

Morphometric discrimination of Black Legume Aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae), populations associated with different host plants

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Abstract. Morphometric analyses were carried out on some populations of *Aphis craccivora* Koch (Hemiptera: Aphididae) associated with different host plants. Twelve morphological characters were measured. Two multivariate discriminant analyses, Canonical variates analysis and Cluster analysis were employed to discriminate individuals of *A. craccivora* populations on a morphological basis. Both Canonical variates analysis and Cluster analysis revealed significant differences among *A. craccivora* collected from different host plants. A stepwise Canonical variates analysis selected four morphological characters (siphunculus length, ultimate rostral segment length, basal diameter of siphunculus and processus terminalis length) from which two (siphunculus length and ultimate rostral segment length) showed the highest contribution to the separation of host-associated populations and 87.6% of individuals were reclassified correctly into their original populations. The result of morphometric analyses performed here revealed the existence of three morphologically differentiated groups within *A. craccivora* associated with different host plants which provide evidence for the presence of host related forms. This study demonstrates that morphometric analysis of morphological characters can discriminate among host related groups based on size variation and clearly separates the individuals of *A. craccivora*. The aphid population associated with *Robinia pseudoacacia* L. was clearly separated from other populations. The most important discriminatory characters of *A. craccivora* and their functions in relation to adaptation of populations with different host plant species are discussed. We concluded that *A. craccivora* populations are not homogeneous morphological entities and represent different host-associated forms. The results presented here for the morphological structure of *A. craccivora* provide the framework to investigate the genetic and biological differences among these entities so that we could be able to exactly evaluate their taxonomic situation.

Keywords: host association, morphometric analysis, multivariate discriminant analyses, aphid, Iran.

Introduction

Aphids are an important group of insects with worldwide distribution. They are a truly interesting group of herbivorous insects and can affect plants directly or indirectly by feeding on the plant's sap (Blackman & Eastop 2007). They have been used in many investigations in the fields of ecology, biodiversity, physiology, behaviour and genetics (Martin & Brown 2008). They have experienced some adaptations in relation to host plants so that many aphid taxa have biologically complex life cycles (Martin & Brown 2008).

Taxonomists have frequently used morphological variations as primary parameters in the differentiation and separation of many natural populations of organisms and many species have been described based on the results of these studies. Phytophagous insects comprise many cryptic spe-

cies that have been recognised during last several decades (Feder et al. 1998, Dres & Mallet 2002). Cryptic pest aphid species are highly dynamic and rapidly evolving groups with diverse ecological characters (Blackman & Eastop 2007, Lozier et al. 2008). Most aphid species comprise a set of closely related populations which may have diverged genetically so that they could be considered as host races, incipient or sibling species (or subspecies) (Blackman & Eastop 2007). The recognition of these divergent populations can help to understand their ecology and evolution so that we could devise effective control programmes.

The genus *Aphis* is one of the most difficult genera of aphids to identify because of morphological conservatism and this problem is complicated by the fact that several species may live on a single host plant. They are recognisably associated with their host plants (Blackman 1975). Several au-

thors in various countries have studied the taxonomy and morphology of *Aphis* species and in some cases have described several new species (e.g. Holman 1966 a, b, Prior & Stroyan 1977, Mier Durante & Nieto Nafria 1991, Holman 1992, Jorg & Lampel 1995, Rakauskas 1996, Nieto Nafria et al. 1999, Heie 2000, Mier Durante et al. 2003, Perez Hidalgo & Nieto Nafria 2004, Voegtlin et al. 2004).

Aphis craccivora Koch, 1854 is associated with many host plants in the Leguminosae and also in many other plant families so that it attacks about 50 crops in 19 different plant families (Blackman & Eastop 2007).

Morphotaxonomy can be regarded as a reliable and powerful tool among applied entomologists for discrimination of aphid taxa (Poulios et al. 2007). The computation and application techniques to reveal underlying differentiation based on shape and size differences using numeric statistics become a necessary option as a result of the aforementioned problems. Morphometrics involves the quantitative analysis of forms, using measurements of morphological structures. Morphometrics can be used to summarise morphological data numerically and graphically, and to express and test hypothetical relationships exactly (Daly 1985).

