Development of Species-Specific Markers and Molecular Differences in Mitochondrial and Nuclear Dna Sequences of Aphis Gossypii and Myzus Persicae (Hemiptera: Aphididae)

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DEVELOPMENT OF SPECIES-SPECIFIC MARKERS AND MOLECULAR DIFFERENCES IN MITOCHONDRIAL AND NUCLEAR DNA SEQUENCES OF *APHIS GOSSYPII* AND *MYZUS PERSICAE* (HEMIPTERA: APHIDIDAE)

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Abstract

Aphids are one of the major challenges in the agricultural pest management programmes. A reliable, quick, accurate and life stage-independent method of identification of vectors such as *Aphis gossypii* Glover and *Myzus persicae* (Sulzer) is important with respect to virus transmission, insecticide resistance and biological control. The complex life cycles, significant polymorphism, immature taxonomy and absence of trained manpower make the identification of these pests difficult. On the other hand, molecular identification is not limited by the above factors and can be easily executed by a non-specialist with a little training. Since the mitochondrial cytochrome oxidase-1 (COI) exhibits maternal inherited characteristics and reliable inter-specific variation as compared to other molecular markers, species-specific markers have been developed using existing nucleotide differences in the COI partial sequences of both *A. gossypii* and *M. persicae*. These species-specific markers have proved to be adequate for the molecular identification of these species, and to corroborate their morphological identification. Molecular diversity analyses using both mitochondrial and nuclear markers showed that neither *A. gossypii* nor *M. persicae* has as much genetic variability as expected. An outcome of this investigation is the development of a technique that is useful for the quick identification of *A. gossypii* and *M. persicae*, a critical factor in understanding the epidemiology and management of the potyviruses, and also in facilitating quarantines of these 2 pests.

Key words: *Aphis gossypii*, *Myzus persicae*, species-specific marker, COI

Resumen

Los áfidos son uno de los retos más grandes en los programas de manejo de plagas agrícolas. Un método de identificación de vectores como *Aphis gossypii* Glover y *Myzus persicae* (Sulzer) que es confiable, rápido, preciso e independiente del estadio de la vida presente es importante con respecto a la transmisión de enfermedades por vectores, resistencia a los insecticidas y control biológico. El complicado ciclo de vida el grado de polimorfismo, la taxonomía de los inmaduros y la falta de mano de obra calificada hace difícil la identificación de estas plagas. Por otro lado, la identificación molecular no está limitada por los factores mencionados anteriormente y puede ser ejecutada fácilmente por un no-especialista con un poco de entrenamiento. Puesto que la oxidasa mitocondrial citocromo-1 (COI), exhibe características heredadas maternas y variación inter-específica confiable de en comparación con los otros marcadores moleculares, se han desarrollado marcadores específicos para las especies utilizando las diferencias que existen en los nucleótidos de las secuencias de COI parciales de *A. gossypii* y *M. persicae*. Se han demostrado que estos marcadores específicos son adecuados para la identificación molecular de estas especies, y para corroborar su identificación morfológica. Los análisis de la diversidad molecular utilizando los marcadores mitocondriales y nucleares, demostró que *A. gossypii* y *M. persicae* no tienen mucha variabilidad genética a como se esperaba. Uno de los resultados de esta investigación es el desarrollo de una técnica que es útil para la identificación rápida de *A. gossypii* y *M. persicae,* un factor

crítico para entender la epidemiología y el manejo de los potivirus, y también el facilitar la cuarentena de estas 2 plagas.

Palabras Clave: *Aphis gossypii*, *Myzus persicae*, marcadores especie-específicos, COI

Predominant among the many challenges in sustaining productivity of food and nutritional security are the direct and indirect damages caused by insect pests. Management of the plant pathogens vectored by insect pest is all the more complex because of the insect factors influencing the epidemiology of the disease. Aphid transmitted viruses are numerous and predominant among the many plant viruses transmitted by insects. Aphids transmit only plant viruses, and mostly in a non-persistent manner. However, some plant viruses are transmitted in semi-persistent and persistent way. Among the aphid vectors of viruses, the melon aphid or cotton aphid, *Aphis gossypii* Glover, and the green peach aphid, *Myzus persicae* (Sulzer) are predominant. While hundreds of aphid species infest plants, only a few are predominant vectors; and it is still not clear why species, such as *Aphis gossypii* and *Myzus persicae*, are effective vectors of many potyviruses transmitted in a non-persistent way, nor what factors determine these species' competence to transmit a virus.

