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Systematics

Morphometric Analysis of Three Putative Species of *Bemisia tabaci* (Hemiptera: Aleyrodidae) Species Complex From India

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ABSTRACT The *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a pest of agricultural and horticultural crops. The *B. tabaci* species complex consists of 36 morphologically indistinguishable putative species. This study evaluates the morphometric variations of developmental stages, puparia, and adults in three putative species of *B. tabaci* occurring in India. The genetic identity of these studied populations were confirmed by mtCO1 analysis and revealed that the population from Amravati, Ludhiana, and Delhi were clustered with Asia I, Asia II1, and Asia II7 putative species, respectively. The morphological comparisons showed that fourth instar and adult of Asia-II1 was comparatively larger than Asia-I and Asia-II7. The positioning of sensorial cone on antennal segment 7 is much apart, and away from the sensorium in Asia-II1 while these are comparatively adjacent in Asia-I and Asia-II7 for both the sexes. The multivariate statistical analyses reveal that 31 measurements in puparia, 23 of male and 22 of female show significant variations ($P \leq 0.01$). This was supported by scatter graphs derived from principal components and canonical discriminant analysis (CDA), and separate clustering was obtained for Asia-I, Asia-II1, and Asia-II7. Overall 91 and 99% of the classifications were correctly attributed by CDA for puparia and adults which confirmed the distinction of these groups. The characters brought out in this study could be used as a population/putative species specific markers in *B. tabaci* species complex and these variations might enable distinguishing the other genetic groups too.

KEY WORDS *Bemisia tabaci*, developmental stage, puparia, adult, morphometric

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) is a haplodiploid, sap feeding arthropod pest in field crops of warm to hot climates between 30° N and S of the equator. It is polyphagous and reported feeding on more than 900 hosts (Hsieh et al. 2006). In the last two decades, it is becoming a serious pest in short-lived herbaceous hosts including numerous agricultural dicotyledon, horticultural and commodity crops (Brown et al. 1995). Phylogenetic studies by De Barro et al. (2011) suggested that *B. tabaci* is a cryptic species complex consisting of 24 morphologically indistinguishable species in 11 well-defined high-level groups. These were later concluded as belonging to 36 putative species comparing their differences in genetic structure, host plant preferences, bacterial symbionts (Hu et al. 2011; Boykin et al. 2012, 2013; Liu et al. 2012; Tay et al. 2012; Boykin 2013, 2014; Lee et al. 2013; Wang et al. 2013; Ashfaq et al. 2014; Boykin and De Barro 2014) and interbreeding capabilities (Wang et al. 2010, Xu et al. 2010).

In India *B. tabaci* was first reported from cotton fields of Punjab in 1905 (Misra and Lambda 1929). The presence of invasive biotype B was reported from the field of Kolar and Bangalore (Banks et al. 2001). Later the Asian *B. tabaci* was differentiated into Asia I, Asia II, Asia II1, China, and MEAM 1 (Boykin et al. 2007, Ahmed et al. 2011). Chowda Reddy et al. (2012) further resolved these and showed that the Asia I, Asia II1, Asia II5, Asia II7, Asia II8, and MEAM1 putative species are present in the Indian subcontinent and Asia.

At present, biochemical and molecular biological techniques are very helpful for differentiation of these putative species within the species complex as well as for identification of some morphologically indistinguishable species or for construction of phylogenetic trees (Cervera et al. 2000, Calvert et al. 2001, Khasdan et al. 2005, Hsieh et al. 2007, Gueguen et al. 2009, McKenzie et al. 2009, Papayiannis et al. 2009, Dinsdale et al. 2010, Sun et al. 2011). However, in terms of taxonomy or systematics, morphology is considered the foremost basis for species separation (Gill and Brown 2010), not only due to the relationship between morphological characteristics and phylogeny, but due to convenience for identification as well (Yan 2001). Therefore, it is necessary to conduct thorough investigations of *B. tabaci* species complex for their morphological characters (Gill and Brown 2010).

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In the 1990s morphology and morphometrics of *B. tabaci* were extensively studied due to the invasion of *B. tabaci* B biotype to the United States. The B biotype was described as silver leaf whitefly *Bemisia argentifolii* based on morphological and allozymic characters (Bellows et al. 1994). And then more characters of fourth-instar nymph (the pupal case) were found, such as absence of the fourth anterior margin setal pair, width of the thoracic tracheal folds, and width of wax extrusions from the folds that could be used to separate biotype B from A. However, the subsequent comparison across 5 of the 11 major groups (Asia I, Asia II, Africa, Middle East Asia Minor, New World) showed that the morphological characters previously used to separate A from B were not useful for the reliable separation of any of the groups (Rosell et al. 1997, Calvert et al. 2001, Liu et al. 2012). As then, there have been few morphological studies on the *B. tabaci* species complex. Gill and Brown (2010) examined the species status and the morphological variations in puparia within *Bemisia* and the relatives and concluded that these biotypes/putative species are a complex of cryptic species which have evolved over time in isolation probably without the need to change morphologically, but they suggested conducting more thorough morphological studies at least in the puparia and adults.

Tay et al. (2012) stated that Mediterranean is the real *B. tabaci* and illustrated the puparium and antennae of male and female, genitalia of male and variation in the cement gland of three adult female collected by Gennadius. Li et al. (2013) conducted a thorough study on the morphological characters and morphometrics of six biotypes from China and reported that length of operculum, length and width of lingula, and length of antennal segments can be used to distinguish these. This study also suggested that such results to be applied to more biotypes from more plants and more locations to test their stability and reliability. Hence, this study focusing on the morphology of developmental stages, and morphometrics of puparia and adults of three putative species viz., Asia-I, Asia-II1 and Asia-II7 of the *B. tabaci* species complex from India.

Materials and Methods

Populations of *B. tabaci* were collected from different locations (Amravati, N 21° 01′ 604″ E 77° 55′ 004″, 367 m; Ludhiana, N 30° 53′ 932″ E 75° 48′ 229″, 240 m, and Delhi, N 28° 38′ 28.1″ E 77° 10′ 12.2″, 213 m asl) in various agroclimatic zones of India from their host plants viz., cotton (*Gossypium* spp.; Malvales: Malvaceae) and leucaena (*Leucaena* spp.; Fabales: Fabaceae). These were reared up to six generations on cotton in the insect proof climate control chamber at the Indian Agricultural Research Institute, New Delhi, India, under controlled conditions of temperature (28 ± 2°C), humidity (60 ± 5%) and photoperiods (10 L × 14D). The samples drawn from these cultures were subjected to morphometric studies after the required molecular studies to confirm their genetic groups.

Genomic DNA was extracted from single *B. tabaci* adult using DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol. The mtCOI region was amplified using the universal described by Frohlich et al. (1999) and Simon et al. (1994). All PCR analyses were conducted with *Taq* polymerase (Invitrogen, Sao Paulo, Brazil) in a PTC-200 thermocycler (Biorad, Germany). These amplified PCR products were purified and sequenced in DNA analyzer (Scigenomics, Cochin, India).

The mtCO1 sequence obtained from each population was subjected to homology search using Basic Local Alignment Search Tool (nBLAST) algorithm (Altschul et al. 1997, Schaffer et al. 2001) at NCBI (http://www.ncbi.nlm.nih.gov, last accessed 2 February 2014). The haplotypes of the consensus sequences of the all putative species identified in the previous reviews were retrieved from GenBank (Chowda Reddy et al. 2012, De Barro and Boykin 2013, Ashfaq et al. 2014, Boykin and De Barro 2014) and aligned using the MUSCLE programme with default parameters (Edgar 2004).