The application of morphometric methods was proposed in the early 1960s by taxonomists who argued that taxonomy and systematics should be based on the use of multivariate statistical analysis of morphological characters as opposed to the use of underlying evolutionary or biological information (see Sokal & Crovello 1970). Morphometrics has the advantage over other methods such as electron microscopy or biochemical techniques (electrophoresis, protein and DNA analysis), that the organisms need not be destroyed, and can therefore be used for future studies. Insects are eminently suitable subjects for morphometric studies because their hard exoskeleton is easily measured and is largely free from the physical distortions suffered by many soft-bodied animals. Morphometric techniques can be used to identify and distinguish between morphologically similar groups of organisms when no single diagnostic character is available. Morphological variation within and between populations has been and still is the most widespread and practical method of classification of living and preserved organisms. Studies on variation among populations can be carried out using the morphometric technique. It can also be used to assess affinities and in many

cases justify synonymy or recognise new taxa at the species level.

Two types of morphometric analysis techniques are commonly used by researchers: firstly techniques based on multivariate statistical analyses, i.e. traditional morphometrics and secondly, techniques based on graphically oriented geometrical analysis of size and shape, geometric morphometrics (Rohlf 1990). Morphometric analysis has become the cutting-edge in morphological analysis in a wide variety of organisms and has been found useful in differentiating population variation between closely related or sibling species.

Morphometric approaches have been used widely to differentiate between closely related species, populations and biotypes in various insects (e.g. Punglerl 1986, Barari et al. 2005, Barahoei et al. 2011). In aphids alone, there have been a series of studies involving the application of multivariate techniques to the study of morphological variation (e.g. Blackman & Paterson 1986, Blackman 1987, Blackman & Spence 1994, Rakauskas 1998, Smith et al. 1998, Margaritopoulos et al. 2000, Barbagallo & Cocuzza 2003, Gorur 2003, Margaritopoulos et al. 2006, Poulios et al. 2007, Lozier et al. 2008, Madjdzadeh & Mehrparvar 2009).

The number of aphid taxa associated with a particular host plant differs from one to many aphid species. Some aphid species are associated with a particular host plant or may have several host plants, for example *Brachycaudus cardui* (L.) and *Brachycaudus helichrysi* (Kaltenbach) do not only live on plum (*Prunus* spp.) but are also associated with several plant species belonging to Asteraceae family. Identification of host plants is very useful in determination of aphid species that are close to each other morphologically, but live on different host plants and are biologically different entities. Some aphid species show a wide range of host plant associations. This is the case for *A. craccivora* which lives on 50 host plants of different families.

Phytophagous insects such as aphids have undergone morphological and physiological changes in order to live on different host plant species. In other words they show a very high degree of host-specific behavioural adaptations. The degree of phenotypic plasticity varies among phytophagous insects, because they have different capacities for morphological, physiological and behavioural adaptations in response to the nutri-

tional, chemical and physical structure of their host plants (Scheiner 1993, Via et al. 1995). Morphological characters of aphids are very important in adaptation to various host plants.

Aphids display a wide range of morphological variability so that it is difficult to identify them based on morphological features alone. Taxonomists are dealing with interpreting observed variation among species under study. Different factors contribute to morphological diversity among aphids, including effects of environmental and biological factors (Margaritopoulos et al. 2000).

Environmental factors such as day length or crowding and various biological factors such as variation in developmental stages, differences in the number of nymphal instars, different foods, individual growth rates etc. have complicated effective use of morphological characters in population differentiation (Atchley & Martin 1971). Temperature can have considerable effects on morphological characters such as body size and allometry (Blackman & Spence 1994, Mopper & Strauss 1997). Other factors such as season, host plant and climate are also very important.

The aim of this study was to investigate morphological variation in field samples of *A. craccivora* populations associated with different host plants using morphometric analysis.