The complex life cycles and significant polymorphism makes the identification of *A. gossypii* and *M. persicae* difficult. Their rapid and parthenogenetic reproduction and feeding behavior of aphids results inconsiderable crop damage. Their feeding can cause the induction of premature leaf senescence (Shah et al. 2005), secondary pathogen infection through fungal growth on aphid honeydew and the transmission of plant viruses, which remains the greatest threat for agricultural crops. *Aphis gossypii* and *M. persicae* are known to be capable of transmitting more than 75 plant viruses, and they are most versatile of insect vectors of plant viruses (Blackman & Eastop, 2000). In India *Papaya ringspot virus* (PRSV) is transmitted by both *A. gossypii* and *M. persicae* (Kalleshwaraswamy et al. 2007). Accurate and timely identification not limited by life stage of aphid vectors is important in elucidating the epidemiology of potyviruses, their management and also in quarantine.

Various molecular markers have been employed by researchers for species identification and molecular phylogeny studies, viz., Cytochrome b (Raboudi et al. 2005), 16S rRNA (von Dohlen & Moran 2000), 18S rRNA, 28S rRNA, 5.8S rRNA (Ji et al. 2003), internal transcribed spacers, elongation factor-1α (Jernaes & Arngaad 2006; Ji et al. 2003), mitochondrial cytochrome oxidase (mtCOI) (von Dohlen et al. 2006), etc., in aphids. Mitochondrial genes, because of maternal inheritance and reliable inter-specific variation as compared to other markers (Savolainen et al. 2005), have been widely employed in studying the molecular systematics of insects (Simon et al. 1994). In our study molecular diversity analyses were carried out using both mitochondrial (mitochondrial cytochrome oxidase-1) and nuclear (elongation factor-1 α) genes.

The present investigation was carried out to develop the species-specific markers for *A. gossypii* and *M. persicae* based on COI for life-stage independent identification, and also to carry out phylogenetic analyses based on mitochondrial cytochrome oxidase I and II (COI and COII) and elongation factor-1α (EF1α).

Materials and Methods

Maintenance of Stock Culture and Morphological Identification

For developing species- specific markers, the *A. gossypii* and *M. persicae*, were collected on cotton (*Gossypium hirsutum* L.; Malvales: Malvaceae), and brinjal (*Solanum melongena* L; Solanales: Solanaceae) respectively, at the experimental farm of the Indian Institute of Horticultural Research (IIHR), Bangalore, India. Pure cultures of both species were maintained on cotton and on brinjal at room temperature (30-32 °C) and 70-90% RH. In addition to these two species, 33 different species of aphids viz. *Aphis fabae* (Theobald), *Aphis punicae* Passerini, *Aphis craccivora* Koch, *Aphis nerri* (Boyer de Fonscolombe), *Aphis spireacola* Patch, *Melanaphis sacchari* (Zehntner), *Melanaphis donacis* (Passerini), *Acyrthosiphon pisum* (Harris), *and Macrosiphum rosea* (L.), etc. were collected from the Division of Entomology, National Bureau of Agriculturally Important Insects (NBAII), Bangalore for testing the species-specific primers, which were developed in the present study. In molecular diversity studies, *A. gossypii* and *M. persicae* were collected from different host plants from different geographical locations in India (Tables 1 and 2) samples preserved in ethyl alcohol (100%) and stored at -20 °C until further use. Morphological identifications of *A. gossypii* and *M. persicae* were carried out according to Blackman (2010) prior to molecular studies.

DNA Isolation and Polymerase Chain Reaction

Total DNA was isolated from individual *A. gossypii* and *M. persicae* from the stock culture using

TABLE 1. ANALYZED SAMPLES OF *APHIS GOSSYPII* WITH DESCRIPTION OF THE HOST PLANT, SITE OF COLLECTION AND NCBI-GENBANK ACCESSION NUMBERS.

