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First Molecular Identification of *Tetranychus malaysiensis* and *Sancassania* sp. in Bangladesh

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ABSTRACT

The mite samples were collected from different host plants in southern part of Bangladesh during January, 2017 to December, 2017. Morphological identification was performed in Systematic Entomology laboratory of Patuakhali Science and Technology University, Bangladesh. Consequently, molecular work was carried out in the Insect Molecular Physiology Laboratory of Kyungpook National University, Korea using both of ribosomal internal transcribed spacer 2 (ITS2) and mitochondrial cytochrome oxidase subunit I (mtCOI) primers. Molecularly three species (*Tetranychus truncatus*, *Tetranychus malaysiensis* and *Sancassania* sp.) were identified by those nucleotide sequences which make different clades in phylogenetic tree with distinct distance. Analysis of pairwise distance of nucleotides and different clades in a phylogeny were shown great host range diversity of red acaroid mite among 9 samples from different host plants. The red acaroid mite collected from sesame, sunflower, mungbean, okra and jute which were morphologically little bit different but molecularly identical, which were clustered in same clade of phylogeny. *Sancassania* sp. were shown another clade in phylogenetic tree which were collected from aroid and cucurbits. In this study, using mtCOI sequences of *Tetranychus malaysiensis* and ITS2 sequences of *Sancassania* sp. provide molecular identification for first time in Bangladesh.

Key words: Red acaroid mite, *Sancassania*, *Tetranychus malaysiensis*, *Tetranychus truncatus*

Introduction

The mites are microscopic arthropods which belong the members of several groups in the subclass Acari under the class Arachnida. The phylogeny of the Acari has been relatively little studied, but molecular information from ribosomal DNA is being extensively used to understand relationships between groups. The 18 S rRNA gene provides information on relationships among phyla and superphyla, while the ITS2, and the 18S ribosomal RNA and 28S ribosomal RNA genes, provide clues at deeper levels (*Dhooria, 2016).*

Mites are the most diverse and abundant group of arachnids (Thomas 2002) which play an important role in agriculture as many species are plant feeders causing various types of direct damages like loss of chlorophyll, appearance of stipplings or bronzing of foliage, stunting of growth, producing various types of plant deformities and reduction of yield.

In Bangladesh, many vegetables are grown throughout the year but most of them are prone to the attack of variety of insect and mite pests. Among them, spider mites have recently emerged as a major pest of vegetables which causing serious economic loss (Dutta et al., 2012). Some other preliminary works on litchi mite, spider mite, red spider mite, tea red spider mite of Bangladesh have been done by Alam et al., 2007; Jahan et al., 2011 and Jahan et al., 2013. However, the species of different mites are of great concern to agriculture because several species are significant pests of a number of important crop plants, and they reach high population densities thereby damaging the crops as a major pest. In the field level of Bangladesh, people are treating such species as simply red mite based on color without confirming their identity at species level. For these reason, molecular methods are highly support to traditional taxonomic work and nowadays are widely employed for insect species identification.

As it is known to all that Bangladesh is a humid and subtropical country favoring luxuriant growth of various mite species with rich diversity. A very few taxonomic

work had been done in Bangladesh due to lack of mite taxonomist and therefore, the mite taxonomy is not completely understood. There are still many undescribed species, and some known species are difficult to identify accurately because of within species variation, the strong similarity among closely related species, the difficulty of properly mounting male specimens, the rarity of males and the overwintering phenology. As a result, many species are treated with misidentification that is a core problem for further advanced research work. Besides, some rare species are going to be extinct due to ecological changes without any record. Therefore, detection, collection and identification of such mite species are barely necessary in Bangladesh.

Materials and Methods

This study was conducted for surveying and identifying agriculturally important mites based on morphological and molecular features known from Southern part of Bangladesh (especially, Barisal, Patuakhali, Barguna, Jhalokathi, Pirojpur and Bhola district) in Systematic Entomology Laboratory, Department of Entomology, Patuakhali Science and Technology University, Dumki, Patuakhali, Bangladesh and Insect Molecular Physiology Lab, Kyungpook National University, Korea during the period from January, 2017 to December, 2017.

