

MOLECULAR CHARACTERIZATION AND BARCODING OF WHITEFLIES INFESTING TUBER CROPS

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ABSTRACT

Most of the world's poorest smallholders depend on tropical roots and tuber crops as their principal source of food and nutrition. Sucking pests like whiteflies are major limiting factors in their production. Although whiteflies are important pests of tuber crops, their taxonomic diversity is poorly understood in these crops. As DNA barcoding is an effective tool for resolving species complexes and analyzing species distributions, the approach was used to analyze genetic diversity in the whitefly species infesting tuber crops such as cassava (*Manihot esculenta*), sweetpotato (*Ipomoea batatas*) and arrowroot (*Maranta arundinacea*). Among the whiteflies characterized, a 'distinct' genetic variant/biotype was identified as a pest in sweetpotato and is found to be quite different when compared with all available 44 whitefly genetic variants of Bayesian phylogenetic tree inferred from mtCOI data using the GTR+G model.

Keywords: *Aleurodicus*, Barcoding, *Bemisia*, Biotype, Tuber crops, Whiteflies,

Tuber crops are important food crops serving either as subsidiary or subsistence food in different parts of the world and are rich sources of starch besides many vitamins and minerals (Moorthy, 2004). Whiteflies are one of the main pests of these crops belonging to family Aleyrodidae. They typically feed on the undersides of leaves by piercing and sucking sap from a plant, causing the leaves to go yellow and mottled. More than 1550 species have been described. Most species have a wingspan of less than 3 mm and a body length of 1 mm to 2mm (Martin, 2007). The insects and their wings are variously marked or mottled according to species, and many species are covered with fine wax powder, giving most species a floury, dusted appearance (Jaeger, 1955). They develop rapidly in warm weather and populations can build up quickly in situations where the natural enemies are ineffective and when weather and host plants favour outbreaks. In warm or tropical climates whiteflies present a major problem in crop protection and worldwide economic losses are estimated at hundreds of millions of dollars annually (Capinera, 2008; Sundararaj, 2021). Although several species of whitefly may cause crop losses by sucking sap when they are very numerous, the major harm they do is indirect. They secrete large amounts of honey dew that support harmful infestations of sooty mold. Secondly, they inject saliva that may harm the plant more than either the mechanical damage of feeding as they transmit viruses and cause severe diseases to the plant. There are a number of different types of whiteflies infesting tuber crops such as cassava, sweetpotato, arrowroot etc., and most of them are not yet fully studied.

Identification of an insect using phenotypic characters can often lead to misdiagnosis. This limitation inherent in the morphology-based identification system can be overcome using a novel micro-genomic method called DNA barcoding (Hebert *et al.*, 2003). This technique involves sequencing a short fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene, the DNA barcode, from a taxonomically unknown sample and making comparisons to a reference library of barcodes of known species origin to generate a species-level identification (Wilson, 2012).

The present study focuses on the molecular characterization and barcoding of whiteflies infesting tuber crops such as cassava, sweet potato, arrowroot and comparison of their genetic variations.

MATERIALS AND METHODS

All the analytical grade chemicals were obtained from Sigma Aldrich Corporation, USA. Media components were purchased from Himedia Laboratories, India and SD Fine Chemicals, India. Various enzymes and molecular markers used in PCR were purchased from Bangalore, Genei, India. From cultivated areas of cassava, sweetpotato and arrowroot in Kerala (Thiruvananthapuram, Thrissur and Idukki districts), whitefly samples were collected using an aspirator and transferred to Eppendorf tubes containing 70% alcohol until analysis. Total samples collected were 27 (Three types of whiteflies from three locations each in these districts). Each of the sample had 10 whiteflies per sample.

