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HOST SPECIFIC MOLECULAR VARIATIONS IN *BEMISIA TABACI* (G.) AS REVEALED BY USING MITOCHONDRIAL AND RIBOSOMAL MARKER

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ABSTRACT

Bemisia tabaci is globally important pest and also vector of Gemini viruses on various economically important crops worldwide. Presently there are lots of debates going on the species status and cryptic nature of this species which is complex as challenging. Therefore, in this study we analysed the genetic diversity of *B. tabaci* (18 samples collected on various host plants). The diversity analysis was performed using two well known markers such as mitochondrial COI gene and the ribosomal ITS1. The phylogenetic trees were constructed separately for two dataset using Neighbor-Joining method. Our result confirmed presence of four putative species of *B. tabaci* such as Asia I, Asia II 1, Asia II 5 and Asia II 8 in India. The Asia I genetic group found most widely distributed and shows relatively polyphagus, which has mtCOI consensus sequence identity of 84.32% to 86.76% with Asia II sub groups. Our work has shown the genetic boundary of *B. tabaci* which helped in understanding host specificity across Karnataka, India.

INTRODUCTION

The whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a widely distributed polyphagous pest infesting wide range crops in India. It was first recorded on cotton in the year 1905 in India (Misra and Lambda, 1929). It assumed serious proportion during last two decades necessitating intensive research in the management of the same. It is of particular concern, because it can rapidly increase in numbers which causes direct feeding damage, and also an important vector of plant viruses. In this regard, *B. tabaci* transmitted Cotton Leaf Curl virus (CLCuV), Mungbean Yellow Mosaic virus (MYMV) and other Begomoviruses that can reduce the vigor and growth of the plant (Jones, 2003; PalashChandrapaul *et al.,* 2013). Incidentally *B. tabaci* has developed high level of resistant to insecticides. *B. tabaci* populations display reproductive incompatibilities and a large amount of biological, physiological and genetic variation which has led to the characterization of numerous biotypes within the species complex (Perring, 2001). However, it was difficult to differentiate the *B. tabaci* species complex by morphological characters, which could otherwise be resolved by molecular methods. In this regard, merely 28 putative species were reported worldwide so far (Boykins *et al.*, 2007). Genetic ways of species analyses exploit the diversity among DNA sequences to distinguish species complex. DNA sequences data from mitochondrial and ribosomal gene are increasingly being used to estimate phylogenetic relationships and for elucidating inter and intra species variations in insects (Ellango *et al.*, 2012). Particularly, Mitochondrial COI gene has been extensively used as a molecular marker to identify *B. tabaci* species variants that exhibits rich biological differences. Similarly, ITS1 which evolves at a much higher rate and are highly variable (De Barro *et al.,* 2000) is also employed to understand the genetic structure. Both mitochondrial and nuclear datasets provide an opportunity to examine across a broad array of *B. tabaci* genetic groups.

Therefore, studies were carried out to identify the presence of various genetic groups of *B. tabaci* species, Perring (2001) described I, G and H biotypes using esterase profile; Banks *et al*. (2001) and Rekha *et al*. (2005) discovered B-biotype in South India; Lisha *et al*. (2003) described the cassava and sweet potato whitefly population; Meena et al., (2013) reported *B. tabaci* on Chili and recently Chowda-reddy *et al*. (2012) found a new genetic group Asia I-India. Indeed to ensure the current status of distribution of *Bemisia tabaci* on horticultural crops and other crops, we analyzed the *B. tabaci* samples collected from four different host plant families, Solanaceae (Tomato and Brinjal); Malvaceae (Cotton and Okra); Fabaceae (Cowpea); and Asteraceae (Sunflower) across Karnataka using both mitochondrial and nuclear marker.