Mite Collection

Mites were collected from plants in several ways. When collecting was done in the field, foliage or plant parts was **(a)** beaten so that mites fall off onto a funnel leading to a collecting jar, or onto a(black or white) plastic tray where mites was picked with a moist hair brush; or **(b)** cut and placed into plastic bags (may include blotting paper to avoid excess moisture) for later removal of the mites. Mite specimens was extracted from plant material (foliage, fruits) in the lab by:

(1) Plant parts were examined by using a hand lens (preferably 20X) or a stereoscope. Live mites were the most easily detected because of their movement. Potential hiding places (e.g. leaf domatia, bark cracks, fruit calyx) was inspected and sometimes dissected as mites hide in tight shelters. Mites were collected using a moist, fine hair brush (preferably 00, 0 or 1) and stored in vials containing 70–95% alcohol for later slide-mounting or DNA extraction. When foliage was not to be examined soon, it was stored at about 5℃ to keeping mites live for several days.

(2) Foliage was washed with 70–95% alcohol. Mites were extracted from foliage by manually dipping and shaking leaves into a jar of alcohol. Leaves were then disposed of, and mites dropped at the bottom of the jar was transferred into a petridish (for specimen examination and preparation), using a spray bottle to push the mites out after having decanted the top fluid out.

Some specimens were preserved in 95% alcohol, at ≤15°C, for the purpose of DNA analysis; genetic markers may confirm species identity, especially in the case where no males were present in the samples.

Specimen preparation

After collection, mites were immediately preserved with 99% ethanol (alcohol) until their morphological identification and /or DNA extraction. When present, both females and males of spider mites were mounted on microscopic slides. Immature stages (larva, protonymph, deutonymph), and in many cases also females, can be identified to genus only. For many species (e.g. *Tetranychus*, *Oligonychus* spp.), males are required to determine the species because in those cases, the aedeagus is the most diagnostic character. Microscope specimen slides were prepared directly from live mites picked using a moist brush or metal probe, while specimens kept in alcohol was picked up using a probe ending with a looped tipped minute pin. When no clearing was deemed necessary, the mite was placed directly into a droplet of mounting medium (e.g. Hoyer's medium, or a mixture of PVA or polyvinyl alcohol) on the centre of a slide, and a (preferably 12–15 mm round) cover slip was slowly placed onto the mounting medium. Slides were dried for 2 days to 2 weeks in an oven at 40–50°C. After the drying stage, slides made with water-soluble medium (e.g. Hoyer's medium; not PVA) was sealed ('ringed') around the cover slip using insulating paint (e.g. Glyptal), applied with a brush or a polyethylene bottle applicator, to prevent water from getting into the medium and ruining the mount in high-humidity environments. For larger or dark specimens, or when urgent species identification was required. It cleared by a clearing agent (e.g. lactic acid, Nesbitt's fluid) before slide-mounting, into a small dish (e.g. cavity block). These clearing agents were more effective than the mounting media (Hoyer's, PVA), which also contribute to the clearing of specimens (during the drying process). Specimens were mounted dorso-ventrally (venter down) on the slide, and with mouthparts placed closer to the user (as a compound scope will reverse the image), except for males of the subfamily Tetranychinae which were placed laterally so that the aedeagus was in lateral profile. However, if a specimen could not be placed side-ways, it was often possible to turn the aedeagus laterally by pressing the cover slip and checking and readjusting its position by alternate examination from a stereoscope to a compound scope. Slides were labelled with origin, host, habitat, collecting method, date and collector.

Molecular analysis

DNA extraction and PCR analysis

Genomic DNA was extracted from single adult individuals which preserved in 99% ethanol to avoid cross-contamination between species using a PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) without the sample homogenization with some modification of the manufacturer's instruction. A single mite was transferred into a 1.5 mL sample tube containing 200 µl of digestion buffer and 20 µl of proteinase K (50 µg/mL). Then samples were incubated for more than 12 hrs at 55℃ subsequently, transferred the supernatant to the new Eppendorf tube and kept the spider mite in another new E- tube with 99% ethanol for making the permanent slide. After that RNase 20 µl was added into the supernatant and gently mix by vortex mixture, and then kept for 3 min at room temperature.