Isolation of genomic DNA and quality checking

The genomic DNA was isolated using the modified

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cetyltrimethyl ammonium bromide buffer (CTAB) method (Gawel and Jarrett, 1991). Insect sample of five insects was homogenized with 500µl lysis buffer (CTAB 2%, 100mM Tris-HCl pH 8.0), 1.4M sodium chloride, 20mM EDTA, 0.1% of 2-mercapto ethanol) and suspended in the same buffer. The suspension was incubated at 65°C for 1 hr and centrifuged at 10,000 rpm for 10 min. Then an equal volume of chloroform: isoamyl alcohol (24: 1) was added and the suspension was centrifuged at 6000 rpm for 15 min at room temperature. The upper aqueous layer was transferred to a fresh micro centrifuge tube and DNA was precipitated by adding 40µl of sodium acetate, 600 µl of 95% ethanol. The tubes were kept at -20°C for 20 minutes and centrifuged at 8000 rpm for 10 min. The supernatant was discarded and the resultant pellet was washed with 70% ethanol, dissolved in 50 µl DNase free water. The quantity of DNA was checked using Nanodrop spectrophotometer. 1 µl of the sample was placed on the spectrophotometer and the amount of DNA present was recorded. Nucleic acids show absorption maxima at 260 nm.

PCR, gel electrophoresis and purification

Good quantity genomic DNA isolated from the whitefly species was amplified using PCR in a 25 µl reaction. The polymerase chain reaction (PCR) was carried out in a thermal cycler (AB-Applied Biosystems, Veriti) with the following conditions: initial denaturation -94°C for 5 min, denaturation -94°C for 45 sec, 35 cycles, primer annealing -47°C for 45 sec, primer extension -72°C for 45 sec, final extension -72°C for 10 min and hold at 4°C. The primers used were specific to mitochondrial cytochrome oxidase (COX-1) LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3', HCO2198; 5'-TAAACTTCAGGGTGA CCAAAA AAT CA-3' (Folmer *et al.*, 1994). PCR was performed in 25 µL total reaction volume containing 20 pico moles of each primer, 1.0 µL of 20 mM dNTP, 2.5 µL of 10X buffer and 1.0µL of 1.0 UT aq DNA polymerase (Fermentas Life Sciences, Maryland, USA).

Success of the PCR was confirmed by agarose gel electrophoresis and the ladder used was 100 bp (Thermo Scientific). The amplified products were resolved in 1% agarose gel, stained with ethidium bromide (10µg/mL). Agarose (0.8 g) was dissolved in 80ml 1X TAE buffer by heating in microwave oven. Electrophoresis was performed at 100 volts until the tracking dye reaches 3/4th of the gel. The DNA bands were visualized in gel documentation system (UVP).

The PCR amplified fragments were eluted using GeneJET PCR purification kit according to the manufacturer's protocol (Thermo Scientific, USA). The purified DNA was stored at -20°C for further use. The good quantity DNA was used for cloning.

Cloning, Colony PCR, Plasmid DNA Isolation

The PCR amplified fragments were ligated in to the T/A cloning vector, (PTZ57R/T) InsT/Aclone PCR cloning kit according to the manufacturer's protocol (Thermo Scientific, USA). The transformed cells were spread on LB agar (LBA) plates containing X-gal (300 µg/mL), IPTG (120 µg/mL) and ampicillin (100µg/mL). Colony PCR was performed for white colonies and for each 20µl reaction, the following reagents were mixed - pJET1.2 forward sequencing primer 10µM-0.4µL, pJET1.2 reverse sequencing primer 10µM-0.4µL, Nuclease free water-9.2µL, PCR master mix - 10µL. A small amount of individual colony was picked from the master plate and resuspended in 20µl PCR master mix without touching the agar. PCR was performed at 95°C for 3min, 60°C for 30 seconds and 72°C for 1 min for 25 cycles. The presence of PCR product was analyzed by agarose gel electrophoresis. The white colonies with prominent PCR bands were plated on new LBA containing ampicillin (100 mg/ ml), incubated at 37°C over night and stored at 4°C until further use.

Plasmids were isolated and purified from the overnight culture of the positive colonies using GeneJET Plasmid Miniprep Kit according to manufacturer's protocol (Thermo Scientific, USA). They were then stored at -20°C.