MATERIALS AND METHODS

Sample collection and DNA Extraction

A total of 18 *B. tabaci* samples were collected on 6 different

host plant species that belong to four plant families from various locations of Karnataka (India) (Table 1) using a hand-held aspirator and preserved immediately in 95% ethanol and stored at 4°C for future use. From all 18 samples, we took three specimens each for the marker analysis. Total genomic DNA was extracted from individual adults. The specimens were washed briefly in sterile distilled water to remove alcohol prior to homogenization. The grinding was carried out in 0.5 mL microcentrifuge tube containing 15 μL of the lysis buffer (10 mM Tris pH 8.4, 1 mM EDTA, 0.30% Triton X-100 and 60 μg/mL proteinase K). The homogenate was then incubated at 65°C for 15 min and at 95°C for 5 min to inactivate the proteinase K. Spin the tube at 8000 rpm for 5 minutes and collect the supernatant to new tube. The samples were then stored at -20°C for future use.

Mitochondrial COI gene amplification

A portion of mitochondrial COI gene (approx 850bp) fragment was amplified in a 25-ul reaction mix, containing 1X PCR buffer (10 mM Tris-HCl pH 8.0), 2.5 mM MgCl2, 0.2 mM dNTPs, 10 pmole each of Forward and reverse primers [C1 - J -2195 (forward) 5′ - TTGATTTTTT GGTCATCCAGAAGT- 3′ and TL2 - N-3014 (Reverse) 5′-TCCAATGCACTAATCTGCCATATTA - 3′ (Simon *et al.*, 1994)], 1.25U *Taq* DNA polymerase (Bioline, UK) and 100ng of DNA template. PCR was performed in Veriti 96well thermo-cycler (Life technologies-AB, USA) according to the following cycling condition: Initial denaturation at 94°C for 3 min,followed by 35 cycles of 94°C for 30 sec; annealing temperature 54°C for 40 sec; extension at 72°C for 50 sec and final extension at 72°C for 10 min to extend the incomplete fragment.

Ribosomal ITS 1 amplification

Approximately 500bp fragment, spanning from the 3′ end of the 18S rRNA to the 5.8S rRNA gene, including the ITS1 (Internal transcribed spacer 1) in between, were PCR amplified in a 25-uL reaction mix, contains 1X PCR buffer (10 mM Tris-HCl pH 8.0), 2.5 mM MgCl2, 0.2 mM dNTPs, 10 pmole each of forward and reverse primers [TW81 (forward) 5′-GTTTCCGTAGGTGAACCTGC-3′ & B.tabaci specific 5.8R (Reverse) 5′-ATCCGCGAGCCGAGTGATCC-3′], 7% dimethyl sulfoxide, 1.25U *Taq* DNA polymerase (Bioline, UK) and 100ng of DNA template. PCR was performed in Veriti 96well thermo-cycler (Life technologies-AB, USA) according to the following cycling condition: Initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec; annealing temperature 54°C for 40 sec; extension at 72°C for 40 sec and final extension at 72°C for 10 min to extend incomplete fragment.

PCR products were visualized in 1.2% agarose gel. The expected band was eluted using PCR clean-up gel extraction kit (Macherey-Nagel, Germany). Purified PCR products were cloned using pTZ57-T/A plasmid vector system and the vector was transformed into *Escherichia coli* DH5α according to the manufacture protocol (Fermentas life science, USA). The plasmid DNA was isolated from three randomly selected clones selected from each samples using GeneJET Plasmid miniprep kit (Fermentas life science, USA). Presence of the insert was checked by colony PCR using gene specific primer and visualized in 1.2% agarose gel. The sequencing was performed using M13 universal primers at Eurofins MWG Operon, India. The sequence homology was determined using BLASTn (http://www.ncbi.nlm.nih.gov), and the selected sequences were edited by manual using the sequence alignment editor 'BioEdit' version 7.0. A total of 36 sequences each of mitochondrial COI and ribosomal ITS1 were deposited in GenBank-NCBI (Table 1).

Phylogenetic analyses

Phylogenetic analyses were carried out for two separate datasets on mitochondrial and nuclear marker. The sequences were aligned separately in CLUSTAL W program. Independent alignment was carried out for each taxon sample, resulting in marker dataset. Neighbor-Joining method was used to construct phylogenetic tree with kimura-2-parameter model. In addition, the number of substitutions, Transition (Ti)/ Transversion (Tv) ratio and nucleotide compositions for mitochondrial COI and ribosomal ITS1 were also determined using MEGA version 5.0.