Materials and Methods

Sampling, preserving and collecting data

The study was based on 89 individuals of *A. craccivora* collected from different host plants. The host plant species include *Astragalus* sp., *Atriplex leucoclada* Boiss., *Chenopodium album* L., *Robinia pseudoacacia* L., *Tripleurospermum disciforme* (C. A. Mey) Schultz Bip. and *Zygophyllum eurypterum* Boiss. & Buhse (Table 1). Different methods were used to collect aphids. The most satisfactory way of obtaining aphids is by examining plants to find colonies. Sampling data such as host plant name, feeding site, colour of the live specimens, locality, date, biological information and GPS data were recorded at the time of collection. It was also noted whether there are ants in attendance or not.

To obtain aphids, host plant foliage was carefully examined for colonies. The infested parts were cut and placed into plastic bags. Adult apterous viviparous specimens of *A. craccivora* were cleared and mounted in Canada balsam on microscope slides.

All of the specimens preserved in ethanol and/or on slide mounts are deposited in the Aphid collection of Ecology Department of International Center for Science, High Technology & Environmental Sciences (ICST), Kerman, Iran.

Character choice

Twelve morphological characters were scored altogether. Some characters have been previously used in the taxonomy of Aphidoidea which is standard practice in traditional morphometric study on this group of insects (Rakauskas 1998, Blackman & De Biose 2002, Gorur 2003, Margaritopoulos et al. 2006, Poullos et al. 2007, Lozier et al. 2008). All morphological measurements were made using a phase contrast microscope (ZEISS, Axiostar, Germany) fitted with a calibrated micrometer eyepiece. Before the measurements were made, the microscope magnification was calibrated and kept unchanged throughout the measurements and a consistency test was performed in order to check the accuracy and consistency of measurements. Descriptions of all measured characters are given in Table 2.

Data analysis

Multivariate morphometric analyses were conducted using the statistical package SPSS Base 16 (SPSS Inc. 2007). Two common multivariate methods were used in morphometric analysis, Canonical Variates Analysis (CVA) and Cluster Analysis (CA). Canonical variates analysis was carried out on all specimens using all 12 variables to determine those variables which contributed most to separation of the host-related groups and also to determine whether any host-related differences exist between each aphid population (Tabachnick & Fidell 2006). The phenotypic relationships among populations from different host plants were examined using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) hierarchical cluster analysis (Sneath & Sokal 1973) based on squared Euclidean distances.

Results

A summary of Canonical variates analysis is shown in Table 3 which considers 12 morphological characters for *A. craccivora* individuals collected from six host plant species including the levels of variance and the significance for three CVs. The table indicates that CV1 carries 78.9% of the total variance, CV2 shows 14.6% and CV3 carries 4.9% of the total variance. All three CVs together therefore accounted for 98.4% of total variation in the data. Low values of Wilks' Lambda test (the smaller the lambda, the greater the difference among groups) and statistically significant Chi-square values show that, in particular, all three CVs are significant predictors.

Table 4 represents the standardised canonical coefficients that determine the relative importance of the characters in discriminating the populations. As the table shows, the characters SIPH (Siphunculus length) and URS (Ultimate rostral segment length) show the highest magnitudes (0.904 and 0.585 respectively) at CV1, the character BDSIPH

Table 1. Information on the host plant, locality, elevation and collection date of the *Aphis craccivora* populations used in the present study.

Host Plant	Family	Locality	Elevation (m)	Date
<i>Astragalus</i> sp.	Leguminosae	N29° 56' E56° 23'	2209	21 April 2007
<i>Atriplex leuoclada</i>	Chenopodiaceae	N30° 15' E57° 33'	1418	24 April 2007
<i>Chenopodium album</i>	Chenopodiaceae	N30° 44' E56° 23'	1750	14 April 2007
<i>Robinia pseudoacacia</i>	Leguminosae	N30° 15' E57° 06'	1759	24 April 2007
<i>Tripleurospermum disciforme</i>	Asteraceae	N29° 32' E56° 36'	2730	7 May 2007
<i>Zygophyllum eurypterum</i>	Zygophyllaceae	N29° 56' E56° 23'	2202	21 April 2007

Table 2. Morphological characters used for morphometric analysis of populations of *Aphis craccivora*.