Sequencing

Sequencing was carried out in an automated sequencer (ABI Prism 310; Applied Biosystems, USA) using M13 universal primers both in forward and reverse directions. Homology search was carried out using BLAST (<http://www.ncbi.nlm.nih.gov>) and the sequences were submitted to NCBI database.

Barcoding

The sequences generated using DNA barcoding primer and specimen details were submitted to Barcode of Life Database (BOLD system v4). An account was opened in work bench session of BOLD systems v4 database and a new project was created. Specimen data *viz.*, specimen identifiers, specimen taxonomy, specimen details, collection details was submitted and an autogenerated process ID was obtained. Further, primer details, high resolution specimen images, mitochondrial DNA sequences (fasta) and the trace files (.ab1) obtained from sequencer were uploaded to the database and the corresponding barcode of whiteflies were generated.

RESULTS AND DISCUSSION

Molecular approach was adopted for the accurate identification of whitefly samples collected from the tuber crop plants as conventional methods had several

disadvantages.

From the samples, large and small whiteflies were obtained from cassava (CL and CS) and quantity of DNA extracted were 82.80 ng/μl and 48.10 ng/μl, respectively; whereas, 35.18 ng/μl of DNA was extracted from the whitefly sample obtained from sweet potato (SS). The amount of DNA obtained from the whitefly collected from arrowroot (AL) was 41.80 ng/μl.

Figure 1 shows the PCR amplification of insect DNA at 658 bp. The amount of DNA present in PCR elute were from CL and CS, 47.45 ng/μl and 52.62 ng/μl, respectively. 23.80 ng/μl of DNA was obtained from SS and 18.30 ng/μl from AL. The quantity was good enough to proceed with cloning.

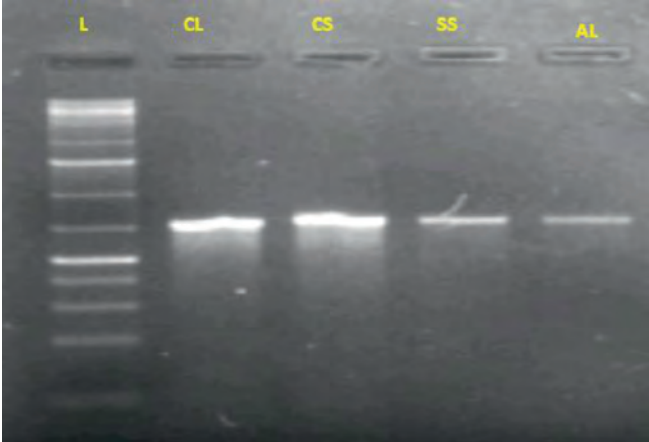


Fig. 1. PCR amplification of whitefly DNA

L: Ladder (100bp); CL: Cassava Large; CS: Cassava Small; SS: Sweet potato Small; AL: Arrowroot Large

The concentration of the plasmids was in the range of 80-150 ng/μl. The homology searches of the sequences were done using National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST). The sequences were submitted to NCBI and the accession numbers obtained were OP303313 (*Aleurodicus dispersus* infesting cassava), OP303264 (*Bemisia tabaci* infesting cassava), OP303265 (*Bemisia tabaci* infesting sweetpotato) and OP303261 (*Aleurodicus rugioperculatus* infesting arrowroot) (Table 1).