RESULTS

The mitochondrial COI gene (657bp consensus sequence) was analyzed to check genetic variation among the *B. tabaci* population in India. All mitochondrial COI sequences were translated into amino acids before the sequence analysis to check for stop codon; which showed no stop codon suggesting all are functional copies. The complete data set of mtCOI DNA sequence shows considerable variation among *B. tabaci* samples. There were a total of 162 polymorphic sites, of which 49 were singletons and 113 were parsimony informative sites. The mtCOI gene nucleotide frequencies were 24.22% (A), 43.42% (T), 18.70% (C), and 13.66% (G). The maximum composite likelihood (MCL) estimate of the pattern of nucleotide substitution with overall transition/transversion bias is R = 3.137 (Table 2a). The phylogenetic tree based on Neighbor-Joining method with bootstrap support (1000 replicates) resulted in a consensus tree with well supported nodes were shown in Fig. 1. The *B. tabaci* mtCOI reference sequence belong to Asia I, Asia II and Middle East-Asia Minor, genetic group based on the report of Boykins *et al.* (2007) were drawn from GenBank as reference sequences. The COI sequence of *Bemisia afer* (Priesner and Hosny) (AF418673) and *Bemisia berbericola* (Cockerell) (FN821792) were used as outgroup. Among the 18 Asian putative species Asia I, Asia II-1, Asia II-5, and Asia II-8 were found in this study. The mtCOI sequence identity of these phylogenetic groups was listed in Table 3.

The analysis of the ITS1 rDNA region was also performed in an attempt to obtain another method for the identification of population variations in *B. tabaci*. The ITS1 rDNA region is a conserved region and is useful in distinguishing recently divergent taxa especially species like *B. tabaci* (De Barro *et al.*, 2000; Boykin *et al.*, 2007). The ITS1 sequence length ranged from 496 to 502 bases. In the alignment 470 sites are constant and there were only 22 parsimony informative sites. The ITS1 nucleotide frequencies are 14.76% (A), 11.75% (T/U), 35.56% (C) and 37.93% (G). The MCL estimate of the pattern of nucleotide substitution with overall transition/transversion bias is R = 0.716 (Table 2b). The *B. tabaci* ITS1 sequence belong to Asia I, unresolved Asia II genetic group and other genetic

Table 1: *Bemisia tabaci* sample details

S.no/ID	Locations	Host Plant	mtCOI	ITS1
KA01	Hessaraghatta	Tomato	JQ995265	JQ995269
KA02	Hessaraghatta	Brinjal	JN410793	JN410680
KA03	Hebbal	Cotton	JQ995266	JQ995270
KA04	Hosapete	Tomato	JN410807	JN410700
KA05	Irakumpalli	Cotton	JN410802	JN410699
KA06	Agrahara	Brinjal	JN410792	JN410679
KA07	Tadkod	Cotton	JN410806	HQ446127
KA08	Budurkatti	Brinjal	HM590152	HQ446152
KA09	Nayanagar	Cowpea	HM590178	JN410708
KA10	Auradhi	Brinjal	HM590153	HQ446117
KA11	Devlinkopa	Brinjal	HM590165	HQ446118
KA12	Dumawada	Tomato	HM590181	HQ446153
KA13	Arvani	Cowpea	HM590184	HQ446128
KA14	Palacrosshossanagar	Okhara	HM590185	HQ446125
KA15	Hobbali	Cotton	HM590186	HQ446121
KA16	Therlapur	Sunflower	HM590169	HQ446123
KA17	Yammanur	Cotton	HM590167	HQ446122
KA18	Saunsi	Cotton	HM590188	HQ446130

Table 2: Maximum composite likelihood estimate of the pattern of nucleotide substitution from different populations of *Bemisia tabaci.*

a) mtCOI sequence

	A	T	C	G
A	-	*4.64*	*1.46*	13.69
T	*2.59*	-	11.29	*2*
C	*2.59*	35.9	-	*2*
G	17.73	*4.64*	*1.46*	-

b) ITS1 sequence

	A	T	C	G
A	-	*3.06*	*9.89*	18.29
T	*3.85*	-	16.76	*9.27*
C	*3.85*	5.19	-	*9.27*
G	7.59	*3.06*	*9.89*	-

Each entry shows the probability of substitution (r) from one base (row) to another base (column) [1]. For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in *italics*. Evolutionary analyses were conducted in MEGA 5.