Phylogenetic trees were constructed by using the GTR + I + G (General Time Reversible model with a proportion of invariable sites and a gamma shaped distribution of rates across sites) DNA substitution which showed the best Bayesian information criterion score (Posada 2008) and graphically displayed in a maximum likely hood tree by using the program MEGA 6 (Tamura et al. 2013). To assess the phylogenetic supports for groupings on the tree, we performed a bootstrap resembling analysis (1,000 replication). The pair wise distances were calculated using the Kimura-2 parameter model.

Developmental stages were identified according (Malumphy et al. 2009, Chaubey et al. 2010); measurements and photographs were taken in Leica M205FA stereozoom microscope attached with DFC425 digital camera at 350× to 1,600× ($n = 30$). Puparia and adults were processed for mounting as recommended earlier (Mound 1963, Mohanty and Basu 1986, Chaubey et al. 2010). The mounted specimens ($n = 30$) were observed under the Leica DM100 phase contrast research microscope at 400× for studying the essential characters. Photographs were taken on Leica DM500 stereozoom microscope attached with DFC290 digital camera.

Adult antennal sensilla were subjected to analysis in scanning electron microscope (SEM). Specimen preparations were adopted from Calvert et al. (2001). The adults were dehydrated in a series of 70%, 90% and absolute ethyl alcohol for 20 min in each and treated in carbolxylene for 2–4 h to remove the microscopic wax particle. After that specimens were transferred to absolute ethyl alcohol for 10 min and then to hexamethyl disilazane (chemical dryer) for 2–3 min. SEM studies were done with Zeiss EVOMA10 SEM at 20 Kv/EHT and at 3.20–6.40 kx after 24 nm palladium coating. A sample size of $n = 30$ for male and female from each putative species was used and measurements were carried out by Smart Tiff V1.0.0.12 software.

Univariate one way single factor ANOVA was performed individually for all characters to select the significant characters as a prelude to identifying the

significant ones (Kalaisekar et al. 2012). After this the pattern of clustering was analyzed using multivariate statistical approaches (Tabachnick and Fidell 2007); Principal Component Analysis (PCA; SAS procedure; PRINCOMP; SAS version 9.1.3, SAS Institute Inc., Cary, NC, USA) was used without any prior assumption of groupings which assesses the components for total variation among the specimens by calculating linear combination of variables that explain the maximum of total variation. Canonical Discriminant Analysis (CDA; SAS procedure; CANDISC) was used for calculating the linear combinations of variables that maximize the separation of means of previously defined classes. Contribution of the variables best summarizing the differences between classes is revealed by this technique. As, Discriminant Function Analysis (DFA; SAS procedure; DISCRIM) maximizes the variation among groups, it was used to separate putative species of *B. tabaci* based on morphometric data.

Results

Assigning of Populations to Genetic Groups. The global phylogenetic analysis with the partial mtCO1 sequences revealed that these are assignable to the Asia I, and Asia II groups namely Asia II1 and Asia II7. The Amravati (KF298442) populations clustered with Asia I, while those from Ludhiana (KF298443) and Delhi (JQ023501) clustered with Asia II1 and Asia II7, respectively (Fig. 1). The pair wise distance between the putative species Asia II1 versus Asia II7 were found to be 0.098 while in Asia I versus Asia II1and Asia II7 it was 0.156 and 0.137, respectively.

Morphometrics.

Developmental Stages. Morphology of developmental stages was studied especially the pattern and size of wax fringe in the instars (Fig. 2). Morphometrics of developmental stages is shown in Table 1 which reveals that the Asia I is comparatively smaller than the Asia II1 and Asia II7 except for the length of fourth instar which was larger in Asia II1 followed by Asia I and Asia II 7. The breadth of wax fringe in first (0.028 ± 0.008, 0.021 ± 0.001, and 0.017 ± 0.001) and in second instars (0.19 ± 0.006, 0.016 ± 0.001, and 0.015 ± 0.005) was more in Asia II7 as compared with Asia I and Asia II1, respectively. The length of anterior wax fringe in fourth instar was comparatively more in Asia 1 (0.120 ± 0.002) followed by Asia II1 (0.091 ± 0.002) and Asia II7 (0.063 ± 0.003), while that of posterior wax fringe was more in Asia II7 (0.080 ± 0.001) than Asia I (0.072 ± 0.006) and Asia II1 (0.069 ± 0.001). All these measurements of developmental stages and their wax fringe vary and are statistically significant at $P \leq 0.001$ except length of fourth instar which is significant at $P \leq 0.05$.

Puparia. The morphometrics measurement of important characters of puparia, and their significance are listed in Table 2. The puparia from Asia II1 was longer and wider than the Asia I and Asia II7. The length of tracheal fold (0.045) was longer while breadth (0.104) was shorter in Asia II7. The vasiform orifice was shorter (0.068) in Asia II7 while it was broader (0.06) in Asia I; and a longer and wider operculum and

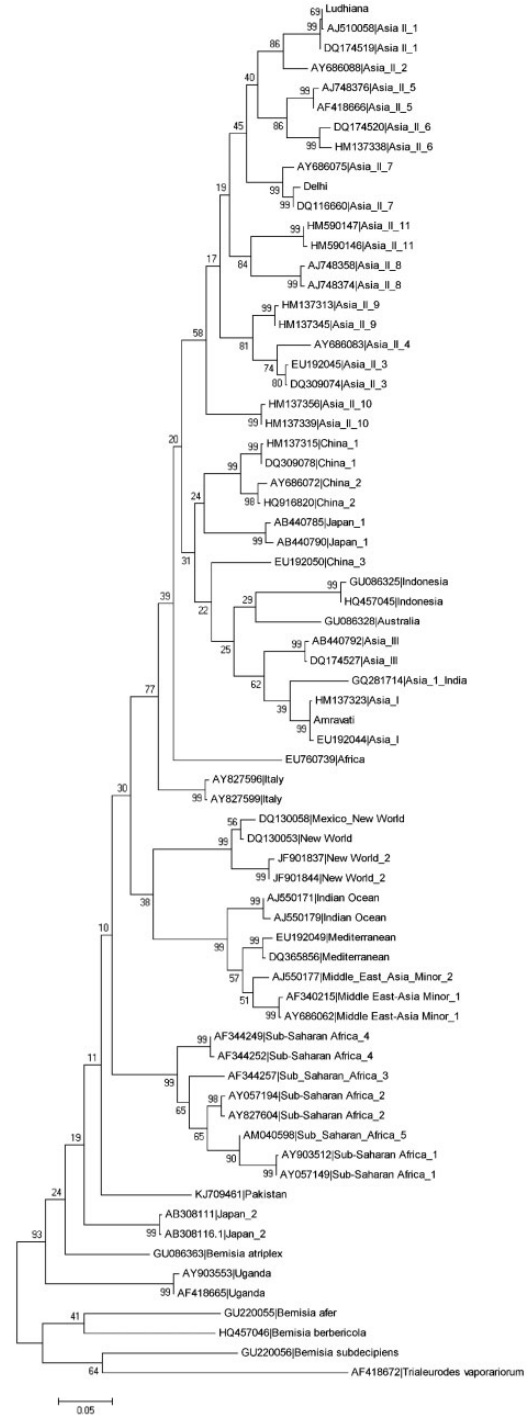

Fig. 1. Phylogenetic tree of mtCO1 gene sequences of the studied putative species of *B. tabaci* species complex (representative tree). Numbers above the branches indicate bootstrap value.

