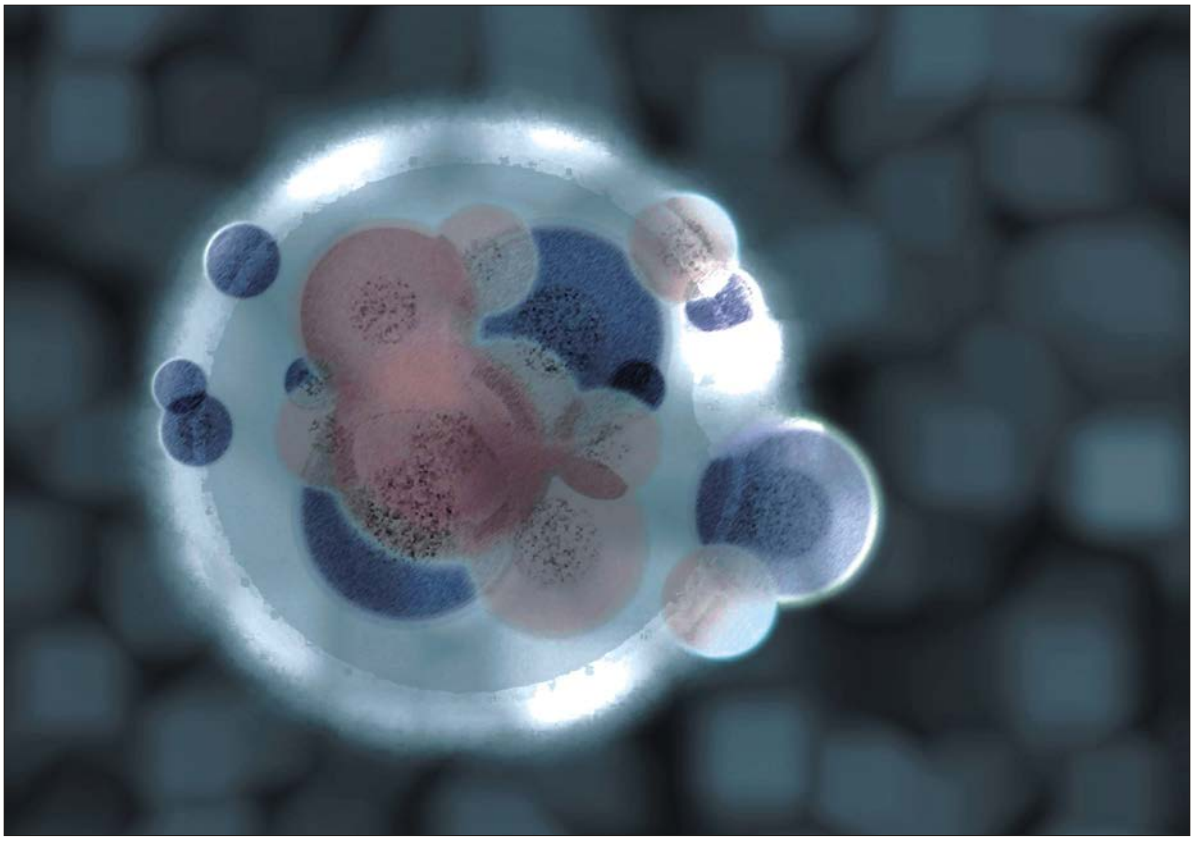

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Molecular Identification of the Biotype of Whitefly (*Bemisia tabaci*) Inhabiting the Eastern Region of Saudi Arabia

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ABSTRACT
The whitefly *Bemisia tabaci* (Genn.) is a worldwide important insect pest and vector of plant viruses. Recently, the whitefly populations upsurges dramatically in Al-Ahsa region of Saudi Arabia due to the expansion in greenhouse cropping system and it causes severe economic damage to many vegetable crops in greenhouses. Proper classification of the whitefly that prevails in the area is a crucial step towards efficient management of the pest. The whitefly is highly polymorphic with extreme plasticity in key morphological characters that vary according to the host which makes the taxonomic identity of existing biotype is difficult and sometimes ambiguous. The specimens for this study were collected from nine different locations of the whitefly populations in Al-Ahsa region. The study was done on the basis of three molecular techniques namely: (1) PCR amplification and polymorphic analysis of restriction enzyme digestion of mitochondrial cytochrome oxidase I gene (mtCOI), (2) Cloning, sequencing and phylogenetic analysis of mitochondrial 16S rRNA gene and (3) Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD PCR) analysis. The results of these molecular techniques clearly indicated the dominance of B biotype of the whitefly on crops grown in greenhouses in Al-Ahsa region of Saudi Arabia.

