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Molecular Characterization of Cryptic Species of *Bemisia tabaci* Associated with Cucumber in Eastern Dry Zone of Karnataka

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Abstract

The whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: *Aleyrodidae*) is posing major havoc on vegetable production jeopardizing Indian agriculture. During, April 2023, whitefly, *B. tabaci* sample was collected from the cucumber fields of Muthagadahalli village in the Bengaluru North taluk, Bengaluru district of Karnataka. The total genomic DNA was extracted from a single adult whitefly using the Chelex 100 method. The identification of whitefly cryptic species was performed by amplifying the mitochondrial cytochrome oxidase subunit one (*mtCOI*) gene using gene-specific primers of *B. tabaci.* PCR results revealed expected amplificon size of 800 bp. Subsequently, the PCR product was subjected to Sanger sequencing. The nucleotide (nt) sequence of the whitefly sample collected from Muthagadahalli exhibited 98-100 per cent identity with sequences belonging to Asia I whitefly cryptic species known to infect different crops worldwide. The sequence demarcation graph and phylogenetic analysis provided substantial evidence for the present findings.

Keywords : Molecular characterisation, White fly, Cucumber, Sequence analysis

Cucumber (*Cucumis sativus* L.) belonging to the *Cucurbitaceae* family is a popular and extensively grown warm-season vegetable crop across the world. It was known to be originated in India and widely grown in the tropical and subtropical regions of the country. It is regarded to be one of the oldest vegetable crops, having been farmed for over 3000 years in India (De Candole, 1982) and is a global vegetable crop farmed for its immature fruits, which are consumed fresh as salads and can also be cooked as vegetables, processed or pickled. The fruits of cucumber are also used as an astringent and antipyretics (Harshitha and Shyamalamma, 2021). *Cucurbitaceae* family is known to be comprised of 118 genera and 825 species out of which India is home to 36 genera and 100 species (Christenhusz and Byng, 2016).

The total area and production of cucumber in India are 119.1 thousand hectares and 1694.2 thousand metric tonnes, respectively (Anonymous, 2022). Andhra Pradesh, Assam, Bihar, Jammu and Kashmir, Karnataka, and Telangana states contribute more than 80 per cent of total output in India. In Karnataka, cucumber covers an area of 8.61 thousand hectares and produces 124.6 thousand metric (Anonymous, 2022). The major cucumber-growing districts of Karnataka State include Belagavi, Haveri, Mandya, Hassan, Chikkaballapur, Bagalkote, Dharwad, Mysuru and Ramanagara districts.

The production and quality of cucumber are hampered by many pests and diseases. Thrips, whiteflies and aphids are sucking insect pests that not only cause direct damage to plants but also serve as significant vectors for the transmission of plant viruses, resulting in disastrous impact on various agricultural crops. Viruses belonging to the genus Begomovirus, Potyvirus, Nepovirus, Polerovirus, Cucumovirus, Tymovirus and Tobamovirus are the potential threats to cucurbits cultivation worldwide (Nagendran *et al.,* 2017). Depending on the kind of cucurbit crop being grown and the prevailing season in various regions of the world, the amount of yield loss from the pest to cucurbitaceous vegetables ranged from 30 to 100 per cent (Dhillon *et al.,* 2005).

The whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: *Aleyrodidae*) is an established polyphagous sucking pest of the tropical and subtropical zones of the world which is prevalently reported in Europe, Asia, Africa, North, Central and South America and Oceania. The plant becomes weaker and dries as a result of the adult and nymphs sucking the cell sap from the phloem and also by secreting honeydew (Das *et al*., 2017). *B. tabaci* is also a vector of several viruses, of which, begomoviruses stand out as the most abundant and economically most significant group (Rajeswari and Reddy, 2014). Reportedly, *B. tabaci* is a complex species comprised of 46 cryptic species worldwide (De barro *et al.,* 2011 and Rehman *et al*., 2021). Despite the absence of morphological differences, cryptic species can be accountably distinguished in their genetics, development and behavioural characteristics. The individual cryptic species within the complex differ in their capacity to adapt to different hosts, resistance to chemical treatments, degree of fecundity and notably, in their ability to transmit the viruses. Owing to these factors, various molecular methods have been applied over the past two decades to delimit the members of the *B. tabaci* species complex. The most often used technique in recent years has been based on mitochondrial cytochrome oxidase gene subunit-1 (*mtCOI*). In the present study, the *mtCOI* gene of whitefly species, *B. tabaci* associated with cucumber crop was analysed and the identification of cryptic species was determined through *mtCOI* gene sequence analysis.