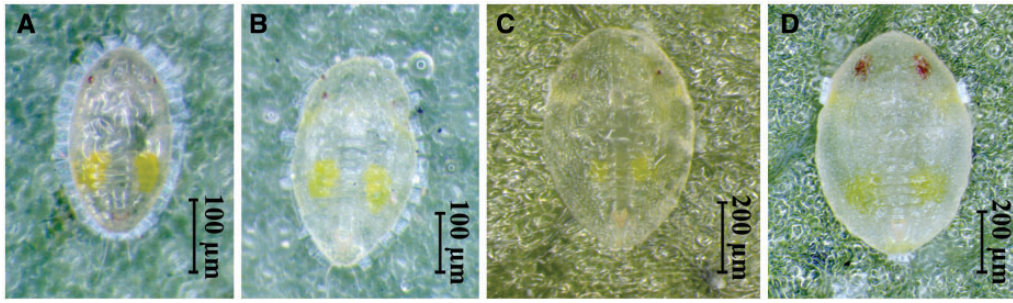

Fig. 2. Developmental stages of *B. tabaci* showing the wax fringe pattern; A, first instar; B, second instar; C, third instar; D, fourth instar.

Table 1. Morphometric measurements of developmental stages and there wax fringe Asia-I, Asia-II1, and Asia-II7 genetic group of *B. tabaci* (mean ± SE mm; $n = 30$)

Characters	Amravati Asia I	Ludhiana Asia I-1	Delhi Asia II-7
First instar (L × B)	0.212 ± 0.003 × 0.130 ± 0.002	0.264 ± 0.003 × 0.136 ± 0.002	0.232 ± 0.005 × 0.144 ± 0.003
Wax fringe breadth	0.021 ± 0.000	0.017 ± 0.000	0.028 ± 0.008
Second instar (L × B)	0.314 ± 0.003 × 0.199 ± 0.002	0.325 ± 0.004 × 0.182 ± 0.002	0.333 ± 0.007 × 0.214 ± 0.006
Wax fringe breadth	0.016 ± 0.000	0.015 ± 0.005	0.019 ± 0.006
Third instar (L × B)	0.423 ± 0.003 × 0.263 ± 0.004	0.442 ± 0.004 × 0.258 ± 0.003	0.454 ± 0.006 × 0.322 ± 0.009
Fourth instar (L × B)*	0.687 ± 0.004 × 0.481 ± 0.011	0.725 ± 0.011 × 0.495 ± 0.012	0.665 ± 0.009 × 0.438 ± 0.009
Anterior wax fringe (L × B)	0.120 ± 0.002 × 0.022 ± 0.000	0.091 ± 0.002 × 0.021 ± 0.006	0.063 ± 0.003 × 0.022 ± 0.002
Posterior wax fringe (L × B)	0.072 ± 0.001 × 0.016 ± 0.004	0.069 ± 0.006 × 0.017 ± 0.004	0.080 ± 0.001 × 0.021 ± 0.005

All the measurements were statistically significant at $P < 0.001$ and *was significant at $P < 0.05$.

Table 2. Statistically significant characters of *B. tabaci* puparia from Asia-I, Asia-II1, and Asia-II7 genetic groups (mean ± SE mm; $n = 30$) in which $P < 0.01$

Sr. No.	Characters	Amravati Asia-I	Ludhiana Asia II-1	Delhi Asia II-7	*F*-value	*P*-value
1	Length of the dorsal seta 3	0.126 ± 0.008	0.130 ± 0.004	0.014 ± 0.010	73.008	9.03 × 10-34
2	Length of the dorsal seta 1	0.132 ± 0.010	0.138 ± 0.005	0.019 ± 0.009	64.769	2.55 × 10-31
3	Length of the dorsal seta 5	0.122 ± 0.006	0.075 ± 0.000	0.009 ± 0.011	38.861	4.44 × 10-22
4	Length of the antennae	0.061 ± 0.000	0.058 ± 0.000	0.051 ± 0.000	38.428	6.71 × 10-22
5	Breadth of the vasiform orifice	0.060 ± 0.004	0.057 ± 0.006	0.052 ± 0.006	36.485	4.42 × 10-21
6	Length of the dorsal seta 2	0.117 ± 0.006	0.087 ± 0.000	0.007 ± 0.012	35.141	1.68 × 10-20
7	Length of the dorsal seta 6	0.052 ± 0.001	0.005 ± 0.002	0.006 ± 0.008	28.138	2.67 × 10-17
8	Length of caudal furrow	0.058 ± 0.007	0.058 ± 0.001	0.048 ± 0.001	24.669	1.37 × 10-15
9	Breadth of operculum	0.049 ± 0.004	0.047 ± 0.005	0.043 ± 0.005	24.427	1.82 × 10-15
10	Length of the posterior submarginal setae 5	0.048 ± 0.000	0.005 ± 0.000	0.005 ± 0.000	22.818	1.23 × 10-14
11	Length of the operculum	0.037 ± 0.002	0.036 ± 0.003	0.033 ± 0.009	22.794	1.26 × 10-14
12	Length of the dorsal seta 4	0.059 ± 0.012	0	0	21.897	3.74 × 10-14
13	Breadth of the pupal case	0.576 ± 0.005	0.549 ± 0.008	0.515 ± 0.008	19.252	1.01 × 10-12
14	Length of first abdominal segment	0.043 ± 0.001	0.044 ± 0.001	0.042 ± 0.000	18.506	2.63 × 10-12
15	Length of eighth abdominal segment from pocket to caudal pore	0.193 ± 0.001	0.189 ± 0.003	0.169 ± 0.002	18.142	4.22 × 10-12
16	Distance of vasiform orifice from anterior end	0.618 ± 0.004	0.609 ± 0.008	0.598 ± 0.007	16.935	2.05 × 10-11
17	Length of the lingula	0.055 ± 0.004	0.052 ± 0.001	0.047 ± 0.003	14.525	5.43 × 10-10
18	Distance of vasiform orifice from dorsal seta 6	0.021 ± 0.002	0.018 ± 0.004	0.016 ± 0.007	14.243	7.89 × 10-10
19	Breadth of the tracheal fold	0.106 ± 0.001	0.106 ± 0.002	0.104 ± 0.003	13.997	1.11 × 10-09
20	Length of the pupal case	0.796 ± 0.005	0.744 ± 0.011	0.720 ± 0.009	12.842	5.65 × 10-09
21	Distance of transverse moulting suture from anterior end	0.376 ± 0.002	0.351 ± 0.004	0.348 ± 0.004	12.526	8.86 × 10-09
22	Distance of transverse moulting suture from posterior end	0.388 ± 0.003	0.394 ± 0.007	0.373 ± 0.006	10.495	1.7 × 10-07
23	Length of si × th abdominal segment	0.044 ± 0.000	0.043 ± 0.002	0.042 ± 0.000	8.941	1.76 × 10-06
24	Length of the vasiform orifice	0.073 ± 0.003	0.073 ± 0.009	0.068 ± 0.003	6.518	7.48 × 10-05
25	Distance between caudal seta	0.045 ± 0.006	0.045 ± 0.007	0.041 ± 0.007	6.343	9.87 × 10-05
26	Distance between posterior submarginal seta 1 from base of caudal seta	0.043 ± 0.000	0.044 ± 0.000	0.040 ± 0.000	6.074	1.5 × 10-04
27	Distance between the dorsal seta 5	0.126 ± 0.002	0.128 ± 0.002	0.127 ± 0.002	5.6611	2.91 × 10-04
28	Distance between ventral seta	0.156 ± 0.006	0.146 ± 0.006	0.127 ± 0.008	4.556	1.7 × 10-03
29	Distance between dorsal seta 1 and base of proleg	0.144 ± 0.001	0.146 ± 0.002	0.140 ± 0.003	4.1418	3.3 × 10-03
30	Length of the tracheal fold	0.043 ± 0.001	0.038 ± 0.001	0.045 ± 0.000	3.496	9.3 × 10-03
31	Length of the ventral seta	0.032 ± 0.004	0.027 ± 0.007	0.018 ± 0.002	3.029	9.52 × 10-02

lingula were found in Asia I as compared with those of Asia II1 and Asia II7.