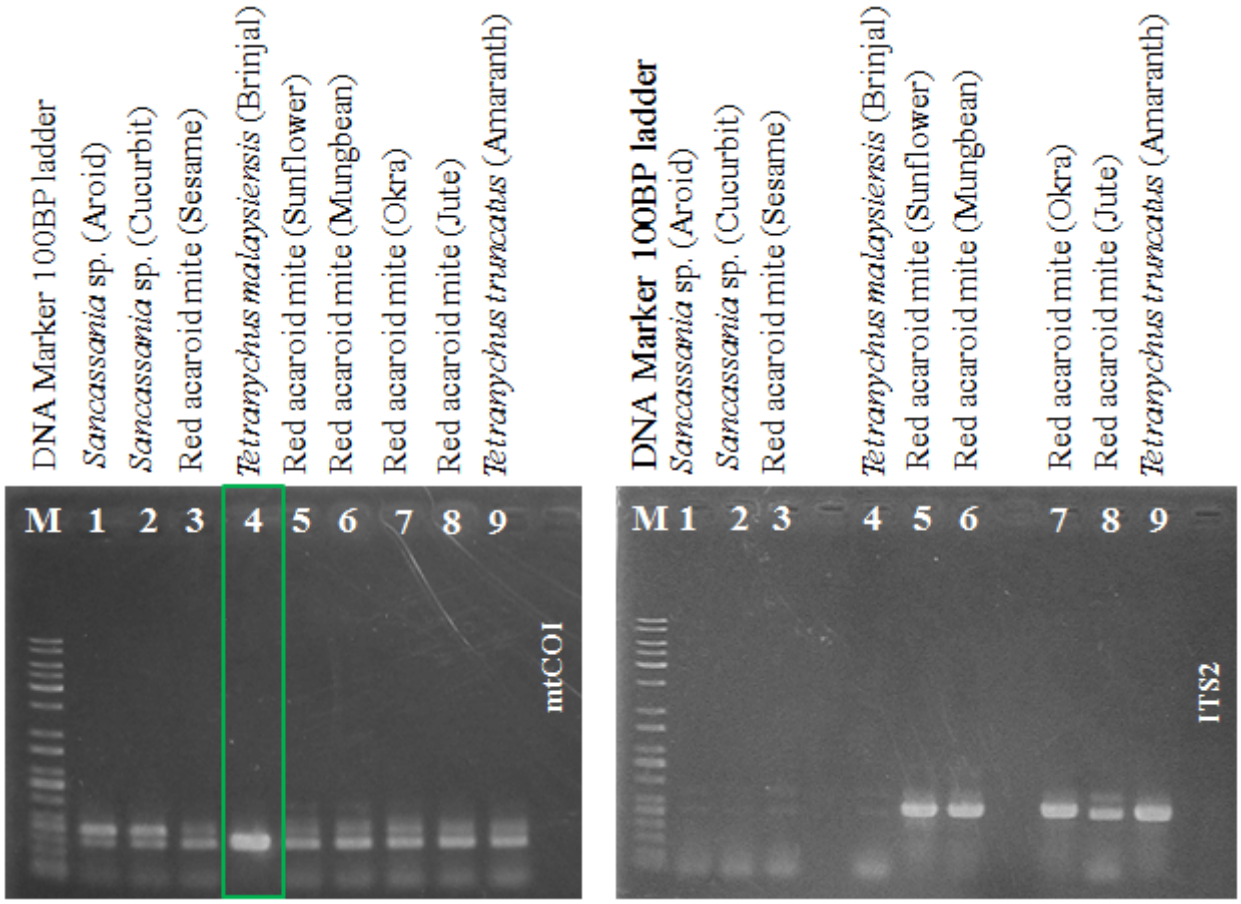

Figure 1. Amplified DNA by PCR using mtCOI and ITS2 primer with 1% agarose gel electrophoresis and visualized by UV light after staining.

The solution was mixed with the Genomic lysis/binding buffer (200 µl) and washed with 100% ethanol (200 µl) through the Genomic spin column by centrifugation at 10000 rpm for 1 min. After washing the spin column two times with the Wash buffer, DNA was eluted using the Elution buffer (20 µl) into a new tube by centrifugation at 12 000 rpm for 1 min. Concentrations of purified DNA samples were determined using a Nanophotometer (Implen, Schatzbogen, Germany). Extracted DNA kept at -20℃ immediately for later use.

PCR amplification

Amplification of both ITS2 and COI regions was carried out from the same DNA samples using universal primer sets of astigmatid mites. Primer set for the ITS2 region is as follows: forward (5′-CGA CTT TCG AAC GCA TAT TGC-3′) and reverse (5′-GCT TAA ATT CAG GGG GTA ATCTCG-3′) primers which amplified the complete ITS2 region and portions of the flanking 3′ end of the 5.8S and 5′ end of the 28S rDNA coding regions of a stigmatid mite (Noge *et al*. 2005). Primer set for COI region is as follow: forward (5′-GTT TTG GGA TAT CTC TCA TAC-3′) and reverse (5′-GAG CAA CAA CAT AAT AAG TAT C-3′) primers which amplified the central region of COI (Yang *et al*. 2011). PCR was performed in 25 µl of Smart Taq Pre-Mix (Solgent, Daejeon, Korea) containing 40 ng of DNA as a template and 10 pmol of each primer. The mixtures were amplified by the following conditions: an initial denaturation at 94℃ for 3 min followed by 35 cycles at 94℃ for 30 s, (either 55℃ for ITS2 or 50℃ for COI) for 30 s, and 72℃ for 1 min, and a final elongation step at 72℃ for 7 min. PCR products were analyzed by 1% agarose gel electrophoresis and visualized by UV light after staining with ethidium bromide solution (Figure 1).