Key words: Whitefly, mtCOI, 16S rRNA, RAPD, Al-Ahsa, Saudi Arabia

INTRODUCTION

The whitefly *Bemisia tabaci* is attacking most high value and economically important crops that grown in greenhouses in Saudi Arabia causing severe damage through direct feeding on plants cells sap and transmitting plant viruses (Al-Zahrani, 2010; McKenzie *et al*., 2012). Among *B. tabaci* species complex, the B biotype (formerly known as *B. argentifolii*) is the only known whitefly transmitter of geminiviruses (Brown, 2010). The whitefly is capable of establishing excessive populations, particularly in irrigated arid lands grown with field and greenhouse crops (Brown, 1994). The whitefly has the potential to colonize a wide range of approximately 700 species of plants that are primarily vegetables and fibers of economic importance worldwide. Recent studies indicated that numerous populations of *B. tabaci* vary rather in their capacity to develop high population densities and cause direct feeding damage but they can transmit geminiviruses (Brown and Idris, 2005; Brown and Bird, 1992; Czosnek and Brown, 2010). Viral transmission is most likely semi-persistent as discussed for *Beet pseudoyellows virus* (Pusag *et al*., 2012; Moreno-Delafuente *et al*., 2013). In Saudi Arabia, a number of plant diseases were caused by viruses that transmitted by the whitefly including *Tomato yellow leaf curl virus* (TYLCV) (Mazyad *et al*., 1977; Al-Shahwan *et al*., 1997; Alhudaib *et al*., 2013). The *Squash leaf curl virus* (SqLCV)

was reported in different locations of Saudi Arabia in 2002 (Al-Shahwan *et al*., 2002). When identifying and describing insect taxon, the morphology has been used historically to separate species. Among several groups of insects, however, morphological characters can vary with respect to environmental factors within a single species, or be convergent and cryptic among closely related species as to be of limited usefulness (Rosell *et al*., 1997; Hu *et al*., 2011). The study of insects' biology and molecular profiles become essential to define species and characterize populations. At the molecular level, protein and DNA polymorphisms can be combined with the study of biological characteristics by using either one or combinations of different technological approaches. The most common practically approaches were the electrophoresis of allozymes, analysis of Random Amplified Polymorphic DNAs (RAPDs) and nucleic acid sequence comparisons of nuclear or mitochondrial DNA markers (De Barro and Driver, 1997). Protein polymorphism was also utilized to investigate natural populations of *B. tabaci*. Differences in esterase isozyme patterns were used to describe two biotypes of the whitefly, the native form A biotype and the other form B biotype which exhibited high population density, wide host range, high insecticide resistance and capable of inducing silverleaf symptoms on some plants (Brown *et al*., 1995a, b). The whitefly *B. tabaci* A biotype was previously the predominant biotype in most regions of the Mediterranean and Middle East. Many of these populations had been displaced by the B biotype. A new Q biotype of whitefly spread rapidly into several states of the USA. It is less susceptible to many of the insecticides currently used to manage the A and B biotypes of whitefly. The Q biotype of whitefly was believed to have been originated in the Mediterranean region and have been associated with the whitefly control problems (Hadjistylli *et al*., 2010; Czosnek and Brown, 2010). Other reports suggested that there might be additional biotypes, eighteen biotypes from throughout the world have been described based on esterase banding patterns alone (Brown *et al*., 1995a). DNA sequence comparisons can be made between individuals or populations using a PCR generated marker genes from either the nuclear or the mitochondrial DNA (Calvert *et al*., 2001). The latter offers some advantages; it is maternally inherited and non-recombining and contains known and predictable gene sequences for which universal primers for insects have been designed. The sequences of some genetic markers are sufficient for population level analysis and for molecular characterization that help to solve the taxonomy puzzle of *B. tabaci* complex (Gill and Brown, 2010). In this study, several molecular techniques were used to identify the biotype of whitefly populations in Al-Ahsa region of Saudi Arabia. Unveiling the identity of the predominant whitefly populations in the region will help to design an efficient whitefly management strategy to combat this cryptic insect pest.