The evaluation of morphometrics of puparia through univariate single factor one-way ANOVA revealed that 31 of them had statistically significant variations at $P \leq 0.01$ (Table 2). These 31 characters when subjected to multivariate analysis, it was observed that the first four Principal Components (PCs) with an eigen values >1 account for 76.8% of the total variation obtained through PCA (Table 3), in which characters with maximum loadings were considered as the major sources of variation. The plot for PC1 and PC2 shown in Figure 3 brings out the grouping of these populations. Separate clustering was observed for Asia II7 with slight overlapping between Asia I and Asia II1. CDA was carried out with prior grouping and using the populations as classification variables. The projection of morphometric data onto the first two canonical discriminant axes is shown in Figure 4. This clustering confirmed the grouping brought out by the PCA. The first canonical root clearly discriminates the groups with major contribution being from the length of operculum, breadth of vasiform orifice, breadth of operculum, and length of caudal furrow; the second canonical root is able to discriminate due to the contribution from length of abdominal segment 1, length of vasiform orifice, length of caudal furrow, breadth of vasiform orifice and distance of vasiform orifice from base of dorsal setae 6 (Table 3). Overall 91% of the classifications were correctly attributed and the result of cross validation accurately classifies 93.3% of Asia II1 followed by 90% of those of Asia I, and 90% of Asia II7 (Table 8).

Adults. The morphometrics of male and female shown in Tables 4 and 5 reveal that, in males body length was significantly varying at $P \geq 0.01$ between the three putative species and it was comparatively larger in Asia I (1.0) than Asia II7 (0.997) and Asia II1 (0.951). The length of antennae was also significantly varying between the three putative species, and it was

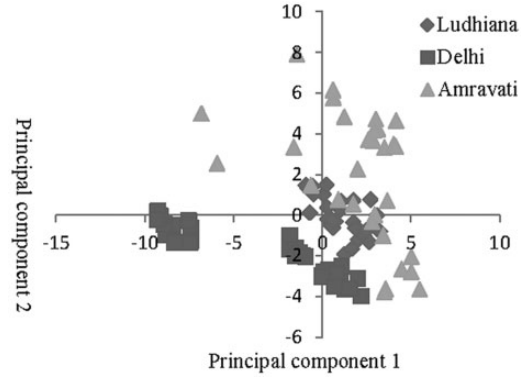

Fig. 3. PC coordinate of *B. tabaci* puparia from Asia-I, Asia-II1, and Asia-II7 genetic groups based on the analysis of 31 morphological variables onto first and second principal axis.

Table 3. Contributions of variable coefficients of first four eigenvectors for PC analyses from measurement of *B. tabaci* puparia of Asia-I, Asia-II1, and Asia-II7 genetic groups with its significance and total sample standardized canonical coefficients for CDA

Proportion of total variation	Prin1 45.3%	Prin2 21.3%	Prin3 6.5%	Prin4 3.5%	Can1	Can2
PCL	0.242	−0.129	0.095	−0.065	−26.385	−44.197
PCB	0.244	−0.093	0.056	0.090	7.860	18.147
DTMSAM	0.198	−0.097	0.171	−0.115	−0.131	43.250
DTMSPM	0.231	−0.133	0.026	−0.054	−23.209	−2.214
DVOAM	0.231	−0.141	0.140	−0.046	17.635	34.388
LABS1	0.183	−0.114	0.022	−0.172	−72.612	−145.603
LABS6	0.204	−0.131	0.157	0.037	29.027	32.004
L8thABPC	0.261	0.008	−0.034	0.017	−23.977	85.963
TFL	0.008	−0.078	0.402	0.588	−15.150	42.952
TFB	0.192	−0.177	0.095	0.040	−11.580	−46.325
AL	0.175	0.180	−0.150	−0.018	77.517	−37.364
LVO	0.243	−0.071	−0.005	−0.043	−70.202	−129.830
LO	0.242	0.050	−0.094	−0.003	360.730	196.960
LL	0.232	0.004	−0.033	0.103	−60.391	−20.004
BVO	0.227	0.024	−0.056	0.162	249.277	237.401
BO	0.234	0.033	−0.067	0.158	237.043	−13.422
LCF	0.245	0.029	−0.068	−0.007	218.887	−152.297
DCS	0.176	0.073	−0.158	0.097	−79.228	44.742
LDS1	0.117	0.257	−0.302	0.035	14.733	−12.336
DDS1PL	0.159	−0.069	−0.138	−0.200	−17.346	26.458
LDS2	0.064	0.313	−0.108	0.128	−2.416	−21.593
LDS3	0.109	0.280	−0.290	0.102	−5.004	−13.195
LDS4	0.058	0.290	0.369	−0.150	0.868	−2.831
LDS5	0.074	0.306	−0.097	0.141	10.625	28.368
DDS5	0.122	−0.225	0.043	0.229	−27.359	−39.586
LDS6	0.069	0.291	0.373	−0.133	−16.520	17.746
LDS7	0.058	0.303	0.353	−0.132	7.331	30.694
DVODS7	0.135	0.267	0.189	−0.124	51.723	−191.974
LVS	0.098	0.265	0.040	0.040	7.621	−26.988
DVS	0.105	−0.118	−0.063	−0.544	−2.052	−81.607
DPSMS1CS	0.172	−0.075	−0.113	0.079	−26.397	−91.943
P-value	<0.001	<0.001	<0.001	<0.001		

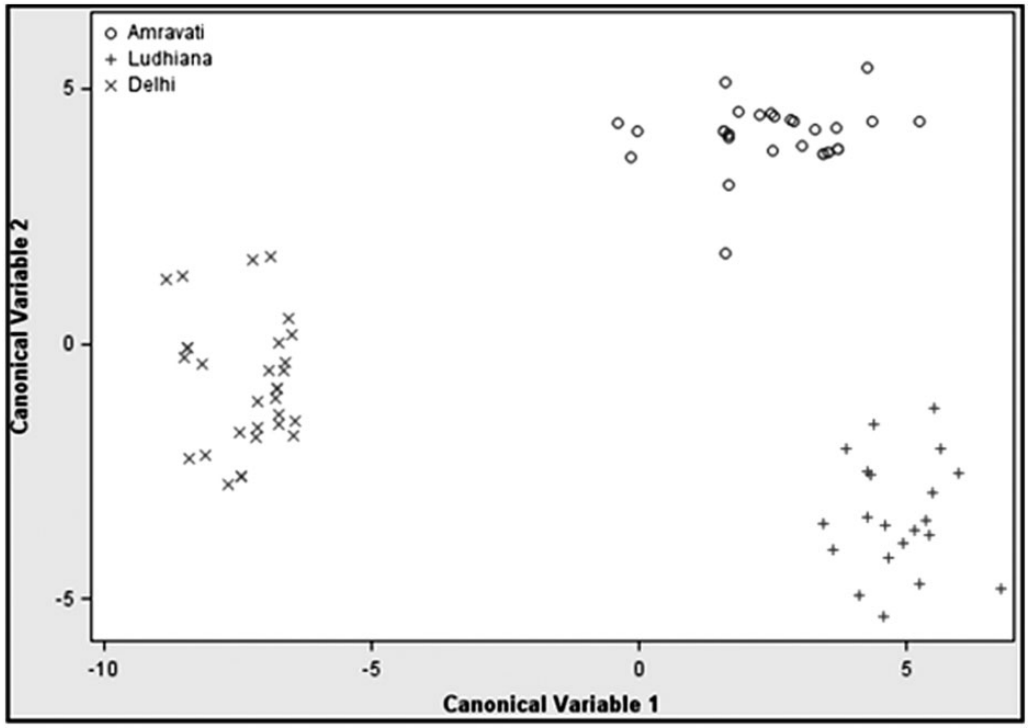

Fig. 4. CDA showing the coordinate of *B. tabaci* puparia from Asia-I, Asia-II1, and Asia-II7 genetic groups based on the analysis of 31 morphological variables onto first and second canonical axis.

