580498.1

Table 6 Homology of sequences – BLASTn analysis

SI No.	Accession number	Locus	Name of speceis	Query coverage (%)	Identity (%)	E-value
1	AdVk1236	*ITS2*	*Tetranychus okinawanus*	100	99	0.00
2	PapOk2736	*ITS2*	*Tetranychus okinawanus*	99	99	0.00
3	BrTv0356	*ITS2*	*Tetranychus okinawanus*	97	99	0.00
4	DaId30316	*ITS2*	*Tetranychus truncatus*	98	99	0.00
5	PapKa1256	*ITS2*	*Tetranychus okinawanus*	98	99	0.00
6	CpVk19116	*COI*	*Tetranychus truncatus*	97	98	0.00
7	AgOl21116	*ITS2*	*Tetranychus okinawanus*	98	100	0.00
8	TpMk29116	*COI*	*Tetranychus truncatus*	97	97	0.00
9	TpOk12126	*COI*	*Tetranychus truncatus*	99	97	0.00
10	OkEr12126	*COI*	*Tetranychus udaipurensis*	77	99	0.00
11	TpKK0227	*COI*	*Tetranychus udaipurensis*	77	99	0.00
12	PmMK0927	*COI*	*Tetranychus truncatus*	97	99	0.00
13	TpCm1827	*COI*	*Tetranychus udaipurensis*	77	98	0.00
14	BaPo2327	*COI*	*Tetranychus udaipurensis*	78	98	0.00
15	CpVl2827	*ITS2*	*Tetranychus okinawanus*	100	99	0.00
16	BaKo1037	*COI*	*Tetranychus truncatus*	96	98	0.00

4.3.3 Barcode gap analysis

The sequences were aligned using Clustal W tool provided by Mega 7 software seperately for *COI* and *ITS2* sequences. The maximum aligned length of *COI* sequences were 920 bp and the barcode gaps were identified at alignment positions; 436 (T → C), 446 (T → C), 527 (C → T), 538 (A →G) and 761 (A→ T) for *T. udaipurensis* from base sequence of *T. truncatus*, however at alignment position 745 Adenine (A) was substituted by Thymine (T) in *T. truncatus*. For *ITS2* sequences maximum length after alignment was 662 and the barcode gaps were identified at alignment positions 326 (T → A), 335 (T → A), 340 (C →T), 458 (T→ A), 461 (T → C), 463 (C → T) and 467 (T → C) for the species *T. truncatus* from the base sequence of *T. okinawanus*. The existence of barcode gaps were confirmed in both the loci (Fig. 8 & 9; Table 7 & 8).

4.3.4 Pairwise distances of sequences

After initially aligning the sequences with clustal W (neighbor joining clustering method), the pairwise distances were computed for both *COI* and *ITS2.* The results represent the variation in substitution levels between two sequences. The overall mean distance between the sequences of all the accessions was 0.735. The interspecific distances between the sequences for both *COI* and *ITS2* ranged from 0.03- 1.05. The accessions PmMk0927 (*T. truncatus* on pumpkin) and BrTv0356 (*T. okinawanus* on brinjal) showed maximum variation from other sequences and the value of divergence ranged form 0.79- 0.84 and 0.83- 1.05 respectively. However the intraspecific distances for all accessions were found to be less than 1.0 indicating more similarity than divergence (Fig. 10).

4.3.5 Distance summary of sequences

The distance summaries between the sequences were analysed using sequence analysis tool provided by BOLD (Barcode of Life Data Systems). The maximum and minimum nucleotide divergence within species was found to be 0.31 and 0.00 per cent for *ITS2* (*T. okinawanus* and *T. truncatus*) and 2.88 and 0.96 per cent for *COI* (*T. truncatus* and *T. udaipurensis*), respectively (Fig. 11 and 12).

4.3.6 Analysis based on phylogenetic tree

The phylogenetic trees were constructed with MEGA 7 software utilizing the Neighbor Joining Method. A separate phylogenetic tree were constructed for both the *COI* and *ITS2* loci formed two different clusters. The phylogenetic tree formed is represented in the Fig. 13 and 14.

The phylogenetic tree constructed for *COI* locus showed that accessions CpVK19116 (cowpea, Vellanikkara), Bako1037 (banana, Kodali), TpMk29116 (tapioca, Manjakunnu), and TpOk12126 (tapioca, Olavakkod) formed a single clade and represented the species *T. truncatus*. The accession PmMk0927 (pumpkin, Manjakunnu) however formed an outgroup despite representing the same species *T. truncatus*. The accessions BaPo2327 (banana, Potta), OkEr12126 (okra, Ernakulam), TpCm1827 (tapioca, Chuvnnamannu) and TpKk0227 (tapioca, Kakkani) formed another clade representing the species *T. udaipurensis*. All the above accessions had a common node, indicating common ancestory.

In the phylogenetic trees constructed with *ITS2* sequences, the accessions PapOk2736 (papaya, Olavakkod), AgOl21116 (ashgourd, Vellanikkara) and PapKa1256 (papaya, Kannara) formed a clade representing the taxa *T. okinawanus* and accession DaId30316 (dahlia, Idukki) identified as *T. truncatus* formed a separate clade from the above. However the accession BrTv0356 formed an outgroup, though represented the species *T. okinawanus*.

Fig. 8 Barcode gaps *ITS2* sequences

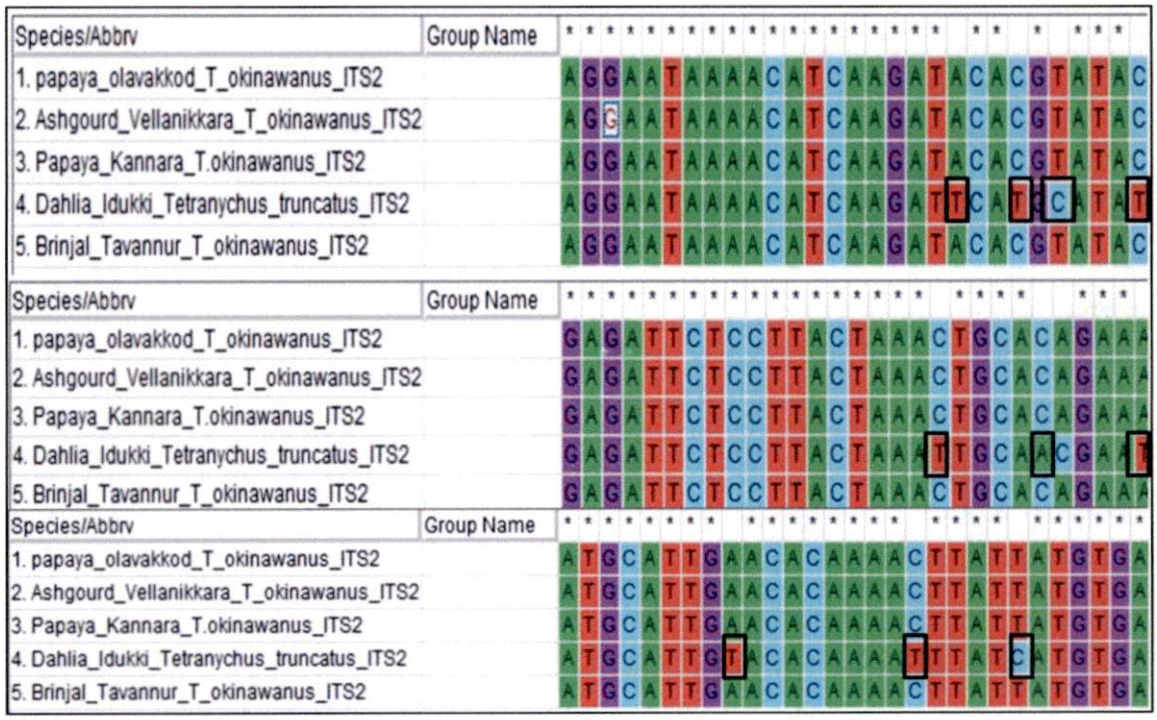


Fig. 9 Barcode gaps *COI* sequences

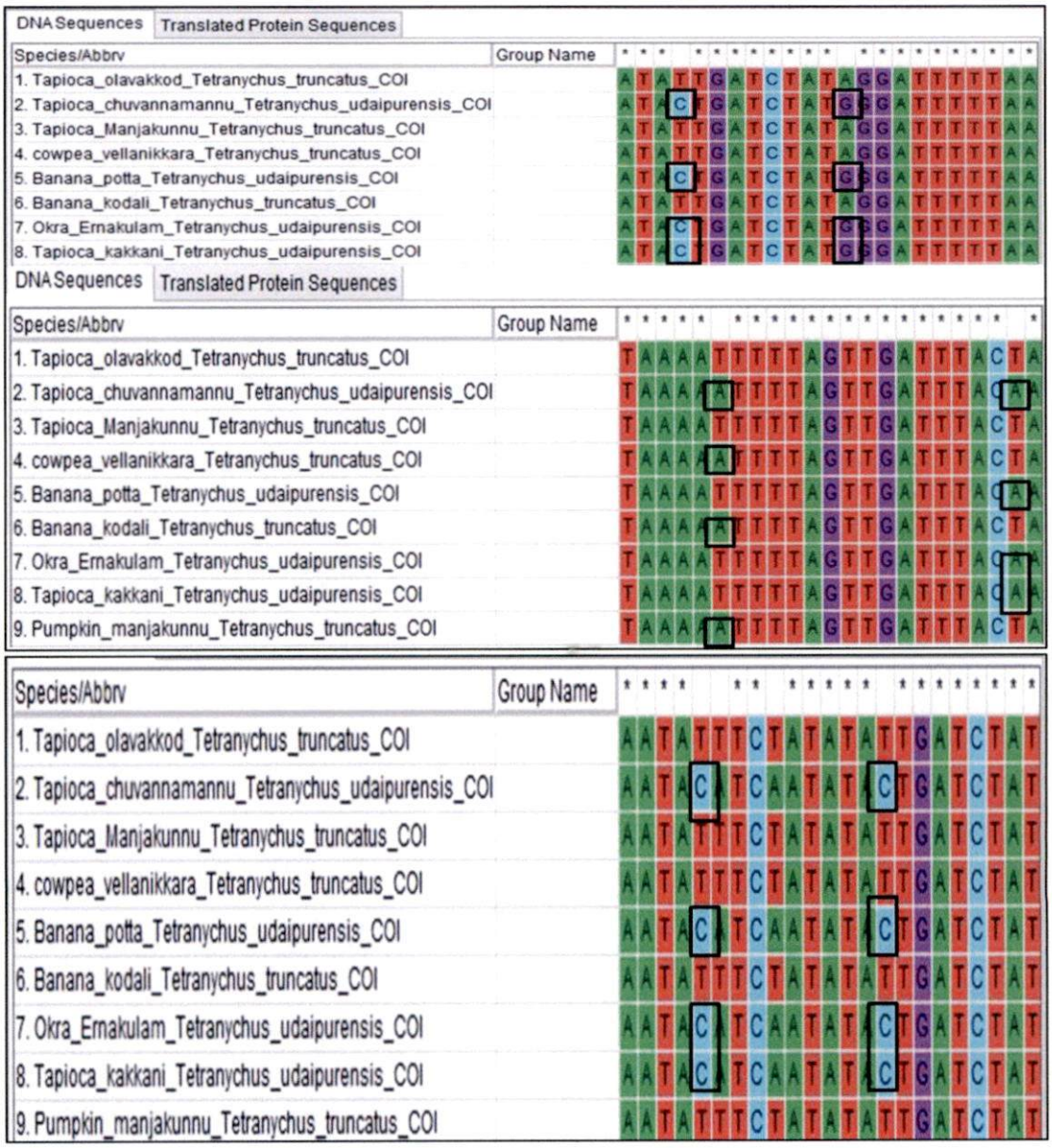


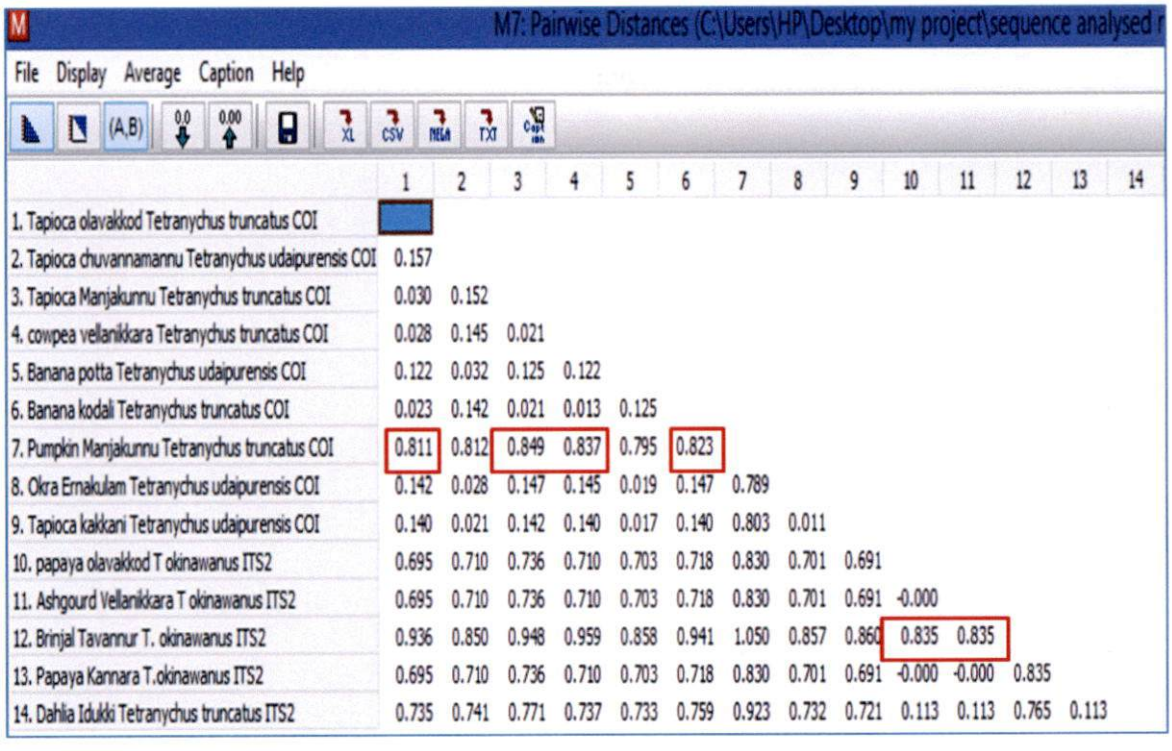
Table 7 Alignment position of barcode gaps and type of substitution for *ITS2* sequences

SI No.	Alignment position	substitution	Type of substitution	Species
1	326	T→ A	Transversion	*T. truncatus*
2	335	T→ A	Transversion	*T. truncatus*
3	340	C→ T	Transition	*T. truncatus*
4	458	T→ A	Transversion	*T. truncatus*
5	461	T→ C	Transversion	*T. truncatus*
6	463	C→ T	Transition	*T. truncatus*
7	467	T→ C	Transition	*T. truncatus*

Table 8. Alignment position of barcode gaps and type of substitution for *COI* sequences

SI NO.	Alignment position	Substitution	Type of substitution	Species
1	436	T→ C	Transition	*T. udaipurensis*
2	446	T→C	Transition	*T. udaipurensis*
3	527	C→T	Transition	*T. udaipurensis*
4	538	A→ G	Transition	*T. udaipurensis*
5	745	A→T	Transversion	*T. truncatus*
6	761	A→T	Transversion	*T. udaipurensis*

Fig. 10 Pairwise distance alignment of *COI* and *ITS2* sequences



M7: Pairwise Distances (C:\Users\HP\Desktop\my project\sequence analysed r

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Tapioca olavakkod Tetranychus truncatus COI														
2. Tapioca chuvannamannu Tetranychus udaipurensis COI	0.157													
3. Tapioca Manjakunnu Tetranychus truncatus COI	0.030	0.152												
4. cowpea vellanikkara Tetranychus truncatus COI	0.028	0.145	0.021											
5. Banana potta Tetranychus udaipurensis COI	0.122	0.032	0.125	0.122										
6. Banana kodali Tetranychus truncatus COI	0.023	0.142	0.021	0.013	0.125									
7. Pumpkin Manjakunnu Tetranychus truncatus COI	0.811	0.812	0.849	0.837	0.795	0.823								
8. Okra Ernakulam Tetranychus udaipurensis COI	0.142	0.028	0.147	0.145	0.019	0.147	0.789							
9. Tapioca kakkani Tetranychus udaipurensis COI	0.140	0.021	0.142	0.140	0.017	0.140	0.803	0.011						
10. papaya olavakkod T okinawanus ITS2	0.695	0.710	0.736	0.710	0.703	0.718	0.830	0.701	0.691					
11. Ashgourd Vellanikkara T okinawanus ITS2	0.695	0.710	0.736	0.710	0.703	0.718	0.830	0.701	0.691	-0.000				
12. Brinjal Tavannur T. okinawanus ITS2	0.936	0.850	0.948	0.959	0.858	0.941	1.050	0.857	0.860	0.835	0.835			
13. Papaya Kannara T.okinawanus ITS2	0.695	0.710	0.736	0.710	0.703	0.718	0.830	0.701	0.691	-0.000	-0.000	0.835		
14. Dahlia Idukki Tetranychus truncatus ITS2	0.735	0.741	0.771	0.737	0.733	0.759	0.923	0.732	0.721	0.113	0.113	0.765	0.113	

Fig. 11 Distance summary within species for *ITS2* sequences

Distance Summary Tables

The distribution of sequence divergence at each taxonomic level is summarized below. Detailed distance tables can be downloaded by clicking on Details button for each rank.