MATERIALS AND METHODS

Source of specimen: Whitefly populations on tomato were separately collected from different locations in Al-Ahsa region of Saudi Arabia.

Genomic DNA extraction: Genomic DNA was extracted from 5 individual adults from each of the 9 populations using a modified extraction method previously described by Anfoka *et al*. (2008). Briefly, 5 whitefly adults were ground in 50 μL extraction buffer (100 mM Tris-HCl, 500 mM NaCl, 10 mM β-mercaptoethanol, 50 mM EDTA) and incubated in water bath at 65°C for 10 min. One-fifth volume of 5 M potassium acetate and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) were added. The mixtures were kept on ice for 10 min and then clarified by centrifugation at 10,000 rpm for 20 min. An equal volume of isopropanol was added to the supernatant of the mixture. Incubated at -20°C for 10 min and then centrifuged at 10,000 rpm for 10 min. After air dry, the pellets were washed with 70% ethanol and suspended in deionized distilled water (ddH_2O).

Characterization of the whitefly species by PCR and restriction enzyme digestion: PCR was used to amplify mitochondrial cytochrome oxidase I (mtCOI) gene using primers 2195F and 3014R (Table 1) (Frohlich *et al*., 1999; Khasdan *et al*., 2005; Cifuentes *et al*., 2011). The thermocycling conditions were as follows: 1 cycle of preheating for 5 min at 95°C followed by 35 cycles each for 30 sec at 94°C, 45 sec at 52°C and 1 min at 72°C, then followed by an extension at 72°C for 5 min. The PCR products were digested with *VspI* restriction enzyme by adding 2 μL of enzyme buffer to 10 μL of PCR product and 0.5 μL of the *Vsp*I enzyme then the total mixture was completed to 20 μL by adding 7.5 μL ddH_2O. The digestion reaction mixture was incubated at 37°C for 2 h, electrophoresed on 1% agarose gel

Table 1: Primers* used in this study

Primer	Sequence (5'→ 3')	Reference
2195 F	TTGATTTTTTGGTCATCCAGAAGT	Frohlich *et al*. (1999)
3014 R	TCCAATCGACTAATCTGCCATATTA	Frohlich *et al*. (1999)
WF-F	CGCCTGTTTAACAAAAACAT	Frohlich *et al*. (1999)
WF-R	CCGGTCTGAACTCAGATCACGT	Frohlich *et al*. (1999)
F2	GAGGATCCCT	De Barro and Driver (1997)
F12	ACGGTACCAG	De Barro and Driver (1997)
H9	TGTAGCTGGG	De Barro and Driver (1997)
H16	TCTCAGCTGG	De Barro and Driver (1997)

*Pair of primers 2195 F and 3014 R were used to amplify the whitefly mitochondrial cytochrome oxidase I gene (mtCOI) fragment. The pair of primers WF-F and WF-R were used to amplify the whitefly mitochondrial 16S rRNA gene fragment. The primers F2, F12, H9 and H16 were individually used to amplify and analyze polymorphism in whitefly genomic DNA via RAPD PCR

in 0.5×TBE buffer and the DNA was stained with ethidium bromide. The gel was visualized with UV illumination using Gel Documentation System (SYNGENE).

Amplification and sequencing of the whitefly mitochondrial 16S rRNA gene: The PCR was used to partially amplify the mitochondrial 16S rRNA. The experiment was done on the specimens collected from 9 different locations using primers WF-F and WF-R (Table 1) (Frohlich *et al*., 1999). The PCR amplifications were performed in a total volume of 25 μL containing 2.5 μL of genomic DNA, 2.5 μL of 10×*Taq* polymerase buffer, 2.5 μL of 25 mM $MgCl_2$, 2.5 μL of 10 mM dNTPs, 1 μL of both the forward and reverse primers (10 μM each), 0.3 μL of *Taq* polymerase (5 U μL^{-1}) and ddH_2O to complete reaction volume. Amplifications were performed for 30 cycles in a thermal cycler started at 95°C for 1 min for denaturation, annealing at 50°C for 50 sec, extension 72°C for 50 sec and a final one cycle for extension at 72°C for 10 min. The PCR products were electrophoresed on 1% agarose gel in 0.5×TBE buffer. The 1 kb DNA ladder (Promega) was used as marker to determine the size of the PCR products. The gel was stained with ethidium bromide and then visualized with UV illumination using Gel Documentation System (SYNGENE).