larger in Asia II1 (0.224) and shorter in Asia II7 (0.174). The length of sensorial cone on antennal segment (LSCAS) 3, 6, and 7 and distance of sensorial cone on 5 and 7 were significantly varying and LSCAS 3 was larger in Asia II1, and LSCAS 6 and 7 was smaller Asia II7 (Table 4). For females as in males, size was found to be significantly varying at $P \geq 0.01$ and it was larger in Asia I (1.094) as compared to Asia II1 (0.974) and Asia II7 (1.050). Also as regards length of antennae, LSCAS 3, operculum and lingula and positioning of sensorial cone on antennal segment 7 this was true. Critical observations revealed that the positioning of sensorial cone on antennal segment 7 is below the sensorium in Asia II1, while it is adjacent to the sensorium in Asia I and Asia II7 (Fig. 5) for both the sexes.

The PCA and CDA. The morphometric evaluation of adults through univariate single factor one-way ANOVA revealed that of the measurements/observations explored, 23 of male and 22 of female, had statistically significant variations at $P \leq 0.01$ (Tables 4 and 5). These significant characters were subjected to PCA, and the first five PCs with an eigen values >1 were observed to account for 74.2% in male and 77.1% in female, of the total variation (Tables 6 and 7). The scatter plot for the PC1 and PC2 shown in Figures 6 (male) and 7 (female) brings out the grouping of these putative species. Separate clustering was observed for Asia II7 with slight overlapping between Asia I and Asia II1. The projection of CDA groups onto the first two canonical discriminant axes is shown in Figures 8 (male) and 9 (female). This clustering obtained from CDA confirmed the grouping brought out by PCA. The first canonical root clearly discriminates the groups with major contribution being from LSCAS 6, 7, and 3, antennal segment 6 and distance of sensorium from base of antennal segment 5 (in male); and also with regard to LSCAS 3, antennal segment 4, and lingula (in female). The second canonical root is able to discriminate from spacing between wax plate 2 and 3, LSCAS 3 and distance of sensorial cone on antennal segment 6 (for male), and LSCAS 7, antennal segment 4 and 1, and distance of sensorium on antennal segment (for female) (Tables 6 and 7). A cross validation of group membership was performed. The result of cross validation accurately classifies Asia II1 and Asia II7 populations (100%) followed by Asia I (96.67%) (Table 8).

Discussion

The *B. tabaci* species complex consists of multiple haplotypes and number of well characterized behaviorally differing variants (Brown et al. 1995, Perring 2001). For the separation of putative species of the *B. tabaci* species complex ultimately molecular data had been in vogue so far. The present analyses have used the >3.5% divergence limits observed in mtCO1 gene for identifying the genetic groups/putative species (Dinsdale et al. 2010, De Barro et al. 2011). The interpretations utilizing the global phylogenetic analysis reveal that these populations cluster with Asia I (Amravati), Asia II1 (Ludhiana), and Asia II7 (Delhi),

Table 4. Statistically significant characters of *B. tabaci* adults male from Asia-I, Asia-II1, and Asia-II7 genetic groups (mean ± SE mm; $n = 30$) in which $P < 0.01$

S. No.	Characters	Amravati Asia-I	Ludhiana Asia-II1	Delhi Asia-II7	*F* value	*P*-value
1	Spacing between wax plate 1	0.048 ± 0.002	0.042 ± 0.004	0.049 ± 0.006	63.597	<0.001
2	LSCAS 3	0.007 ± 0.002	0.008 ± 0.002	0.006 ± 0.002	39.896	<0.001
3	Length of antennae	0.217 ± 0.024	0.224 ± 0.030	0.174 ± 0.037	38.564	<0.001
4	Spacing between wax plate 3	0.041 ± 0.001	0.041 ± 0.001	0.046 ± 0.001	27.235	<0.001
5	LSCAS 7	0.010 ± 0.001	0.010 ± 0.002	0.008 ± 0.002	27.212	<0.001
6	Length of wax plate 1	0.049 ± 0.001	0.045 ± 0.000	0.045 ± 0.001	26.394	<0.001
7	Breadth of wax plate 1	0.098 ± 0.001	0.092 ± 0.001	0.089 ± 0.001	22.368	<0.001
8	Length of antennal segment 2	0.036 ± 0.004	0.036 ± 0.004	0.040 ± 0.001	22.271	<0.001
9	LSCAS 6	0.005 ± 0.001	0.005 ± 0.002	0.004 ± 0.001	21.786	<0.001
10	Breadth of wax plate 2	0.098 ± 0.001	0.092 ± 0.002	0.089 ± 0.001	19.997	<0.001
11	Length of clasper	0.094 ± 0.001	0.092 ± 0.000	0.097 ± 0.001	19.609	<0.001
12	Spacing between wax plate 2	0.043 ± 0.001	0.042 ± 0.002	0.046 ± 0.003	15.442	<0.001
13	Length of wax plate 3	0.041 ± 0.000	0.039 ± 0.001	0.044 ± 0.002	14.981	<0.001
14	Length of adeagus	0.086 ± 0.001	0.091 ± 0.001	0.092 ± 0.002	14.655	<0.001
15	Length of wax plate 2	0.043 ± 0.001	0.040 ± 0.001	0.045 ± 0.001	13.797	<0.001
16	Body Length	1.000 ± 0.003	0.951 ± 0.032	0.997 ± 0.008	13.187	<0.001
17	Length of lingula	0.028 ± 0.001	0.027 ± 0.000	0.025 ± 0.000	12.875	<0.001
18	Length of antennal segment 6	0.023 ± 0.005	0.023 ± 0.004	0.020 ± 0.002	7.615	<0.001
19	Distance of sensorial cone from base of antennal segment 6	0.016 ± 0.004	0.016 ± 0.003	0.014 ± 0.005	7.347	<0.001
20	Distance of sensorium from base of antennal segment 5	0.020 ± 0.003	0.021 ± 0.005	0.020 ± 0.002	6.564	<0.001
21	Breadth of clasper	0.027 ± 0.001	0.028 ± 0.000	0.026 ± 0.001	6.315	<0.001
22	Length of antennal segment 5	0.025 ± 0.003	0.026 ± 0.005	0.024 ± 0.002	5.272	<0.001
23	Spacing between wax plate 4	0.041 ± 0.000	0.041 ± 0.001	0.046 ± 0.001	3.772	<0.001

