Color Polymorphism does not Affect Species Diagnosis of the Melon Aphid, Aphis gossypii (Hemiptera: Aphididae)

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COLOR POLYMORPHISM DOES NOT AFFECT SPECIES DIAGNOSIS OF THE MELON APHID, *APHIS GOSSYPII* (HEMIPTERA: APHIDIDAE)

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Abstract

The melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) has become a serious pest of wide spectrum of crops and is considered to be a potential vector of numerous plant pathogenic viruses worldwide. This pest exhibits color polymorphism in which dark green, pale green and yellow forms exist within a single colony on the same plant and also on different host plants. The co-occurrence of different color forms makes species diagnosis and identification of cryptic species difficult, while they have immense importance in quarantine and biosecurity. Therefore, establishing a tool that facilitates quick and accurate identification of *A. gossypii* is an urgent need. Molecular diagnosis is the most appropriate and becomes handy because it is independent of sex, polymorphism and life stage, and could be effectively used by person with no or little knowledge in aphid taxonomy. In this study, two mitochondrial markers i.e. cytochrome oxidase subunit gene I (COI) and the tRNA-leucine + cytochrome oxidase subunit gene II (tRNA/COII) were tested for species diagnosis. Alignment of partial COI and tRNA/COII sequences from various color forms of *A. gossypii* collected from 6 different host plants viz., cotton, okra, cucumber, watermelon, aubergine and chili pepper revealed maximum sequence identity (99.7% and 99.4%) suggesting that molecular identification is independent of the color polymorphism in this species. Thus, it is concluded that, color polymorphism does not affect species diagnosis of *A. gossypii.* The study has unambiguously proved the utility of COI and tRNA/COII for a quick and accurate diagnosis of *A. gossypii*, which is a critical factor in elucidating virus transmission and implementing biological control.

Key Words: *Aphis gossypii*, COI, color forms, melon aphid, tRNA/COII

Resumen

El áfido (pulgón) del melón, *Aphis gossypii* Glover (Hemiptera: Aphididae) se ha convertido en una plaga grave de un amplio espectro de cultivos y se considera que es un vector potencial de numerosos patógenos virus de plantas en todo el mundo. Esta plaga exhibe un polimorfismo cromático en el cual existen diferentes formas de color incluyendo verde oscuro, color verde pálido y amarillo dentro de una sola colonia en la misma planta y también en diferentes plantas hospederas. La co-ocurrencia de formas de diferentes colores hace difícil el diagnóstico de la especie y la identificación de especies crípticas. Las distintas formas de color y posibles especies crípticas tienen una enorme importancia en la cuarentena y bioseguridad. Por lo tanto, el establecimiento de una herramienta que facilita la identificación rápida y precisa de *A. gossypii* es una necesidad urgente. A estas alturas, el diagnóstico molecular se hace útil, ya que es independiente del sexo, polimorfismo y la etapa de la vida, y se podría utilizar con eficacia por un no especialista. En este sentido, se probaron dos marcadores, específicamente el gen I de la subunidad de la citocromo oxidasa (COI) y el gen II de la subunidad oxidasa ARNt-leucina + citocromo (ARNt/COII) para el diagnóstico de la especie. La alineación de las secuencias de COI parcial y ARNt/COII de diversas formas de color de *A. gossypii* recogidos de 6 plantas hospederas diferentes, específicamente el algodón, okra, pepino, sandía, la berenjena y el chile verde reveló un máximo de identidad de

secuencia (99.7% y 99.4%) lo que sugiere que la identificación molecular es independiente del polimorfismo de color de esta especie específica. Así, el polimorfismo de color no afectan el diagnóstico de las formas de color de la especie *A. gossypii*. El estudio ha demostrado de manera inequívoca la utilidad de la COI y ARNt/CO II para el diagnóstico rápido y preciso de *A. gossypii,* que es un factor crítico en elucidar la transmisión del virus y la implementación de control biológico.