Material and Methods

Source of Whiteflies

Adult whiteflies (*B. tabaci*) were collected from a cucumber field using hand held aspirator and transferred to a 1.5 mL eppendorf microcentrifuge tube containing 70 per cent ethanol. Parafilm was used to adequately seal the eppendorf microcentrifuge tube and the sample was labelled with the sample number, location, date of collection, host and additional details of the samples were given in the Table 1. The remaining sample was taken to the laboratory and stored at 4 ^{0}C until further processing. The sample was designated as K54 Muthagadahalli isolate.

Table 1

Details of the whitefly sample collected

Sample detail	
Sample name	K54
Place of collection	Muthagadahalli, Bangalore North taluk, Bangalore District
Host	Cucumber
GPS coordinates	13.186456N,77.672008E
Year of collection	2023
Age of the crop (days)	70 days
Cropping system	Monocropping
Area (Acre)	1.5
Surrounding crops	Maize,Chilli,Crossandra

Extraction of DNA and PCR Amplification of *mt*COI gene

The total DNA from whitefly, *B. tabaci* sample was extracted by modified Chelex 100 method as described earlier by Rua *et al*. (2006). Using a camel hair brush, single whitefly was picked up from a collecting tube and placed on a piece of parafilm to speed up the ethanol's evaporation. Following this, flies were transferred to a petridish and subjected to single rinse with sodium hypochlorite (0.1%) and two subsequent rinses with sterile distilled water (SDW). A single whitefly was transferred to a 1.5 mL microcentrifuge

tube. Each whitefly was homogenized in 100 µL TE buffer solution containing 5 per cent Chelex 100 resin and 300 µg Proteinase K. The homogenised sample was incubated at 60 ^{0}C for three hours, followed by protein denaturation at 96 ^{0}C for 10 minutes. Further, the homogenised sample was centrifuged for 10 minutes at 13,000 rpm. The resulting upper aqueous supernatant, with DNA was carefully transferred into a new tube and stored at -20 ^{0}C. The DNA sample was subjected to PCR using the *mtCOI* gene-specific primers to *B. tabaci.* The details of the primer used, PCR cyclic conditions and expected amplicon size are given in the Table 2 and the components of PCR mixture are provided in Table 3. Four microliters of PCR product was electrophoresed on one per cent agarose gel stained with ethidium bromide and visualized under gel documentation system. The amplified PCR product of *mtCOI* gene specific primers was purified from agarose gel using QIA quick gel extraction kit (Qiagen, Hilder, USA) and purified sample was bidirectionally sequenced at Barcode Biosciences Pvt. Ltd., Bangalore, India.

TABLE 2

Details of *mtCOI* gene specific primers and PCR conditions used in the current study

Primer sequences (5' to 3')	PCR Cycles	Product size (bp)
F-TTGATTTTT TGGTCATCCA GAAGTR-TCCA ATGCACTAATC TGCCATATTA	Initial denaturation : 94ºC for 1min. Denaturation : 94ºC for 1 min. Annealing : 55ºC for 1 min. Extension : 72ºC for 1 min. Final extension : 72ºC for15 min. Number of cycles: 35	800 bp

TABLE 3

Different components used in PCR

Sterile distilled water	17.7µL
10 x PCR buffer	2.5 µL
25 mM $MgCl_2$	1.5 µL
2.5 mM dNTP mixture	0.5 µL
Primer-F (10 mM)	0.625µL
Primer-R (10 mM)	0.625µL
Taq polymerase (5 units/iL)	0.3 µL
Template DNA (100 ng)	1.25 µL
Total	25.0 µL

Sequence Analysis

The *mtCOI* gene sequence of K54 Muthagadahalli isolate obtained after sequencing was subjected to BLASTn analysis to retrieve the similar sequences in the National Center for Biotechnology Information (NCBI) database. The sequences showing maximum percent identity with *mtCOI* gene sequence of K54 Muthagadahalli isolate were retrieved from the NCBI database and aligned using the BioEdit program (Hall, 1999). The sequences with maximum similarity were retrieved from Gen Bank to calculate pairwise per cent identity between *B. tabaci* K54 Muthagadahalli isolate and the retrieved sequences using Sequence Demarcation Tool version 1.2 (SDTv1.2). Phylogenetic tree was constructed using the Neighbor-Joining method with 1000 boot strapped replications in MEGA X software to study the relations among different cryptic species of *B. tabaci* reported so far (Kumar *et al.,* 2016).