Sequencing of amplified DNA

The PCR products were purified from the amplification tube using PCR clean-up purification kit (Promega, WI, USA). The sequences amplified using the ITS2 primers were cloned using the pGEM-Teasy vector by following the procedures suggested by the manufacturer plasmid mini-prep kit (Promega, WI, USA) and inserted into *Escherichia coli* cells. Bacteria were cultured in LB medium after blue/white selection. The ITS2 purified products were sequenced by company (Solgent, Daejeon, Korea).

Alignment of sequences

DNA sequences were aligned using CLUSTAL W (Thompson et al. 1994). The aligned sequences were checked and compared the sequences similarity with the online published sequences using BLAST in the National Centre of Biotechnology Information (NCBI). Sequences were aligned and arranged using the Clustal W multiple alignments in BioEdit (version7.0). The sequences divergences calculated by Molecular Evolutionary Genetics Analysis (MEGA) among intraspecific, interspecific species, based on Kimura-2-

parameter (K2P) distances (Tamura et al.2007). Phylogenetic relationships were inferred byMEGA Software Version 4.0 (Tamura et al. 2007) using Neibour-joining method (NJ). Bootstrap valueswere obtained from 1000 replicates. The sequences were deposited in the GenBank database.

Results

Identification of acaroid mites using ITS2 and CO1 marker

The length of obtained PCR products of the ITS2 and CO1 were 307 bp and 265 bp respectively, in all examined samples. Species were identified by identical or less than 0.1% variation values of ITS2 and COI sequences. In ITS2 identity, *Sancassania sp* (aroids) and *Sancassania sp* (cucurbit) was 100% identical with strains *Sancassania* sp (AB104963). Red acaroid mite collected from sesame, mungbean, okra, jute and sunflower that are still taxonomically unidentified because it shown 91% identical with strains *Aceria guerreronis* (DQ060617). But all are belonging in a same species by their single clade in phylogeny. *Tetranychus truncatus* was 100% identical with strains of *Tetranychus truncatus* (KT070710). *Tetranychus malaysiensis* was shown 100% identical with the strains *Tetranychus malaysiensis* (KJ729019) using mtCO1 sequences (Figure 2). Thus, identified three species, including *Sancassania sp., Tetranychus truncatus* and *Tetranychus malaysiensis* in Acaridae. These recorded unidentified species which DNA sequence and pairwise distance was calculated with these known species. The pairwise genetic distances among three known species and four unknown species based on ITS2 and mtCO1 nucleotide sequences were calculated by the Kimura-2-parameter model in MEGA 7.

Analysis of ITS2 gene sequence of spider mite

Eight internal transcribed spacer-2(ITS2) gene sequences of different acaroid mites in various host plants from different places of patuakhali district were analyzed. These sequences had shown the propotion of A+T and G+C in residues composition 52.2% and 47.8% respectively. The average proportion of T:C:A:G was 26.4: 24.2: 25.8: 23.6 with a narrow standard error around means but base composition varied substantially in different portions within the sequence of species.

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spider_mite_(Brinjal_Bangladesh)        TATATATTTTAATTTTACCAGGATTTGGGATAATTTCACATATTATTAGATATAATTTAG   60
Tetranychus_malaysiensis_(KJ729019)     TATATATTTTAATTTTACCAGGATTTGGGATAATTTCACATATTATTAGATATAATTTAG   60
                                        ************************************************************

spider_mite_(Brinjal_Bangladesh)        GGAAAAAAGAAGTATTTGGAAAAATTGGTATAATATTTGCTATAATATCAATTGGTTTAT   120
Tetranychus_malaysiensis_(KJ729019)     GGAAAAAAGAAGTATTTGGAAAAATTGGTATAATATTTGCTATAATATCAATTGGTTTAT   120
                                        ************************************************************