Character No.	Acronym	Character
1	ANTIII	Antennal segment III length
2	ANTVIb	Basal part of antennal segment VI length
3	PT	Processus terminalis length
4	URS	Ultimate rostral segment length
5	BDURS	Basal diameter of ultimate rostral segment
6	HT	2 nd segment of hind tarsus length
7	SIPH	Siphunculus length
8	BDSIPH	Basal diameter of siphunculus
9	CD	Cauda length
10	HFEM	Hind femora length
11	HTIB	Hind tibia length
12	CDH	Number of caudal hairs

(Basal diameter of siphunculus) shows the highest magnitude (1.133) at CV2 and the character PT (Processus terminalis length) shows the highest magnitude (0.914) at CV3 that contribute more to the separation between populations. A graphical presentation of the first two canonical variates is shown in Figure 1, indicating the degree of inter-population differences. Here we can see that aphid samples associated with *Robinia pseudoacacia* and *Atriplex leuoclada* were completely separated from other groups while there were different levels of overlapping between samples associated with other populations. Table 5 shows CVs at group centroids (group means are centroids) for *A. craccivora* populations associated

with six host plant species.

Table 6 shows the results of reclassification of individuals according to the original CVs, derived with *a priori* specified group membership. As a whole 87.6% of original grouped cases were correctly classified. The UPGMA dendrogram of cluster analysis based on data of squared Euclidean distances between six populations of *A. craccivora* (Fig. 2) revealed three major clusters. The first contained samples from *Astragalus* sp., *Zygophyllum eurypterum* and *Tripleurospermum disciforme*, the second contained those from *Chenopodium album* and *Atriplex leuoclada* and the last one contained

Robinia pseudoacacia.

Discussion

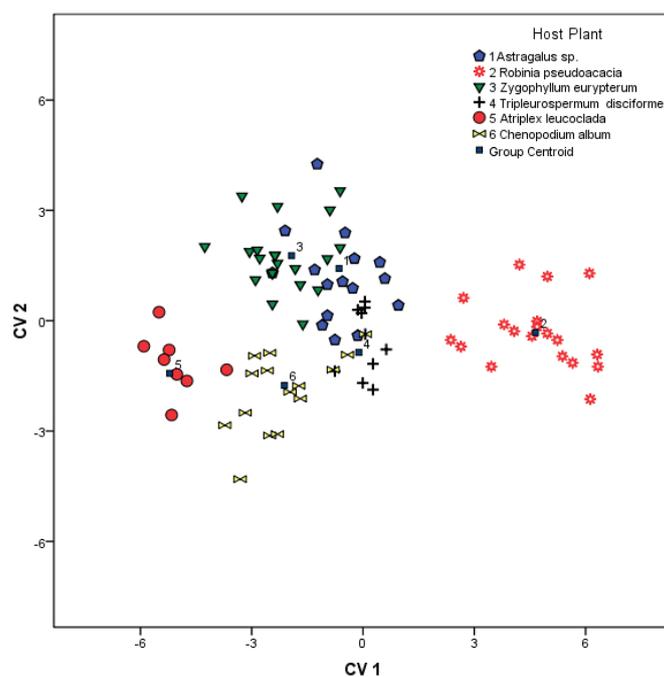
The population associated with *Robinia pseudoacacia* showed a large phenotypic distance from other populations. The result of morphometric analysis performed here revealed the existence of three morphologically differentiated groups within *A. craccivora* associated with six host plant species which provides evidence for the presence of three host-associated groups. The first contained samples from *Robinia pseudoacacia*, the second those from *Atriplex leuoclada* and *Chenopodium album* and the third, samples from other populations. The separation by host plant was impressive along CV1, the *Robinia pseudoacacia* group was strongly separated from all other groups along this axis. Along CV2, however, there is no clear separation between populations. The aphid population associated with *R. pseudoacacia* was separated from other populations on CV1. The most important characteristics on this CV are the length of siphunculi and ultimate rostral segment. The siphunculi length in aphids associated with *R. pseudoacacia* is considerably larger than that of other aphid populations. This could be a characteristic in which the

Table 3. Summary of Canonical variates analysis for six populations of *Aphis craccivora* ($p < 0.001$).