Sl. No.	Species	Host plant	Site of collection	NCBI-GenBank accessions		
				COI	COII	EF1α
1	*A. gossypii*	Sweet orange	Nagpur (NRCC-1)	JQ067095	JQ067109	JQ690307
2	*A. gossypii*	Sweet orange	Nagpur (NRCC-2)	JQ067096	JQ067110	JQ690308
3	*A. gossypii*	Sweet orange	Nagpur (1)	JQ067097	JQ067111	JQ690309
4	*A. gossypii*	Sweet orange	Nagpur (2)	JQ067098	JQ067112	JQ690310
5	*A. gossypii*	Cotton	Bangalore	JQ067099	JQ067113	JQ690311
6	*A. gossypii*	Watermelon	Bangalore	JQ067100	JQ067114	JQ690312
7	*A. gossypii*	Cotton	Shimoga	JQ067101	JQ067115	JQ690313
8	*A. gossypii*	Watermelon	Calicut (IISR)	JQ067102	JQ067116	JQ690314
9	*A. gossypii*	Watermelon	Cochin	JQ067103	JQ067117	JQ690315
10	*A. gossypii*	Watermelon	Kasaragod (CPCRI)	JQ067104	JQ067118	JQ690316
11	*A. gossypii*	Watermelon	Calicut (Balussery)	JQ067105	JQ067119	JQ690317
12	*A. gossypii*	Hibiscus	Calicut (Balussery)	JQ067106	JQ067120	JQ690318
13	*A. gossypii*	Cotton	Pune	JQ067107	JQ067121	JQ690319
14	*A. gossypii*	Cotton	Gujarath	JQ067108	JQ067122	JQ690320
15	*A. gossypii*	Cotton	Kolar	JQ690329	JQ690299	JQ690321
16	*A. gossypii*	Cotton	Coimbatore (TNAU)	JQ690330	JQ690300	JQ690322
17	*A. gossypii*	Cotton	Akola, Maharashtra	JQ690331	JQ690301	JQ690323
18	*A. gossypii*	Sponge guard	Jaipur, Rajasthan	JQ690332	JQ690302	JQ690324
19	*A. gossypii*	Brinjal	Jaipur, Rajasthan	JQ690333	JQ690303	JQ690325
20	*A. gossypii*	Pumpkin	Jaipur, Rajasthan	JQ690334	JQ690304	JQ690326
21	*A. gossypii*	Cotton	Kauroli, Rajasthan	JQ690335	JQ690305	JQ690327
22	*A. gossypii*	Cotton	Dausa, Rajasthan	JQ690336	JQ690306	JQ690328

the 'salting out' procedure adapted from Rugman Jones et al. (2006). Individual aphid specimens were pierced through one side of the abdomen by using a sterilized minute pin and placed in 0.5 mL PCR tubes containing 100 µL of TNES (10 mM Tris-HC1, pH 7.5, 1 rnM EDTA, l00 mM NaC1, 5 % Sucrose). Tubes were incubated at 37 °C for 24 h, and somewhat later, proteins were precipitated with the addition of 5 M NaCl and vigorous shaking for 30 s. Proteins were pelleted in a microfuge at 13,000 rpm for 5 min, and the supernatant was transferred to a new microfuge tube. DNA was

TABLE 2. ANALYZED SAMPLES OF *MYZUS PERSICAE* WITH DESCRIPTION OF THE HOST PLANT, SITE OF COLLECTION AND THE NCBI-GENBANK ACCESSION NUMBERS.

Sl No.	Species	Host plant	Site of collection	NCBI-GenBank accessions		
				COI	COII	EF1α
1	*M. persicae*	Egg plant	Nagpur	JQ808454	JQ808469	JQ808484
2	*M. persicae*	Okra	Nagpur	JQ808455	JQ808470	JQ808485
3	*M. persicae*	Egg plant	Bangalore (IIHR)	JQ808456	JQ808471	JQ808486
4	*M. persicae*	Okra	Bangalore (IIHR)	JQ808457	JQ808472	JQ808487
5	*M. persicae*	Watermelon	Bangalore	JQ808458	JQ808473	JQ808488
6	*M. persicae*	Watermelon	Bangalore (IIHR)	JQ808459	JQ808474	JQ808489
7	*M. persicae*	Egg plant	Shimoga	JQ808460	JQ808475	JQ808490
8	*M. persicae*	Egg plant	Calicut (IISR)	JQ808461	JQ808476	JQ808491
9	*M. persicae*	Egg plant	Calicut (Balussery)	JQ808462	JQ808477	JQ808492
10	*M. persicae*	Okra	Gujarath	JQ808463	JQ808478	JQ808493
11	*M. persicae*	Egg plant	Jaipur	JQ808464	JQ808479	JQ808494
12	*M. persicae*	Egg plant	Coimbatore (TNAU)	JQ808465	JQ808480	JQ808495
13	*M. persicae*	Okra	Maharashtra (Akola)	JQ808466	JQ808481	JQ808496
14	*M. persicae*	Okra	Kolar (Karnataka)	JQ808467	JQ808482	JQ808497
15	*M. persicae*	Egg plant	Kasaragod (CPCRI)	JQ808468	JQ808483	JQ808498

precipitated from the supernatant by adding of one volume of ice-cold 100% ethanol and incubation for 1 h at -20 °C. DNA was then pelleted by centrifugation, washed in ice-cold 70% ethanol, air-dried, and finally dissolved in 30 µL of sterile distilled water. The original specimens were preserved as specimen vouchers in Entomology Division, Indian Institute of Horticultural Research.