Palabras Clave: formas de color, áfido de melón, *Aphis gossypii*, COI, ARNt/COII

The melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) is the most important cosmopolitan, extremely polyphagous invasive aphid species that affects numerous agricultural, horticultural and green house crops worldwide (Blackman & Eastop 1984). It is capable of transmitting more than 75 plant viruses and is considered to be the most versatile of insect vectors of plant viruses (Blackman & Eastop 2000). It exhibits greater diversity in terms of host suitability, life cycle and geographical range than any other aphid (Van Emden et al. 2007). It has an extremely complex clonal structure and great phenotypic plasticity. *Aphis* spp. are known to exhibit different color forms, as are evident in *Aphis frangulae* Kaltenbach (Thomas 1968), *Aphis fabae* Scopoli (Muller 1979), *Aphis triglochinis* Theobald (Hille Ris Lambers & Dicker 1965), *Aphis schneideri* (Born) (Rakauskas & Turcinaviciene 1998) and *Aphis odinae* Van der Goot (Blackman et al. 2011).

Extreme color variation has been observed to be a constant feature in colonies of *A. gossypii,* the variation being chiefly exhibited by the apterous forms which range from light yellow to blackish green (Wall 1933; Blackman & Eastop 1984; Dixon 1998). The species is best known for its heritable color polymorphism. It has been observed that apterous adult females of *A. gossypii* may exhibit three distinct colors, i.e. varieties of green to greenish black (dark green, pale green) with irregular darker shadings and lemon yellow (Pergande 1895) (Fig. 1), as a result of abiotic, viz., environmental (light intensity, photoperiod and temperature) (Markkula &Rautapaa 1967), nutritional (host plant) (Honek 1982; Weber 1985; Nevo & Coll 2001) and biotic factors (bacterial symbionts, predator attack and infection by pathogens) (Tsuchida et al. 2010).

The co-occurrence of different color forms of a single species on different hosts, at different time makes it difficult to identify morphologically similar *Aphis* spp. using conventional taxonomic keys (Patch 1926). Species in the genus, *Periphyllus*, are difficult to distinguish since they exhibit variable colors and are morphologically similar, e.g., misidentification of *P. coracinus* color forms (brown, brown-greenish and dark-green) as *P. lyropictus* (Kessler),which is amber colored with a brown design (Mackos 2007). Similarly, misidentification of the African sorghum head bug, *Eurystylus marginatus* Odhiambo (Hemiptera: Miridae) as *E. oldi* was due to variation in color (Sharma & Ratnadass 2000).

The variable form of *A. gossypii* has made it difficult to place the species in generic keys and the diverse coloring has been a further confusing circumstance (Wall 1933). At this juncture, molecular identification becomes handy as it is not limited by polymorphism, sex and life stage of the target species (Hebert et al. 2003; Asokan et al. 2011, 2013). Molecular techniques provide reliable data in the form of DNA sequences to identify target species and construct phylogenetic relationships (von Dohlen et al. 2006). The present study was conducted to identify *A. gossypii* in their various colors viz., dark green, pale green and yellow infesting 6 different host plants, namely, cotton (*Gossypium hirsutum* L.; Malvales: Malvaceae), okra (*Abelmoschus esculentus* Moench; Malvales: Malvaceae), cucumber (*Cucumis sativus* L.; Cucurbitales: Cucurbitaceae), watermelon (*Citrullis lanatus*(Thunb.) Matsum & Nakai; Cucurbitales: Cucurbitaceae), aubergine (*Solanum melongena* L.; Solanales: Solanaceae) and chili pepper (*Capisicum frutescens* L.; Solanales: Solanaceae) using mitochondrial markers.

Various molecular markers have been employed by researchers for species identification and molecular phylogeny studies in aphids, viz., mitochondrial cytochrome oxidase I (mtCOI) (Foottit et al. 2008; Wang et al. 2011), tRNA-leucine + cytochrome oxidase II (tRNA/COII) (Kim et al. 2010), Cytochrome b (Raboudi et al. 2005), 16S rRNA (vonDohlen & Moran 2000), etc. Since, mitochondrial markers are based on maternally inherited characteristics having subjected to relatively less rapid rates of intra-specific evolutionary change and more reliably reflect inter-specific variation compared to nuclear molecular markers (Savolainen et al. 2005), they have been widely employed in molecular systematics of insects. In the present study, mitochondrial cytochrome oxidase subunit gene I (COI) "barcode" (658bp) and tRNA-leucine + cytochrome oxidase II (tRNA/COII) (813bp) regions were used for species diagnosis.