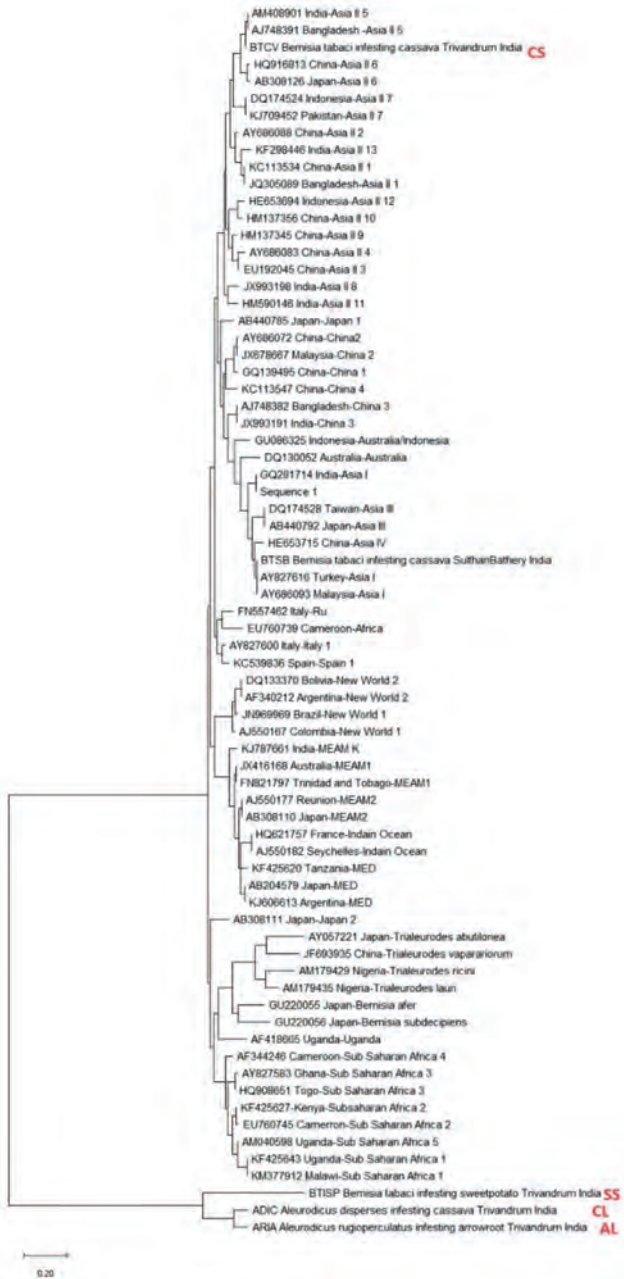


Fig. 2. Whitefly genetic variants of Bayesian phylogenetic tree inferred from mtCOI data using the GTR+G model (Kanakala and Ghanim, 2019)

Table 1. Whiteflies infesting tuber crops

Insect sample	Organism identified	Host	Biotype	NCBI accession number
CL	<i>Aleurodicus dispersus</i>	Cassava	Not reported	OP303313
CS	<i>Bemisia tabaci</i>	Cassava	Asia II 5	OP303264
SS	<i>Bemisia tabaci</i>	Sweet potato	New	OP303265
AL	<i>Aleurodicus rugioperculatus</i>	Arrowroot	Not reported	OP303261

From the whiteflies collected and analyzed for genetic variability from different tuber crop plants, a 'distinct' genetic variant/ biotype was identified in sweet potato fields of Thiruvananthapuram, Kerala (SS/ OP303265) and is found to be quite different when compared with all available 44 whitefly genetic variants of Bayesian phylogenetic tree inferred from mtCOI (mitochondrial cytochrome oxidase I) data using the GTR+G model (Kanakala and Ghanim, 2019) (Fig. 2). Among the whiteflies, Asia II 5 is already reported as the prevalent biotype in cassava (Harish *et al.*, 2019) and it was confirmed in the present study. Comparable to the present study, Munguti *et al.* (2021) also found similarity in phylogenetic distribution of different types of whitefly species colonizing cassava in Kenya.

Per cent identity matrix values were calculated based on the similarity in their mtCO1 sequences. All the 4 sequences were aligned using online software 'Clusta I2.1' and the respective values for their similarity were obtained by pair wise arrangement. The calculated values ranged from 45.30 to 100 (Table 2).