spider_mite_(Brinjal_Bangladesh)        TAGGGTTTATTGTATGAGCACATCATATATTTACAGTAGGAATAGATGTAGACACTCGAG   180
Tetranychus_malaysiensis_(KJ729019)     TAGGGTTTATTGTATGAGCACATCATATATTTACAGTAGGAATAGATGTAGACACTCGAG   180
                                        ************************************************************

spider_mite_(Brinjal_Bangladesh)        CTTATTTTACAGCTGCTACAATAATTATTGCTATCCCAACTGGAATTAAAATTTTTAGTT   240
Tetranychus_malaysiensis_(KJ729019)     CTTATTTTACAGCTGCTACAATAATTATTGCTATCCCAACTGGAATTAAAATTTTTAGTT   240
                                        ************************************************************

spider_mite_(Brinjal_Bangladesh)        GGTTTACTACTTTAATAAACTCACATATTAATTTTAATGTATCCATATTTTGAGCCATAG   300
Tetranychus_malaysiensis_(KJ729019)     GGTTTACTACTTTAATAAACTCACATATTAATTTTAATGTATCCATATTTTGAGCCATAG   300
                                        ************************************************************

spider_mite_(Brinjal_Bangladesh)        GTTTCTTAATTATATTTTCTATTGGAGGATTTACGGGAATTGTAGCATCAAATTCCTGTT   360
Tetranychus_malaysiensis_(KJ729019)     GTTTCTTAATTATATTTTCTATTGGAGGATTTACGGGAATTGTAGCATCAAATTCCTGTT   360
                                        ************************************************************

spider_mite_(Brinjal_Bangladesh)        TAGATATTAATTTACATGATACTTATTATATTGTAGCTCATTTTCATTAT     410
Tetranychus_malaysiensis_(KJ729019)     TAGATATTAATTTACATGATACTTATTATATTGTAGCTCATTTTCATTAT     410
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Figure 2. DNA sequence (mtCOI) of brinjal spider mite from Bangladesh compare with *Tetranychus malaysiensis* (KJ729019) through multiple sequence alignment using CLUSTAL Omega (ClustalW2).

Among these 305 bp nucleotide, 8 characters were conserved and 297 characters were variable. The sequence divergence in pairwise comparisons clearly shown that collected acaroid mite samples are much divergence and make three (3) distinct clades in phylogenetic tree where pairwise distance of value was shown 1.072, 1.111 and 1.171 among all examined sequences. The genetic relationship among eight collected samples sequences were extracted from neighbor –joining method (NJ) and shown three distinct clades that indicates three different species. One is *Sancassania* sp, the second one is *Tetranychus truncatus* and another one is still unknown (needs more further study). Analysis was run with kimura -2 parameter distance model using the MEGA-7 program.

Phylogenetic analysis of acaroid mites

The neighbor-joining (NJ) phylogenetic tree reconstructed based on eight internal transcribed spacer -2 (ITS2) sequences were clustered in three distinct clades. It revealed that there are three different mite species from in all eight collected samples (Figure 3).

Discussion

In the present study, three species of acaroid mites collected from the southern part of Bangladesh were identified by the comparison of nucleotide sequences of

Figure 3. Neighbor –joining (NJ) tree based on kimura 2-parameter distance with complete deletion of gap/missing data, using partial ribosomal ITS2 sequences. The number on each branch is the bootstrap support (1,000 replicates)