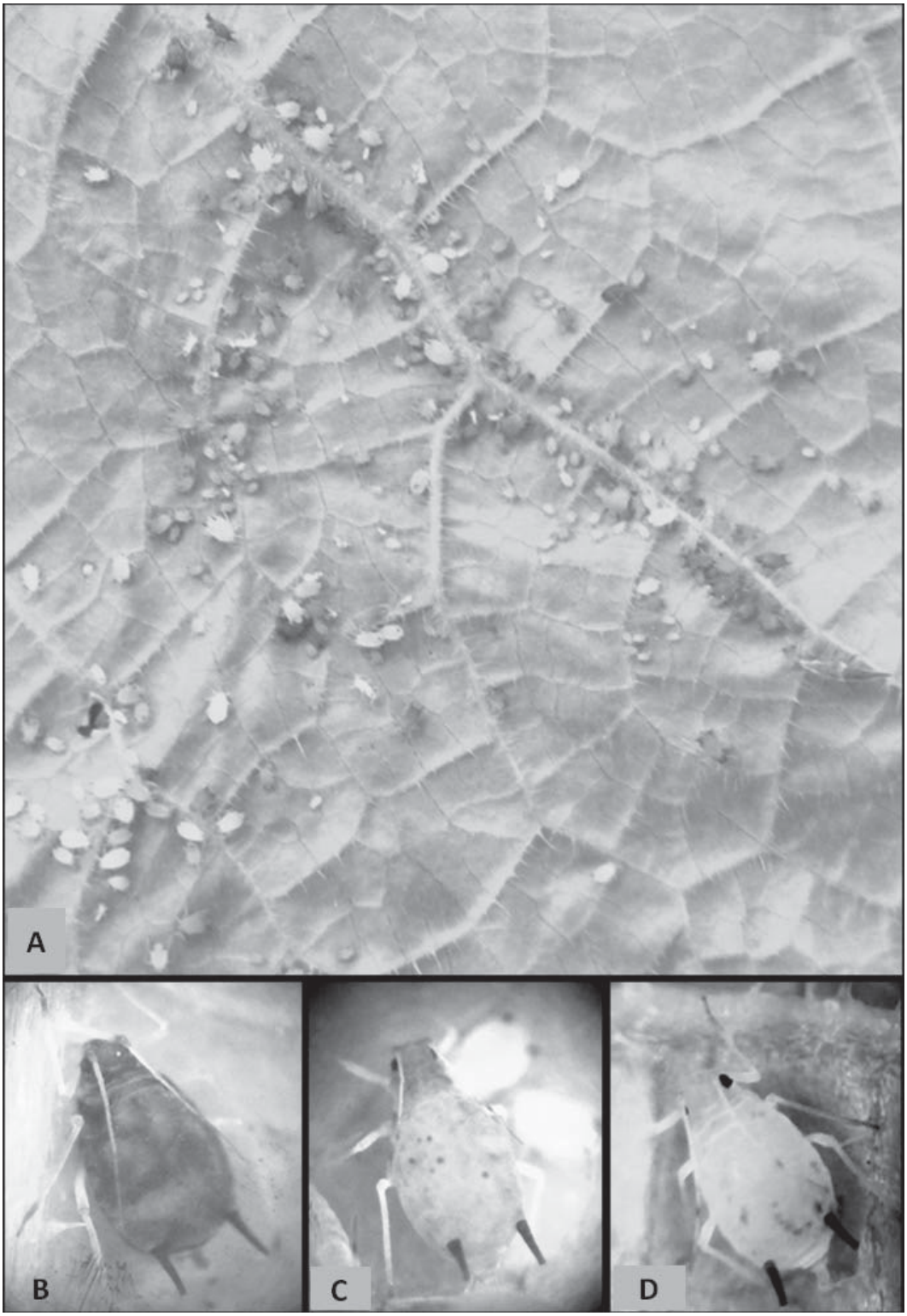

Fig. 1. General view of *Aphis gossypii* Glover exhibiting color variations (A; 20x); and dark green, pale green and yellow forms (B, C & D respectively; 400x). A color version of this graphic can be seen online in supplementary material for this article in Florida Entomologist 97(3) (September 2014) at http://purl.fcla.edu/fcla/entomologist/browse .