Table 2. Per cent identity matrix (Clusta I2.1) for whiteflies (mtCO1 sequences) infesting tuber crops

Whitefly type	CS	SS	CL	AL
CS	100.00	45.30	46.92	45.50
SS	45.30	100.00	63.12	70.38
CL	46.92	63.12	100.00	89.55
AL	45.50	70.38	89.55	100.00

(CS: *Bemisia tabaci* infesting cassava, SS: *Bemisia tabaci* infesting sweetpotato, CL: *Aleurodicus dispersus* infesting cassava, AL: *Aleurodicus rugioperculatus* infesting arrowroot)

In between, *Aleurodicus dispersus* and *Aleurodicus rugioperculatus*, there was 89.55% similarity and in between *Bemisia tabaci* infesting sweet potato and *Aleurodicus rugioperculatus* 70.38% similarity was observed. Less than 50% similarity was observed in between other samples in pair wise comparison.

Morphological characterization of the adult whitefly is very difficult and limits the capacity of identifying new species. Almost universally, whitefly taxonomical characterization is performed at the nymphal stage, which is labor intensive and time consuming. Because of the limitations for a rapid identification of whitefly species by conventional means, a molecular approach was adopted to assess its use for rapid identification of whitefly adult samples (Dobrajc *et al.*, 2020). Analysis of this mitochondrial DNA provides insight to understand the natural genetic diversity and population structures in organisms (Avisé, 1994). Many researchers (Frohlich *et al.*, 1999; Legg and Thresh, 2000) used the primers for the genetic variability studies of whiteflies. The results

of the present study reveal that sequence analysis is a very accurate and effective tool in the identification of whitefly species.

In barcoding, specimen data viz., specimen identifiers, specimen taxonomy, specimen details, collection details were submitted and an autogenerated process ID BTICV001-19 and ADIC001-19 for the cassava samples, BTISP001-19 for the sample from sweetpotato and ARIA001-19 for the sample from arrowroot were obtained. Further, primer details, high resolution specimen images, mitochondrial DNA sequences (fasta) and the trace files (.ab1) obtained from sequencer were uploaded to the database and the corresponding barcodes were generated (Figures 3-6). DNA barcode can assign the unknown specimen at different developmental stages either as a complement to morphological analysis or as the primary diagnostic indicator in cases where the requisite morphological keys are unavailable (Hebert *et al.*, 2003). In the present study, mtCO1 gene of different whiteflies infesting tuber crops was used to reveal genetic variability in the insects. The superiority of DNA barcoding over traditional methods for the detection and distribution analysis of invasive species is now well established (Briski *et al.*, 2011; Dejean *et al.*, 2012). Ovalle *et al.* (2014) compared the morphometric traits against DNA barcode sequences to develop and implement a diagnostic molecular kit based on a RFLP-PCR method using the COI gene for the rapid identification of whiteflies and this study allowed the rapid diagnosis of the diverse community of whiteflies attacking plants of economic interest. It also provided access to the COI sequence that could be used to develop predator conservation techniques by establishing which predators have a trophic linkage with the local whitefly pest species. DNA barcode successfully discriminated cryptic species in *B. tabaci* complex and the dominant haplotypes in the *B. tabaci* complex were present in India and Pakistan (Ashfaq *et al.*, 2014).

This type of study expands the identification repertoire and allows for rapid taxonomic identification as well as comparison of the genetic diversity of different whitefly species around the world. Correct species identification is crucial for accurate detection of new and existing invasive species, identification of genetic variations, their range of distribution and also for compatible management outcomes.

Authors' Contribution

Conceptualization of research work and designing of experiments (HER, SBG, HN); Execution of field/ lab experiments and data collection (HN, HER, SBG); Analysis of data and interpretation (HER), Preparation of manuscript (HER, HN)

BTICV - Barcoding of Bemisia tabaci infesting cassava [BTICV]



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Process ID: BTICV001-19
Identification: Bemisia tabaci (Gennadius, 1889)
Identified by
Collected in India, Kerala
by
Institution Storing: ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE
Field ID: BTICV1
Museum ID: BTICV2