Table 5. Statistically significant characters of *B. tabaci* adults female from Asia-I, Asia-II1, and Asia-II7 genetic groups (mean ± SE mm; $n = 30$) in which $P < 0.01$

S. No.	Characters	Amravati Asia-I	Ludhiana Asia-II1	Delhi Asia-II7	*F* value	*P*-value
1	LSCAS 6	0.004 ± 0.001	0.005 ± 0.00	0.004 ± 0.001	89.865	<0.001
2	Length of lingula	0.036 ± 0.000	0.034 ± 0.001	0.031 ± 0.001	86.511	<0.001
3	Length of gonapophyses	0.141 ± 0.012	0.126 ± 0.001	0.126 ± 0.013	74.437	<0.001
4	LSCAS 3	0.008 ± 0.001	0.009 ± 0.002	0.006 ± 0.001	59.629	<0.001
5	Body Length	1.094 ± 0.009	0.974 ± 0.011	1.050 ± 0.010	38.184	<0.001
6	Length of wax plate 2	0.073 ± 0.000	0.063 ± 0.001	0.062 ± 0.001	38.149	<0.001
7	Length of wax plate 1	0.073 ± 0.001	0.065 ± 0.001	0.064 ± 0.001	32.479	<0.001
8	Length of antennal segment 1	0.012 ± 0.001	0.011 ± 0.002	0.010 ± 0.000	30.807	<0.001
9	Length of antennae	0.237 ± 0.023	0.251 ± 0.026	0.197 ± 0.023	25.368	<0.001
10	Length of operculum	0.034 ± 0.001	0.032 ± 0.001	0.031 ± 0.001	20.902	<0.001
11	Breadth of gonapophyses	0.156 ± 0.001	0.145 ± 0.002	0.145 ± 0.001	17.312	<0.001
12	Length of cement gland	0.058 ± 0.000	0.057 ± 0.000	0.053 ± 0.001	17.199	<0.001
13	LSCAS 7	0.010 ± 0.001	0.011 ± 0.002	0.009 ± 0.001	16.152	<0.001
14	Distance of sensorium from base of antennal segment 5	0.022 ± 0.003	0.024 ± 0.004	0.023 ± 0.003	13.041	<0.001
15	Spacing between wax plate 1	0.064 ± 0.001	0.058 ± 0.002	0.067 ± 0.002	12.945	<0.001
16	Breadth of wax plate 2	0.121 ± 0.001	0.110 ± 0.002	0.110 ± 0.002	11.708	<0.001
17	Spacing between wax plate 2	0.063 ± 0.001	0.058 ± 0.001	0.066 ± 0.002	10.969	<0.001
18	Length of antennal segment 5	0.027 ± 0.003	0.029 ± 0.005	0.028 ± 0.004	7.296	<0.001
19	Breadth of wax plate 1	0.120 ± 0.001	0.111 ± 0.002	0.111 ± 0.002	7.149	<0.001
20	Length of antennal segment 2	0.039 ± 0.008	0.042 ± 0.004	0.040 ± 0.001	6.028	<0.001
21	Distance of sensorium from base of antennal segment 7	0.021 ± 0.005	0.020 ± 0.004	0.019 ± 0.003	5.397	<0.001
22	Length of antennal segment 4	0.019 ± 0.004	0.017 ± 0.002	0.018 ± 0.002	5.144	<0.001

respectively. The identification of whiteflies depends on the morphology of puparia and the taxonomic status of *B. tabaci* has been problematic due to continuous morphological differences considered by taxonomists to represent intraspecific variations (Russell 1957, Mound 1963, Rosell et al. 1997). This study found that the size of developmental stages was larger in Asia I as compared with Asia II1 and Asia II7 except fourth instar which was longer in Asia II1. The wax fringe of first, second and fourth instars can be used as a reliable character to differentiate the developmental stages of these three putative species. The characteristic feature of wax fringe is used to identify species (Martin 1987) and biotypes/genetic groups/putative species of *B. tabaci* (Rosell et al. 1997, Yuan et al. 2003, Baoli et al. 2009).

Extensive morphology and morphometrics studies of *B. tabaci* showed that the morphological characters used for separation of different biotypes/genetic groups were not useful for their reliable separation (Rosell et al. 1997, Calvert et al. 2001, Liu et al. 2012). In this study, of the characters chosen for morphometrics, 31

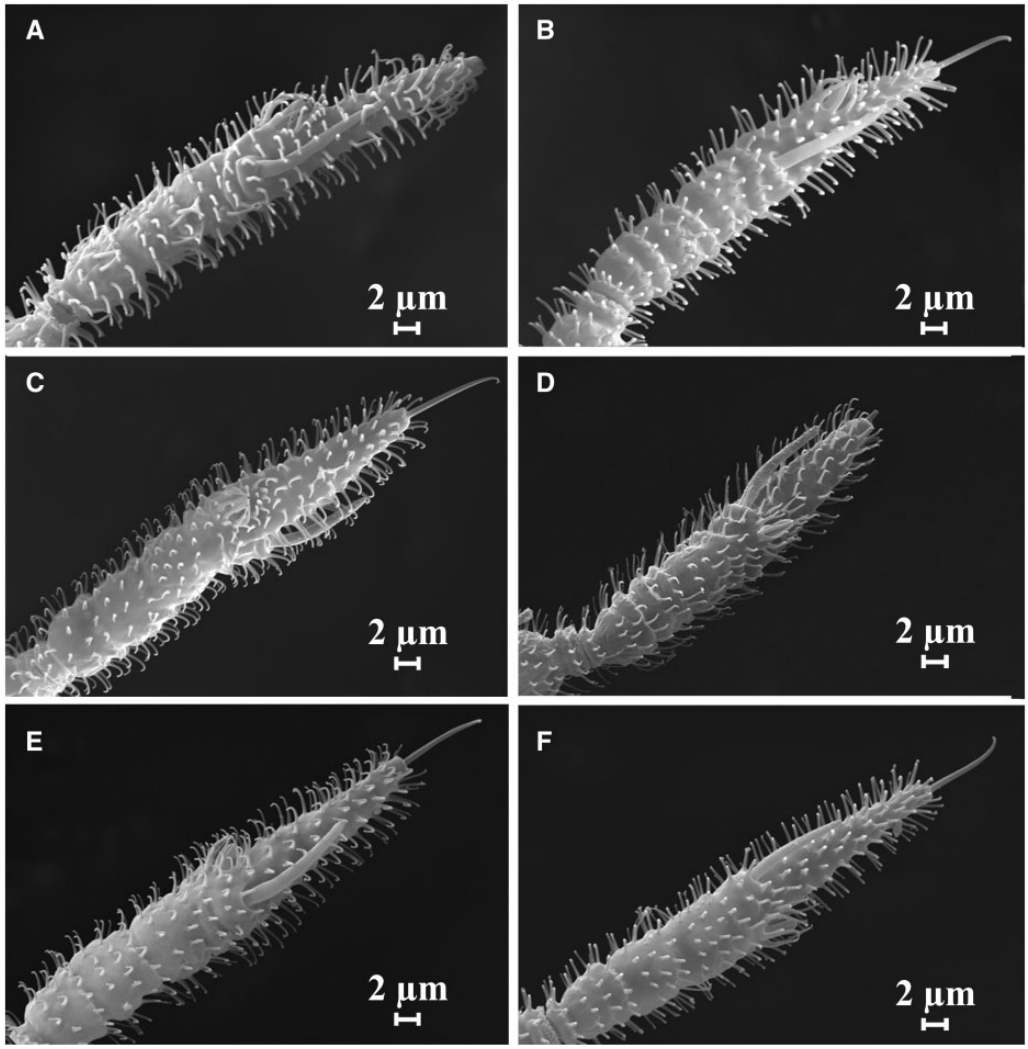

Fig. 5. Apical (seventh) segments of antennae of adult whitefly *B. tabaci*. A, female of Asia-II1; B, male of Asia-II1; C, female of Asia-I; D, male of Asia-I; E, female of Asia-II7; F, male of Asia-II7.