The PCR products from the 7 whitefly specimens were cloned into pGEM®T-Easy vector (Promega) following the supplier's instructions. The ligation mixture contained 5 μL of 2×Rapid buffer, 1 μL of pGEM®T-Easy vector, 2 μL of PCR product and 1 μL of T_4 DNA ligase. The reaction was completed to 10 μL with ddH_2O, mixed gently by pipetting, then incubated at 4°C overnight.

The transformation protocol was as follows: The competent cells DH5α™ were thawed on ice for 30 min. Hundred microliter was transferred into a pre-chilled 5 mL tube and 5 μL of the ligated DNA was added and the tube was incubated on ice for another 30 min. Cells were heat-shocked for 2 min at 42°C in a water bath and then incubated on ice for 2 min. Hundred microliter of LB medium was added and then cells were grown for 1 h at 37°C in a shaking water bath. Transformed cells were plated on LB plates containing 60 μg mL^{-1} ampicillin and the plates were incubated at 37°C overnight (16 h). Single bacterial colonies were inoculated into LB medium with 60 μg mL^{-1} ampicillin and grown overnight in a shaking water bath at 37°C. Recombinant plasmids were extracted using Wizard Plus SV Minipreps DNA Purification System (Promega) following the manufacturer's instructions. Recombinant plasmids were digested with *Eco*RI to confirm the insert size. The digestion reaction was containing 2.5 μL of ddH_2O, 0.5 μL of *Eco*RI restriction enzyme, 1 μL of 10×buffer H and 6 μL of prepared plasmids. The reaction mixture was incubated in water bath at 37°C for 4 h. The digested DNA was electrophoresed on 1.5% agarose gel. The partial nucleotide sequences of the mitochondrial 16S rRNA gene from 7 *B. tabaci* populations were determined. Multiple sequence alignment of the nucleotide sequences were performed using the MEGA6 software (Tamura *et al*., 2013) and clustalx program (Thompson *et al*., 1997). Phylogenetic analysis relationships were done with multiple individuals within populations.

RAPD PCR analysis: RAPD PCR experiment was done on 9 whitefly populations using the primers F2, F12, H9 and H16 (Table 1) (De Barro and Driver, 1997). The PCR amplifications were achieved in a total volume of 25 μL as stated before. The amplification was done using the following thermocycling parameters: one cycle of 5 min at 94°C, 2 min at 40°C and 3 min at 72°C, followed by 39 cycles of 1 min at 94°C, 1.5 min at 40°C and 2 min at 72°C and 1 cycle of a final extension at 72°C for 5 min.

RESULTS AND DISCUSSION

Characterization of Al-Ahsa whitefly populations by PCR and restriction digestion of the mtCOI gene: Amplification of the mtCOI gene using the primers 2195F and 3014R gave a fragment of about 816 bp (Fig. 1a) from all whitefly populations collected from different locations in Al-Ahsa. The products of the primers pair is consistent with previously published results on whiteflies from elsewhere (Khasdan *et al*., 2005; Cifuentes *et al*., 2011). The amplified PCR products were digested with *Vsp*I restriction enzyme. There is a clear evidence that *Vsp*I restriction digestion of the mtCOI of B biotype whiteflies always give a single band of size around

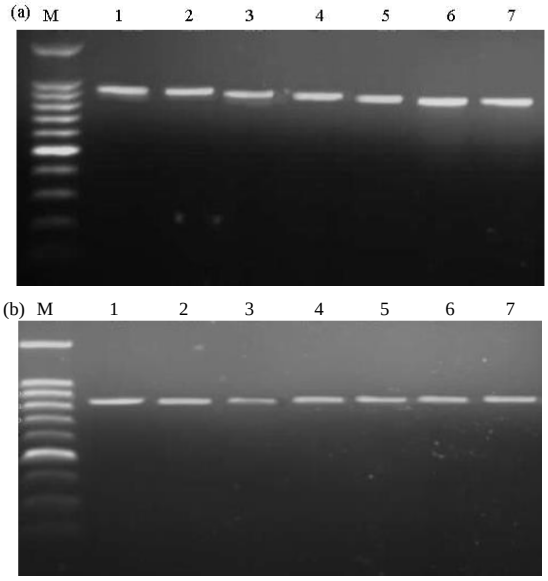

Fig. 1(a-b): Al-Ahsa whitefly mitochondrial cytochrome oxidase I (mtCOI). (a) PCR amplification of mtCOI from 7 representative specimens collected from different locations (lanes 1-7) and (b) The same PCR products were digested with the restriction enzyme *Vsp*I (lanes 1-7). M: 100 bp DNA ladder as a marker