Label	n	Taxa	Comparisons	Min Dist(%)	Mean Dist(%)	Max Dist(%)	SE Dist(%)
Within Species	3	1	3	0.00	0.20	0.31	0.05

Distance Summary Graphs

Sequence divergence for all sequences compared at the species and genus levels.

Within Species

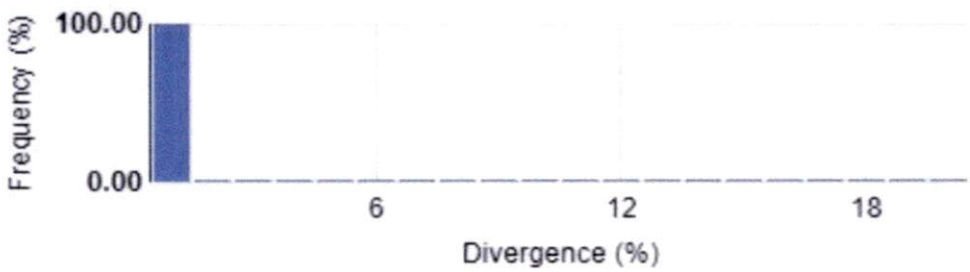


Fig. 12 Distance summary within species for *COI* sequences

Distance Summary Tables

The distribution of sequence divergence at each taxonomic level is summarized below. Detailed distance tables can be downloaded by clicking on Details button for each rank.

Label	n	Taxa	Comparisons	Min Dist(%)	Mean Dist(%)	Max Dist(%)	SE Dist(%)
Within Species	5	1	10	0.96	1.90	2.88	0.07

Distance Summary Graphs

Sequence divergence for all sequences compared at the species and genus levels.

Within Species

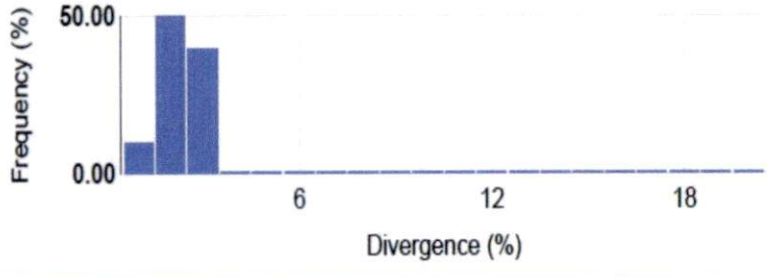


Fig 13 Phylogeny of *COI* sequences

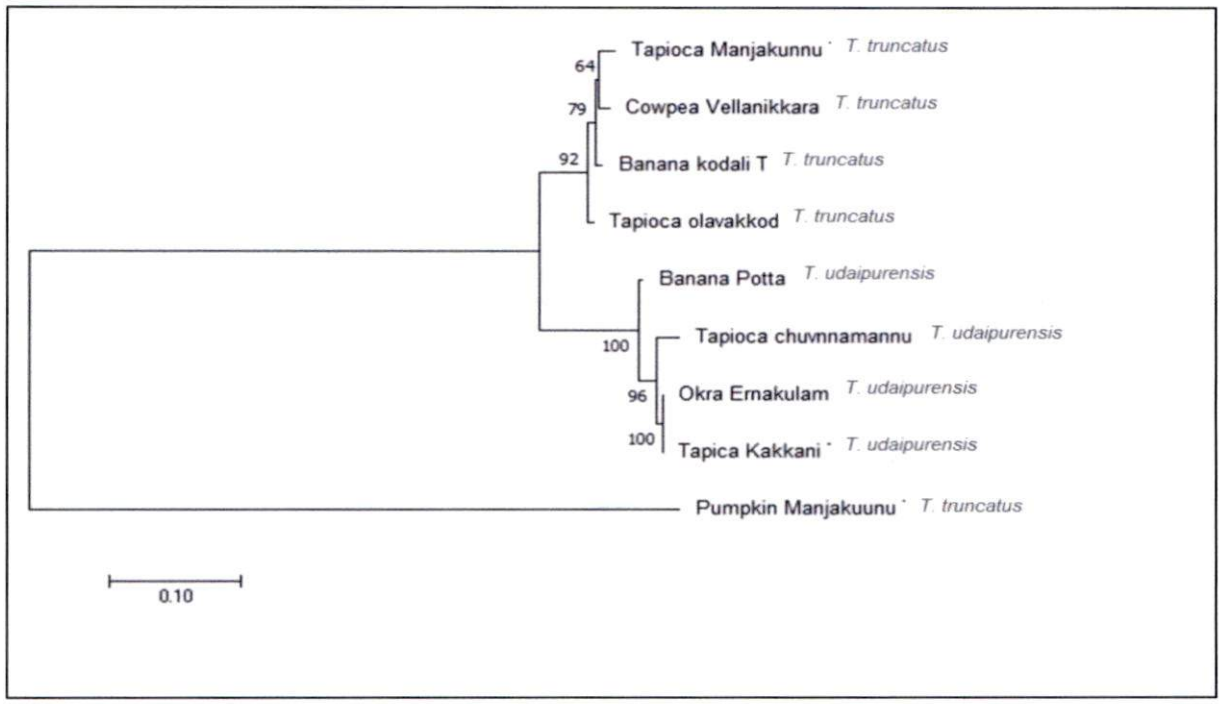
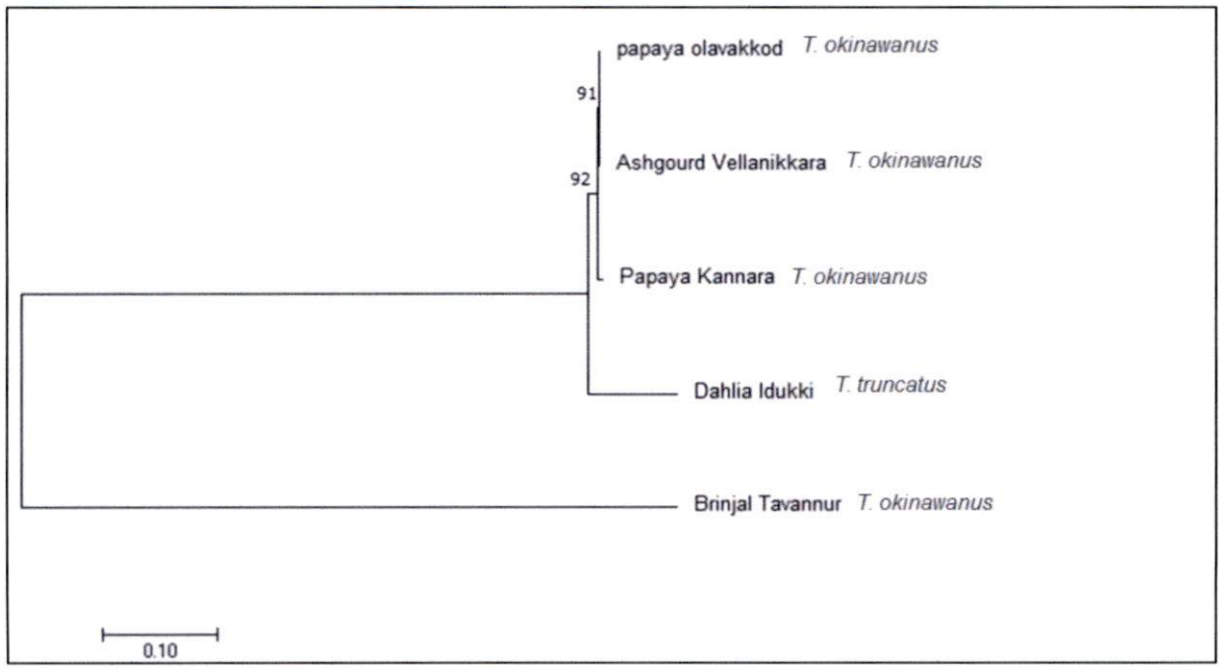


Fig. 14 Phylogeny of *ITS2* sequences



4.3.7. Submission of sequences to NCBI GenBank

Fourteen sequences were submitted to GenBank and the accession numbers were obtained for 14 accessions (nine *COI* sequences and five *ITS2* sequences) and are presented in Table 9.

4.3.8 Submission to BOLD (Barcode of Life Data systems)

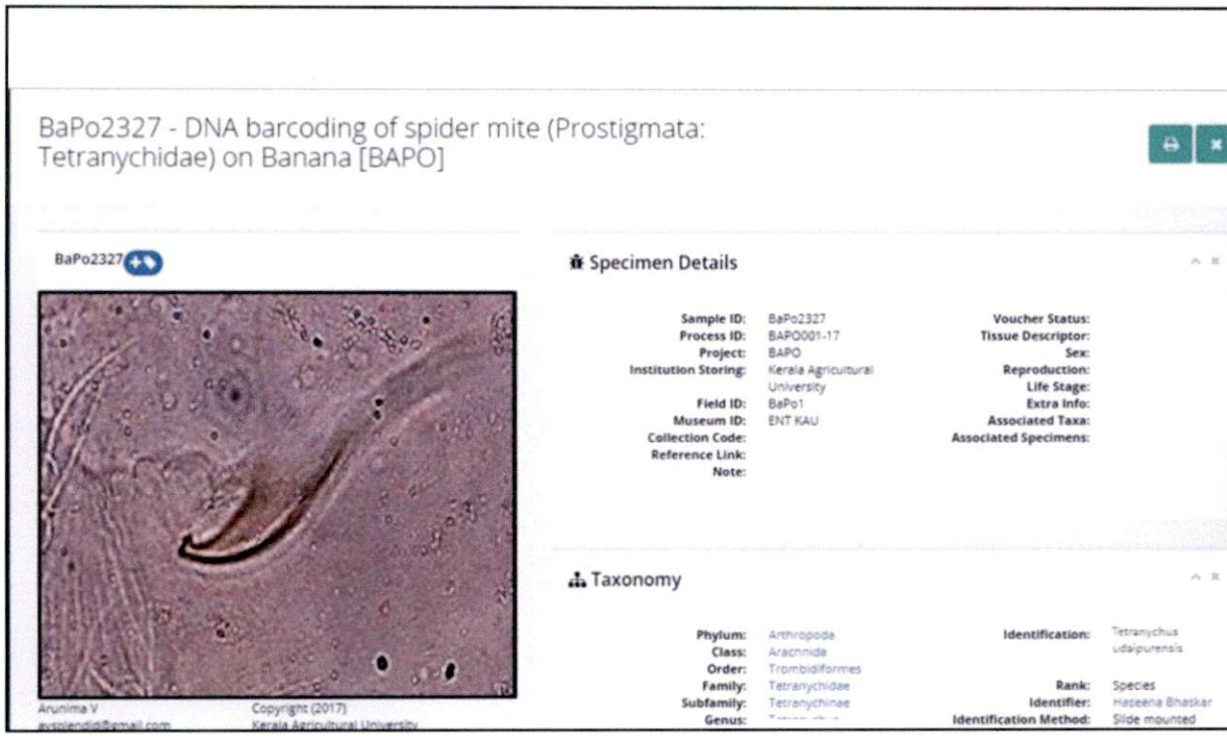
The fourteen sequences amplified with *COI* (nine) and *ITS2* (five) primer were submitted to Barcode of Life Data (BOLD) systems for generation of barcodes by providing specimen data, sequence data, trace files and specimen images (Plate 15). The process IDs obtained is listed in the Table 10.

Table 9 Accession number obtained from NCBI for different accessions

SI No.	Accession number	Loci	NCBI accession number
1	BrTv0356	*ITS2*	MF774625
2	DaId30316	*ITS2*	MF774628
3	PapKa1256	*ITS2*	MF774626
4	AgOl121116	*ITS2*	MF774623
5	TpMk29116	*COI*	MF774632
6	TpOk12126	*COI*	MF774630
7	OkEr12126	*COI*	MF774636
8	TpKk0227	*COI*	MF774629
9	PmMk0927	*COI*	MF774635
10	TpCm1827	*COI*	MF774631
11	BaPo2327	*COI*	MF774637
12	CpVk19116	*COI*	MF774633
13	BaKo1037	*COI*	MF774634
14	PapOk2736	*ITS2*	MF774638

Table 10 Process IDs obtained for the accessions submitted to BOLD

SI No.	Accession number	Loci	Process ID
1	CpVK19116	*COI*	CPVK001-17
2	TpMK29116	*COI*	TPMK001-17
3	TpOk12126	*COI*	TPOK001-17
4	OkEr12126	*COI*	OKER001-17
5	TpKk0227	*COI*	TPKK001-17
6	PmMk0927	*COI*	PMMK001-17
7	TpCm1827	*COI*	TPCM001-17
8	BaPo2327	*COI*	BAPO001-17
9	BaKo1037	*COI*	BAKO001-17
10	PaOk2736	*ITS2*	SAFI001- 17
11	BrTv0356	*ITS2*	SAFI001- 17
12	AgOl21116	*ITS2*	SAFI001- 17
13	PapKa1256	*ITS2*	SAFI001- 17
14	DaId30316	*ITS2*	SAFI001- 17



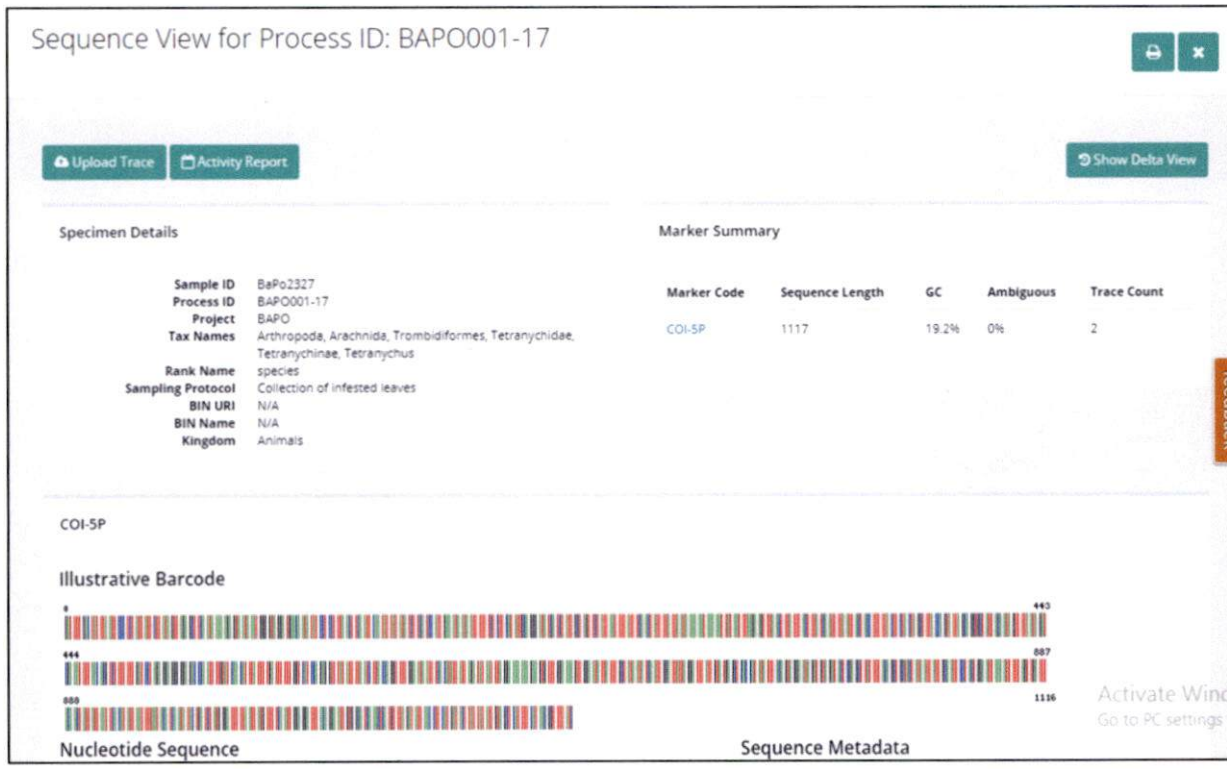


Plate 15 Details of BOLD submission

Discussion

5. Discussion

5.1. Morphology based identification of spider mites

The study recorded three species of *Tetranychus* (Prostigmata: Tetranychidae) *viz.*, *Tetranychus truncatus* Ehara, *Tetranychus okinawanus* Ehara and *Tetranychus udaipurensis* Gupta and Gupta associated with different crops in Thrissur, Malappuram, Ernakulam and Palakkad districts of Kerala. The three species were distinguished by careful examination of morphological features of taxonomic importance particularly distinct aedeagal knob.