characters were found to vary significantly, and PCA and CDA confirmed their distinction as separate clustering was obtained for Asia I, Asia II1, and Asia II7. Amongst these, length of operculum, breadth of vasiform orifice, breadth of operculum, and length of caudal furrow discriminate the putative species with maximum contribution in canonical correlation. CDA with a higher classification (93.3 and 90%) values proposes to establish the probable validity of studied morphological characters towards delineation of *B. tabaci* species complex and its putative species. An observation onto the morphology of puparia among the putative species revealed that the length of vasiform orifice and tracheal fold was smaller in Asia II7 as compared with Asia II1 and Asia I. These results are in agreement with those of Lozier et al. (2008), Jayasekera et al. (2010), and Thomas et al. (2011, 2014). It could be observed that some previous conclusions of David and Ananthakrishnan (1976), Mohanty and Basu (1986), and Mound (1963) are also in agreement with this study.

Adult morphology is neglected in whitefly taxonomy, probably due to less morphological differences (Mound and Halsey 1978). In this study we observed that both male and female of Asia I was longer as compared to Asia II1 and Asia II7. LSCAS s shows significant variation among these putative species which is similar to those observed by Calvert et al. (2001). In the morphometrics, 23 of male and 22 of female measurements were found to be statistically significantly varying. Similarly PCA and CDA also revealed and confirmed the distinction of these genetic groups as separate clustering was obtained for Asia I, Asia II1, and Asia II7. Length of sensorial cone and distance of sensorium on

Table 6. Contributions of variable coefficients of first four eigenvectors for PC analyses from measurement of *B. tabaci* adult male of Asia-I, Asia-II1, and Asia-II7 genetic groups with its significance and total sample standardized canonical coefficients for CDA

Proportion of total variation	Prin1 29.6%	Prin2 17.1%	Prin3 10.9%	Prin4 10.1%	Prin5 6.5%	Can1	Can2
L	0.207	0.367	0.054	0.013	−0.026	4.430	17.505
LWP1	0.036	0.377	−0.019	−0.180	0.205	467.484	−337.283
BWP1	−0.050	0.465	−0.038	−0.070	−0.125	242.478	141.763
SWP1	0.282	0.041	0.116	−0.151	0.321	−244.952	910.658
LWP2	0.235	0.261	0.146	−0.001	0.040	−280.075	237.553
BWP2	−0.041	0.456	−0.082	−0.076	−0.131	−320.386	−3.620
SWP2	0.282	0.015	0.284	0.036	0.027	27.041	−425.970
LWP3	0.279	0.163	0.224	0.125	0.105	−145.811	−3.651
SWP3	0.312	−0.076	0.255	0.114	0.037	461.819	−472.239
SWP4	0.166	−0.032	0.261	0.370	−0.023	−102.217	116.042
LC	0.261	0.053	−0.083	0.156	0.124	−151.083	−70.557
BC	−0.074	0.060	0.029	0.352	−0.445	324.758	76.641
LA	0.120	0.008	0.147	0.363	−0.338	−127.767	−165.548
LL	−0.113	0.348	0.045	−0.036	−0.110	343.611	−68.159
AL	−0.299	0.098	0.273	−0.054	−0.010	156.655	17.206
LSC3	−0.261	0.102	0.055	0.352	0.053	521.554	−418.793
LAS5	−0.117	−0.034	0.415	−0.317	−0.280	127.851	−113.309
DSAS5	−0.144	−0.068	0.451	−0.252	−0.253	−668.608	104.951
LAS6	−0.224	0.019	0.284	0.005	0.364	−537.787	383.060
LSCAS6	−0.202	0.031	0.130	0.302	0.241	1,188.748	70.664
DSCAS6	−0.228	0.010	0.281	0.012	0.299	279.845	−429.726
LSCAS7	−0.255	0.099	0.021	0.304	0.203	−764.600	279.355
LAS2	0.182	−0.174	0.171	−0.105	−0.018	−343.429	−73.777
P-value	<0.001	<0.001	<0.001	<0.001	<0.001		

Table 7. Contributions of variable coefficients of first four eigenvectors for PC analyses from measurement of *B. tabaci* adult female of Asia-I, Asia-II1, and Asia-II7 genetic groups with its significance and total sample standardized canonical coefficients for CDA

Proportion of total variation	Prin1 30.5%	Prin2 21.9%	Prin3 10.8%	Prin4 7.9%	Prin5 6.1%	Can1	Can2
L	0.306	−0.134	0.048	−0.139	0.060	−14.277	2.444
LWP1	0.352	0.022	−0.011	0.059	0.031	15.180	−21.448
BWP1	0.330	−0.058	0.065	0.207	0.046	6.863	−45.550
SWP1	0.223	−0.241	0.291	0.171	−0.169	−73.325	43.858
LWP2	0.358	0.039	0.020	0.018	0.004	69.904	139.050
BWP2	0.349	−0.045	0.054	0.150	0.037	71.244	−2.822
SWP2	0.200	−0.241	0.325	0.191	−0.193	−47.353	−99.518
LO	0.295	0.044	−0.015	0.201	−0.184	95.237	276.437
LL	0.216	0.265	−0.193	0.082	−0.101	302.747	60.135
LCG	0.089	0.217	−0.168	0.006	−0.027	112.564	−0.241
LGP	0.265	0.110	−0.111	−0.324	0.286	24.768	156.244
BGP	0.219	0.093	−0.038	−0.315	0.391	−3.189	−46.902
AL	0.051	0.399	−0.050	0.152	0.042	47.253	−12.494
LSCAS3	−0.054	0.360	−0.011	0.141	−0.193	1,291.248	−144.993
LAS4	0.061	0.121	0.349	−0.435	−0.076	−734.072	304.156
LAS5	−0.108	0.185	0.499	0.025	0.228	107.280	91.459
DSAS5	−0.115	0.149	0.478	0.117	0.313	182.074	−445.843
LSCAS6	−0.051	0.352	0.005	0.323	−0.067	143.564	−82.879
LSCAS7	0.009	0.325	0.109	−0.039	−0.302	76.933	−353.992
DSAS7	0.072	0.261	0.299	−0.211	−0.187	−35.648	160.995
LAS1	0.171	0.246	−0.131	−0.019	0.184	−110.586	390.224
LAS2	−0.060	−0.008	0.002	0.444	0.536	−30.046	−59.106
P-value	<0.001	<0.001	<0.001	<0.001	<0.001		

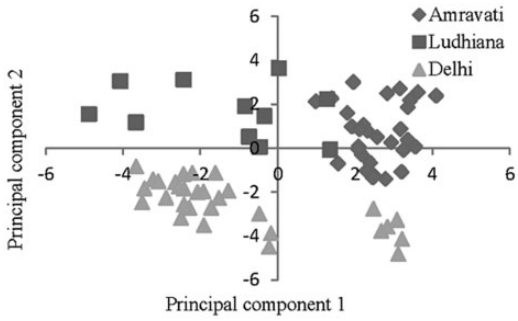

Fig. 6. PC coordinate of *B. tabaci* adult male from Asia-I, Asia-II1, and Asia-II7 genetic groups based on the analysis of 23 morphological variables onto first and second principal axis.