Table 3: Mitochondrial COI Sequence identities of the four *B. tabaci* phylogenetic groups

	Asia I	Asia II 1	Asia II 5	Asia II 8
Asia I	98.48% - 99.70%			
Asia II 1	85.69% - 86.61%	100%		
Asia II 5	84.32% - 85.24%	90.26% - 100%	100%	
Asia II 8	85.39% - 86.76%	86.91% - 87.52%	87.82% - 88.28%	99.09% - 99.85%

groups based on the report of Boykins *et al.* (2007) were downloaded from GenBank as reference sequences. The ITS1 sequence of NAW - New Australian Whitefly, also known as *Lipaleyrodes atriplex* (Froggatt) (AF213988) was used as outgroup. According to the tree topology, the results indicate that *B. tabaci* population from all the locations falls into two major clades, Asia I and Asia II genetic group (Fig. 2).

DISCUSSION

A detailed analysis of molecular data offer an opportunity to consider more closely the question of where species level separation may lie within *B. tabaci* (Dinsdale *et al.,* 2010). Above stated terms are openly accepted in the case of *B. tabaci* population collected in Karnataka on various host plant species, which were segregated into four genetic groups, called Asia I, Asia II-1, Asia II-5 and Asia II-8.

Analysis of nuclear DNA sequence (ITS1 region) resulted the different clusters with lack of clarity in Asian genetic groups as compared to mitochondrial phylogenetic tree. Diversity among the populations were the most determine factors in the partitioning of the total genetic diversity. Particularly, indigenous Asia I population shows inter genetic diversity range from 0.30-1.52%, which is higher than Asia II-8 genetic group has 0.15-0.91% comparably. All the populations recorded here had less than 3.5% sequence divergence from the other *B. tabaci* mtCOI consensus sequences (Dinsdale *et al.,* 2010).

Distribution of the different indigenous species in are shown on the tree, especially Asia I were widely spread on different part of Karnataka, about 61% (11 of the 18) samples belong to this genetic group. Asia II-1 was found in the specimens collected from Irakumpalli, but in previous studies they prescribed that it was found only in northern part of India, which spread from Pakistan and China (Simon *et al.*, 2003; Singh *et al.,* 2012). According to our result, the current status of distribution pattern of native Asia II-8 group was found only in North West region of Karnataka, and Asia II-5 was found in Hosapete. The MEAM1 (previously B-biotype) and Asia II-7 genetic groups was not found in our survey, but it was previously reported in Karnataka on tomato, okra, bean and cotton (Rekha *et al.,* 2005; Chowda-Reddy *et al.*, 2012). This may have been due to the limited number of mtCOI sequences that were examined.