In the study, *T. truncatus* was recorded on five different crops namely, cowpea, pumpkin, tapioca, banana and *Dahlia* from eight of the twelve localities surveyed. *T. truncatus* was first recorded in India from the Northwestern Himalayan regions of Jammu and Kashmir and Himachal Pradesh on *Dahlia* sp. (Rather, 1983). Later, the mite species was reported from Karnataka infesting mulberry leaves (Srinivasa *et al.*, 2012). Bennur *et al.* (2015) reported *T. truncatus* as the predominant species of spider mite on vegetable crops namely, cowpea, amaranthus and cucumber in Thrissur district. Recently, *T. truncatus* was also reported to be a predominant mite species infesting cucumber and amaranthus grown in polyhouses of Kerala (Lenin and Bhaskar, 2016). The present study records three new hosts for *T. truncatus* from Kerala, India namely tapioca, banana, and pumpkin.

The present study brings to light the predominance of the spider mite species, *T. truncatus* in different crops of Kerala, which was only recorded recently as a major pest in this region. Mites associated with vegetable crops of Kerala have been studied earlier by several workers and *T. urticae* Koch was reported as the predominant species on brinjal, bhindi, amaranthus and cowpea (Sudharma and Nair, 1999; Binisha and Bhaskar, 2013). Study on diversity of spider mites on vegetable crops of Thrissur district, Kerala conducted by Bennur *et al.* (2015) reported *T. macfarlanei* on brinjal, cowpea and okra. However, in the present study, *T. urticae* and *T. macfarlanei* was not recorded from any of the crops surveyed. This gives an indication on the possible displacement of *T. urticae* and

T. macfarlanei by *T. truncatus* on different crops in the region. Competitive displacement of *T. urticae* after the invasion of *T. evansi* in the Mediterranean region has been reported by Ferragut *et al.* (2013) recently. Srinivasa *et al.* (2012) repoted that that when *T. urticae* and *T. truncatus* were cultured in the laboratory on mulberry leaves, *T. truncatus* frequently contaminated the culture of *T. urticae* due to its distinct behaviour of prolific multiplication. Further, the wide range of host records of *T. truncatus* which includes 61 species of plants as reported by Bolland *et al.* (1998) is suggestive that the species can widen its host range in Kerala and become invasive.

During the study *T. okinawanus* was recorded on papaya, ashgourd, cowpea, *Adenium*, and brinjal from five localities.This species was first reported on *Pueraria lobata* from Okinawa Islands of Japan by Ehara (1995) and hitherto known from Japan and Taiwan on more than 90 host plants. It was first reported from India on the ornamental plant, *Adenium obesum* from Kerala (Zeity *et al.*, 2016) and later on cucumber from Thrissur district, Kerala (Bennur *et al.*, 2015; Lenin *et al.*, 2015 and Lenin and Bhaskar, 2016). The present study records four new hosts (papaya, ashgourd, brinjal and cowpea) for *T. okinawanus* from Kerala, India suggesting the potential of the species to widen its host range in a short span, since its first record in 2015. This can lead to the speculation that the alien species, *T. okinawanus* can turn invasive in its new natural or semi natural ecosystem and probably can replace the existing spider mite species.

The study documented occurrence of *T. udaipurensis* on three crops namely, okra, banana and tapioca. *Tetranychus udaipurensis* was first described by Gupta and Gupta (2004) on brinjal from Udaipur, Rajasthan. Recently, this species was recorded on papaya from Trivandrum, Kerala (Srinivasa *et al.*, 2016). Okra, banana and tapioca are new host records for *T. udaipurensis* mite in this study.

5.2 Molecular based identification of spider mites

In the context of increasing popularity of molecular systematics in acarology, present study was conducted to generate barcodes for spider mite species found on major crops of Kerala. Seven *ITS2* sequences and *nine* COI sequences were obtained from sixteen accessions used in this study. Both *ITS2* and *COI* have been reported to be suitable for the phylogenetic analysis up to the lowest taxonomic level of species (Cruickshank, 2002).

5.2.1 DNA isolation

Regardless of the source of DNA sample and the marker system involved, good quality of DNA needs to be extracted for successful molecular studies. In the present study modified CTAB method was successfully employed for the extraction of high quality DNA from all the spider mite accessions. Many DNA isolation techniques were proposed for isolation of DNA from arthropods which includes; SDS method, DNAzol reagent, DNAeasy columns, chelex resin (100), Qiagen DNA extraction, DTAB and CTAB method (Philip and Simon, 1995; Wang and Wang, 2012). Chen *et al.* (2010) proposed CTAB method of DNA isolation as ideal for corn root worm beetles and Per and Ercan (2015) successfully used CTAB method for DNA isolation from Oribatid mites. Navajas *et al.* (1998) utilised 2 per cent CTAB method of DNA isolation from *T. urticae* to analyse the species wide homogeneity of ITS2 sequences in the species. Fournier *et al.* (1994) also proposed a differential centrifugation based technique to isolate just the mitochondrial DNA of *T. urticae*. Ros and Breeuwer (2007) successfully used 2 per cent CTAB for the isolation of DNA from spider mites for the analysis of molecular phylogeny using *COI* sequences. In the current study, since one locus (*ITS2*) was of nuclear origin and the other was of mitochondrial origin (*COI*), the total genomic DNA was isolated using the modified CTAB method from all accessions in order to amplify both the loci. Ten to fifteen live mites were crushed in pre-warmed CTAB buffer instead of liquid Nitrogen to obtain higher concentration of DNA. Since oxidative browning was not an issue due to lack of phenols in spider mites, antioxidants like PVP and β mercaptoethanol were not added while crushing. Navajas *et al.* (1992) obtained whole genomic DNA from

10-30 adult spider mites for rDNA sequence analysis while Ben-David (2008) proposed the use of 10 female mites for the isolation of DNA from various species of spider mites in Israel. Pre-incubation of mites in warm CTAB buffer for one hour, prior to DNA isolation as proposed by Bennur (2015) was shown to increase the yield of DNA obtained in this study also. An additional alcohol wash (100 %) increased the purity of the DNA obtained.

5.2.2 Quality of DNA

In molecular biology, the reliable quantification of DNA (in solution) in measures of picogram, nanogram, and microgram is essential and absorbance analysis of micro volumes of samples has become of paramount importance which gives accurate quantification of nucleic acids with minimal sample consumption (Gallangher and Desjardins, 2006). In the present study, quality of DNA was assessed with Nano-Drop Spectrophotometer and the readings obtained for most of the accessions were in the range of 1.8-2.0 for A260/ A280 and 1.8 to 2.3 for A260/A230. Murray and Thompson (1980) suggested similar values for the quality DNA while proposing a CTAB based rapid isolation of high molecular weight DNA for plants. Bennur *et al.* (2015) isolated DNA from spider mites collected from different vegetable crops using the CTAB method and obtained values for absorbance in the range of 1.8-2.0, establishing CTAB method of DNA isolation as a suitable technique for obtaining high quality DNA from spider mites. The readings obtained in the present study were also in the range of 1.8-2-0, confirming the high quality of isolated DNA.

5.2.3. DNA amplification with Polymerase Chain Reaction (PCR)

In the present study two loci, *ITS2* and *COI* were chosen for the molecular identification of spider mites. *ITS* (Internal Transcribed spacer) are fast evolving regions occurring between the highly conserved regions of rDNA (18SrDNA, 5.8S rDNA and 28Sr DNA) and *ITS2* is situated between 5.8S rDNA region and 28S r DNA (Hillis and Dixon, 1991). Being a protein coding gene, the impact of functional constraints is less in *COI* and it also possess a greater range phylogenetic signal than any other mitochondrial gene. The availability of universal primers

which can robustly amplify this region, adds to the advantage that *COI* can be used for phylogenetic analysis and generation of barcodes (Herbert *et al.*, 2003). The DNA amplification was initially attempted with the protocol standardized by Bennur (2015) for both *COI* and *ITS2* loci, which failed to amplify the regions in any of the accessions studied. Hence, the protocol proposed by Li *et al.* (2010) for the genus *Tetranychus* was successfully employed for the amplification of both the loci. In case of *ITS2,* annealing temperature was also standardized with gradient PCR which showed that 55.3° C was optimum for amplification of the locus as opposed to 52.1 ° C suggested by Bennur (2015).

5.2.4 Quality of PCR products

The quality of PCR products were assessed with 2 per cent agarose gel electrophoresis. It was seen that the size of the amplicons obtained for *ITS2* and *COI* were in the range of 600-700 bp and 800- 900 bp respectively, when compared with 100 bp ladder. In a study conducted to analyze the species wide homogeneity of *ITS2* sequences in spider mites, Navajas *et al.* (1998) obtained *ITS2* sequences from spider mites varying in the range of 445-805 and found that the length of the sequences varied from species to species. Marcilla *et al.* (2001) found that the base length varied from 454-696 for various species in Triatominae (Reduviidae: Hemiptera). The amplification of *ITS2* sequences of different spider mite species from Israel resulted in amplicon size ranging from 474- 542bp (Ben- David *et al.*, 2007). Almeida and Stouthamer (2014) when analyzed the base pair length of *ITS2* sequences for *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) found that the length varied from 379-632bp. Bennur (2015) obtained size range of 620bp for *ITS2* and 868 for *COI* sequences in spider mites. A 546 bp region of *COI* was used by Toda *et al.* (2000) to investigate the phylogenetic relationships between *Panonychus* spp. (Prostigmata: Tetranychidae) in Japan. Hajibabaei *et al.* (2006) used a 658 bp fragment of *COI* to generate barcodes for the various species of tropical Lepidoptera. Prosser *et al.* (2015) obtained 453- 610 bp long COI sequences from century old type specimens of Lepidoptera to build the DNA barcode library.

5.2.5 Sequence analysis using *in silico* tools

Basic Local Alignment Search Tool (BLAST) utilizes BLAST algorithim via a web interface for the sequence similarity analysis, which can be executed with default parameters or with customized settings (McGinnis and Madden, 2004) Many of the BLAST databases are not consistently organized on taxonomic basis (Johnson *et al.*, 2008) yet BLAST is a reliable tool for the homology search among sequences.

Using the BLASTn tool provided by NCBI, the sequences obtained after sequencing were compared with the existing sequences in GenBank and sequence homology was ascertained. According to McGinnis and Madden, (2004) mega BLAST is the fastest programme for comparing similar sequences and is appropriate for comparison up to 80 per cent identity. The homology search using BLASTn for the sixteen accessions resulted in the identification of three species *viz*, *Tetranychus truncatus*, *T. okinawanus* and *T. udaipurensis*. The majority of accessions which were amplified for the loci COI showed identity (97- 99%) towards the species *T. truncatus* among which the accessions TpMk29116, CpVk19116, BaKo1037, PmMk0927 showed maximum hit towards the accessions KR072563.1 and KRO63238.1 reported by Bennur *et al.* (2015).

The Accessions TpKk0227, OkEr12126, TpCm1827 and BaPo2327 showed 98- 99 per cent identity and 77 per cent query coverage towards the species *T. udaipurensis.* There were only two accessions (KU310624.1 and KU738616.1) in GenBank database for *T. udaipurensis* for the loci *COI* and both were reported by Srinivasa *et al.* (2016) on the host papaya. However in this study, *T. udaipurensis* was recorded on okra, tapioca and banana. For the *ITS2* loci the accessions PapOk2736, AgOl12116, BrTv0356, CpVl2827, AdVk1236 and PapKa1256 showed 99-100 per cent identity towards the species *T. okinawanus* and one accession DaId30316 showed 99 per cent identity towards *T. truncatus*. The BLAST results obtained were in consensus with the morphological data. de-Mendonca *et al.* (2011) compared 18 COI and ITS2 sequences from spider mites morphologically identified as *T. urticae* for the BLASTn analysis and found that BLASTn analysis confirmed the morphological data.

As a part of further analysis of the sequences, barcode gap analysis, distance summary analysis and phylogenetic tree construction was carried out for the fourteen sequences which formed contigs.

Presence of barcode gaps were analysed in the sequences by the technique proposed by Mathew (2015) in *Momordica* spp. The sequences aligned with Clustal W tool in MEGA 7 was analysed for the presence of barcode gaps manually and the positions with the absence the symbol * was analysed for the variation in nucleotide composition. In the species *T. udaipurensis*, the type of substitution was observed to be transition (C→T & A→G) as well as transversion at *COI* locus. Similarly for the species *T. truncatus* (*ITS2* locus) also both transversions (T→C & A→T) and transitions were observed. Navajas *et al.* (1996) analysed a part of COI for 20 species of phytophagous mites and found that most variations in sequences were transition substitutions, but transversions were also shown to accumulate at steady rate. Candek and Kuntner (2015) analysed the existence of barcode gaps in three families of spider species in the *COI* locus, by computing genetic distances using Kimura 2- parameter to arrive at intra and interspecific distances and concluded that barcode gap existed for the families Tetragnathidae and Lycosidae.

Ben-David (2008) studied pairwise distances between the 16 species of spider mites in Israel for the *ITS2* locus and found that in sub family Tetranychinae, genetic distances ranged from 0.11- 0.5 amongst species. In the present study the genetic distance within *T. truncatus* was found to be in the range of 0.013- 0.157, within *T. okinawanus* 0.00- 0.13 and within *T. udaipurensis* 0.01-0.03. However the accessions PmMk0927 (pumpkin, Manjakunnu) identified as *Tetranychus truncatus* and the accession BrTv0356 idetnified as *T. okinawanus* showed divergence within the species as high as 0.84 and 0.835, respectively. The morphological study of the Cassava green mite collected from the various locations in Africa showed variation in length of dorsal setae, however the sequence divergence for the loci *COI* and *ITS2* was only 0.0- 2.1 and 0.0- 4.0 and the molecular data was not in consensus with the morphological data (Navajas *et al.*, 1994). In an another study conducted by Navajas *et al.* (1992) despite the slight

difference in shape of aedeagus *T. mcdanieli* and *T. pacificus* displayed only little sequence divergence. In this study also, despite having distinct shape for the aedeagal knob, the pair wise distance alignment of *ITS2* sequences between the species *T. okinawanus* and *T. truncatus* showed nucleotide divergence of only 0.113 suggesting that they may be closely related species. Bennur (2015) also suggested that *T. truncatus* and *T. okinawanus* may be closely related species based on the molecular taxonomy study conducted on the spider mites on vegetable crops of Thrissur district, Kerala. Gotoh *et al.* (1998) utilized the marker *ITS2* for the molecular comparison of *T. urticae* and *T. puerariсola* which showed the similarity between species as high as 93.8 per cent but had species specific restriction sites.