800 bp (Khasdan *et al*., 2005). On the other hand, *Vsp*I restriction digestion of the mtCOI of Q biotype whiteflies give two distinct bands of sizes about 300 and 500 bp (De Barro *et al*., 2000; Horowitz *et al*., 2005; Bosco *et al*., 2006; Chiel *et al*., 2007; Ma *et al*., 2009; Al-Shehi and Khan, 2013). The present results of *Vsp*I restriction enzyme digestion of the PCR products showed a single band (Fig. 1b). This result indicates the predominance of the B biotype whitefly in Al-Ahsa region of Saudi Arabia. None of the collected whitefly populations from Al-Ahsa region contains either the Q biotype or any other whitefly biotype.

Characterization of Al-Ahsa whitefly populations by partial sequencing of 16S rRNA: The PCR amplification of mitochondrial 16S rRNA with the primers WF-F and WF-R gave a fragment of about 500 bp from all tested whitefly specimens. PCR products obtained from 7 whitefly templates were cloned and sequenced to confirm the biotype of the whitefly under study. The sequences obtained from 7 whitefly populations were identical, therefore only 1 representative sequence was submitted to the GenBank® (Benson *et al*., 2013) under the accession number JN033570. The identified DNA sequence of the whitefly mitochondrial 16S rRNA gene was aligned with the other sequences available in GenBank® using neighbor joining method implemented in MEGA6 (Tamura *et al*., 2013). The phylogenetic tree showed clustering of Al-Ahsa whitefly 16S rRNA gene with the B biotype genetic sequences (Fig. 2). These results are in general agreement with the data that shown by other investigators (Frohlich *et al*., 1999; De Barro *et al*., 2000; Tahiri *et al*., 2013).

RAPD PCR analysis: Due to its several advantages, RAPD PCR is commonly practiced to distinguish between the most common whiteflies. The technique is reliable, does not require a foreknowledge about any particular gene in the target organism and facilitates the use of more than one primer at a time to increase confidence. Here, 4 random primers were used, namely F1, F12, H9 and H16 (Table 1) in the RAPD PCR amplifications to distinguish between the indigenous Saudi Arabia whitefly populations and others possibly alien biotypes. The banding pattern shown in Fig. 3 reveals the consistency of random primer amplifications of the whitefly populations from Al-Ahsa region. All used random primers showed uniform banding patterns with all tested whitefly populations. It has been reported that the region between 600 and 900 bp demonstrated some polymorphism between different B biotype individuals when amplified with F12 primer but the region between 300 and 600 bp gave of size around 800 bp (Khasdan *et al*., 2005). On the other hand, *Vsp*I restriction digestion of the mtCOI of Q biotype whiteflies give two distinct bands of sizes about 300 and 500 bp (De Barro *et al*., 2000; Horowitz *et al*., 2005; Bosco *et al*., 2006; Chiel *et al*., 2007; Ma *et al*., 2009; Al-Shehi and Khan, 2013). The present results of *Vsp*I restriction enzyme digestion of the PCR products showed a single band (Fig. 1b).