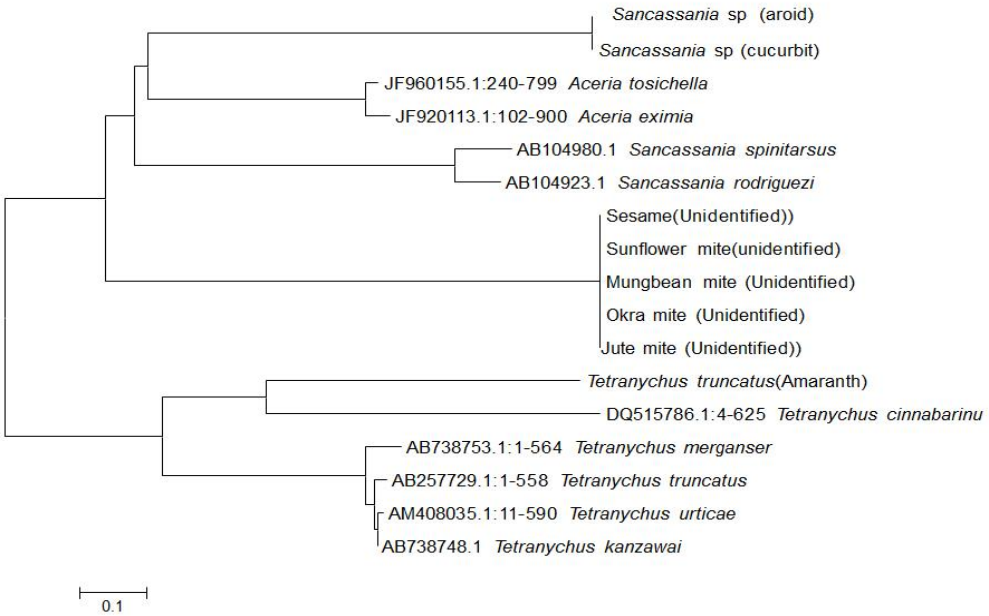

Figure 4. Neighbor –joining (NJ) tree based on kimura 2-parameter distance with complete deletion of gap/missing data, using partial ribosomal ITS2 sequences. The number on each branch is the bootstrap support (1,000 replicates)

ITS2 and one species was identified by the mtCO1 sequence. One species in the genus *Sancassania* was identified. *Sancassania sp* was identified from the host aroid and cucurbit (Figure 4). Several species in this genus *Sancassania* have been morphologically identified in Korea including *S. rodionovi* (=*S. berlesei*) (Lee & Choi 1980; Klimov & Tolstikov 2011), *S. phyllophagianus* from house dusts (Ree *et al*. 1997), and *Sancassania* sp2 and *S. sphaerogaster* (Klimov & Tolstikov 2011). This identified sequence was identical with *Sancassania* sp (accession number AB104963). The genus *Sancassania* is known to be one of the most diverse groups of mites (Noge *et al*. 2005; Klimov & Tolstikov 2011). This suggests the possible presence of various species within this genus in Bangladesh. Further study is required on the species distribution of this genus at molecular level. The red acaroid mite collected from sesame, jute, sunflower, okra and mungbean which were shown 91% similarity with *Aceria guerreronis*the accession number DQ060617. But these are might be different genus of mite because in case of molecular identification, it is 98% or less than 98% similarity indicate different species or species variation. However, genetic distance of these species was clearly separated by the phylogenic analysis of ITS2. Further study is required on these mites for their correct identification.

Tetranychus truncatus was identified by molecular markers which were collected previously by Jahan et al. 2011. Though it was previously identified using both morphological and molecular techniques. Molecular diagnosis is very important to identify the spider mite especially analyzing the sequence of ITS2 region. That region is very conservative for each species (Navajas *et al.*, 1998; Osakabe *et al*., 2008; Ros and Breeuwer 2007; Ben-David *et al*., 2007). These ITS2 sequences were

compared with online published sequences of spider mites on NCBI and exposed that 100% identical with *Tetranychus truncatus* the accession number KT070710. Results were confirmed that no variation was found in ITS2 among all *Tetranychus truncatus* collected from Southern part of Bangladesh. The results of ITS2 sequences were highly confirmed than morphology-based identification using the taxonomic key (Bolland *et al*., 1998 and Lee and Koh, 2010). ITS2 sequences of Bangladeshi *Tetranychus truncatus* collected from amaranth are very closely related species with *Tetranychus truncatus* of different countries published in NCBI database.

Nucleotide sequences of mtCO1 regions were determined from *Tetranychus malaysiensis* species and it was collected from Brinjal (eggplant). In this study, the identified *Tetranychus malaysiensis* was shown 100% similarity with *Tetranychus malaysiensis (*accession number KJ729019*).* These ITS2 sequences were compared with online published sequences of spider mites on NCBI and exposed that 100% similarity with *Tetranychus malaysiensis* (Figure 2). Results were confirmed that no variation was found in CO1 among all *Tetranychus malaysiensis* collected from southern part of Bangladesh.

Conclusion

In this research work, a base line survey and taxonomic study was done on different mites(Acari) from various host plants in southern part of Bangladesh(Patuakhali, Barishal, Bhola, Pirojpur, Jhalokati and Barguna district). It was very hard job to identify them morphologically. So that we depend to identify molecularly and three species (*Tetranychus truncatus*, *Tetranychus malaysiensis* and *Sancassania* sp.) were identified by using both ITS2 and COI nucleotide sequences. Using mtCOI sequences of *Tetranychus malaysiensis* and ITS2 sequences of *Sancassania* sp. provides molecular identification for first time in Bangladesh.

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