For molecular diversity analyses, we used both mitochondrial (mitochondrial cytochrome oxidase-I and II) and nuclear (elongation factor-1 α) genes (primer details in Table 3). The species- specific markers were developed using the mitochondrial cytochrome oxidase I (COI) gene, which resulted in the amplification of 658 bp fragment. PCR was carried out in a thermal cycler (ABI-Applied Biosystems, Veriti, USA) with the following cycling parameters; 94 °C for 4 min as initial denaturation followed by 35 cycles of 94 °C 30 s, (46 °C-54 °C) for 40 s, 72 °C for 40 s and 72 °C for 20 min as final extension. PCR was performed in 25 µL total reaction volume containing 20 picomoles of each primer, 10 mM Tris HCl (pH-8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5U of Taq DNA polymerase (Fermentas Life Sciences, Maryland, USA). The amplified products were resolved in 1.0% agarose gel, stained with ethidium bromide (10µg/mL) and visualized in a gel documentation system (UVP). For the species-specific primers mentioned in Table 4, PCR mix and PCR cycling parameters were the same except for the annealing temperature viz., 64 °C for 45 s for both *A. gossypii* and *M. persicae*.

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 GmBH, St. Leon-Rot, Germany) according to the manufacturer's protocol. Blue/white selection was carried out and all the white colonies were maintained on LBA containing ampicillin (100 mg/ml), incubated at 37 °C overnight and stored at 4 °C until further use. Plasmids were prepared from the overnight culture of the positive colonies cultured in LB broth (enzymatic casein- 10 g, yeast extract-5 g, NaCl-5 g in 1000 mL of water, pH-7.0) using GeneJET™ Plasmid Miniprep Kit (Fermentas GmBH, St. Leon-Rot, Germany) according to manufacturer's protocol, from the overnight cultures of the 5 randomly selected clones grown in LB broth. Sequencing was carried out in an automated sequencer (ABI Prism® 3730 XL DNA Analyzer; Applied Biosystems, Maryland, USA) using M13 universal primers both in forward and reverse directions.

Development of Species-Specific Markers and Molecular Diversity

Homology search was carried out using BLAST (http://www.ncbi.nlm.nih.gov), and the differences in COI sequences of *A. gossypii* and *M. persicae* were determined using the sequence alignment editor BioEdit version 7.0.5.3 (Hall 1999). Sequences for *A. gossypii* and *M. persicae*

Table 3. Primers employed in the current study.

Region	Gene Location	Primer Name	Sequence	Reference
Mitochondria	COI	LCO1490 HCO2198	5'-GGTCAACAAATCATAAAGATATTGG-3' 5'- TAAACTTCAGGGTGACCAAAAAATCA-3'	Hebert et al. 2003a,b
Mitochondria	tRNA/COII	2993+ A3772	5'-CATTCATATTCAGAATTACC-3' 5'-GAGACCATTACTTGCTTTCAGTCATCT-3'	Stern 1994 Normark 1996
Nuclear DNA	EF1α	EF3 EF6	5'-GAACGTGAACGTGGTATCAC-3' 5'-TGACCAGGGTGGTTCAATAC-3'	von Dohlen et al. 2002

Table 4. Species specific molecular markers developed for *Aphis gossypii* and *Myzus persicae*.

Species	Primer	Binding Region (bp)	Product size (bp)
A. gossypii	KBR(AG)-F- 5'- TTCTTCTCTTAGAATTTTAATCCGATTA -3' KBR(AG)-R- 5'- AAGAATAGGGTCTCCCCCACCT -3'	43-70 616-643	600
M. persicae	KBR(MP)-F- 5'- ATCATCACTTAGAATCTTAATTCGTCTT -3' KBR(MP)-R- 5'- TGGTATTATATTTAAGATTGTACAAATA -3'	43-70 445-472	429

were deposited with the NCBI database, and the accession numbers are recorded in Tables 1 and 2. To develop the species-specific markers for *A. gossypii* and *M. persicae*, 5 sets of forward and reverse primers were synthesized based on the variable regions in the aligned sequences of the same (Fig. 1). The primers thus designed were validated both on identified *A. gossypii* and *M. persicae* (8 specimens each) and 5 each of the unidentified test specimens collected on cotton and brinjal. The PCR amplified fragments resulting from species-specific markers for *A. gossypii* and *M. persicae* were further cloned, sequenced and analyzed as above. In order to validate the species-specific primer specificity, we tried cross amplification through PCR for both the primer sets developed in the present study with 33 different species of aphids viz. *Aphis fabae* (Theobald), *Aphis punicae* Passerini, *Aphis craccivora* Koch, *Aphis nerii* Boyer de Fonscolombe, *Aphis spiraecola* Patch, *Melanaphis sacchari* (Zehntner), *Melanaphis donacis* (Passerini), *Acyrthosiphon pisum* (Harris), *and Macrosiphum rosea* (L.), etc., which were morphologically identified and collected from National Bureau Agriculturally Important Insects, Bangalore (DNA barcodes for all these 35 species were developed and submitted to both iBOL and NCBI, unpublished data).