The acceptance of certain plant species by *B. tabaci* can vary

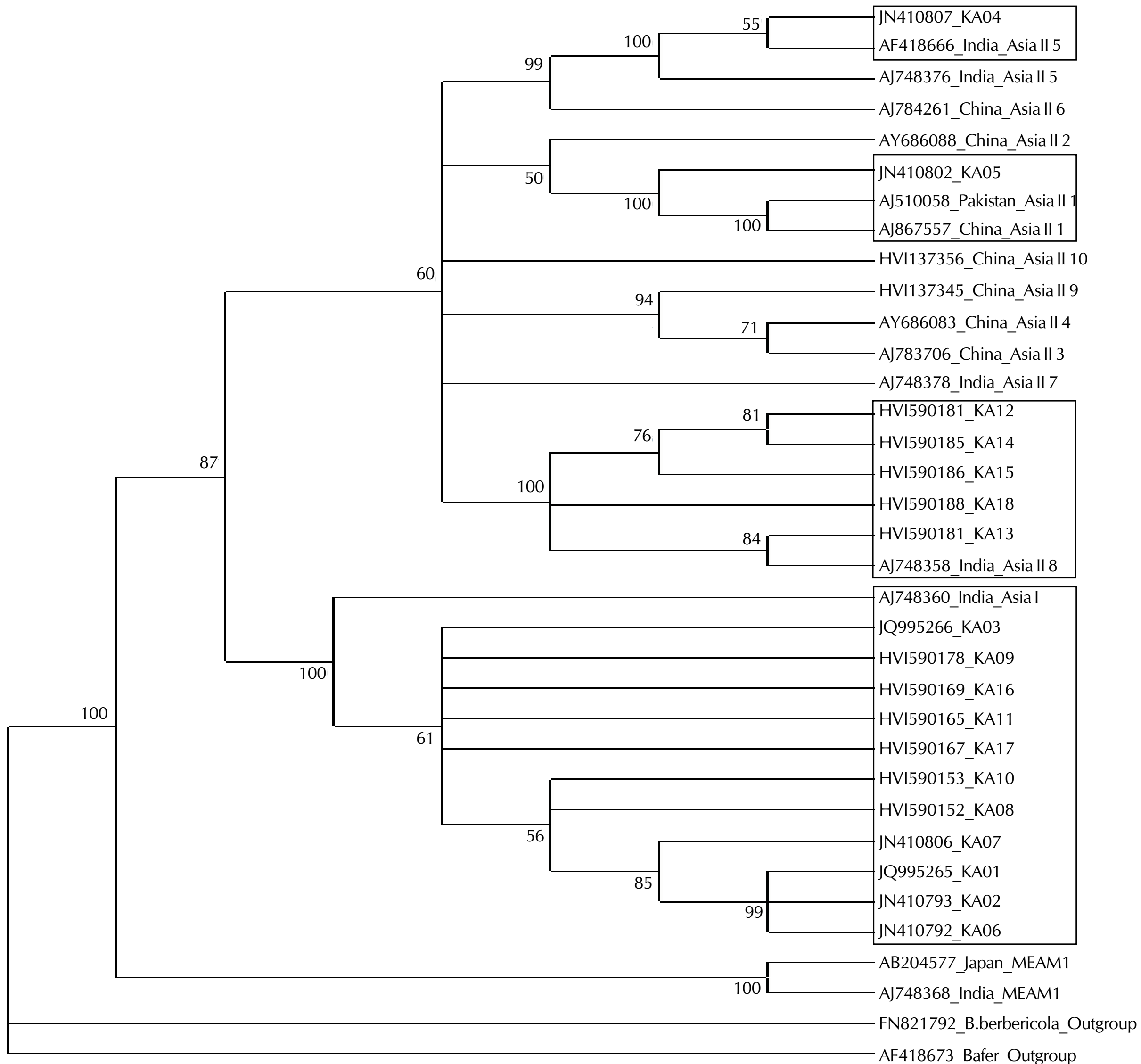

Figure 1: Neighbor-Joining method tree using mitochondrial COI consensus sequences (657bp) with bootstrap support (1000 replicates) showing phylogram for *Bemisia tabaci* collected from different hosts and different geographical locations in Karnataka (MEGA 5.0)

within and among locations, and ecological and biological factors may help dictate new host infestation (Omondi *et al.*, 2005). This statement is truly accepted through our study where Asia I was found on four different host plant families across Karnataka viz., Malvaceae (Cotton), Solanaceae (Brinjal and Tomato), Fabaceae (Cowpea) and Asteraceae (Sunflower). This genetic group has relatively polyphagous while it most frequently found in eggplants. Initially, the existence of Asia II-5 group was pertained only on Euphorbiaceae (Cassava), but later researchers found on other host plant, tobacco (Chowda-Reddy *et al.*, 2012). However, in addition to the above, we also found the existence of this group on other economically important host plant, tomato. Asia II-8 was found on tomato, okra, cotton and cowpea and Asia II-1 was found in specimen collected on cotton. Interestingly, host plants belonging to Solanaceae serve as common hosts for three Asian genetic groups.

Finally, we conclude that all Asian putative groups exhibit relatively polyphagous by adapting to new host plant species on different locations. Our result shows that large genetic differences exist between populations of *B. tabaci* by applying a mitochondrial marker with good resolution of genetic structure. This would encourage our interest to establish the analyses on other crops with extensive field data support to extend the knowledge of the genetic variation within whitefly populations. The patterns of spread and impacts on species diversity with host plant species will provide useful insights into the invasion process and in the discovery of newly evolving biotypes that would help in the management of pest.