The Distance Summary tool provides a report on the sequence divergence between barcode sequences at the conspecific and congeneric level. It is desirable for barcodes to show minimum divergence within species. In this study the maximum and minimum intra specific divergence in *COI* sequences were in the range of 0.96- 2.88 per cent with a mean divergence of 1.90 per cent. For *ITS2* sequences the maximum and minimum divergence were observed to be 0.31 and 0.00 per cent respectively. Similar values were suggested by Bennnur (2015) for the *COI* locus (0- 3.50 %) in spider mites. In a study conducted by lv *et al.* (2014) on ticks (Acari: Ixodidae) it was seen that the average intra- specific distances for *ITS2* and COI loci were 0.014 and 0.003 respectively, while inter-specific distances were 0.174 and 0.417, respectively.

A phylogenetic tree was constructed for 46 sequences of *ITS2* loci of spider mites in Israel, which formed a monophyletic group for all the species with a bootstrap value of 100, indicating that *ITS2* sequences can provide sufficient resolution and can serve as barcodes (Ben- David, 2008). In this study seperate phylogenetic trees were constructed for *ITS2* and *COI* sequences. Ros and Breeuwer (2007) constructed phylogenetic tree from the COI sequences of spider mites collected from Europe and North America and it was found that the resulting tree separated *T. urticae*, *T. kanzawai* and *T. truncatus* into three different clades. In this study also, the accessions separated into distinct clades for *T. truncatus*, *T. okinawanus* and *T. udaipurensis*. The accessions BaPo2327 (banana, Potta),

OkEr12126 (okra, Ernalulam), TpCm1827 (tapioca, Chuvannamannu) and TpKk0227 (tapioca, Kakkani) formed a distinct clade and all belonged to same species *T .udaipuresis* and had mimium divergence at sequence level from each other. All the accessions representing the species *T. truncatus* formed a single clade (Accessions: CpVK19116, BaKo1037, TpMk29116 and TpOk12126) with an exception of accession PmMk0927 which formed out group. The contig length for the accession (864 bp) was found to be much less than the other accessions (920-932 bp), which may be due to the errors in sequencing and resulted in the formation of outgroup. Phylogenetic relationships between six species of economically important tetranychid mites were analysed using the 300 bp region of *ITS2* and it was found that the resulting tree expressing the interspecific relationships in genus *Tetranychus* was in agreement with morphological data (Navajas *et al*., 1992).

The present study identified three species of spider mites from the ten different crops *viz*., *T. truncatus*, *T. okinawanus* and *T. udaipurensis*. *Tetranychus truncatus* and *T. okinawanus* were earlier reported by Bennur *et al*. (2015) on vegetable crops of Thrissur districts, however *T. udaipurensis* was recorded in this study on okra, banana and tapioca and are new host records for the species. The molecular data obtained from the *COI* and *ITS2* sequence from the sixteen accessions supported the morphological identification. However, the absence of contigs in the accessions AdVk1236 and CpVl2827 (*ITS2*) resulted in loss of data and the smaller contigs formed by the accessions PmMk0927 and BrTv0356 resulted in bias in the values obtained in pairwise distance alignment, distance summary and phylogeny analysis. The fourteen sequences which formed contigs were submitted to NCBI as well as to BOLD for generation of barcode data for the species *T. truncatus* and *T. udaipurensis* and *T. okinawanus*. The study was affirmative on the utility of *ITS2* and *COI* loci as efficient tool in delineating spider mites up to species level.

Summary

Summary

In the context of increasing popularity of molecular techniques in molecular taxonomy of spider mites, present study entitled "DNA barcoding of spider mites (Prostigmata: Tetranychidae) on major crop plants of Kerala" was carried out at Centre for Plant Biotechnology and Molecular Biology and Department of Agricultural Entomology (Under AINP on Agricultural acarology), College of Horticulture, Vellanikkara, during the period 2015 - 2017. The objective of the study was to generate DNA barcodes for different species of spider mites associated with major crop plants of Kerala as well as to find out the genetic variability among species using molecular techniques. The survey was conducted mainly in four districts of Kerala *viz*., Thrissur, Palakkad, Ernakulam and Malappuram and spider mites associated with ten crops (cowpea, okra, pumpkin, brinjal, ashgourd, banana, papaya, Adenium, Dahlia and tapioca) were collected from eleven different localitions. Separate isolines were maintained for each collection and were assigned unique accession numbers. Morphological and molecular characterization revealed that the spider mites collected from dfferent crops belonged to three different species of a single genus *Tetranychus* namely, *T. truncatus*, *T. okinawanus* and *T. udaipurensis*.

Salient findings of the study are furnished below:

1. Three different species of spider mites *viz*., *T. truncatus*, *T. okinawanus* and *T. udaipurensis* were found associated with ten different crops from eleven different locations across four distrits of Kerala *viz*., Thrissur, Palakkad, Malappuram and Ernakulam.

2. The study recorded new host records for all the three species. Pumpkin, banana, tapioca were new host records for *T. truncatus*; papaya, cowpea, ashgourd and brinjal were new host records for *T. okinawanus* and banana, okra and tapioca were new host records for *T. udaipurensis*.

3. Use of ten to fifteen adult female mites for crushing; one hour incubation at 65 C after crushing and 100 per cent alcohol wash increased the quantity as well as quality of DNA obtained.

4. Polymerase chain reaction for both the *ITS2* and *COI* loci were carried out by following the Protocol proposed by Li *et al.* (2010). The annealing temperature for *ITS2* locus was standardized with gradient PCR and was ascertained to be 55.3° C.

5. The size of amplicons obtained for *ITS2* and *COI* loci were in the range 600- 700 bp and 800- 900 bp, respectively, which were in the expected range and were suitable for barcoding.

6. The BLASTn analysis of both *COI* and *ITS2* loci revealed an identity per cent in the range of 97-99 for all the accessions and results obtained were in consensus with the morphological identification.

7. The barcode gap analysis of *COI* and *ITS2* sequences confirmed the existence of bardcode gaps at both the loci to differentiate *T. truncatus* from *T. udaipurensis* as well as *T. okinawanus.*

8. The pairwise distance analysis of the sequences revealed that the intraspecific nucleotide divergence was less than one. The distance between *T. truncatus* and *T. udaipurensis* was 0.140 – 0.142, while between *T. truncatus* and *T. okinawanus* was 0.113.

9. On distance summary analysis of sequences it was found that the divergence (%) within species for *ITS2* and *COI* loci were in the range of 0.00 - 0.31 and 0.96 -2.88 respectively.

10. Phylogeny analysis of *COI* and *ITS2* sequences revealed that the same species within a monophyletic group formed a single clade.

11. The study brought to light the predominance of *T. truncatus* and *T. okinawanus* from the crops sampled for spider mite.

12. *Tetranychus okinawanus* which was first reported from *Adenium* in Kerala, was seen to have expanded its host range and was reported from four new hosts indicating its potential to widen its host range and is also suggestive that this alien species may turn invasive.

13 The pairwise distance analysis, distance summary analysis and phylogeny analysis point out that *T. truncatus* is closely related to *T. udaipurensis* as well as *T. okinawanus.*

14 Present study validates and confirms the earlier reports that both *ITS2* as well as *COI* as reliable tools to differentiate closely related species of spider mites.

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Annexures

ANNEXURE I

Materials used in collection of spider mites and rearing

Transparent polythene bags

Rubber Bands

Paint Brush

Plastic trays (Small)

Absorbent sponge

Materials used in permanent slide preparation

Hoyer's medium

Slides

Cover slip

Insulin Syringe

Composition of Hoyer's medium

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

ANNEXURE II

Reagents used for DNA isolation

1. CTAB extraction buffer (100ml)

CTAB (Cetyl Trimethyl ammonium bromide)	: 2g
Tris HCl (1M, pH- 8)	: 10ml
EDTA (0.5 M, pH- 8)	: 2ml
NaCl (5M)	: 30ml
Distilled water	: 54 ml

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

To twenty four parts of chloroform, one part of isoamyl alcohol was added and mixed properly.

3) Sodium acetate (3M)

40.8g of sodium acetate put in 100ml distilled water and mixed well. The mixture was stored in refrigerator at 9° C.

4) Ethanol (70%)

To seventy parts of absolute ethanol thirty parts of distilled water was added and mixed well. 70% alcohol stored at 9° C.

ANNEXURE III

Composition of buffers and dyes used for gel electrophoresis

1. TAE buffer (50X)

Tris base	: 242g
Galcial aceitic acid	: 57.1ml
0.5M 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 10mg/ml; working concentration 0.5ug/ml (Genie)) and was stored at room temperature in a dark bottle.

4. Agarose gel

Gels with two different compositions were made: 1.5 per cent for Genomic DNA and 2 per cent for PCR samples.

ANNEXURE IV

List of laboratory equipment used for the study

SI NO.	Equipment	Stage used	Company
1	Accublock digital dry bath	DNA isolation	Labnet, international Inc.
2	Vortexer	DNA isolation	GeNei ™
3	High speed refrigerated centrifuge	DNA isolation	Kubota 6500
4	Nanodrop® spectrophotometer ND- 1000	Qualitative assessment of nucleic acids	GeNei
5	Laminar Air Flow Cabinet	Preparation of PCR reaction mixture	Rotek, B&C
4	Proflex PCR system	Polymerase chain reaction	Applied Biosystems
5	Sure Cycler 8800	Gradient PCR	Agilent technologies
6	Electrophoresis unit	Agarose Gel Electrophoresis (AGE)	GeNei
6	Gel Doc ™ XR+	Gel documentation	BIO-RAD
7	Ultra low temperature freezer	Storage of DNA samples	Haier BIO-MEDICAL
8	Phase contrast microscope	Morphological identificaton	Leica DM 500

Annexure V

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

>Tapioca Olavakkod forward *COI* locus

GGGCGTTGGGTACCTGTAGATTTATGTTTTCACGAATTAATAATATAAGATTTTGACTATTAATTC
CTTCTCTTATCTTAATAATTAGTTCTTCCATAAAAAGAGTTATAAATGGAGTTGGATGAACAATAT
ATCCTCCATTAACTTCAATTCAATATTTTATATCTTCTTCTATTGAAATAATAATTTTTTCTTTACAT
GTTGCAGGAATTTCATCAATTGCTAGATCTATTAATTTTATTTCAACTATTTTATTAATAAAAAAT
AAAAACTATTATTTAAGTAATTTAACATTGTTTTCTTTATCAATTTTAATTACTACTTTATTACTTT
TATTAGCTTTACCTGTATTAGCAGGTGCTATTACAATAATTTTAATAGATCGAAACTTTAATACAT
CATTTTTTGATCCTAGAGGAGGAGGAGACCCAATTTTATATCAACATTTATTCTGATTTTTTGGGC
ATCCAGAAGTTTATATTTTAATTTTACCAGGTTTTGGAATGATTTCACACATTATTAGATATAATTT
AGGTAAAAAAGAAGTTTTTGGTAAAATTGGAATAATATTTGCTATAATATCGATTGGTTTATTAG
GTTTTATTGTATGAGCTCACCACATATTTACAGTAGGAATAGATGTTGATACACGAGCTTACTTTA
CTGCTGCTACAATAATTATTGCTATTCCTACTGGAATTAAAATTTTTAGTTGATTTACTACAATTTT
AAATTCACATATTAATTTTAATATTTCTATATATTGATCTATAGGATTTTTAATTATATTTTCTATT
GGAGGATTTACAGGAATTGTAGCTTCAAATTCATGTTTGGATATTATTTACATGACTCATATTATA
TTGTAGCTCATTTTCATAACGTTTTATCAAGA

>Tapioca Olavakkod reverse *COI* locus

TAAAAAATATAATTAAAAATCCTATAGATCAATATATAGAAATATTAAAATTAATATGTGAATTT
AAAATTGTAATAAATCAACTAAAAATTTTAATTCCAGTAGGAATAGCAATATTTATTGTAGCAGC
AGTAAAGTAAGCTCGTGTATCAACATCTATTCCTACTGTAAATATGGGGGGAGCTCATACAATAA
AACCTAATAAACCAATCGATATTATAGCAAATATTATTCCAATTTTACCAAAAACTTCTTTTTTAC
CTAAATTATATCTAATAATGTGTGAAATCATTCCAAAACCTGGTAAAATTAAAATATAAACTTCT
GGATGCCCAAAAAATCAAAATAAATGTTGATATAAAATTGGGTCCCCTCCTCCTCTAGGATCAAA
AAATGATGTATTAAAGTTTCAATCTATTAAAATTATTGTAATACCACCGGCTAATACAGGTAAAG
CTAATAAAAGTAATAAAGTAGTAATTAAAATTGATAAAGAAAACAAGGTTAAATTACTTAAATA
ATATTTTTTATTTTTTATTAATAAAATATTTGAAATAAAATTAATAGATCTAGCATTTGATGAATTT
CCTGCAACATGTAAAGAAAATATTATTATTTCAATAAAAAAAAATATAAAATATTGAATTGAA

>Tapioca Chuvannamannu forward *COI* locus

GCAGATTTATGTTTTCCTCGTATTAACAATATAAGATTTTGGCTTTTAATTCCCTCTATTATTCTTA
TAGTTAGAGCTTCAATAAAAAGAGTAATAAATGGGGTAGGATGAACAATATATCCTCCTTTAACT
TCAATTCAATTTTATATATCTTCATCCATTGAAATAATAATTTTTTCTCTTCATATTGCAGGGTTAT
CATCTATTGCTAGTTCTATTAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAATTATATTTT
TAGAAGTTTAACTTTATTTACTTTATCAATTTTAATTACTACCTTTTTACTTCTACTAGCTTTACCA
GTATTAGCAGGTGCTATTACAATAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCT
AGAGGAGGGGGAGATCCTATTTTGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTAT
ATTTTAATCTTACCAGGTTTTGGAATAGTTTCACATATTATTAGATATAATTTAGGAAAAAAAGAA
GTGTTTGGAAAAGTTGGAATATTATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGA
GCTCATCATATATTTACAGTAGGAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATA
ATTATTGCAATTCCTACTGGAATTAAAATTTTTAGTTGATTTACAACTATTATTAATTCACATATTA
ATTTTAATACATCAATATACTGATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAG
GAATTGTGGCATCTAACTCTTGTTTAGATATTAACCTTCATGATACTTATTA

>Tapioca Chuvannamannu reverse *COI* locus

TCCTGTAAATCCTCCAATTGAAAACATAATTAAAAATCCCATAGATCAGTATATTGATGTATTAA
AATTAATATGTGAATTAATAATAGTTGTAAATCAACTAAAATTTTTATTTCCAGTAGGAATTGCAA
TAATTATAGTAGCAGCTGTAAAATAAGCTCGAGTATCTACATCTATTCCTACTGTAAATATATGAT
GAGCTCATACAATAAATCCTAATAATCCATTTGATATTATAGCAAATAATATTCCAACTTTTCCAA
ACACTTCTTTTTTTCCTAAATTATATCTAATAATATGTGAAACTATTCCAAAACCGGGAAAAATTA
AAATATAACCCTCGGGATGACCAAAAAATCAAAATAAATGTTGATACAAAATAGGATCCCCCCCT
CCTCTAGGATCAAAAAAAGACGTGTTAAAATTTCAATCTATTAAAACTATTGTAATAGCCCCTGC
TAATACTGGTAAAGCTAGTAAAAGTAAAAGGGTAGTAATTAAAATTGATAAAGTAAATAAAGTT
AAACTTCTAAAAATATATTTTTTATTTTTTATTAATAAAATAGTTGAAATAAAATTAATAAAACTA
GCAATAGATGATACCCCTGCAATATGAAAAAAAAAAATTATTATTTCAAGGGATGAAGATATATA
AAATTGATTTGAATTTAAGGGAGGATATATTGTTCATCCTACCCCATTAATTACTCTTTTTATGGA
AGCTCTAACTATAAAAATAATAGAGG