RESULTS AND DISCUSSION

The isolated genomic DNA of *B. tabaci* (K54 Muthagadahalli) was subjected to PCR for the amplification of *mtCOI* gene using specific primers (Dinsdale *et al*., 2010, Himler *et al*., 2011; Ashwathappa *et al*., 2020). The 800 bp PCR amplification product (Fig.1) was sequenced

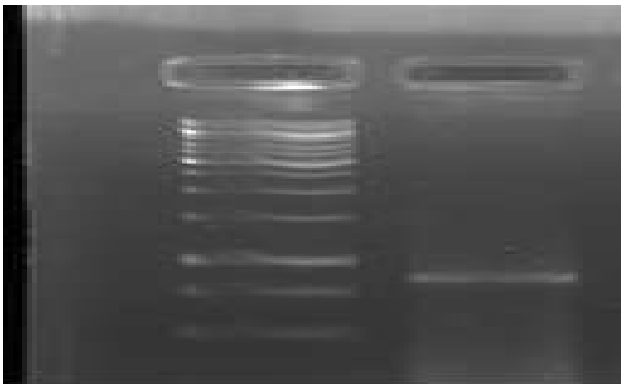

Fig. 1 : PCR amplification of *mtCOI* gene of *B. tabaci* K54 Muthagadahalli isolate using specific primers

bi-directionally and the consensus sequences (Accession number OR523367) was deposited in GenBank. The *mtCOI* gene sequence of *B. tabaci* K54 Muthagadahalli isolate was compared with the corresponding region of 39 different cryptic species of whiteflies retrieved from the NCBI database. Sequence comparison results demonstrated that the current *B. tabaci* Muthagadahalli isolate *mtCOI* gene sequence shared 98 to 100 per cent identity with sequences of Asia I cryptic species reported earlier from Pakistan (HG315654, HF934996), Thailand (KR110117, AF164671) and other parts of India (AJ748370) (Table 4). Hence, the *B. tabaci* Muthagadahalli population collected was designated to be Asia I cryptic species. *B. tabaci* K54 Muthagadahalli isolate was also compared with 20 additional *B. tabaci mtCOI* gene sequences of other cryptic species that were obtained from the NCBI database using SDTv1.2. The pairwise identity of query sequence with retrieved consensus sequences used are provided in Table 6. The phylogenetic analysis of *mt*COI gene sequences with selected reference cryptic species (Table 5) indicated that the

TABLE 4

Nucleotide sequence similarity of *B. tabaci* K54 Muthagadahalli isolate with selected *mtCOI* gene nucleotide reference sequences

Cryptic species	Reference sequence Accession No.	Maximum nucleotide similarity (%)
Asia I	HF934996	100
Asia I	KR110117	98.3
Asia I	HG315654	99.8
Asia I	AJ748370	98.9
Asia I	AF164671	98.8