Materials and Methods

Maintenance of Stock Culture and Morphological Identification

The aphid samples used in this work were collected from cotton, okra, cucumber, watermelon, aubergine and chili pepper cultivated at Indian Institute of Horticultural Research (IIHR), Bengaluru, India (N 12° 58' E 77°35') during 2011-2012. Color variants of green apterous viviparous females were observed with the yellow ones. Live aphids along with the plant material were transferred to the laboratory, where a single apterous parthenogenetic viviparous adult female was used to establish a stock culture of this material in the laboratory and were maintained on respective hosts under glasshouse insectary conditions. Aphids were identified morphologically by Dr. Sunil Joshi of the National Bureau of Agriculturally Important Insects (NBAII), Bengaluru, India prior to molecular studies. The aphid specimens used for morphological as well as molecular analyses were collected and preserved in 70% ethanol at -20 °C until further use. Three individuals of each color form (dark green, pale green and yellow) of *A. gossypii* were randomly selected from different hosts as replications and were subjected to molecular analysis to evade sequencing error, if any. A color version of the graphics in this report can be seen online in supplementary material for this article in Florida Entomologist 97(3) (September 2014) at http://purl.fcla.edu/fcla/entomologist/browse .

DNA extraction and Polymerase Chain Reaction

Total genomic DNA was extracted from individual aphid using modified CTAB method (Saghai et al. 1984) and the sample details are given in Table 1. An individual aphid was homogenized in 100 µL of extraction buffer containing CTAB-2%, 100 mM Tris-HCl (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA, 0.1% of 2-mercaptoethanol using a sterile micropestle in 1.5 mL microcentrifuge tube. The suspension was incubated at 65°C for 60 min and then an equal volume of chloroform: isoamylalcohol (24:1) was added. The suspension was centrifuged at 10,000 rpm for 10 min at 8°C. The upper aqueous layer was transferred to a fresh microcentrifuge tube. DNA was precipitated by adding an equal volume of ice-cold isopropanol. The precipitated DNA was spun at 10,000 rpm for 10 min. The resultant DNA pellet was washed with 70% ethanol and dissolved in 30 µL DNase, RNase and Protease free molecular biology water. 5 µL of the extracted DNA was used as template for polymerase chain reaction (PCR).

The mitochondrial COI partial gene fragment was amplified using universal barcode primers (Folmer et al. 1994) (LCO1490F) 5'- GGTCAACAAATCATAAAGATATTGG -3' and (HCO2198R),

Table 1. Details of *Aphis gossypii* color forms, its hosts and NCBI-GenBank accession numbers.

		NCBI Accessions	
Host plant	Color	COI	COII
Cotton (Malvaceae)	Dark green	KF446143	KF446161
	Pale green	KF446149	KF446167
	Yellow	KF446155	KF446173
Okra (Malvaceae)	Dark green	KF446144	KF446162
	Pale green	KF446150	KF446168
	Yellow	KF446156	KF446174
Cucumber (Cucurbitaceae)	Dark green	KF446145	KF446163
	Pale green	KF446151	KF446169
	Yellow	KF446157	KF446175
Watermelon (Cucurbitaceae)	Dark green	KF446146	KF446164
	Pale green	KF446152	KF446170
	Yellow	KF446158	KF446176
Aubergine (Solanaceae)	Dark green	KF446147	KF446165
	Pale green	KF446153	KF446171
	Yellow	KF446159	KF446177
Chili pepper (Solanaceae)	Dark green	KF446148	KF446166
	Pale green	KF446154	KF446172
	Yellow	KF446160	KF446178

5'-TAAACTTCAGGGTGACCAAAAAATCA-3'. Similarly, the tRNA/COII was amplified using 2993+ (5'-CATTCATATTCAGAATTACC-3'; Stern 1994) and A3772 (5'-GAGACCATTACTTGCTTTCAGTCATCT-3'; Normark 1996). PCR was carried out in a thermal cycler (Eppendorf, New York, USA) with the following cycling parameters; For COI, 94 °C for 3 min as initial denaturation followed by 35 cycles of 94 °C for 30 s, 47° C for 45 s, 72 °C for 45 s and 72 °C for 10 min as final extension. For tRNA/COII, 94 °C for 3 min as initial denaturation followed by 35 cycles of 94°C for 30 s, 46 °C for 60 s, 72 °C for 60 s and 72 °C for 10 min as final extension. PCR was performed in 25 µL total reaction volume containing 10 picomoles of each primer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5U of Taq DNA polymerase (Thermoscientific, USA). The amplified products were resolved in 1.0% agarose gel, stained with ethidium bromide (10 µg/mL) and visualized in a gel documentation system (Syngene, USA).