Sequence Analysis

All the sequences generated in the present study, corresponding to COI, COII and EF1α was aligned using BioEdit. 4. 0 program using Clustal W. The alignment was further analyzed using MEGA.4.0 (Kumar et al. 1993). Maximum- Parsimony (MP) and Neighbour-Joining (NJ) trees were constructed using the Kimura-2-parameter (K2P) distance model (Kimura 1980; Saitou & Nei 1987). All the corresponding sequences for different mitochondrial and nuclear markers of *A. gossypii* and *M. persicae* were deposited in the NCBI-GenBank. Phylogenetic analyses were carried out by Maximum Parsimony (MP) and Bayesian approach using PAUP v4b10 (Swofford, 1998).

Results and Discussion

The PCR amplicon of the same size (approx.700 bp) was amplified for both *A. gossypii* and *M. persicae* and the sequences showed that the total nucleotide length obtained was 709 bp for both *A. gossypii* and *M. persicae.* The BLAST search for all of the sequences generated in the present study showed that the sequences had the similarity for the respective species. Alignment of the COI sequences for *Aphis gossypii* (HM237329) and *Myzus persicae* (HM237331) in BioEdit v.7.0 showed that there were variations in 60 nucleotides out of 658 amounting to 9% difference between *A. gossypii* and *M. persicae* (Fig. 1). Species-specific markers require only customary PCR which is readily available, rapid and inexpensive. Out of 5 primer sets identified each for *A. gossypii* and *M. persicae*, 1 primer set, viz., KBR(AG)-F & KBR(AG)-R and KBR(MP)-F & KBR(MP)-R, could successfully identify *A. gossypii* and *M. persicae*, respectively (Table 4, Fig. 2). These species-specific markers amplified an expected fragment size of 600 bp and 429 bp

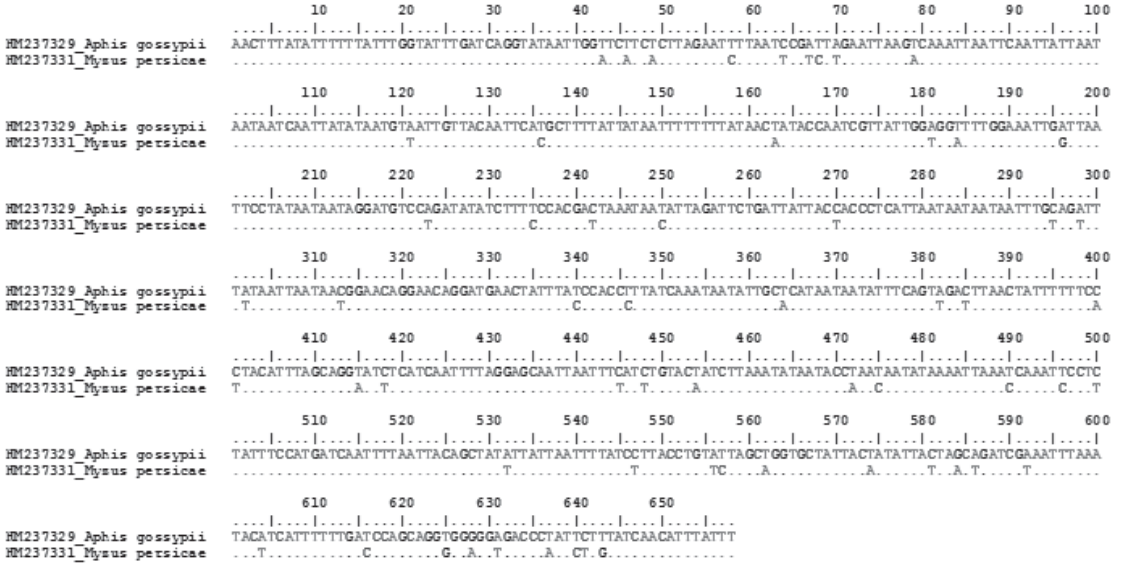

Fig 1. Consensus sequence of 658 bp from the mitochondrial cytochrome oxidase I (COI) gene for the Bangalore strain of *Aphis gossypii* and *Myzus persicae.*