>Tapioca Manjakunnu forward *COI* locus

AATTAATAATATAAGATTTTGACTATTAATTCCTTCTCTTATCTTAATAATTAGTTCTTCCATAAAA
AGAGTTATAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATATCT
TCTTCTATTGAAATAATAATTTTTTCTTTACATGTTGCAGGAATTTCATCAATTGCTAGATCTATTA
ATTTTATTTCAACTATTTTATTAATAAAAAATAAAAACTATTATTTAAGTAATTTAACATTGTTTTC
TTTATCAATTTTAATTACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCAGGTGCTATTACA
ATAATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAGACCCAAT
TTTATATCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACCAGGTTTT
GGAATGATTTCACACATTATTAGATATAATTTAGGTAAAAAAGAAGTTTTTGGTAAAATTGGAAT
AATATTTGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATTTACAGT
AGGAATAGATGTTGATACACGAGCTTACTTTACTGCTGCTACAATAATTATTGCTATTCCTACTGG
AATTAAAATTTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATAT
TGATCTATAGGATTTTTAATTATATTTTCTATTGTAGGATTTACAGGAATTGTAGCTTCAAATTCAT
GTTTGGATATTAATTTACATGACTCATATTATATTGTAGCTCATTTTCATAC

>Tapioca Manjakunnu reverse *COI* locus

TCCTCCAATAGAAAATATAATTAAAAATCCTATAGATCAATATATAGAAATATTAAAATTAATAT
GTGAATTTAAAATTGTAGTAAATCAACTAAAAATTTTAATTCCAGTAGGAATAGCAATAATTATT
GTAGCAGCAGTAAAGTAAGCTCGTGTATCAACATCTATTCCTACTGTAAATATGGGGTGAGCTCA
TACAATAAAACCTAATAAACCAATCGATATTATAGCAAATATTATTCCATTTTTACCAAAAACTTC
TTTTTTACCTAAATTATATCAAATAATGTGTGAAATCATTCCAAAACCGGGAAAAATTAAAATAT
AAACTTCGGGATGCCCAAAAAATCAAAATAAGTGTTGATATAAAATTGGGTCCCCCCCTCCTCTA
GGATCAAAAAATGATGTATTAAAGTTTCAATCTATTAAAATTATTGAAATAGCACCTGCTAATAC
GGGTAAAGCTAATAAAAGTAATAAAGTAGTATTTAAATTTGATAAAGAAAACAGTGTTAAATTAC
TTAAATAATATTTTTTATTTTTTATTAAAAAAATAGTTGAAATAAAATTAATAGATCTAGCATTTG
ATGAATTTCCTGCAACATGTAAAGAAAAAATTATTATTTCAATAGAAGAA

>Cowpea Vellanikkara forward *COI* locus

CGAATTAATAATATAAGATTTTGACTATTAATTCCTTCTCTTATCTTAATAATTAGTTCTTCCATAA
AAAGAGTTATAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATAT
CTTCTTCTATTGAAATAATAATTTTTTCTTTACATGTTGCAGGAATTTCATCAATTGCTAGATCTAT
TAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAACTATTATTTAAGTAATTTAACATTGTTT
TCTTTATCAATTTTAATTACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCAGGTGCTATTA
CAATAATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAGACCCA
ATTTTATATCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACCAGGTT
TTGGAATGATTTCACACATTATTAGATATAATTTAGGTAAAAAAGAAGTTTTTGGTAAAATTGGA

ATAATATTTGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATTTACA
GTAGGAATAGATGTTGATACACGAGCTTACTTTACTGCTGCTACAATAATTATTGCTATTCCTACT
GGAATTAAAATTTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATAT
ATTGATCTATAGGATTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCAAATT
CATGTTTGGATATTAATTTACATGACTCATATTATATTGTAGCTCAT

>Cowpea Vellanikkara reverse *COI* locus

TTTGAAGCTACAATTCCTGTAAATCCTCCAATAAAAAATATAATTAAAAATCCTATAGATCAATA
TATAGAAATATTAAAATTAATATGTGAATTTAAAATTGTAGTAAATCAACTAAAATTTTTATTTCC
AGTAGGAATAGCAATAATTATTGTAGCAGCAGTAAAGTAAGCTCGTGTATCAACATCTATTCCTA
CTGTAAATATGTGGTGAGCTCATACAATAAAACCTAATAAACCAATCGATATTATAGCAAATATT
ATTCCATTTTTACCAAAAACTTCTTTTTTACCTAATTTATATCTAATAATGTGTGAAATCATTCCAA
AACCTGGAAAAATTAAAATATAAACTTCGGGATGCCCAAAAAATCAAAATAAATGTTGATATAA
AATTGGGTCTCCTCCTCCTCTAGGATCAAAAAATGATGTATTAAAGTTTCAATCTATTAAAATTAT
TGAAATAGCACCTGCTAATACAGGTAAAGCTAATAAAAGTAATAAAGTAGTATTTAAAATTGATA
AAAAAAACAATGTTAAATTACTTAAATAATATTTTTTATTTTTTATTAATAAAATAGTTGAAATAA
AATTAATAGATCTAGCATTTGATGAAATTCCTGCAACATGTAAAGAAAAAATTATTATTTCAATA
GAAGAAGATATAAAATATTGAATTGAAGTTAGTGGAGGATATATTGTTCATCCAACTCCATTATA
TATCTCTTTTTATGGAAGATCTATTTATTAAGATAAGAGAAGGATTTAATAGTCAAA

>Banana Potta forward *COI* locus

TTTATGTTTTCCTCGTATTATAATATAAGATTTTGACTATTTAATTCCCTCTCTTATTCTTATAGTTA
GAGCTTCAATAAAAAGAGTAATAAATGGGGTAGGATGAACAATATATCCTCCTTTAACTTCAATT
CAATTTTTTATATCTTCATCCATTGAAATAATAATTTTTTCTCTTCATGTTGCAGGGTTATCATCTA
TTGCTAGTTCTATTAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAATTATATTTTAAGAA
GTTTAACTTTATTTACTTTATCAATTTTAATTACTACCTTTTTACTTCTACTAGCTTTACCAGTATTA
GCAGGTGCTATTACAATAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCTAGAGGA
GGGGGAGACCCTGTTTTGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTATATTTTAA
TTTTACCAGGTTTTGGAATGATTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGTTTG
GAAAAGTTGGAATATTATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGAGCTCACC
ATATATTTACAGTAGGAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTATTG
CAATTCCTACTGGAATTAAAATTTTTAGTTGATTTACAACTATTATTAATTCACATATTAATTTTAA
TACTTCAATATACTGATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAGGAATTGT
GGC

>Banana Potta reverse *COI* locus

GCCACAATTCCTGTAAATCCTCCAATTGAAAACATAATTAAAAATCCCATAGATCAGTATATTGA
TGTATTAAAATTAATATGTGAATTAATAATAGTTGTAAATCAACTAAAAATTTTAATTCCAGTAGG
AATTGCAATAATTATAGTAGCAGCTGTAAAATAAGCTCGAGTATCTACATCTATTCCTACTGTAA
ATATATGATGAGCTCATACAATAAATCCTAATAATCCAATTGATATTATAGCAAATAATATTCCA
ACTTTTCCAAACACTTCTTTTTTTCCTAAATTATATCTAATAATATGTGAAACTATTCCAAAACCTG
GTAAGATTAAAATATAAACCTCTGGATGACCAAAAAATCAAAATAAATGTTGATACAAAATTGG
ATCTCCCCCTCCTCTAGGATCAAAAAAAGACGTGTTAAAATTTCGATCTATTAAAACTATTGTAAT
AGCACCTGCTAATACTGGTAAAGCTAGTAGAAGTAAAAAGGTAGTAATTAAAATTGATAAAGTA
AATAAAGTTAAACTTCTAAAATAATAATTTTTATTTTTTATTAATAAAATAGTTGAAATAAAATTA
ATAGAACTAGCAATAGATGATATCCCTGCAATATGAAGAGAAAAAATTATTATTTCAATGGATGA
AGATATATAATATTGAATTGAAGTTAAAGGAGGATATATTGTTCATCCTACCCCATTTATTACTCT
TTTTATTGAAGCTCTAACTATAAGAATAATAGAGGGAATTAA

>Banana Kodali forward *COI* locus

CGAATTAATAATATAAGATTTTGACTATTAATTCCTTCTCTTATCTTAATAATTAGTTCTTCCATAA
AAAGAGTTATAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATAT
CTTCTTCTATTGAAATAATAATTTTTTCTTTACATGTTGCAGGAATTTCATCAATTGCTAGATCTAT
TAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAACTATTATTTAAGTAATTTAACATTGTTT
TCTTTATCAATTTTAATTACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCAGGTGCTATTA
CAATAATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAGACCCA
ATTTTATATCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACCAGGTT
TTGGAATGATTTCACACATTATTAGATATAATTTAGGTAAAAAAGAAGTTTTTGGTAAAATTGGA
ATAATATTTGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATTTACA
GTAGGAATAGATGTTGATACACGAGCTTACTTTACTGCTGCTACAATAATTATTGCTATTCCTACT
GGAATTAAAATTTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATAT
ATTGATCTATAGGATTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCAAATT
CATGTTTGGATATTAATTTACATGACTCATATTATAT

>Banana Kodali reverse *COI* locus

TTGAAGCTACAATTCCTGTAAATCCTCCAATAGAAAATATAATTAAAAATCCTATAGATCAATAT
ATAAAAATATTAAAATTAATATGTGAATTTAAAATTGTAGTAAATCAACTAAAATTTTTATTTCCA
GTAGGAATAGCAATATTTATTGTAGCAGCAGTAAAGTAAGCTCGTGTATCAACATCTATTCCTAC
TGTAAATATGTGGTGAGCTCATACAATAAAACCTAATAAACCAATCGATATTATAGCAAATATTA
TTCCATTTTTACCAAAAACTTCTTTTTTACCTAAATTATATCTAATAATGTGTGAAATCATTCCAAA
ACCTGGAAAAATTAAAATATAAACTTCTGGATGCCCAAAAAATCAAAATAAATGTTGATATAAA
ATTGGGTCCCCCCCTCCTCTAGGATCAAAAAATGATGTATTAAAGTTTCAATCTATTAAAATTATT
GTAATACCACCTGCTAATACAGGTAAAGCTAATAAAAGTAATAAAGTAGTAATTAAAATTGATAA
AAAAAACAATGTTAAATTACTTAAATAATATTTTTTATTTTTTATTAATAAAATAGTTGAAATAAA
ATTAATAAATCTAGCATTTGATGAATTTCCTGCAACATGTAAAGAAAAAATTATTATTTCAATAG
AAGAAGATATAAAATATTGAATTGAAGTTA

>Pumpkin Manjakuunnu forward *COI* locus

CACGAATTAATAATATAAGATTTTGACTATTAATTCCTTCTCTTATCTTAATAATTAGTTCTTCCAT
AAAAAGAGTTATAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTAT
ATCTTCTTCTATTGAAATAATAATTTTTTCTTTACATGTTGCAGGAATTTCATCAATTGCTAGATCT
ATTAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAACTATTATTTAAGTAATTTAACATTG
TTTTCTTTATCAATTTTAATTACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCAGGTGCTAT
TACAATAATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAGACC
CAATTTTATATCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACCAGG
TTTTGGAATGATTTCACACATTATTAGATATAATTTAGGTAAAAAAGAAGTTTTTGGTAAAATTGG
AATAATATTTGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATTTAC
AGTAGGAATA

>Pumpkin Manjakunnu reverse *COI* locus

AACATGAATTTGAAGCTACAATTCCTGTAAATCCTCCAATAGAAAATATAATTAAAAATCCTATA
GATCAATATATAGAAATATTAAAATTAATATGTGAATTTAAAATTGTAGTAAATCAACTAAAAAT
TTTAATTCCAGTAGGAATAGCAATAATTATTGTAGCAGCAGTAAAGTAAGCTCGTGTATCAACAT
CTATTCCTACTGTAAATATGTGGTGAGCTCATACAATAAAACCTAATAAACCAATCGATATTATA
GCAAATATTATTCCAATTTTACCAAAAACTTCTTTTTTACCTAAATTATATCTAATAATGTGTGAA
ATCATTCCAAAACCTGGTAAAATTAAAATATAAACTTCTGGATGCCCAAAAAATCAGAATAAATG
TTGATATAAAATTGGGTCTCCTCCTCCTCTAGGATCAAAAAATGATGTATTAAAGTTTCGATCTAT
TAAAATTATTGTAATAGC

>Okra Ernakulam forward *COI* locus

GATTTATGTTTTCCTCGTATTAACAATATAAGATTTTGGCTTTTAATTCCCTCTATTATTCTTATAG
TTAGAGCTTCAATAAAAAGAGTAATAAATGGGGTAGGATGAACAATATATCCTCCTTTAACTTCA
ATTCAATTTTATATATCTTCATCCATTGAAATAATAATTTTTTCTCTTCATATTGCAGGGTTATCAT
CTATTGCTAGTTCTATTAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAATTATATTTTTAG
AAGTTTAACTTTATTTACTTTATCAATTTTAATTACTACCTTTTTACTTCTACTAGCTTTACCAGTAT
TAGCAGGTGCTATTACAATAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCTAGAG
GAGGGGGAGATCCTATTTTGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTATATTTT
AATCTTACCAGGTTTTGGAATAGTTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGT
TTGGAAAAGTTGGAATATTATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGAGCTC
ATCATATATTTACAGTAGGAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTA
TTGCAATTCCTACTGGAATTAAAATTTTTAGTTGATTTACAACTATTATTAATTCACATATTAATTT
TAATACATCAATATACTGATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAGGAAT
TGTGGCATCTAACTCTTGTTTAGATATTAACCTTCATGATACTTATTA

>Okra Ernakulam reverse *COI* locus

ATCTAAACAAGAGTTAGATGCCACAATTCCTGTAAATCCTCCAATTGAAAACATAATTAAAAATC
CCATAGATCAGTATATTGATGTATTAAAATTAATATGTGAATTAATAATAGTTGTAATCAACTAA
AAATTTTAATTCCAGTAGGAATTGCAATAATTATAGTAGCAGCTGTAAAATAAGCTCGAGTATCT
ACATCTATTCCTACTGTAAATATATGATGAGCTCATACAATAAATCCTAATAATCCAATTGATATT
ATAGCAAATAATATTCCAACTTTTCCAAACACTTCTTTTTTTCCTAAATTATATCTAATAATATGTG
AAACTATTCCAAAACCTGGTAAGATTAAAATATAAACCTCTGGATGACCAAAAAATCAAAATAA
ATGTTGATACAAAATAGGATCTCCCCCTCCTCTAGGATCAAAAAAAGACGTGTTAAAATTTCGAT
CTATTAAAACTATTGTAATAGCACCTGCTAATACTGGTAAAGCTAGTAGAAGTAAAAAGGTAGTA
ATTAAAATTGATAAAGTAAATAAAGTTAAACTTCTAAAAATATAATTTTTATTTTTTATTAATAAA
ATAGTTGAAATAAAATTAATAGAACTAGCAATAGATGATAACCCTGCAATATGAAGAGAAAAAA
TTATTATTTCAATGGATGAAGATATATAAAATTGAATTGAAGTTAAAGGAGGATATATTGTTCAT
CCTACCCCATTTATTACTCTTTTTATTGAAGCTCTAACTATAAGAATAATAGAGGGAATTAAAAGC
CAAAATCTTATATTGTTAATACGAGGAAAACATAAATCTGCAGTATTAATTATAAATG