Molecular Cloning and Sequencing

The PCR amplified fragments were eluted using Nucleospin® Extract II according to the manufacturer's protocol *(*Macherey-Nagel, Duren, Germany) and ligated into the general purpose-cloning vector, InsT/Aclone (Fermentas Life Sciences, Germany). Transformation was carried out according to manufacturer's protocol (Fermentas Life Sciences, Germany) and blue/white selection was done. All the white colonies (with insert) were maintained on LBA containing ampicillin (100 mg/mL), incubated at 37 °C overnight and stored at 4 °C. Plasmids were isolated from the overnight culture of 5 randomly selected positive colonies cultured in LB broth using GeneJET™ Plasmid Miniprep Kit (FermentasLife Sciences, Germany) according to manufacturer's protocol. Sequencing was carried out in an automated sequencer (ABI Prism® 3730 XL DNA Analyzer; Applied Biosystems, USA) using M13 universal primers both in forward and reverse directions.

Sequence Analysis

Homology search was carried out using BLAST (http://www.ncbi.nlm.nih.gov), compared with published sequences available in the NCBI and matched with the corresponding region of mitochondrial COI and tRNA/COII. The differences in COI and tRNA/COII sequences of *A. gossypii* color forms were determined using the sequence alignment editor BioEdit (Hall1999) version 7.0.5.3. All the corresponding sequences of *A. gossypii* color forms were deposited with the National Center for Biotechnology Information (NCBI), GenBank with accession numbers KF446143-KF446160 for COI and KF446161-KF446178 for tRNA/COII (Table 1). Sequences generated in the present study along with other widely distributed major aphid vectors of genus *Aphis* which are morphologically similar differing significantly with only few morphological characters, viz., *Aphis glycines* Matsumura, *Aphis craccivora* Koch, *Aphis spiraecola* Patch, *Schizaphis graminum* Rondani(as outgroup) (Retrieved from NCBI) were aligned using Clustal W algorithm in BioEdit v7.2.5. Of an 813 bp region determined for tRNA/COII, a 589 bp region could be aligned with sequences obtained from GenBank. Phylogenetic analysis of aligned sequences was done using MEGA. v5. 0. (Tamura et al. 2011). The method of neighbor-joining (NJ) with the Kimura two-parameter model (Kimura 1980) was utilized to build the phylogenetic tree. To assess the robustness of the tree, 1000 bootstrap replicates were selected. The maximum composite likelihood estimate of the pattern of nucleotide substitution for COI and tRNA/COII sequences of *Aphis* spp. was performed using MEGA v5. 0. (Tamura et al. 2011).

Results and Discussion

Sequencing partial COI and tRNA/COII genes yielded an approximately 700 and 850bp long fragment respectively, for three color forms of *A. gossypii* infesting 6 crops. Upon sequencing the fragment, 658 and 813bp nucleotides were obtained for COI and tRNA/COII, respectively. A comparison of the replicate sequences for all samples of *A. gossypii* showed no mismatch, indicating that there were no sequencing errors. BLAST search for the sequences obtained showed highest hits for the respective species. Pair wise alignment of COI and tRNA/COII gene sequences revealed maximum sequence identity (99.7% and 99.4%) with very few variable sites among the color forms which proved that the molecular identification is independent of color polymorphism of the target species.

Further, the phylogenetic tree generated using COI and tRNA/COII sequences of morphologically similar *Aphis* spp., demonstrated genetic distinction of the species with bootstrap values greater than 85% (Figs. 2 and 3). The sequence divergence among the genus *Aphis* comprising of 3 morphologically similar species i.e., *Aphis glycines, A. craccivora* and *A. spiraecola* based on COI sequences (658 bp) ranged between 3.8–7.6% (mean divergence of 6.13%, SE 0.55%). Similarly, the sequence divergence based on tRNA/COII sequences (589bp) ranged 3.1–4.6% (mean divergence of 3.87%, SE 0.20%) indicating *A. gossypii* is more closely related to *A. glycines* than to *A. spireacola* and *A. craccivora*. In addition, the study showed that COI and tRNA/COII sequences are consistent among species and is able to differentiate the species well; thus mitochondrial