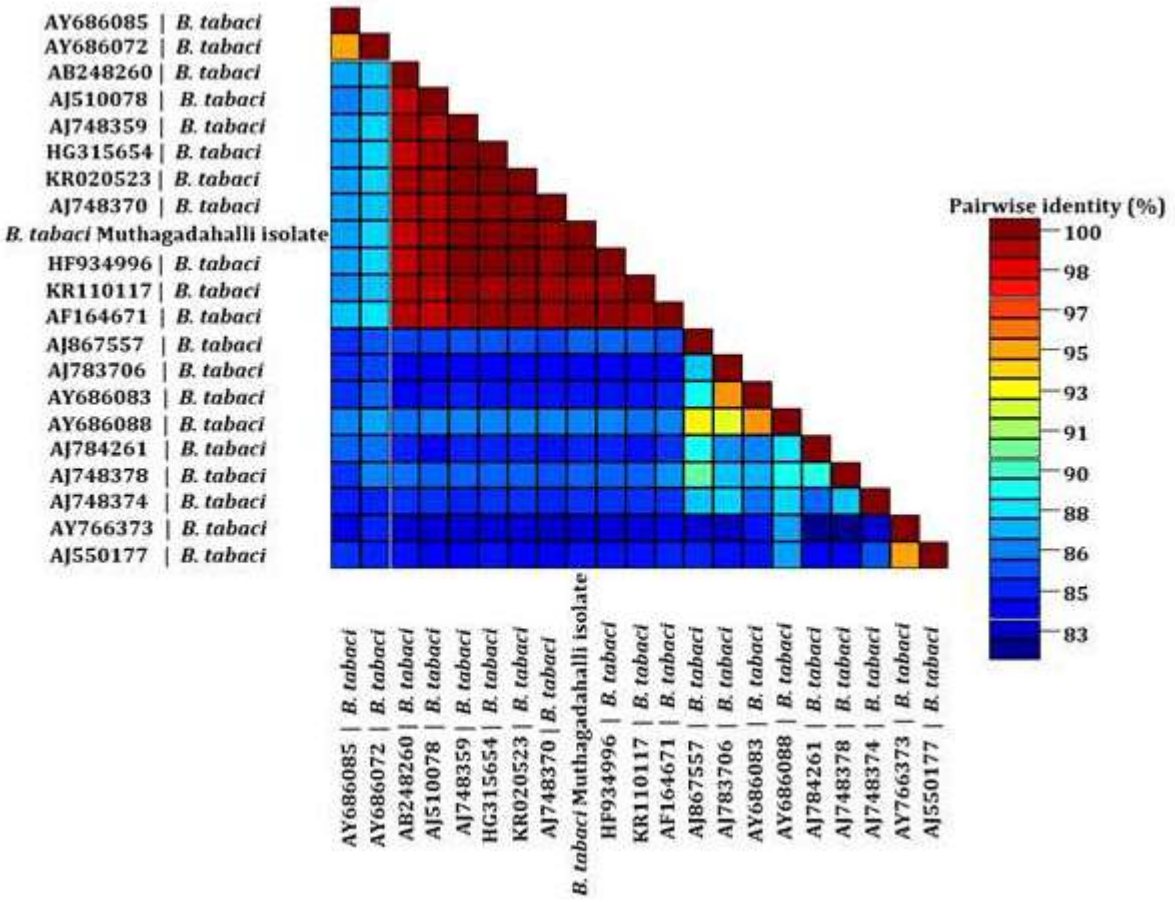

Fig. 2 : Graphical representation of percentage pairwise genomic scores and nucleotide identity plot of *B. tabaci* K54 Muthagadahalli isolate collected from cucumber plot compared with reference

TABLE 5

The *mtCOI* gene sequences of *B. tabaci* cryptic species employed in the phylogenetic analyses

Accession Number	Organism	Country	Genetic sub group of reference whitefly
HF934996	*B. tabaci*	Pakistan	Asia I
KR110117	*B. tabaci*	Thailand	Asia I
HG315654	*B. tabaci*	Pakistan	Asia I
KR020523	*B. tabaci*	India	Asia I
AF164671	*B. tabaci*	Thailand	Asia I
AJ748370	*B. tabaci*	India	Asia I
AJ748359	*B. tabaci*	India	Asia I
AJ510078	*B. tabaci*	Pakistan	Asia I
AB248260	*B. tabaci*	Indonesia	Asia I
AJ867557	*B. tabaci*	China	Asia II-1
AJ783706	*B. tabaci*	China	Asia II-3
AY686083	*B. tabaci*	China	Asia II-4
AY686088	*B. tabaci*	China	Asia II-2
AJ784261	*B. tabaci*	China	Asia II-6
AJ748378	*B. tabaci*	India	Asia II-7
AJ748374	*B. tabaci*	India	Asia II-8
AY686085	*B. tabaci*	China	China-1
AY686072	*B. tabaci*	China	China-2
AY766373	*B. tabaci*	Israel	Middle East Asia Minor-1 (MEAM-1)
AJ550177	*B. tabaci*	Reunion	MEAM-2
AY827598	*B. tabaci*	Italy	-
AY057181	*B. tabaci*	Uganda	SubsabAf 1
AF344257	*B. tabaci*	Cameroon	SubsabAf 3
AF344249	*B. tabaci*	Sub-Saharan Africa	SubsabAf 4
AJ550167	*B. tabaci*	Colombia	New world
AF418665	*B. tabaci*	Uganda	-
AB308116	*B. tabaci*	Japan	JPL
EU192050	*B. tabaci*	China	China 3
GU086326	*B. tabaci*	Indonesia	Aust/ Indonesia
HM137313	*B. tabaci*	China	AsiaII-9
HM137356	*B. tabaci*	China	AsiaII-10
JF901836	*B. tabaci*	Argentina	New World-2
HQ622855	*B. tabaci*	Seychelles	Indian Ocean
GU220056	*B. sub-decipiens*	Spain	-
AF418673	*B. afer*	Uganda	-
AJ842039	*B. afer*	Tanzania	Zanzibar 5
AY057220	*B. tuber-culata*	Africa	-
GU086363	*B. atriplex*	Spain	-
AJ550183	*Trialeurodes vaporariorum*	Reunion	-