CV	Eigenvalue	% of Variance	Wilks' Lambda	Chi-square	df	Sig.
1	10.837	78.9	.014	261.426	20	.000
2	2.001	14.6	.163	110.683	12	.000
3	.667	4.9	.489	43.654	6	.000

Table 4. Standardised canonical coefficients for the four CVs.

Character	CV1	CV2	CV3	CV4
PT	-.130	.422	.914	.578
URS	.585	.054	-.639	.606
SIPH	.904	-.597	.105	-.737
BDSIPH	-.321	1.133	-.084	-.204

**Figure 1.** Scatter-plot of scores of the first two canonical variates for *Aphis craccivora* populations inhabiting six host plant species.**Table 5.** CVs at group centroids (group means) for *Aphis craccivora* populations associated with six host plant species.

Host plant	CV1	CV2	CV3	CV4
<i>Astragalus</i> sp.	-.647	1.419	-1.530	.112
<i>Robinia pseudoacacia</i>	4.646	-.321	.157	-.179
<i>Zygophyllum eurypterum</i>	-1.927	1.770	.946	-.107
<i>Tripleurospermum disciforme</i>	-.114	-.862	.486	1.540
<i>Atriplex leuoclada</i>	-5.213	-1.430	.181	-.389
<i>Chenopodium album</i>	-2.119	-1.757	-.209	-.161

population could be adapted to environmental conditions. The siphunculi release alarm pheromone, so probably aphids with longer siphunculi are able to release the alarm pheromone in a wider space. Since the microclimatic conditions of *R. pseudoacacia* are different from other host plants

Table 6. Results of a reclassification of individuals of six populations of *Aphis craccivora*. 87.6% of original grouped cases were correctly classified. Percentages of individuals that were correctly classified are in the diagonal.

Host plant	Predicted Group Membership					
	<i>Astragalus</i> sp.	<i>Robinia pseudoacacia</i>	<i>Zygophyllum eurypterum</i>	<i>Tripleurospermum disciforme</i>	<i>Atriplex leucoclada</i>	<i>Chenopodium album</i>
<i>Astragalus</i> sp.	75.0	.0	12.5	12.5	.0	.0
<i>Robinia pseudoacacia</i>	.0	94.7	.0	5.3	.0	.0
<i>Zygophyllum eurypterum</i>	9.5	.0	90.5	.0	.0	.0
<i>Tripleurospermum disciforme</i>	10.0	.0	.0	90.0	.0	.0
<i>Atriplex leucoclada</i>	.0	.0	.0	.0	87.5	12.5
<i>Chenopodium album</i>	6.7	.0	.0	6.7	.0	86.7

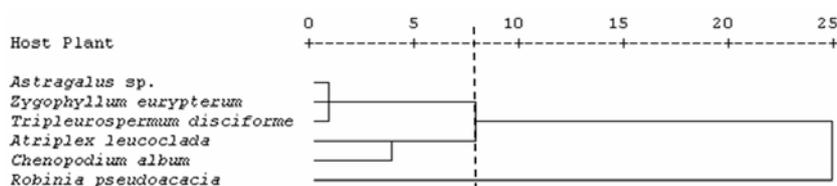


Figure 2. The UPGMA dendrogram based on squared Euclidean distance using average linkage between *Aphis craccivora* populations collected from six host plant species.

the length of siphunculi might be in accordance with a population adaptation to environmental conditions. The ultimate rostral segment in aphid individuals associated with *R. pseudoacacia* is noticeably larger than aphids associated with other host plants studied here. This could be the result of adaptation to the relevant host plant. The *R. pseudoacacia* is a tree, so it is different from the shrubby plants in having thick bark. The tree bark has several layers, so the length of rostrum in aphids must be longer in order to penetrate deeply into the plant tissue. This facilitates the feeding function of aphids so that they are able to access the plant sap easily. Some evidence show that variation between herbivorous insects is related to the ranges of host plants they use. Patterns of host plant utilisation have often given rise to the idea that they are the final step of host plant associated sympatric speciation and it is certain that complexes of host-specific sibling species may exist within polyphagous species (Claridge 1988). Morphological variation could be heritable or non-heritable (Falconer 1989). In polyphagous aphids the particular host plant species with its unique chemical and nutritional qualities may have specific effects on subsequent adult morphology. Re-