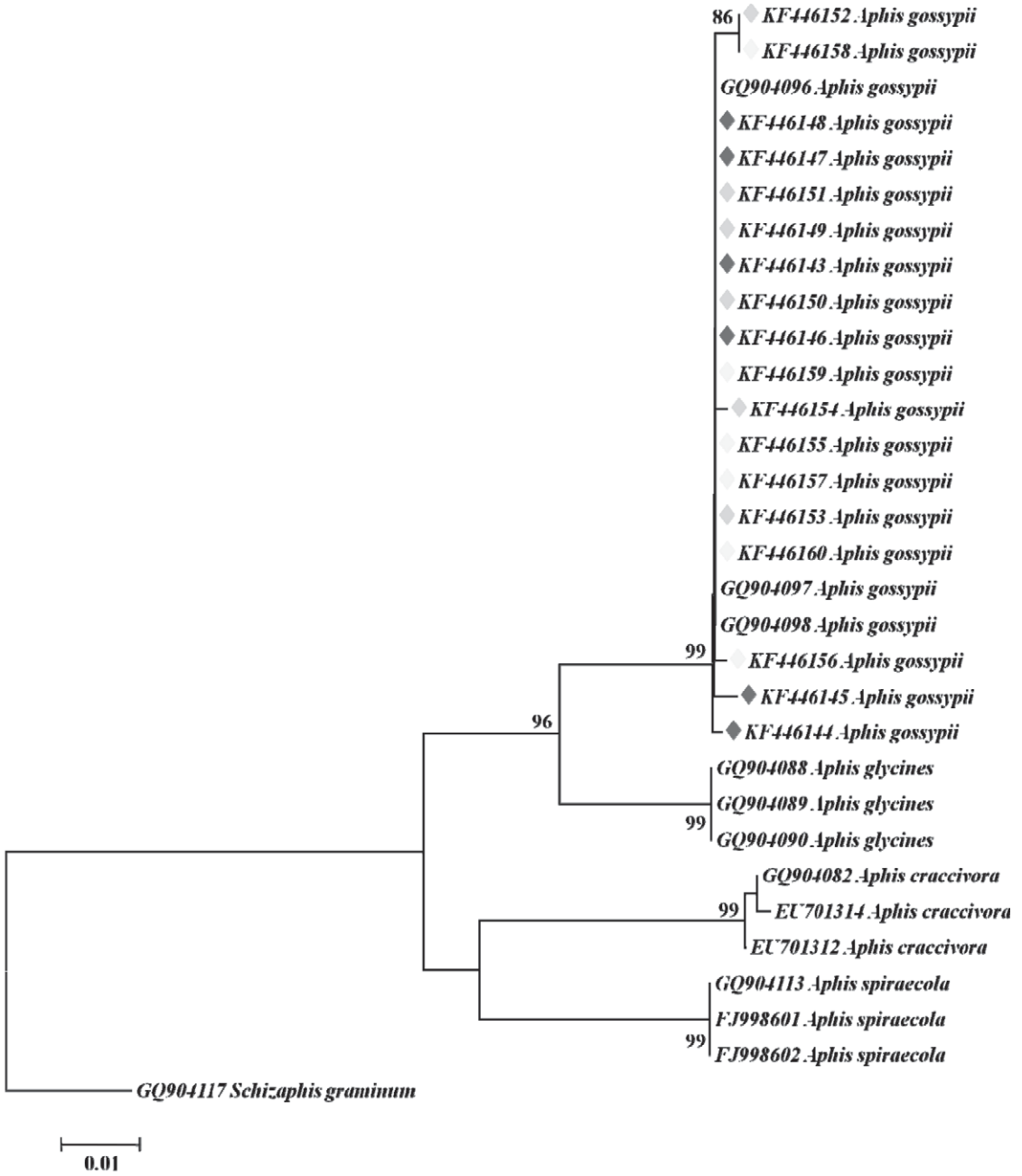

Fig. 2. Neighbor joining tree of *Aphis gossypii* color forms along with other morphologically similar *Aphis* spp. for partial sequences of COI with bootstrap support (1000 replicates). Bootstrap values greater than 85% are shown for branches. *Schizaphis graminum* was used as an outgroup. A color version of this graphic can be seen online in supplementary material for this article in Florida Entomologist 97(3) (September 2014) at http://purl.fcla.edu/fcla/entomologist/browse .

markers proves to be a useful tool for identification of aphids.