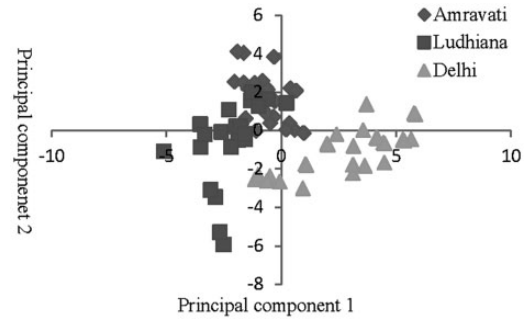

Fig. 7. PC coordinate of *B. tabaci* adult female from Asia-I, Asia-II1, and Asia-II7 genetic groups based on the analysis of 22 morphological variables onto first and second principal axis.

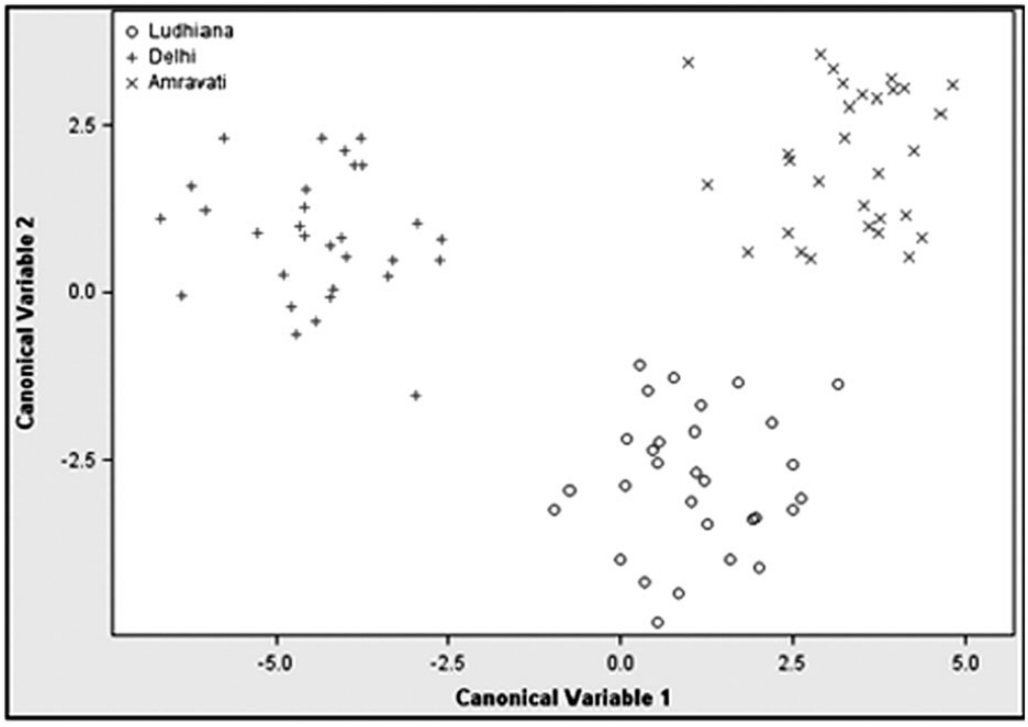

Fig. 8. CDA showing the coordinate of *B. tabaci* adult male from Asia-I, Asia-II1, and Asia-II7 genetic groups based on the analysis of 23 morphological variables onto first and second canonical axis.

Table 8. Cross validation matrix of the DFA from measurement of *B. tabaci* puparia and adults of Asia-I, Asia-II1, and Asia-II7 genetic groups

	Number of observations and percent classified								
	Puparia			Male			Female		
Population	Amravati	Delhi	Ludhiana	Amravati	Delhi	Ludhiana	Amravati	Delhi	Ludhiana
Amravati	27	0	3	29	0	1	29	0	1
	90.00	0	10.00	96.67	0	3.33	96.67	0	3.33
Delhi	0	27	3	0	30	0	0	30	0
	0	90.00	10.00	0	100	0	0	100	0
Ludhiana	2	0	28	0	0	30	0	0	30
	6.67	0	93.33	0	0	100	0	0	100
Total	29	27	34	29	30	31	29	30	31
	32.22	30.00	37.78	32.22	33.33	34.44	32.22	33.33	34.44

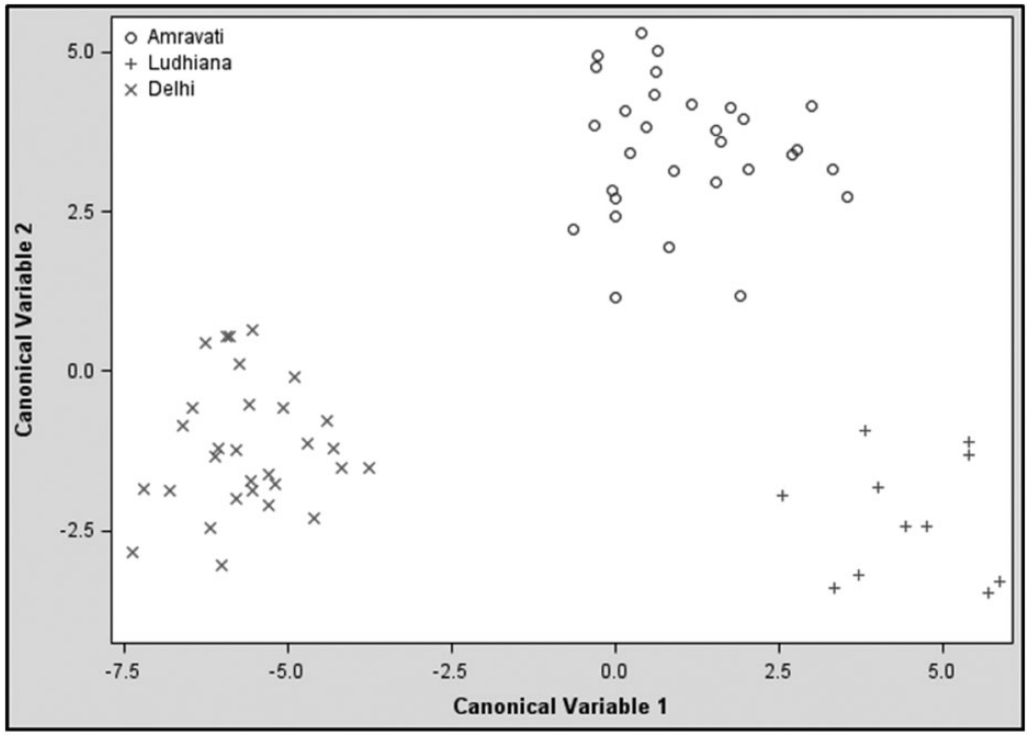

Fig. 9. CDA showing the coordinate of *B. tabaci* adult female from Asia-I, Asia-II1, and Asia-II7 genetic groups based on the analysis of 22 morphological variables onto first and second canonical axis.

antennal segments in both male and female, and spacing between wax plates in female discriminate the genetic groups with maximum canonical correlation. These findings are similar to and corroborate those of Li et al. (2013). The positioning of sensorial cone on antennal segment 7 is below the sensorium in Asia II1, while it is more adjacent in Asia I and Asia II7, in both the sexes. These explicit distinctions might provide support for distinguishing the other putative species of *B. tabaci* too. However, a detailed study warranting more putative species from various hosts and geographical locations might be necessary to validate these findings.

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