Table 5 Continued

TABLE 6

Per cent nucleotide identity of *B. tabaci* K54 Muthagadahalli isolate with *mt*COI gene nucleotide sequences of cryptic species retrieved from NCBI

Accession Number	Per cent nucleotide identity	Accession Number	Per cent nucleotide identity
HF934996	100	AY827598	80.1
KR110117	98.3	AY057181	72.3
HG315654	99.8	AF344257	78.4
KR020523	96.2	AF344249	75.9
AF164671	98.8	AJ550167	82.4
AJ748370	98.9	AF418665	77.8
AJ748359	96	AB308116	77
AJ510078	95.2	EU192050	84.2
AB248260	96.1	GU086326	68.1
AJ867557	85.9	HM137313	76.9
AJ783706	77.6	HM137356	78.5
AY686083	76.9	JF901836	64.4
AY686088	78.4	HQ622855	45.2
AJ784261	78.4	GU220056	61.2
AJ748378	83.2	AF418673	70.2
AJ748374	82.4	AJ842039	52.3
AY686085	78.9	AY057220	70.9
AY686072	79.6	GU086363	66.8
AY766373	78	AJ550183	68.3
AJ550177	82.2		

K54 Muthagadahalli isolate was closely clustering with Asia I cryptic species of *B. tabaci* (Fig. 3).

Same line of work was carried out by Reddy *et al*. (2012), who employed RAPD-PCR and identified the presence of Asia I, Asia II-5, Asia II-7, Asia II-8 and MEAM-1 cryptic species from the whitefly samples collected from 31 locations of India. Based on *mtCOI* gene sequences, cryptic species of whitefly including MEAM-1, Asia I, Asia II-1, Asia II-5, Asia II-7, Asia II-8 and Asia II-11 were also reported (Ellango *et al*., 2015 and Prasanna *et al*., 2015) Further more, the current study's results were reinforced by the outcomes reported by Acharya *et al.* (2020), wherein they identified three cryptic species, namely, Asia I, Asia II-1 and Asia II-5, with considerable inter-specific but minimal intra-specific variation. A previous survey carried out by Sujatha *et al.* (2021) in tomato fields of Tippuru village, Bengaluru Rural district reported the presence of Asia II-5. Similarly, present results were corroborated with previous studies conducted by Roopa *et al.* (2015) who analysed 71 samples of *B. tabaci* to determine the prevalence of various genetic groups (Asia I, AsiaII-7, Asia II-8 and