The nucleotide frequencies for COI of *Aphis* spp. were 34.63% (A), 41.13% (T), 10.25% (C), and 13.98% (G). Similarly, the nucleotide frequencies for tRNA/COII of *Aphis* spp. were 41.10% (A), 39.44% (T/U), 7.49% (C), and 11.97% (G). The base composition of the COI and tRNA/COII gene

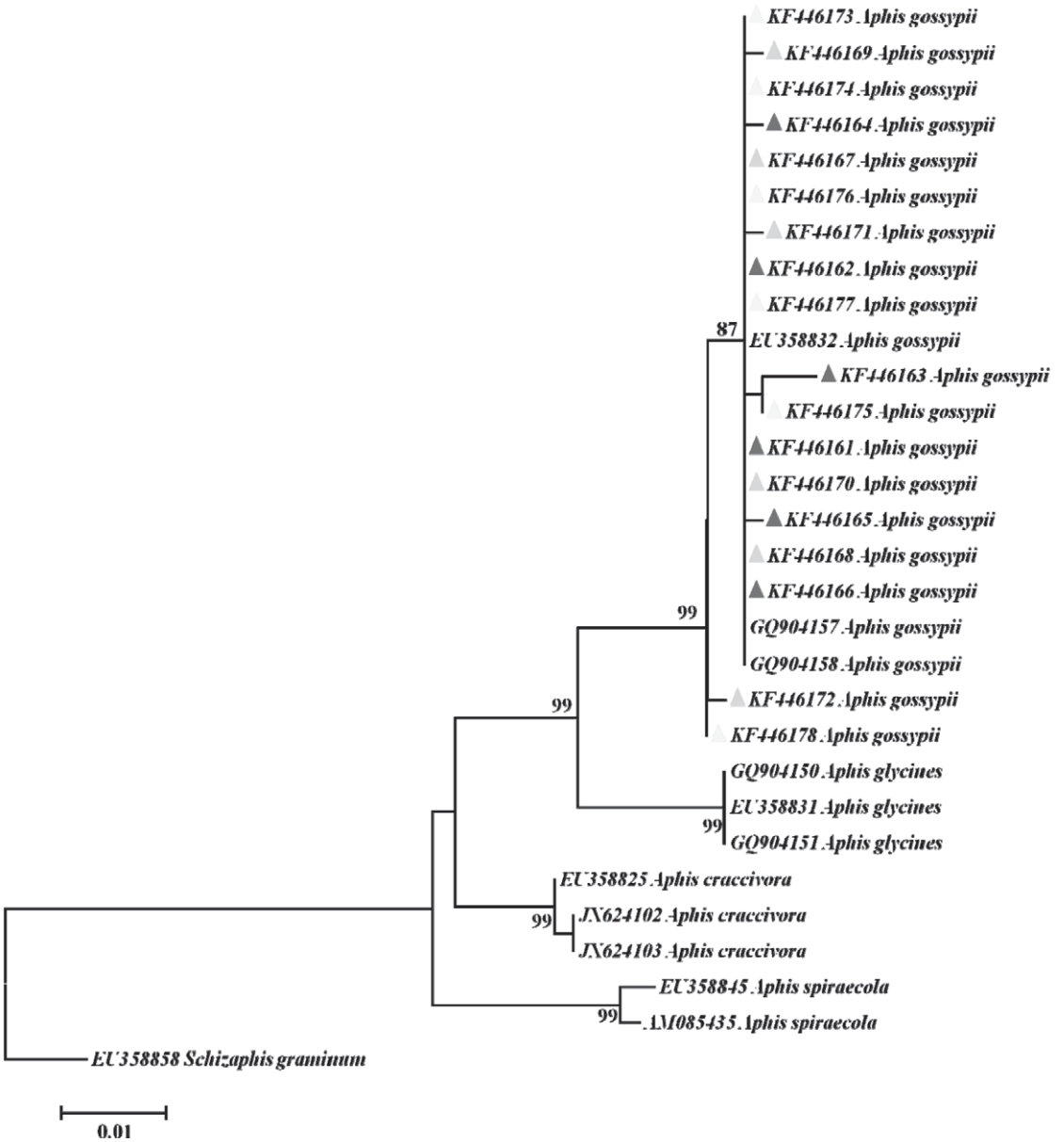

Fig. 3. Neighbor joining tree of *Aphis gossypii* color forms along with other morphologically similar *Aphis* spp. for partial sequences of tRNA/COII with bootstrap support (1000 replicates). Bootstrap values greater than 85% are shown for branches. *Schizaphis graminum* was used as outgroup. A color version of this graphic can be seen online in supplementary material for this article in Florida Entomologist 97(3) (September 2014) at http://purl.fcla.edu/fcla/entomologist/browse .

fragments was biased toward Adenine (A) and Thymine (T). The overall transition (ti)/ transversion (tv) bias of *Aphis* spp.was R = 3.4 for COI and R=6.7 fortRNA/COII, where R = [A*G*k1 + T*C*k2]/ [(A+G)*(T+C)]. Codon positions included were 1st + 2nd + 3rd+Noncoding. All positions containing gaps and missing data were eliminated from the datasets (complete deletion option in MEGA). Summary statistics for the different substitutional changes are shown in Table 2 and 3. Each entry showed the probability of substitution from one base (row) to another base (column) instantaneously. Rates of different transitional substitutions were indicated in bold and those of

TABLE 2. MAXIMUM COMPOSITE LIKELIHOOD ESTIMATE OF THE PATTERN OF NUCLEOTIDE SUBSTITUTION FOR COISEQUENCES OF *APHIS* SPP.

COI	A	T	C	G
A	—	*3.87*	*1.31*	**5.31**
T	*3.26*	—	**14.71**	*0.96*
C	*3.26*	**43.27**	—	*0.96*
G	**17.92**	*3.87*	*1.31*	—

Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

transversionsal substitutions are shown in italics. All these results clearly showed that molecular identification is not limited by color polymorphism of insect pests.

Species diagnosis of prolific aphid pests, especially *A. gossypii,* is very important worldwide to protect agricultural crops from the view point of quarantine and plant protection as this insect pest inflicts economic damage on numerous crops by direct feeding on plant phloem and by vectoring devastating plant diseases (Blackman & Eastop 2000). Due to its complex life cycles, parthenogenetic