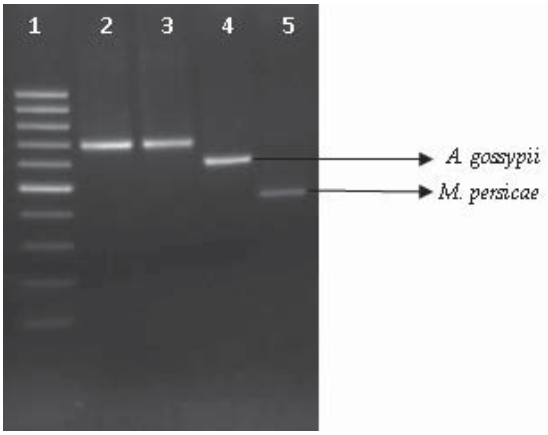

Fig. 2. Validation of species-specific markers for *Aphis gossypii* and *Myzus persicae* {M - 100bp DNA ladder (Fermentas). 1. and 2. PCR amplified product (LCO/HCO) *A. gossypii* and *M. persicae* respectively, 3. *A. gossypii* - molecular specific marker, 4. *M. persicae* - specific molecular marker.

for *A. gossypii* and *M. persicae*, respectively. The same fragments were cloned, sequenced and the BLAST search of these sequences obtained using species-specific markers showed the similarity for the respective species. Similar amplicon sizes were obtained from test specimens of aphids collected on cotton and brinjal.

The validation of the species specific primers, which were developed in the present study, were carried out, by employing PCR using both these primers and genomic DNA isolated from 33 different species of aphids, which were morphologically identified and collected from NBAII. No PCR reactions produced amplification, including non-specific amplicons, where the PCR mix and PCR cycling conditions were kept the same, including the annealing temperature, viz., 64 °C for 45 s for all the reactions. However the applicability of these primers on other populations of *A. gossypii* and *M. persicae* within and outside India will depend on variations in the nucleotide sequences both in forward (43-70 for both *A. gossypii* and *M. persicae*) and reverse primer binding regions (613-643 and 441-472 for *A. gossypii* and *M. persicae* respectively).

Comparison of forward primer binding regions for *A. gossypii* (HM237329) with other 129 existing COI sequences (sequences generated in the present study and from NCBI-GenBank database) showed that there were variations at the 43rd position. One accession had A and another had C instead of T, at the 54th position. One accession had T instead of G at the 59th position. One accession had C instead of T and at the 70th position. Four accessions had G instead of A in all the other sequences. Similarly in the reverse primer binding region, which was compared, and which showed that there were variations at the 622nd position. One accession had T instead of A at the 623rd position. One accession had A instead of G at the 633rd position. Two accessions had T instead of A in all the other sequences.

A comparison of the forward primer binding region for *M. persicae* with 35 existing COI sequences (sequences generated in the present study and from NCBI-GenBank database) showed that there were no variations in any of the nucleotide positions; whereas in the reverse primer binding region such comparison showed that there were variations in the 451st position. Two accessions had C instead of T in all sequences. In this regard, development of degenerate primers would be a valuable tool in identifying the other populations of *A. gossypii* and *M. persicae* throughout the world independent of life stages and sex (Asokan et al. 2011). Other criteria to be taken into consideration while developing a species-specific marker for aphid species are intra- and inter-specific variations (Foottit et al. 2008; Shufran et al. 2004). Molecular identification using a species-specific marker is an advantage where there is polymorphism in the target species. Development of species-specific markers for *A. gossypii* and *M. persicae* would be of immense value to identify these vectors at any of the developmental stages (egg, nymph or adult). Previously Shufran & Puterka (2011) demonstrated the utility of COI in identification of aphid eggs and undescribed morphs found in wheat (*Triticum* spp.) or barley (*Hordeum vulgare* L.), but here the additional cost was that of sequencing. The species-specific markers that have been identified in this study will enable even a non- specialist to identify the target species, *A. gossypii* and *M. persicae* at any developmental stage without the aid of sequencing.

The intraspecific variations associated with host utilization is the most frequent and familiar phenomenon shown by phytophagous insects (Mopper & Strauss 1997; Margaritopoulos et al. 2006). Massutti & Chavigny (1998) reported host associated genetic differentiation in *A. gossypii* using Random Amplified Polymorphic DNA (RAPD) markers. In our present study, we targeted *A. gossypii*, a species in which there is already evidence of intraspecific variation with respect to host associated reproductive performance and related traits i.e., on chrysanthemum, cucumber (*Cucumis sativus* L.) and cotton (Margaritopoulos et al. 2006). Using morphological traits Margaritopoulos et al. (2006) proved the existence of the taxonomic status of the Compositae and Non-Compositae forms of *A. gossypii*, and they suggested the molecular work for the confirmation of the same. In this regard, it is necessary to analyze the molecular diversity in *A. gossypii* and *M. persicae* considering the fact that they are major vectors of many plant viruses. Comparison of the