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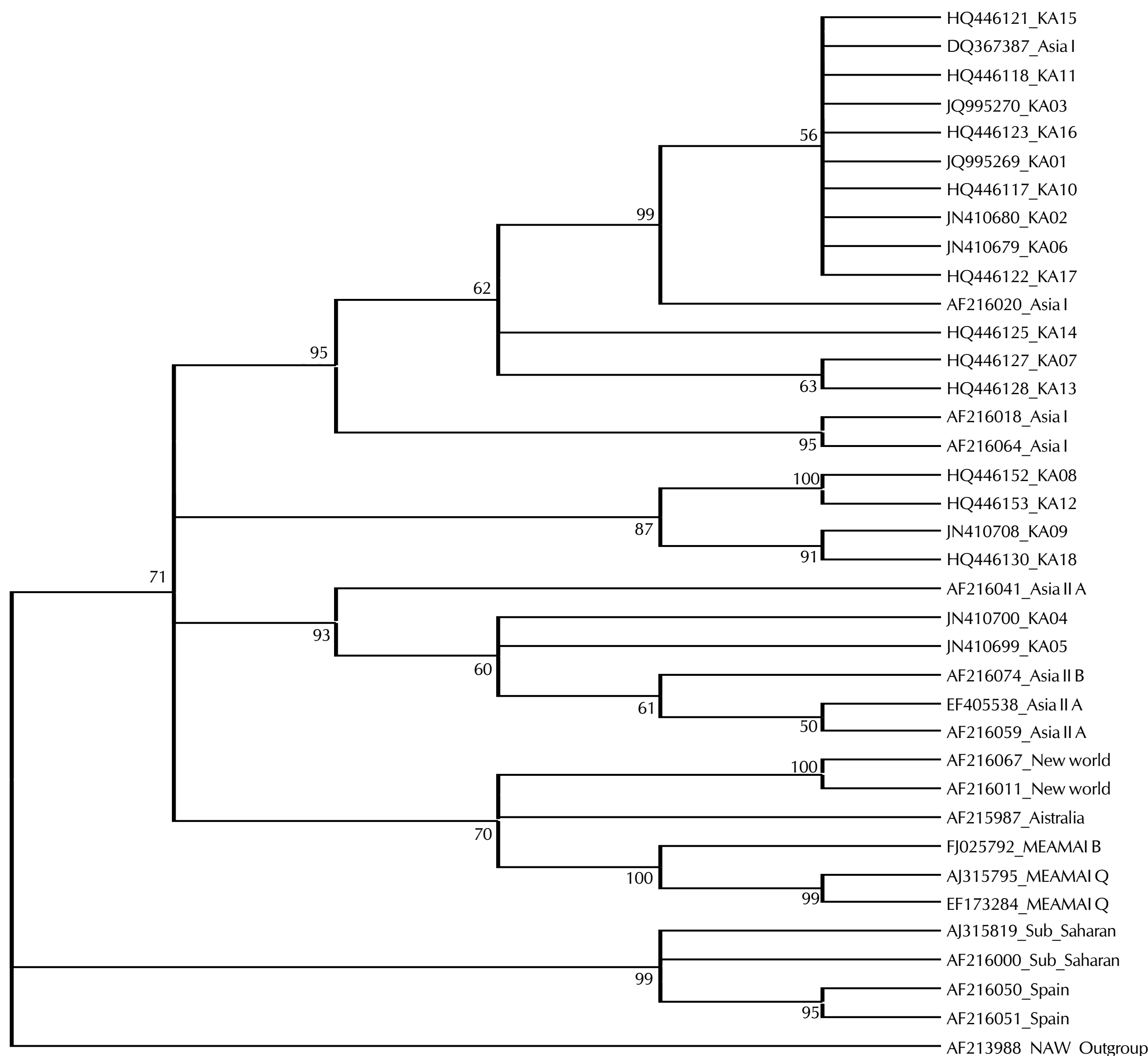

Figure 2: Neighbor-Joining method tree using ITS1 sequences with bootstrap support (1000 replicates) showing phylogram for *Bemisia tabaci* collected from different hosts and different geographical locations in Karnataka (MEGA 5.0)

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