cent studies of morphometric characters in the aphid genus *Hyalopterus* demonstrated that morphological discrimination associated with different host plant species is possible using a sufficient number of characters (Poulios et al. 2007, Lozier et al. 2008). Morphometric studies that have been carried out in Greece showed that the tobacco-feeding form of *Myzus persicae* is morphologically different from those on other crops (Margaritopoulos et al. 2000, Margaritopoulos et al. 2002). The number of taxonomic entities within the *A. craccivora* in Iran as well as other parts of the world and the extent of their host specificity is unclear. The present study confirms the existence of a number of morphologically similar but distinct groups within the *A. craccivora*. However it must be mentioned that detecting morphological variation alone is not adequate for resolving taxonomic relationships among aphids (Poulios et al. 2007). There are no evidence of past morphometric and molecular studies in host-associated taxa within *A. craccivora*. Canonical variates analysis has proved to be a powerful technique that helps to resolve taxonomic difficulties in closely related aphid taxa (Margaritopoulos et al. 2006, 2007, Poulios et al. 2007, Lozier et al. 2008, Madjzadeh et al. 2009).

Aphid samples from field populations have been studied using this technique (Blackman & De Boise 2002, Poullos et al. 2007). Although factors such as environmental conditions and natural enemies may have considerable effect on aphid morphology (Dixon 1998, Margaritopoulos et al. 2000), it seems that the morphological separation in this study is mostly due to host-related differences because samples were collected from host plants in a small scale range with similar climatic conditions during about three weeks in the spring. The separation of populations belonging to *R. pseudoacacia* is mostly due to the fact that this plant is a tree while the other host plants are shrubs, so the former has different microclimatic and physiological conditions in comparison to other shrubby plants. It can be concluded that differences in microclimatic and physiological conditions could be considered as two important factors affecting morphological characters of *A. craccivora* population that inhabit *R. pseudoacacia*. The aphids inhabiting *Chenopodium album* and *Atriplex leucoclada* are more closely related because they are separated from other populations and are located close to each other on CVA graph as well as in cluster analysis. The reason for this close relationship is that both belong to the same plant family and have more similarities based upon host conditions. These two plants are among halophytic plants, living in areas where the soil is rich in salts and minerals. The plant sap is also rich in salts and minerals in the mentioned plants. Generally there is no previous evidence of host-associated morphological variation within taxa under study, especially aphids living on *R. pseudoacacia*, to support our view.

Studies show that the length of ultimate rostral segment responds to selection arising from host characteristics (Moran 1986). Host-specific behavioural adaptations could affect morphological variation in aphids (Via & Shaw 1996). Some early aphid taxonomists identified similar aphids feeding on different host plants as distinct species and provided host-led identification keys. Through the 20th century more investigations have led to many revisions in different aphid species through synonymy and the host-led identification keys seemed to be old (Blackman & Eastop 2000).

The insect's ability to express an alternative morphology, physiology and behaviour in response to environmental changes is called phenotypic plasticity (Gorur 2003). Host plant has considerable effect on morphological variation in

most aphid taxa. It has been demonstrated that changes in host relationships could be considered as the primary isolating mechanisms in speciation process, rather than spatial isolation (Guldmond & Mackenzie 1994, Hawthorne & Via 2001). Thus in rapidly speciating groups of aphids there are incipient species which display early stage of speciation processes and are recognisable by biological rather than morphological criteria (Blackman & Eastop 2007).

Parthenogenesis and assortative mating are two important factors in aphids that tend to maintain the genotypes associated with particular host plants and lead to further specialisation and sympatric speciation (Dixon 1987). Sympatric speciation through host shift and host race formation in aphids has been proposed by Müller (1969, 1971, 1983, 1985) (quoted from Dixon 1987).

The correct identification of different forms of a species that are identified as biotypes, host races or cryptic species is of particular importance in control programmes. The results presented here for the morphological structure of *A. craccivora* provide the framework to investigate the genetic and biological differences among these entities so that we could be able to evaluate exactly their taxonomic situation. So more investigations based on molecular tools such as mitochondrial DNA sequencing and microsatellite DNA genotyping is needed before exact conclusions can be drawn.

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