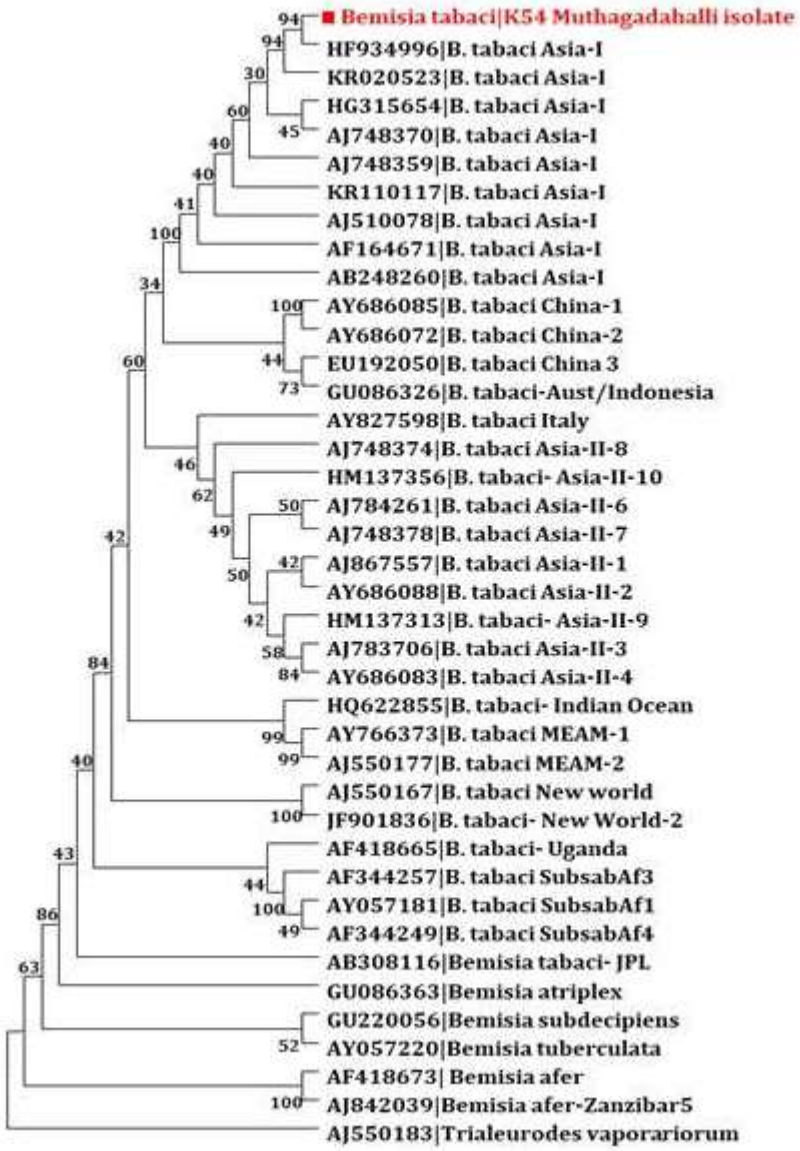

Fig. 3 : Neighbor-Joining tree constructed with *mtCOI* gene nucleotide sequences depicting the phylogenetic relationship of *B. tabaci* K54 Muthagadahalli isolate with other cryptic species of *B. tabaci*

MEAM-1) on various host plants in India and results of Venkataravanappa *et al*. (2023) who reported four cryptic species *viz.,* Asia I, China-3, Asia II-5 and Asia II-1 in various vegetable crops *viz.,* tomato, squash, brinjal, pointed gourd, cucumber and watermelon in two areas of Uttar Pradesh.

This geographical dominance of different cryptic species is governed by many variables, including fertility, egg-to-adult survival, virus transmission efficiency and most critically, pesticide resistance and parasite sensitivity that affect the whitefly population's ability to survive and reproduce. It is reported that various pesticide resistance levels can lead to the redeployment and displacement of particular populations of whiteflies (Crowder *et al*., 2010). The efficiency of transmitting viruses was studied where Asia I cryptic species females were found to be more efficient transmitters (86.6%) of Chilli Leaf Curl Virus, a begomovirus, than males (53.0%) (Gunda *et al.,* 2021). This study consolidates our understanding of the species composition of *B. tabaci* from Muthagadahalli village in Bengaluru North taluk, Bengaluru district, Karnataka. The results of the PCR amplification, the *mtCOI*-based pairwise nucleotide identity analysis and the phylogenetic analysis, provided confirmation for the presence of Asia I cryptic species in Muthagadahalli village, Bengaluru North taluk, Bengaluru district, Karnataka. The data produced here could be valuable for tracking changes in the cryptic species abundance and displacement patterns in the future. It would be intriguing to carry out more thorough surveys in this area to determine whether the species composition of *B. tabaci* cryptic species is greater than what has been previously recorded. Further, it is necessary to identify the cryptic species in different regions of the Karnataka State, as well as to understand the interactions between the virus, and associated symbionts. New emerging approaches such as genomics, proteomics, metabolomics and transcriptomics will open up new avenues for unravelling the complex interactions that occur during virus transmission by vector insects.

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