reproduction, sex, color morphs and the evolutionary tendency towards the loss of taxonomically useful morphological characters, the identification of *A. gossypii* is difficult (Foottit et al. 2008). There are implications for the identification of pest aphid species when individuals of the same species can differ depending on environmental conditions (Van Emden et al. 2007). For non-specialists, identification of aphids using morphology is difficult; because many species look very alike, even when they display strongly different ecology and leads to misidentification of species. Further, identification of aphid species is hampered by a considerable intraspecific color variation and continuous morphological variation.

TABLE 3. MAXIMUM COMPOSITE LIKELIHOOD ESTIMATE OF THE PATTERN OF NUCLEOTIDE SUBSTITUTION FOR TRNA/COII SEQUENCES OF *APHIS* SPP.

tRNA/COII	A	T	C	G
A	—	*1.93*	*0.59*	**0.18**
T	*2.02*	—	**20.74**	*0.37*
C	*2.02*	**68.3**	—	*0.37*
G	**0.97**	*1.93*	*0.59*	—

Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics.

Precision in species identification is the fundamental step for most aspects of biological science. In this regard, molecular identification employing COI and tRNA/COII has an added advantage of not being limited by color polymorphism, sexual forms and life stages of the target species. The present study evaluated the use of mitochondrial markers for quick and reliable identification of *A. gossypii* color forms amongst other *Aphis* spp., which are morphologically similar and genetically close. The *Aphis* spp. were differentiated clearly on the basis of DNA sequence data, which proved to be a valuable tool that could enable accurate identification of these serious insect pests by non specialists and can also be of great interest to detect new invasive species.The study clearly suggests either of COI or tRNA/COII marker can be used as a tool for rapid and reliable identification of *A. gossypii* from other closely related *Aphis* spp. irrespective of color polymorphism. Thus, the present investigation helps in quick, accurate, and timely identification of *Aphis* spp., a critical factor in understanding the fundamentals of species diagnosis, virus transmission, management and devising effective quarantine measures.

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