>Tapioca Kakkani forward *COI* locus

ATTTATGTTTTCCTCGTATTAACAATATAAGATTTTGGCTTTTAATTCCCTCTATTATTCTTATAGTT
AGAGCTTCAATAAAAAGAGTAATAAATGGGGTAGGATGAACAATATATCCTCCTTTAACTTCAAT
TCAATTTTATATATCTTCATCCATTGAAATAATAATTTTTTCTCTTCATATTGCAGGGTTATCATCT
ATTGCTAGTTCTATTAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAATTATATTTTTAGAA
GTTTAACTTTATTTACTTTATCAATTTTAATTACTACCTTTTTACTTCTACTAGCTTTACCAGTATTA
GCAGGTGCTATTACAATAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCTAGAGGA
GGGGGAGATCCTATTTTGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTATATTTTAA
TCTTACCAGGTTTTGGAATAGTTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGTTTG
GAAAAGTTGGAATATTATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGAGCTCATC
ATATATTTACAGTAGGAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTATTG
CAATTCCTACTGGAATTAAAATTTTTAGTTGATTTACAACTATTATTAATTCACATATTAATTTTAA
TACATCAATATACTGATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAGGAATTGT
GGCATCTAACTCTTGTTTAGATATTAACCTTCATGATACTTATTATATTG

>Tapioca Kakkani *COI* locus

CAAGAGTTAGATGCCACAATTCCTGTAAATCCTCCAATTGAAAACATAATTAAAAATCCCATAGA
TCAGTATATTGATGTATTAAAATTAATATGTGAATTAATAATAGTTGTAAATCAACTAAAAATTTT
AATTCCAGTAGGAATTGCAATAATTATAGTAGCAGCTGTAAAATAAGCTCGAGTATCTACATCTA
TTCCTACTGTAAATATATGATGAGCTCATACAATAAATCCTAATAATCCAATTGATATTATAGCAA
ATAATATTCCAACTTTTCCAAACACTTCTTTTTTTCCTAAATTATATCTAATAATATGTGAAACTAT
TCCAAAACCTGGTAAGATTAAAATATAAACCTCTGGATGACCAAAAAATCAAAATAAATGTTGAT
ACAAAATAGGATCTCCCCCTCCTCTAGGATCAAAAAAAGACGTGTTAAAATTTCGATCTATTAAA
ACTATTGTAATAGCACCTGCTAATACTGGTAAAGCTAGTAGAAGTAAAAAGGTAGTAATTAAAAT
TGATAAAGTAAATAAAGTTAAACTTCTAAAAATATAATTTTTATTTTTTATTAATAAAATAGTTGA
AATAAAATTAATAGAACTAGCAATAGATGATAACCCTGCAATATGAAGAGAAAAAATTATTATTT
CAATGGATGAAGATATATAAAATTGAATTGAAGTTAAAGGAGGATATATTGTTCATCCTACCCCA
TTTATTACTCTTTTTATTGAAGCTCTAACTATAAGAATAATAGAGGGAATTAAAAGCCAAAATCTT
ATATTGTTAATACGAGGAAAACATAAATCTGCAGTATTAATTATAA

>Papaya Olavakkod forward *ITS2* locus

CATTACGTAACTGGATCAAAATATTGATTAAGTTGATTGTATATTGGTGTTGTTTGCAAGCAAATC
AACGTAAATCTACTTATGTGTTTACACAAATTTTGGTATCAGCAATAGTCCTTAGCTCTGCTTCAA
ACAGAATGAGATAGTTACCATTTTGTATGTAAATGTATTCATACAAGTGCATTGAGAAATCTCAA
TAGAGAAGGTTAGCTGATCACCATAGTTATCTGCAATACGACTTTAGCGTCGTCAGATAGGCGAC
TAACCTTAGAATCTCATGCTAGTATCTATTCATATATACTGTTTACAGAGATGAAATATGCATTGA
ACACAAAACTTATTATGTGAAACAAGTAGATTTGAGATTCTCCTTACTAAACTGCACAGAAAATG
TGCAATTAAGAAAAGGAATAAAACATCAAGATACACGTATACACGCGAATCTCAAAACAATGCA
TATATGGTAGTATACAAGTGTTTTGTTGATTATTTTACATCTCAACTCTCAGACAGATGTGACCTC
GGAAAAGACCCGAAAGCCGCAATGTGCGTTAGAAGCTCAAGTGCTCGGCGTGTCCTGCAATTCGC
ACCGATTATCGTAGCTAGCTGCGTTCTTCATCGACCA

>Papaya Olavakkod reverse ITS2 locus

GAACCTTATCGGGTGCGATTGCAGGAACGCCGAGCACTTGAGCTTCTAACGCACATTGCGGCTTT
CGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGT
ATACTACCATATATGCATTGTTTTGAGATTCGCGTGTATACGTGTATCTTGATGTTTTATTCCTTTT
CTTAATTGCACATTTTCTGTGCAGTTTAGTAAGGAGAATCTCAAATCTACTTGTTTCACATAATAA
GTTTTGTGTTCAATGCATATTTCATCTCTGTAAACAGTATATATGAATAGATACTAGCATGAGATT
CTAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAGCTAA
CCTTCTCTATTGAGATTTCTCAATGCACTTGTATGAATACATTTACATACAAAATGGTAACTATCT
CATTCTGTTTGAAGCAGAGCTAAGGACTATTGCTGATACCAAAATTTGTGTAAACACATAAGTAG
ATTTACGTTGATTTGCTTGCAAACAACACCAATATACAATCAACTTAATCAATATTTTTGATCTCA
GATCAGGTGAGGTTACCCGCTGAATTTAAAGCATATA

>Cowpea Vellayani forward *ITS2* locus

CCATTAATGAATGAACAAAATATTGATTTAGTTGATTGTTGATATGTGGTTATTGTCTGTTGTTTA
TATTTGTGTGTGGTTAGGAACAACAAAGGAACAACATAAAATCTACTTTTTTATGTTATGTGTGTT
TTTGTGTGTCTCTCTCACACACAACTTTTTTTATCTGCGCTCAACACAAAGTGATATAATAATATA
CCCTTTTTTGTGTGTACACAAATTTTATCTCATACATGCGCACAAAATTCTCATATATACACGGGG
ATTTATATGTGCACCATATTTCTGTGTAACAACTTTTTCGCCTCCACATATACGACAAAACTTATA
AACTCTCGCGATAATCTATAATATAATCTGTGTTACACAGAGATAAAAAATATGTTTAAAACACA
ATCTCAATATCGTGAAAACACATATATATTAGATACTCCCCTCTAAAATTGTGCAATTGTGCACAA
AGTACACTTTTAAAAAGGAGAATAACATTATAAAACTCGTGTGTGTGAGAACATTTTTCTCACAA
CACTGCGTGTGTCTAAAAAAAATATTACGTTTTTACAAGAGTGTTGTGGTTAATATTACATCTCAC
ATCTCACACACATATGAGATCTCAAAAGACACAAAAACCCACAGTGCGTTTTAAACTCACGTGTG
CTCGCGTGTCGTGATTCTCACAGATAATAGCATATATATGTGCTCTTCACATAGACACA

>Cowpea Vellayani reverse *ITS2* locus

GCGAGCGATTCGGGTGCGATTGCAGGACACGCCGAGCACTTGAGCTTCCAACGCACATTGCGGCT
TTCGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTT
GTAAAAACTTGTAATATTACTTATAGACATGCAGTGTTTTGAGAATAATGTATTCATACATACATG
ATTCTTAATGTTTTATTCCTTTTCTTAATTGTACTTTTTGTTCAATTGTACAACTTTAGTGAGGAGA
ATCTCAAATCTACTTGTGTTCACATGATATTGATTTTGTGTTCAAAGCATATTTCATCTCTCTGTAA
AACAGATACTATAATAGATACTAGCATGAGATTCTAAGGTTAGTCGCCTATCTGACGACGCTAAA
GTCGTATTACAGATAACTATGGTACCTATAACTAACCTTGCTTAATTAAGAATTTTTTGTGCACTT
GTATGATTTAATTTTTGTAAACATACAAAATGGTAACTACTATTATTTCATTCTGTTTAGAGCAGA
GCTAAAAAGTATTGCTTGTGAATGAATCAATCAATAACACATATAATATAAAAAAATATATTTTG
TGGTGCTTCTTTGGTGCCCATACCCACACACATATATACACCACACACTATCCACATATATCCACT
CTCCTATAACTCAATATTTTAGCTCACATCACGTGAGAGGATACCCGCAAATTTTTAAAAAAAAA

> Ashgourd Vellanikkara forward *ITS2* locus

CCCCTTAAGTTATGAGACAAAATATTGATTAGTTGATTGTATATTGGTGTTGTTTGCAAGCAAATC
AACGTAAATCTACTTATGTGTTTACACAAATTTTGGTATCAGCAATAGTCCTTAGCTCTGCTTCAA
ACAGAATGAGATAGTTACCATTTTGTATGTAAATGTATTCATACAAGTGCATTGAGAAATCTCAA
TAGAGAAGGTTAGCTGATCACCATAGTTATCTGCAATACGACTTTAGCGTCGTCAGATAGGCGAC
TAACCTTAGAATCTCATGCTAGTATCTATTCATATATACTGTTTACAGAGATGAAATATGCATTGA
ACACAAAACTTATTATGTGAAACAAGTAGATTTGAGATTCTCCTTACTAAACTGCACAGAAAATG
TGCAATTAAGAAAAGGAATAAAACATCAAGATACACGTATACACGCGAATCTCAAAACAATGCA
TATATGGTAGTATACAAGTGTTTTGTTGATTATTTTACATCTCAACTCTCAGACAGATGTGACCTC
GGAAAAGACCCGAAAGCCGCAATGTGCGTTAGAAGCTCAAGTGCTCGGCGTGTCCTGCAATTCGC
ACCGATTATCGTAGCTAGCTGCGTTCTTCATCGACCCA

>Ashgourd reverse *ITS2* locus

TCTTCCTATTCGGGGCGATGCAGGAACGCCGAGCACTTGAGCTTCTAACGCACATTGCGGCTTTC
GGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGTA
TACTACCATATATGCATTGTTTTGAGATTCGCGTGTATACGTGTATCTTGATGTTTTATTCCTTTTC
TTAATTGCACATTTTCTGTGCAGTTTAGTAAGGAGAATCTCAAATCTACTTGTTTCACATAATAAG
TTTTGTGTTCAATGCATATTTCATCTCTGTAAACAGTATATATGAATAGATACTAGCATGAGATTC
TAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAGCTAAC
CTTCTCTATTGAGATTTCTCAATGCACTTGTATGAATACATTTACATACAAAATGGTAACTATCTC
ATTCTGTTTGAAGCAGAGCTAAGGACTATTGCTGATACCAAAATTTGTGTAAACACATAAGTAGA
TTTACGTTGATTTGCTTGCAAACAACACCAATATACAATCAACTTAATCAATATTTTTGATCTCAG
ATCAGGTGAGGTTACCCGCTGAATTTAAGCATATAA

> Brinjal Tavannur forward *ITS2* locus

CCCATCCATAATCTGAATCAAATATTGATTAAGTTGATTGTATATTGGTGTTGTTTGCAAGCAAAT
CAACGTAAATCTACTTATGTGTTTACACAAATTTTGGTATCAGCAATAGTCCTTAGCTCTGCTTCA
AACAGAATGAGATAGTTACCATTTTGTATGTAAATGTATTCATACAAGTGCATTGAGAAATCTCA
ATAGAGAAGGTTAGCTGATCACCATAGTTATCTGCAATACGACTTTAGCGTCGTCAGATAGGCGA
CTAACCTTAGAATCTCATGCTAGTATCTATTCATATATACTGTTTACAAAGATGAAATATGCATTG
AACACAAAACTTATTATGTGAAACAAGTAGATTTGAGATTCTCCTTACTAAACTGCACAGAAAAT
GTGCAATTAAGAAAAGGAATAAAACATCAAGATACACGTATACACGCGAATCTCAAAACAATGC
ATATATGGTAGTATACAAGTGTTTTGTTGATTATTTTACATCTCAACTCTCAGACAGATGTGACCT
CGGAAAAGACCCGAAAGCCGCAATGTGCGTTAGAAGCTCAAGTGCTCGGCGTGTCCTGCAATTCG
CACCGATTATCGTAGCTAGCTGCGTTCTTCATCGACACCAG

>Brinjal Tavannur reverse *ITS2* locus

GAACATTTCGGTGCGATTGCAGGACACGCCGAGCACTTGAGCTTCTAACGCACATTGCGGCTTTC
GGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGTA
TACTACCATATATGCATTGTTTTGAGATTCGCGTGTATACGTGTATCTTGATGTTTTATTCCTTTTC
TTAATTGCACATTTTCTGTGCAGTTTAGTAAGGAGAATCTCAAATCTACTTGTTTCACATAATAAG

TTTTGTGTTCAATGCATATTTCATCTCTGTAAACAGTATATATGAATAGATACTAGCATGAGATTC
TAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAGCTAAC
CTTCTCTATTGAGATTTCTCAATGCACTTGTATGAATACATTTACATACAAAATGGTAACTATCTC
ATTCTGTTTGAAGCAGAGCTAAGGACTATTGCTGATACCAAAATTTGTGTAAACACATAAGTAGA
TTTACGTTGATTTGCTTGCAAACAACACCAATATACAATCAACTTAATCAATATTTTTGATCTCAG
ATCA

>Papaya Kannara forward *ITS2* locus

CCCTAAGAACTGGACAAATATTGATTAAGTTGATTGTATATTGGTGTTGTTTGCAAGCAAATCAA
CGTAAATCTACTTATGTGTTTACACAAATTTTGGTATCAGCAATAGTCCTTAGCTCTGCTTCAAAC
AGAATGAGATAGTTACCATTTTGTATGTAAATGTATTCATACAAGTGCATTGAGAAATCTCAATA
GAGAAGGTTAGCTGATCACCATAGTTATCTGCAATACGACTTTAGCGTCGTCAGATAGGCGACTA
ACCTTAGAATCTCATGCTAGTATCTATTCATATATACTGTTTACAGAGATGAAATATGCATTGAAC
ACAAAACTTATTATGTGAAACAAGTAGATTTGAGATTCTCCTTACTAAACTGCACAGAAAATGTG
CAATTAAGAAAAGGAATAAAACATCAAGATACACGTATACACGCGAATCTCAAAACAATGCATA
TATGGTAGTATACAAGTGTTTTGTTGATTATTTTACATCTCAACTCTCAGACAGATGTGACCTCGG
AAAAGACCCGAAAGCCGCAATGTGCGTTAGAAGCTCAAGTGCTCGGCGTGTCCTGCAATTCGCAC
CGATTATCGTAGCTAGCTGCGTTCTTCATCGACACCA

>Papaya Kannara reverse *ITS2* locus

GGGGATATCGGTGCGATGCAGGACACGCCGAGCACTTGAGCTTCTAACGCACATTGCGGCTTTCG
GGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGTAT
ACTACCATATATGCATTGTTTTGAGATTCGCGTGTATACGTGTATCTTGATGTTTTATTCCTTTTCT
TAATTGCACATTTTCTGTGCAGTTTAGTAAGGAGAATCTCAAATCTACTTGTTTCACATAATAAGT
TTTGTGTTCAATGCATATTTCATCTCTGTAAACAGTATATATGAATAGATACTAGCATGAGATTCT
AAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAGCTAACC
TTCTCTATTGAGATTTCTCAATGCACTTGTATGAATACATTTACATACAAAATGGTAACTATCTCA
TTCTGTTTGAAGCAGAGCTAAGGACTATTGCTGATACCAAAATTTGTGTAAACACATAAGTAGAT
TTACGTTGATTTGCTTGCAAACAACACCAATATACAATCAACTTAATCAATATTTTTGATCTCAGA
TCAGGTGAGGTTACCCGCTGAATTTAAAGCAAAAA

>Dahlia Idukki forward *ITS2* locus

CCCCTCATGACTGAACAAATATTGATTAGTTGATTAATGTATATTTGTGTTGTTTGCAAGCAAGCA
ACGTAAATCTACTTTAACGTTTGCACAAATTTTGCCTTTGCATTACTTCTTAGGTCTGCTTTAACAG
AATGAAATAGCTACTATTTGTATGCTTTATACAAGTGCAATAAGATTCATTATTTAGCAGGTTAGT
TGATCACCATAGTTATCTGCAATACGACTTTAGCGTCGTCAGATAGGCGACTAACCTTAGAATCTC
ATGCTAGTATCTATTCATATATACTGCTTGCAGAGATGAAATATGCATTGTACACAAAATTTATCA
TGTGAAACAAGTAGATTTGAGATTCTCCTTACTAAATTGCAACGAATTGCAATTAAGAAAAGGAA
TAAAACATCAAGATTCATGCATATAAATATGCAATCCTCTCAAAACAATGCATATATGGTAGTAT
GCAAGTGTTTTGTTGATTATTTTACATCTCAACTCTCAGACAGATGTGACCTCGGAAAAGACCCGA
AAGCCGCAATGTGCGTTGAAAGCTCAAGTGCTCGGCGTGTCCTGCAATTCGCACCGATTATCGTA
GCTAGCTGCGTTCTTCATCGACACTAAAA