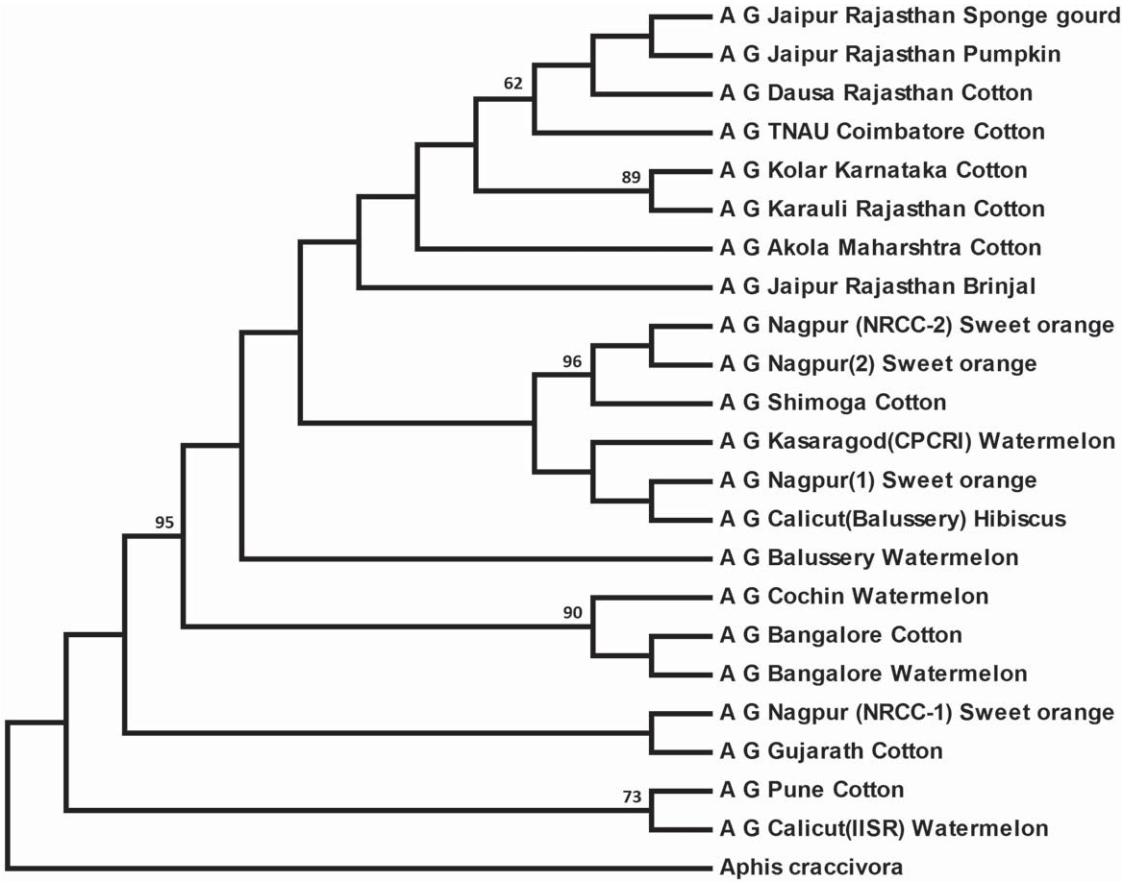

Fig 3. Maximum-Parsimony (MP) tree with bootstrap support (1000 replicates) showing clustering of *Aphis gossypii* (collected from different geographical locations and on different host plants) from the combined datasets of both nuclear and mitochondrial sequences. *Aphis craccivora* used as an out group in the analysis.

COI, COII and EF1α sequences of 22 populations of *A. gossypii* and 15 populations of *M. persicae,* collected from various geographical locations in India and on various host plants showed that were very few nucleotide variations among them, and the phylogram (Figs. 3 and 4) suggested that there are neither cryptic (sub) species nor biotypes existing in India.

The datasets for COI, COII and EF1α were combined based on the ILD test performed using PAUP.4.0. The Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution for *A. gossypii* sequences was performed using MEGA 4.0 (Tamura et al. 2007). The reliability of the clustering pattern in the trees was determined by the bootstrap test, with 1000 replications. The *A. gossypii* nucleotide frequencies were 0.343 (A), 0.359 (T), 0.164 (C) and 0.133 (G). The base composition of these 3 gene fragments was biased toward Adenine (A) and Thymine (T), which together constituted 70.2% of the total. The overall transition (ti)/ transversion (tv) bias of *A. gossypii* nucleotide sequence was R = 1.007, where $R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]$. Codon positions included were 1st + 2nd + 3rd + Noncoding. For *M. persicae*, the nucleotide frequencies were 0.34 4 (A), 0.361 (T), 0.155 (C) and 0.14 (G). As in the previous case the base composition of 3 gene sequences were biased towards Adenine and Thymine, with an estimated frequency of 70.5% of the total. The overall transition/transversion ratio was 0.3. Summary statistics for the different substitutional changes are shown in Tables 5 and 6.