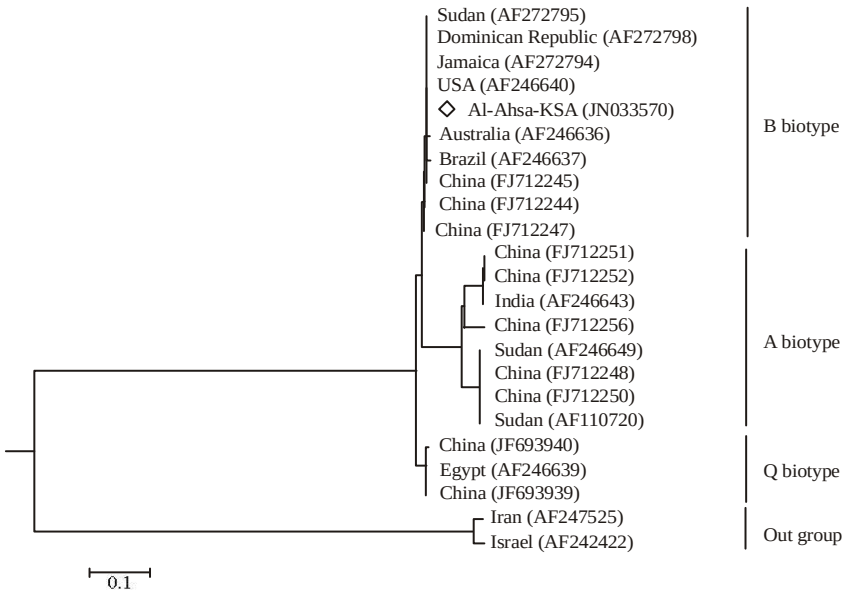

Fig. 2: Phylogenetic tree of the whiteflies mitochondrial 16S rRNA. The evolutionary history and analyses were inferred using the Neighbor-Joining method implemented in MEGA6 (Tamura *et al*., 2013). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences obtained from the GenBank® (accession numbers are in brackets)

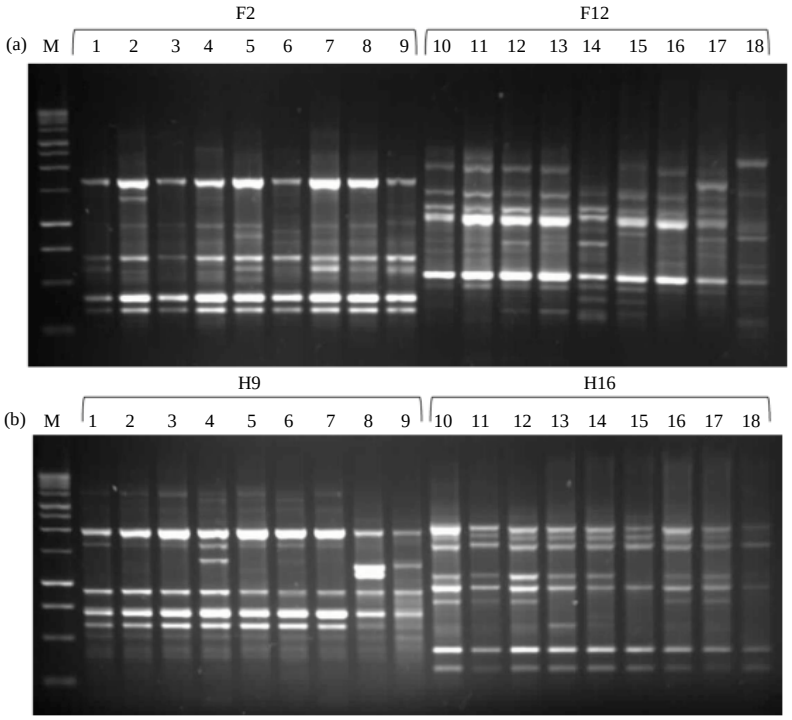

Fig. 3(a-b): RAPD PCR profiles of Al-Ahsa whitefly. (a) RAPD PCR profiles obtained from specimens collected from 9 different locations using the primer F2 for lanes 1-9 and the primer F12 for lanes 10-18 and (b) Using the primer H9 for lanes 1-9 and the primer H16 for lanes 10-18. M: 1 kb DNA ladder as marker

This result indicates the predominance of the B biotype whitefly in Al-Ahsa region of Saudi Arabia. None of the collected whitefly populations from Al-Ahsa region contains either the Q biotype or any other whitefly biotype.

The whitefly biotypes may vary in their efficiency of virus transmission, host utilization, development rate, fecundity, dispersal ability, infestation symptoms, insecticides resistance and the degree of endosymbiosis (Oliveira *et al*., 2001; Sseruwagi *et al*., 2005). Detailed molecular analyses using three techniques revealed the prevalence of the whitefly B biotype in Al-Ahsa region of Saudi Arabia. During the course of this study, it has been reported the existence of A- and Q-like biotypes on cucumber grown in Fayfa region of Saudi Arabia near Yemen border (Ragab, 2013). The current research finding could help the crop protection specialists in the region to design suitable control strategies against this particular biotype and to take suitable preventive measures against any possible invasion by the A- and Q-like biotypes.

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