>Dahlia Idukki reverse *ITS2* locus

AGCATAATCGGTGCGATGCAGGACACGCCGAGCACTTGAGCTTCCAACGCACATTGCGGCTTTCG
GGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGCAT
ACTACCATATATGCATTGTTTTGAGAGGATTGCATATTTATATGCATGAATCTTGATGTTTTATTCC
TTTTCTTAATTGCAATTCGTTGCAATTTAGTAAGGAGAATCTCAAATCTACTTGTTTCACATGATA
AATTTTGTGTACAATGCATATTTCATCTCTGCAAGCAGTATATATGAATAGATACTAGCATGAGAT
TCTAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTATTGCAGATAACTATGGTGATCAACTA
ACCTGCTAAATAATGAATCTTATTGCACTTGTATAAAGCATACAAATAGTAGCTATTTCATTCTGT
TAAAGCAGACCTAAGAAGTAATGCAAAGGCAAAATTTGTGCAAACGTTAAAGTAGATTTACGTT

GCTTGCTTGCAAACAACACAAATATACATTAATCAACTTAATCAATATTTTTGATCTCAGATCAGG
TGAGGTTACCCGCTGAATTTAAAGCATATA

>Adenium Vellanikkara forward *ITS2* locus

TCCCTAATACTGAACAAATATTGATTTAGTTGATTGTTGATATGTGGTTATTGTCTGTTGTTTATAT
TTGTGTGTGGTTAGGAACAACAAAGGAACAACATAAAATCTACTTTTTTATGTTATGTGTTATTGA
GTGTGTCTCTTTCAAGCACTACTTTTTTTCTCTGCGCTCAACACAAAGAGATATAATAATTTACCC
TTTTGTATGTTTACACAAATTATATCTCACACGTGCGCACAAAATTCTTATTTATACACGGTGATT
TATAGGTACCCTATATATCTGTGATACAACTTTTTCGCCTCCACATAGGCGACAAAACTTATAATC
TCACGCGATAATCTATAATAGAATCTGTGTTACACAGAGATAAAATATGCTTTGAAAACAAAATC
TCTATCTTGTGAACACAAATATATTTGAGATTCTCCCCTCTAAAGTTGTGCACTTGTGCACAAAGT
ACAATTATAAAAAGGAATATAACATTTTAAAACTCGTGTGTGTGAGTACATTATTCTCTCAACACT
GCGTGTGTATATGTAATATAACACGTTTTACACGTGTGTTGTTGAGTATATTACATCTCACCTCTC
ACACACATATGACATCTCAGAAGACACGAGAGCCCACTGTGCGTGGGAGCTCTCGTGCGCTCGTG
TCTGTGCTTCGCACAGATATAGCATATCTCTCGTCTCATCCGACACACA

>Adenium Vellanikkara reverse *ITS2* locus

TTGAATTATCGGGTGCGATTGCAGGAACGCCGAGCACTTGAGCTTCCAACGCACATTGCGGCTTT
CGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGAGATGTAAAATAATCAACAAAACACTTGT
AAAAACTTGTAATATTACTTATAGACATGCAGTGTTTTGAGAATAATGTATTCATACATACATGAT
TCTTAATGTTTTATTCCTTTTCTTAATTGTACTTTTTGTTCAATTGTACAACTTTAGTGAGGAGAAT
CTCAAATCTACTTGTGTTCACATGATATTGATTTTGTGTTCAAAGCATATTTCATCTCTCTGTAAAA
CAGATACTATAATAGATACTAGCATGAGATTCTAAGGTTAGTCGCCTATCTGACGACGCTAAAGT
CGTATTACAGATAACTATGGTACCTATAACTAACCTTGCTTAATTAAGAATTTTTTGTGCACTTGT
ATGATTTAATTTTTGTAAACATACAAAATGGTAACTACTATTATTTCATTCTGTTTAGAGCAGAGC
TAAAAAGTATTGCTTGTGAATGAATCAATCAATAACACATATAATATAAAAGAATATTTTATGTT
GTTCCCTTGTTGTGCCCAACCCCACACACATATAAACAACACACACTAAACCCATATCTCCACTCT
CCTATATCTCTATTTTTGAGCTCACATATCGTGAGGGGACACGCTGAAATTTTAACATATATA

Annexure VI

List of contigs formed for *ITS2* and *COI* sequences of spider mites

>Tapioca Olavakkod *Tetranychus truncatus COI*

AAATTGTAGGAATTTGGGAAATGATTAGTTCCCCTAATAGTTAACACCAGTAGATTTATGTTTTTC
ACGAAATAATAATATAAAGATTTTGACTATTAAATCCCTCTCTTATATTAATAAATAGATCTTCCA
TAAAAAGAGTTATAAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTT
ATATCTTCTTCTATTGAAATAATAATATTTTCTTTACATGTTGCAGGAAATTCATCAAATGCTAGA
TCTATTAATTTTATTTCAAATATTTTATTAATAAAAAATAAAAAATATTATTTAAGTAATTTAACA
TTGTTTTCTTTATCAATTTTAATTACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCAGGTGC
TATTACAATAATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAG
ACCCAATTTTATATCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACC
AGGTTTTGGAATGATTTCACACATTATTAGATATAATTTAGGTAAAAAAGAAGTTTTTGGTAAAA
TTGGAATAATATTTGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATT
TACAGTAGGAATAGATGTTGATACACGAGCTTACTTTACTGCTGCTACAATAAATATTGCTATTCC
TACTGGAATTAAAATTTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCT
ATATATTGATCTATAGGATTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCA
AATTCATGTTTGGATATTATTTACATGACTCATATTATATTGTAGCTCATTTTCATAACGTTTTATC
AAGA

>Tapioca Chuvannamannu *Tetranychus udaipurensis COI*

TGAGGGAGTTTGGGAAATGAATAGTTCCCCTTATAAATAATACTGCAGAGATATGTGTTCCTCGT
ATAAACAATATAAGATATTGGCTTTTAATTCCCCTCTATTATTCTTATAGTTAGAGCTTCAATAAA
AAGAGTAATAAATGGGGTAGGATGAACAATATATCCTCCCTTAAATTCAAATCAATTTTATATAT
CTTCATCCATTGAAATAATAATTTTTTCTCTTCATATTGCAGGGGTATCATCTATTGCTAGTTCTAT
TAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAAATATATTTTTAGAAGTTTAACTTTATTT
ACTTTATCAATTTTAATTACTACCCTTTTACTTCTACTAGCTTTACCAGTATTAGCAGGGGCTATTA
CAATAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCTAGAGGAGGGGGAGATCCTA
TTTTGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGGTTATATTTTAATCTTACCAGGTTT
TGGAATAGTTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGTTTGGAAAAGTTGGAA
TATTATTTGCTATAATATCAAATGGATTATTAGGATTTATTGTATGAGCTCATCATATATTTACAG
TAGGAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTATTGCAATTCCTACTG
GAAATAAAAATTTTAGTTGATTTACAACTATTATTAATTCACATATTAATTTTAATACATCAATAT
ACTGATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAGGAATTGTGGCATCTAACT
CTTGTGTAGATATTAACCTTCATGATACTTATTATATTGTAGCTCATTTTCATACAGGTTTTAAACA

>Tapioca Manjakunnu *Tetranychus truncatus COI*

TGGGGGGGTTTGGGAAATGAGTAGTTTCCCTAATAGATAACACAGTAGATATATGTTTTCACGAA
ATAATAATATAAGATTTTGACTATTAATTCCCTCTCTTATCTTAATAAATAGATCTTCCATAAAAA
GAGATATAAAAGGAGTTGGATGAACAATATATCCTCCATTAAATTCAATTCAATATTTTATATCTT
CTTCTATTGAAATAATAATTTTTTCTTTACATGTTGCAGGAAATTCATCAAATGCTAGATCTATTA
ATTTTATTTCAACTATTTTATTAATAAAAAATAAAAAATATTATTTAAGTAATTTAACACTGTTTTC
TTTATCAAATTTAAATACTACTTTATTACTTTTATTAGCTTTACCCGTATTAGCAGGTGCTATTACA
ATAATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAGACCCAAT
TTTATATCAACACTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACCAGGTTTT
GGAATGATTTCACACATTATTAGATATAATTTAGGTAAAAAAGAAGTTTTTGGTAAAAATGGAAT
AATATTTGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATTTACAGT
AGGAATAGATGTTGATACACGAGCTTACTTTACTGCTGCTACAATAATTATTGCTATTCCTACTGG
AATTAAAATTTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATAT
TGATCTATAGGATTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCAAATTCA
TGTTTGGATATTAATTTACATGACTCATATTATATTGTAGCTCATTTTCATACCTTTTTAT

>Cowpea Vellanikkara *Tetranychus truncatus COI*

TTTGAGGGATTGGGAATTGATTAGTTCCCTAATAGTTAACACTGTAGAGTTATGTTTTCACGAACT
AATAATATAAGATTTTGACTATTAAATCCTTCTCTTATCTTAATAAATAGATCTTCCATAAAAAGA
GATATATAATGGAGTTGGATGAACAATATATCCTCCACTAACTTCAATTCAATATTTTATATCTTC
TTCTATTGAAATAATAATTTTTTCTTTACATGTTGCAGGAATTTCATCAAATGCTAGATCTATTAAT
TTTATTTCAACTATTTTATTAATAAAAAATAAAAAATATTATTTAAGTAATTTAACATTGTTTTCTT
TATCAATTTTAAATACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCAGGTGCTATTACAAT
AATTTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAGACCCAATTTT
ATATCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACCAGGTTTTGGA
ATGATTTCACACATTATTAGATATAAATTAGGTAAAAAAGAAGTTTTTGGTAAAAATGGAATAAT
ATTTGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATTTACAGTAGG
AATAGATGTTGATACACGAGCTTACTTTACTGCTGCTACAATAATTATTGCTATTCCTACTGGAAA
TAAAAATTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATATTGA
TCTATAGGATTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCAAATTCATGTT
TGGATATTAATTTACATGACTCATATTATATTGTAGCTCATTTACATACCGTTTTATC

>Banana Potta *Tetranychus udaipurensis COI*

TGGAGGATTTGGGGAATTGATTAGTTCATTTATAGTTAATACTGCAGATTTATGTTTTCCTCGTAT
TAATAATATAAGATTTTGACTATTTAATTCCCTCTATTATTCTTATAGTTAGAGCTTCAATAAAAA
GAGTAATAAATGGGGTAGGATGAACAATATATCCTCCTTTAACTTCAATTCAATATTATATATCTT
CATCCATTGAAATAATAATTTTTTCTCTTCATATTGCAGGGATATCATCTATTGCTAGTTCTATTAA
TTTTATTTCAACTATTTTATTAATAAAAAATAAAAATTATAATTTAAGAAGTTTAACTTTATTTACT
TTATCAATTTTAATTACTACCTTTTTACTTCTACTAGCTTTACCAGTATTAGCAGGTGCTATTACAA
TAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCTAGAGGAGGGGGAGACCCAATTT
TGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTATATTTTAATCTTACCAGGTTTTGG
AATAATTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGTTTGGAAAAGTTGGAATAT
TATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGAGCTCACCATATATTTACAGTAG
GAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTATTGCAATTCCTACTGGAA
TTAAAATTTTTAGTTGATTTACAACTATTATTAATTCACATATTAATTTTAATACATCAATATACTG
ATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAGGAATTGTGGCATCAAACTCATG
TTTAGATATTAACCTTCATGATACTTATTATATTGTAGCTCATTTTCATAACGGTTTTATC

>Banana Kodali *Tetranychus truncatus COI*

TGGGGAATTTGGGGATTGAGTAGTTCCTTAATAGTTAACACTGTAGATTTATGTTTTTCACGAAAT
AATAATATAAGATTTGACTATTAATTCCTCTCTTATCTTAATAAATAGATCTTCCATAAAAAGAGT
TATAAATGGAGTTGGATGAACAATATATCCTCCATTAACTTCAATTCAATATTTTATATCTTCTTCT
ATTGAAATAATAATTTTTTCTTTACATGTTGCAGGAAATTCATCAAATGCTAGATCTATTAATTTT
ATTTCAACTATTTTATTAATAAAAAATAAAAAATATTATTTAAGTAATTTAACATTGTTTTCTTTAT
CAATTTTAATTACTACTTTATTACTTTTATTAGCTTTACCTGTATTAGCAGGTGCTATTACAATAAT
TTTAATAGATCGAAACTTTAATACATCATTTTTTGATCCTAGAGGAGGAGGAGACCCAATTTTATA
TCAACATTTATTCTGATTTTTTGGGCATCCAGAAGTTTATATTTTAATTTTACCAGGTTTTGGAATG
ATTTCACACATTATTAGATATAATTTAGGTAAAAAAGAAGTTTTTGGTAAAAATGGAATAATATT
TGCTATAATATCGATTGGTTTATTAGGTTTTATTGTATGAGCTCACCACATATTTACAGTAGGAAT
AGATGTTGATACACGAGCTTACTTTACTGCTGCTACAATAAATATTGCTATTCCTACTGGAAATAA
AAATTTTAGTTGATTTACTACAATTTTAAATTCACATATTAATTTTAATATTTCTATATATTGATCT
ATAGGATTTTTAATTATATTTTCTATTGGAGGATTTACAGGAATTGTAGCTTCAAATTCATGTTTG
GATATTAATTTACATGACTCATATTATATTGTAGCTCATTTACATACACGTTAATATCA

>Pumpkin Manjakunnu *Tetranychus truncatus COI*

CATTTTTTATGAGTCATGTAATTATATCCAAACATGAATTTGAAGCTACAATTCCTGTAAATCCTC
CAATAGAAAATATAATTAAAAATCCTATAGATCAATATATAGAAATATTAAAATTAATATGTGAA

TTTAAAATTGTAGTAAATCAACTAAAAATTTTAATTCCAGTAGGAATAGCAATAATTATTGTAGC
AGCAGTAAAGTAAGCTCGTGTATCAACATCTATTCCTACTGTAAATATGTGGTGAGCTCATACAA
TAAAACCTAATAAACCAATCGATATTATAGCAAATATTATTCCAATTTTACCAAAAACTTCTTTTT
TACCTAAATTATATCTAATAATGTGTGAAATCATTCCAAAACCTGGTAAAATTAAAATATAAACT
TCTGGATGCCCAAAAAATCAGAATAAATGTTGATATAAAATTGGGTCTCCTCCTCCTCTAGGATC
AAAAAATGATGTATTAAAGTTTCGATCTATTAAAATTATTGTAATAGCACCTGCTAATACAGGTA
AAGCTAATAAAAGTAATAAAGTAGAAATTAAAATTGATAAAGAAAACAATGTTAAATTACTTAA
ATAATAGTTTTTATTTTTTATTAATAAAATAGTTGAAATAAAATTAATAGATCTAGCAATTGATGA
AATTCCTGCAACATGTAAAGAAAAAATTATTATTTCAATAGAAGAAGATATAAAATATTGAATTG
AAGTTAATGGAGGATATATTGTTCATCCAACTCCATTTATAACTCTTTTTATGGAAGAACTAATTA
TTAAGATAAGAGAAGGAATTAATAGTCAAAATCTTATATTATTAATTCGTGAAAACATAAATCTA
CAGGTAGCTTAGCAAG