Further analysis carried out using the acquired sequences from NCBI-GenBank corresponding to COI for both *A. gossypii* and *M. persicae,* revealed that there are very few sequence variations. This suggests that genetic structuring in terms of sub (cryptic) species, biotypes or host-associated genetic differences etc., associated with both *A. gossypii* and *M. persicae* were not evidenced using COI sequences of the world population. Both *A. gossypii* and *M. persicae* can exhibit phenotypic plasticity (Rosenheim et al. 1994; Blackman & Spence 1994) in response to the morphology of the

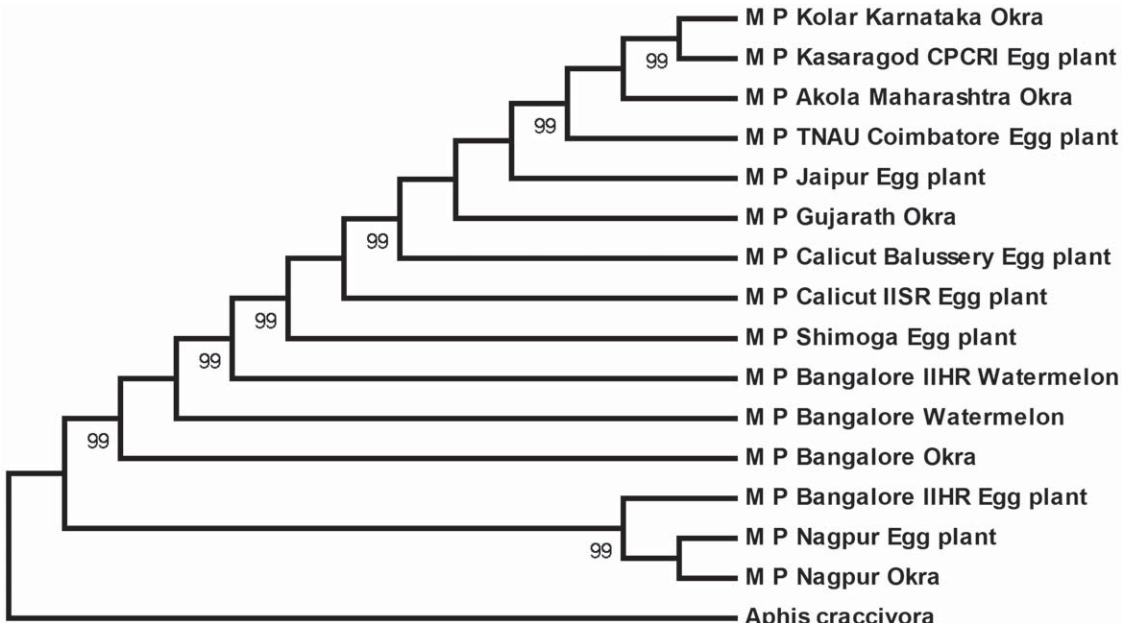

Fig 4. Maximum-Parsimony (MP) tree with bootstrap support (1000 replicates) showing clustering of *Myzus persicae* (collected from different geographical locations and on different host plants) for both nuclear and mitochondrial datasets. *Aphis craccivora* used as an out group.

host plants of the samples; and also temperature can affect both isometric and allometric growth in aphids (Blackman & Spence 1994). Our study undoubtedly proved that these morphological variations of *A. gossypii* and *M. persicae* are not reflected in their genetic structure, and can be the result of host plant morphology and environmental factors affecting aphid growth and development.

Conclusion

In the present study, we showed the utility of species-specific markers which can be used for reliable species identification of *A. gossypii* and *M. persicae* amongst many other aphid species, which are morphologically and genetically close. Thus, our work will help in rapid, accurate, life stage and color morph independent identification of these aphid vectors, which in turn will help in further elucidating the epidemiology of potyviruses, their management and be of value in the operation of quarantines. Also we showed that both *A. gossypii* and *M. persicae* are individual cosmopolitan and polyphagous species, which do not have any cryptic (sub) species or biotypes.

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Table 5. Maximum composite likelihood estimate of the pattern of nucleotide substitution from 22 populations of *Aphis gossypii*.

	A	T	C	G
A	—	*6.69*	*3.06*	**9.95**
T	*6.39*	—	**8.5**	*2.47*
C	*6.39*	**18.58**	—	*2.47*
G	**25.74**	*6.69*	*3.06*	—

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.

Table 6. Maximum composite likelihood estimate of the pattern of nucleotide substitution from 15 populations of *Myzus persicae*.

	A	T	C	G
A	—	*12.03*	*5.18*	**4.66**
T	*11.46*	—	**5.18**	*4.66*
C	*11.46*	**12.03**	—	*4.66*
G	**11.46**	*12.03*	*5.18*	—

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.

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