5/23/2019

BTICV001-19 | BOLDSYSTEMS

Sequence View for Process ID: BTICV001-19

Specimen Details Current

Sample ID: BTICV
Process ID: BTICV001-19
Project: BTICV
Tax Names: Arthropoda, Insecta, Hemiptera, Aleyrodidae, Aleyrodinae, Bemisia, Bemisia tabaci
Taxon: Bemisia tabaci
Rank Name: species
Sampling Protocol: N/A
BIN URI: N/A
Kingdom: Animals

Marker Summary

Marker Code	Sequence Length	GC	Ambiguous	Trace Count
COI-5P	661	33.9%	0%	2

COI-5P

Illustrative Barcode



Nucleotide Sequence

ATTGTTCTCATTAAATAGAGCCGAGCTGGAAACTTGAATATTGGTAAGTTGGGAATAATT
TATGCTATAGTAACATTTGGAATCTAGGTTTATTGTGTGAGGTCATCACAATTTACCGTTGGG
ATAGATGTTGATAGCTGGGCTTATTTACTTCAGCCACTATAATTATCGCTGTTCCGACCGAATT
AAATCTTTAGGTGACTTGTACTCTAGGTGGAATAAATCTAACAGTTTATGTCGCTTGGACTT
TGGTTTACTGGATTCTTTTATTTTACCATGGGTGGGTAACTGGGATCATTCTTGGTAATTC
TCGTGTATGCTGCTTACATGATCTACTTTGTGTGCTCATTTTCATTATGTTCTATCTATA
GGATTATCTTTTGTCTATTTGGGAGGTGTATTTATGATTTCATTAATTTTGGGCTTACACTA
AATAGTCATAGCTGGTATCGGAGTTTACATATGTTTATAGAGTAAATTTAGGTTTTCGA
CAKATTTCTCTGGGTGAGGGGATACCTCGAGGTACTCAGACTATCCGATTGTATCTGATA
TGAATAAAATTTCTCTCGGGGAGAAATTTAAGTGTATTTCTGTTATTTATTTATTTATTT
G

Amino Acid Sequence

IVSHLSSSEADKLEVFGLGMYANVTIGILQFIVNGHWFVNGVDTRAYFTSATKZIAVPTGE
KFPKSLATLQKSNKFSPLUMPTGFLFLFTNGLTGLLNGSSVGVCHDTYTVVWHPHYVLSK
GIZPAIVGVGYMPPLIGLTLNGLVSGFYIMPLGVNLTPFPQHLGLSNGPRYSQYPCYLR
WVKISSAGSILSVISVYFLFIK

Sequence Metadata

Genbank Accession: N/A
Translation Matrix: Invertebrate Mitochondrial
Last Updated: 2019-05-23 07:01:24.227816
Sequence Runsite: ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE

Fig. 3. DNA barcode of *Bemisia tabaci* infesting cassava

ADIC - Barcoding of *Aleurodicus dispersus* infesting cassava [ADIC]

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Process ID:	ADIC001-19
Identification:	Aleurodicus dispersus (Russell, 1965)
Identified by	
♀ Collected in	India, Kerala
by	
Institution Storing:	ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE
Field ID:	ADIC1
Museum ID:	ADIC2

9/25/2019

ADDITION-19: HOLD SYSTEMS

Sequence View for Process ID: ADIC001-19

Specimen Details Current

Sample ID:	ADIC
Process ID:	ADIC001-IS
Project:	ADIC
Tax Names:	Arthropoda, Insecta, Hemiptera, Aleyrodidae, Aleyrodinae, Aleyrodina, Aleyrodia depurata
Taxon:	Aleyrodia depurata
Rank Name:	species
Sampling Protocol:	N/A
BIN URI:	N/A
Kingdom:	Animalia

Marker Summary

Marker Code	Sequence Length	GC	Ambiguous	Trace Count
CC1-MP	1154	30.9%	0%	2

COL-5P ★

Illustrative Barcode



Nucleotide Sequence

[illegible]

Sequence Metadata

Genebank Accession:	N/A
Translation Matrix:	Invertebrate Mitochondrial
Last Updated:	2019-05-22 09:28:04.806645
Sequence Run(s):	ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE

Amino Acid Sequence

[illegible]

Fig. 4. DNA barcode of *Aleurodicus disperses* infesting cassava

BTISP - Barcoding of whitefly, *Bemisia tabaci* infesting sweet potato [BTISP]



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Process ID:	BTISP001-19
Identification:	<i>Bemisia tabaci</i> (Gennadius, 1889)
Identified by	
♀ Collected in	India, Kerala
by	Hizaana Noushad
Institution Storing:	ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE
Field ID:	BTISP1
Museum ID:	BTISP2

6/23/2019

BT/SP001-19 | BOLD SYSTEMS

Sequence View for Process ID: BTISP001-19

Specimen Details Current

Sample ID:	BTGP
Process ID:	BTGP001-19
Project:	BTGP
Tax Name:	Arthropoda, Insecta, Hemiptera, Aleyrodidae, Aleyrodinae, Bemisia, Bemisia tabaci
Taxon:	Bemisia tabaci
Rank Name:	species
Sampling Protocol:	N/A
BIN URI:	N/A
Kingdom:	Animalia

Marker Summary

Marker Code	Sequence Length	GC	Ambiguous	Trace Count
OC1-SP	1026	56.3%	0%	2

COL-5P *

Illustrative Barcode



Nucleotide Sequence

[illegible]

Amino Acid Sequence

[illegible]

Sequence Metadata

Genbank Accession:	N/A
Translation Matrix:	Invertebrate Mitochondrial
Last Updated:	2019-05-23 06:07:16.403590
Sequence Run(s):	ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE

Fig. 5. DNA barcode of *Bemisia tabaci* infesting sweet potato

ARIA - Barcoding of *Aleurodicus rugioperculatus* infesting arrowroot [ARIA]



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Process ID: ARIA001-19
Identification: *Aleurodicus rugioperculatus*
Identified by:
Collected in: India, Kerala
by: Hizaana Noushad
Institution Storing: ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE
Field ID: ARIA1
Museum ID: ARIA2

5/23/2019

ARIA001-19 | BOLDSYSTEMS

Sequence View for Process ID: ARIA001-19

Specimen Details Current

Sample ID: ARIA
Process ID: ARIA001-19
Project: ARIA
Tax Names: Arthropoda, Insecta, Hemiptera, Aleyrodidae, Aleurodicinae, Aleurodicus, *Aleurodicus rugioperculatus*
Taxon: *Aleurodicus rugioperculatus*
Rank Name: species
Sampling Protocol: N/A
BIN URI: N/A
Kingdom: Animals

Marker Summary

Marker Code	Sequence Length	GC	Ambiguous	Trace Count
COI-5P	287	23.7%	0%	2

COI-5P

Illustrative Barcode



Nucleotide Sequence

TATTGGTCTCTATAAGATTATTAATTCGATAGAAATTAAGAATATTAGGAAGTTATATAATAA
TGATCAGTTATATAACATTAGTACATGCTTTTATTATAATTTTATTATACTATACC
TTTAGTTATTGGTGGATTGGAAATTGATTAGTTCCTTAATAATTGGAGCTCCTGATATAGCTTT
TCCTCGTATAAATAATTAAAGTTTGGATTATTAATTCCTCTTTGTTATTTTAATTTTAAGAAT
ATTAATTTCAAGAGGGGGGGGAA

Amino Acid Sequence

IGASMSLLIRHLSPLGSGYNNQQLYNTLVTSNAFIMIFFMTHPLVIGGFGNWLVLNIGAPOMAF
PRMNNLSFWLLIPSLFLILSMLTSSGGGX

Sequence Metadata

Genbank Accession: N/A
Translation Matrix: Invertebrate Mitochondrial
Last Updated: 2019-05-23 01:30:35.13988
Sequence Runsite: ICAR-CENTRAL TUBER CROPS RESEARCH INSTITUTE

Fig. 6. DNA barcode of *Aleurodicus rugioperculatus* infesting arrowroot

Declaration

The authors declare no conflicts of interest.

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