>Okra Ernakulam *Tetranychus udaipurensis COI*

TGTTTAAGGATAGTGGGGAAGATGATAGTTCCATTTATAATTAATACTGCAGATTTATGTTTTCCT
CGTATTAACAATATAAGATTTTGGCTTTTAATTCCCTCTATTATTCTTATAGTTAGAGCTTCAATAA
AAAGAGTAATAAATGGGGTAGGATGAACAATATATCCTCCTTTAACTTCAATTCAATTTTATATAT
CTTCATCCATTGAAATAATAATTTTTTCTCTTCATATTGCAGGGTTATCATCTATTGCTAGTTCTAT
TAATTTTATTTCAACTATTTTATTAATAAAAAATAAAAATTATATTTTTAGAAGTTTAACTTTATTT
ACTTTATCAATTTTAATTACTACCTTTTTACTTCTACTAGCTTTACCAGTATTAGCAGGTGCTATTA
CAATAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCTAGAGGAGGGGGAGATCCTA
TTTTGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTATATTTTAATCTTACCAGGTTTT
GGAATAGTTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGTTTGGAAAAGTTGGAAT
ATTATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGAGCTCATCATATATTTACAGT
AGGAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTATTGCAATTCCTACTGG
AATTAAAATTTTTAGTTGATTTACAACTATTATTAATTCACATATTAATTTTAATACATCAATATAC
TGATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAGGAATTGTGGCATCTAACTCT
TGTTTAGATATTAACCTTCATGATACTTATTATATGTAGCTCATTTCTATAACGTTTTTTAC

>Tapioca kakkani *Tetranychus udaipurensis COI*

TGTGAGGAATTTGGGAATTGATTAGTCCATTTATAATTAATACTGCAGATTTATGTTTTCCTCGTA
TTAACAATATAAGATTTTGGCTTTTAATTCCCTCTATTATTCTTATAGTTAGAGCTTCAATAAAAA
GAGTAATAAATGGGGTAGGATGAACAATATATCCTCCTTTAACTTCAATTCAATTTTATATATCTT
CATCCATTGAAATAATAATTTTTTCTCTTCATATTGCAGGGTTATCATCTATTGCTAGTTCTATTAA
TTTTATTTCAACTATTTTATTAATAAAAAATAAAAATTATATTTTTAGAAGTTTAACTTTATTTACT
TTATCAATTTTAATTACTACCTTTTTACTTCTACTAGCTTTACCAGTATTAGCAGGTGCTATTACAA
TAGTTTTAATAGATCGAAATTTTAACACGTCTTTTTTTGATCCTAGAGGAGGGGGAGATCCTATTT
TGTATCAACATTTATTTTGATTTTTTGGTCATCCAGAGGTTTATATTTTAATCTTACCAGGTTTTGG
AATAGTTTCACATATTATTAGATATAATTTAGGAAAAAAAGAAGTGTTTGGAAAAGTTGGAATAT
TATTTGCTATAATATCAATTGGATTATTAGGATTTATTGTATGAGCTCATCATATATTTACAGTAG
GAATAGATGTAGATACTCGAGCTTATTTTACAGCTGCTACTATAATTATTGCAATTCCTACTGGAA
TTAAAATTTTTAGTTGATTTACAACTATTATTAATTCACATATTAATTTTAATACATCAATATACTG
ATCTATGGGATTTTTAATTATGTTTTCAATTGGAGGATTTACAGGAATTGTGGCATCTAACTCTTG
TTTAGATATTAACCTTCATGATACTTATTATATTGTAGCTCATTTCTAAGGGGTTTTATAACCA

>Papaya olavakkod *Tetranychus okinawanus ITS2*

TATATGCTTTAAATTCAGCGGGTAACCTCACCTGATCTGAGATCAAAAATATTGATTAAGTTGATT
GTATATTGGTGTTGTTTGCAAGCAAATCAACGTAAATCTACTTATGTGTTTACACAAATTTTGGTA
TCAGCAATAGTCCTTAGCTCTGCTTCAAACAGAATGAGATAGTTACCATTTTGTATGTAAATGTAT
TCATACAAGTGCATTGAGAAATCTCAATAGAGAAGGTTAGCTGATCACCATAGTTATCTGCAATA
CGACTTTAGCGTCGTCAGATAGGCGACTAACCTTAGAATCTCATGCTAGTATCTATTCATATATAC
TGTTTACAGAGATGAAATATGCATTGAACACAAAACTTATTATGTGAAACAAGTAGATTTGAGAT
TCTCCTTACTAAACTGCACAGAAAATGTGCAATTAAGAAAAGGAATAAAACATCAAGATACACG
TATACACGCGAATCTCAAAACAATGCATATATGGTAGTATACAAGTGTTTTGTTGATTATTTTACA

TCTCAACTCTCAGACAGATGTGACCTCGGAAAAGACCCGAAAGCCGCAATGTGCGTTAGAAGCTC
AAGTGCTCGGCGTGTCCTGCAATTCGCACCGATTATCGTAGCTAGCTGCGTTCTTCATCGACC

>Ashgourd Vellanikkara *Tetranychus okinawanus ITS2*

TTATATGCTTAAATTCAGCGGGTAACCTCACCTGATCTGAGATCAAAAATATTGATTAAGTTGATT
GTATATTGGTGTTGTTTGCAAGCAAATCAACGTAAATCTACTTATGTGTTTACACAAATTTTGGTA
TCAGCAATAGTCCTTAGCTCTGCTTCAAACAGAATGAGATAGTTACCATTTTGTATGTAAATGTAT
TCATACAAGTGCATTGAGAAATCTCAATAGAGAAGGTTAGCTGATCACCATAGTTATCTGCAATA
CGACTTTAGCGTCGTCAGATAGGCGACTAACCTTAGAATCTCATGCTAGTATCTATTCATATATAC
TGTTTACAGAGATGAAATATGCATTGAACACAAAACTTATTATGTGAAACAAGTAGATTTGAGAT
TCTCCTTACTAAACTGCACAGAAAATGTGCAATTAAGAAAAGGAATAAAACATCAAGATACACG
TATACACGCGAATCTCAAAACAATGCATATATGGTAGTATACAAGTGTTTTGTTGATTATTTTACA
TCTCAACTCTCAGACAGATGTGACCTCGGAAAAGACCCGAAAGCCGCAATGTGCGTTAGAAGCTC
AAGTGCTCGGCGTGTCCTGCAATTCGCACCGATTATCGTAGCTAGCTGCGTTCTTCATCGACCCA

>Brinjal Tavannur *Tetranychus okinawanus ITS2*

CTGGTGTCGATGAAGAACGCAGCTAGCTACGATAATCGGTGCGAATTGCAGGACACGCCGAGCA
CTTGAGCTTCTAACGCACATTGCGGCTTTCGGGTCTTTTCCGAGGTCACATCTGTCTGAGAGTTGA
GATGTAAAATAATCAACAAAACACTTGTATACTACCATATATGCATTGTTTTGAGATTCGCGTGTA
TACGTGTATCTTGATGTTTTATTCCTTTTCTTAATTGCACATTTTCTGTGCAGTTTAGTAAGGAGAA
TCTCAAATCTACTTGTTTCACATAATAAGTTTTGTGTTCAATGCATATTTCATCTCTGTAAACAGTA
TATATGAATAGATACTAGCATGAGATTCTAAGGTTAGTCGCCTATCTGACGACGCTAAAGTCGTA
TTGCAGATAACTATGGTGATCAGCTAACCTTCTCTATTGAGATTTCTCAATGCACTTGTATGAATA
CATTTACATACAAAATGGTAACTATCTCATTCTGTTTGAAGCAGAGCTAAGGACTATTGCTGATAC
CAAAATTTGTGTAAACACATAAGTAGATTTACGTTGATTTGCTTGCAAACAACACCAATATACAA
TCAACTTAATCAATATTTGATTCAGATTATGGATGGG

>Papaya Kannara *Tetranychus.okinawanus ITS2*

TTTTTGCTTTAAATTCAGCGGGTAACCTCACCTGATCTGAGATCAAAAATATTGATTAAGTTGATT
GTATATTGGTGTTGTTTGCAAGCAAATCAACGTAAATCTACTTATGTGTTTACACAAATTTTGGTA
TCAGCAATAGTCCTTAGCTCTGCTTCAAACAGAATGAGATAGTTACCATTTTGTATGTAAATGTAT
TCATACAAGTGCATTGAGAAATCTCAATAGAGAAGGTTAGCTGATCACCATAGTTATCTGCAATA
CGACTTTAGCGTCGTCAGATAGGCGACTAACCTTAGAATCTCATGCTAGTATCTATTCATATATAC
TGTTTACAGAGATGAAATATGCATTGAACACAAAACTTATTATGTGAAACAAGTAGATTTGAGAT
TCTCCTTACTAAACTGCACAGAAAATGTGCAATTAAGAAAAGGAATAAAACATCAAGATACACG
TATACACGCGAATCTCAAAACAATGCATATATGGTAGTATACAAGTGTTTTGTTGATTATTTTACA
TCTCAACTCTCAGACAGATGTGACCTCGGAAAAGACCCGAAAGCCGCAATGTGCGTTAGAAGCTC
AAGTGCTCGGCGTGTCCTGCAATTCGCACCGATTATCGTAGCTAGCTGCGTTCTTCATCGACACCA

> Dahlia Idukki *Tetranychus truncatus ITS2*

TATATGCTTTAAATTCAGCGGGTAACCTCACCTGATCTGAGATCAAAAATATTGATTAAGTTGATT
AATGTATATTTGTGTTGTTTGCAAGCAAGCAACGTAAATCTACTTTAACGTTTGCACAAATTTTGC
CTTTGCATTACTTCTTAGGTCTGCTTTAACAGAATGAAATAGCTACTATTTGTATGCTTTATACAA
GTGCAATAAGATTCATTATTTAGCAGGTTAGTTGATCACCATAGTTATCTGCAATACGACTTTAGC
GTCGTCAGATAGGCGACTAACCTTAGAATCTCATGCTAGTATCTATTCATATATACTGCTTGCAGA
GATGAAATATGCATTGTACACAAAATTTATCATGTGAAACAAGTAGATTTGAGATTCTCCTTACT
AAATTGCAACGAATTGCAATTAAGAAAAGGAATAAAACATCAAGATTCATGCATATAAATATGC
AATCCTCTCAAAACAATGCATATATGGTAGTATGCAAGTGTTTTGTTGATTATTTTACATCTCAAC
TCTCAGACAGATGTGACCTCGGAAAAGACCCGAAAGCCGCAATGTGCGTTGAAAGCTCAAGTGC
TCGGCGTGTCCTGCAATTCGCACCGATTATCGTAGCTAGCTGCGTTCTTCATCGACACTAAAA

DNA BARCODING OF SPIDER MITES (PROSTIGMATA: TETRANYCHIDAE) ON MAJOR CROP PLANTS OF KERALA

By

ARUNIMA V.

(2015-11-003)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE IN AGRICULTURE

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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KERALA, INDIA

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ABSTRACT

Spider mites belonging to the family Tetranychidae are economically important pests of various agricultural and horticultural crops. Recently, spider mites emerged as serious pests of vegetables, banana and rice in Kerala. Though identification of spider mites is primarily based on external morphology, the existence of cryptic species makes precise identification difficult. In this context, the present study entitled "DNA barcoding of spider mite (Prostigmata: Tetranychidae) was conducted in Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Agricultural Entomology, College of Horticulture, Vellanikkara with an objective to generate DNA barcodes for different species of spider mites on major crops of Kerala and to find out genetic variability among them.

A purposive survey was conducted in four districts of Kerala *viz*., Thrissur, Palakkad, Ernakulam and Malappuram during the period of February-May and November- December 2016 and February-May 2017. Spider mites associated with ten different crops *viz*., cowpea, brinjal, okra, ashgourd, pumpkin, banana, papaya, *Adenium*, *Dahlia* and tapioca from 11 different localities were collected. The specimens were maintained in acarology laboratory as sixteen separate isolines assigning unique accession number. Permanent slides were prepared separately for adult female and male mites from each isoline for morphological characterization. The chaetotaxy of hysterosoma and legs as well as empodial characters of female were used for generic level identification. The shape of aedeagal knob in male was the characteristic feature utilised for species level identification.

The study revealed that the spider mites collected from different crops belonged to three species of a single genus *Tetranychus* namely, *T. truncatus*, *T. okinawanus* and *T. udaipurensis*. The host range of *T. truncatus* includes tapioca, banana, pumpkin, *Dahlia* and cowpea *T. okinawanus* was recorded on papaya, ashgourd, *Adenium*, cowpea and brinjal, while *T. udaipurensis* was found only on okra, banana and tapioca.

For molecular characterisation and identification, DNA was isolated from all the sixteen accessions using the modified CTAB method. The DNA was assessed for quality and quantity with NanoDrop spectrophotometer (ND-1000).

The absorbance ratio A260/A280 for all accessions was in the range of 1.8-2.0 and concentration was obtained in the range 106-359 ng/μL indicating good quality. The *COI* loci was amplified with UBC6 (forward) and RCOI (reverse) primers while *ITS2* loci was amplified with the primer ITS2 KAU (forward) and R ITS2 KAU (reverse primer). The reaction mixture proposed by Li *et al.* (2010) was used in study and for *ITS2* loci the annealing temperature was standardised by gradient PCR (Polymerase Chain Reaction). The amplification was confirmed by two per cent Agarose Gel Electrophoresis (AGE) and the amplicon size obtained for *ITS2* and *COI* were in the range of 600-700 bp and 800-900 bp, respectively. The PCR products were sequenced by outsourcing at AgriGenome Lab. Pvt. Ltd. Kochi.

The forward and reverse sequences obtained after sequencing were merged with the CAP3 sequence assembler to obtain contigs. Fourteen out of sixteen sequences formed contigs. Using MEGA 7 software the sequences were analysed for the presence of stop codons. Stop codons were removed with BioEdit software. Nucleotide BLAST (BLASTn) was done for all the accessions and query coverage (%) and identity (%) were obtained in the range of 97-99 for most accessions. The BLASTn result was in consensus with the morphology based species determination. The nine *COI* sequences and five *ITS2* sequences were aligned with Clustal W of MEGA 7 and barcode gaps were identified for both the loci. The sequences were aligned in MEGA 7 using Kimura 2 parameter (K2P) and pairwise distances between the sequences were analysed which showed that intraspecific divergences were always less than one, however accessions PmMk0927 showed maximum divergence within *T. truncatus* (0.812- 0.849) and accession BrTv0356 showed maximum divergence within *T. okinawanus* (0.835). Divergence of *T. truncatus* sequences from *T. okinawanus* (ITS2) and *T. udaipurensis* (COI) were found to be 0.113 and 0.140- 0.142 respectively. The distance summary of sequences when computed with the sequence analysis tool provided by BOLD (Barcode of Life Data systems) showed a sequence divergence within species in the range of 0.00 – 0.31 per cent for ITS2 sequences representing *T. truncatus* and *T. okinawanus* and 0.96 – 2.88 per cent for COI sequences representing *T. truncatus* and *T. udaipurensis*.

A phylogenetic tree was constructed with the fourteen sequences in MEGA 7 using the neighbour joining method and it was seen that the *COI* and *ITS2* sequences branched out into two monophyletic groups. The accessions PmMK0927 (Pumpkin, Manjakunnu) and BrTv0356 (Brinjal, Tavannur) formed out group for *COI* and *ITS2* clusters respectively, as these sequences formed shorter contigs. The sequences were submitted to BOLD (Barcode of Life Data Systems) for generation of barcodes and also to GenBank (NCBI - National centre for Biotechnology Information).

The study brings to light the occurrence of *T. truncatus* and *T. okinawanus* as the predominant species of spider mites on crops of Kerala. The study recorded four new hosts for *T. okinawanus*, a species reported only recently from India, indicating its potential to widen the host range. This is suggestive that this alien species has potential to turn invasive. Three new hosts were also recorded from Kerala for *T. truncatus* and *T. udaipurensis*. The pairwise distance analysis, distance summary analysis and phylogenetic tree are indicative that *T. truncatus* is closely related to *T. okinawanus* at ITS2 loci and to *T. udaipurensis* at COI loci. Present study confirms that ITS2 and COI loci of spider mite DNA provides species level resolution and thus can be used as reliable tool to differentiate closely related species of spider mites.