COMPARATIVE ONTOGENIC MORPHOMETRY: LYGUS HESPERUS VS. LYGUS LINEOLARIS

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Abstract

Lygus hesperus (Knight) and *Lygus lineolaris* (Palisot de Beauvois) are two sympatric species commonly found in the Texas High Plains region. Ontogenic shape change in these two *Lygus* species was evaluated using elliptical Fourier analysis of body outlines of five nymphal stages. The first 15 harmonic Fourier coefficients were used for multivariate statistical analysis to discriminate these species during different developmental stages. The discriminant function analysis (DFA) showed that *L. lineolaris* and *L. hesperus* shapes were two distinct groups with little overlap in their discriminant function (DF) scores and ANOVA revealed that DF scores of *L. lineolaris* and *L. hesperus* were significantly different. A two-way MANOVA test, using the first 10 principal component (PC) scores, revealed that the difference between the two species' PC-scores was highly significant. Likewise, the PC-scores between different growth stages were also significantly different. The UPGMA tree of the mahalonobis distance showed *L. hesperus* and *L. lineolaris* as two distinct clades except for the fifth instar nymph of these two species, which were in a single clade. This study showed that elliptical Fourier analysis of body shape is an effective approach for differentiating immature *Lygus,* especially when they are very small in size and do not have clear landmarks or when only a relatively low resolution image acquisition facility is available.

Introduction

Lygus species (Hemiptera: Miridae), first described by C.W. Hahn in 1833, are an economically and ecologically important group of insects. This genus is composed of 43 species worldwide, of which 34 species are reported from North America (Kelton 1975). Significant revisions of this genus were done by Knight (1917), China (1941), Slater (1950), Leston (1952), Kelton (1955), and Carvalho et al. (1961). Thomas and Lattin (1987) also critically reviewed the taxonomic status of the genus *Lygus* and concluded that the *Lygus* complex exhibits great morphological variation and is therefore very difficult to positively identify. Identification of *Lygus* species in the nymphal stage is not possible due to lack of a nymphal taxonomic key. During a *Lygus* survey in Texas, we also faced similar difficulties in positively identifying some individuals because of the limited taxonomic keys to females and unexpected temporal variation in adult *Lygus* collected from different hosts. Co-existence of multiple species and the difficulty in positive identification to species hinders the development of species specific research and pest management program (Thomas and Lattin 1987).

Identifying the species by the current taxonomic key requires taxonomic expertise. Morphometric quantitative characteristics (shape and size) of different body parts with multivariate data analysis may better delineate the species and result in more accurate identifications than with qualitative keys. With digital images, and their corresponding morphometric data, it may be possible to develop web-based digital taxonomic keys for accurate and easy identification of *Lygus* species. This will lay the foundation for the computer-based, automated, species identification of *Lygus* species in the future. Research scientists and crop producers could both benefit from the development of a web-based digital identification system.

Morphometric analysis is not only for taxa identification but it is also useful in other biological and ecological research such as phylogenetic and phylogeographic studies and evaluation of evolution. In the past, scientists utilizing phenetic and cladistic approaches to phylogenetics have had conflicting findings because phenetic (numeric systematics) and cladistic systematics implied different positions on the tree of life for the same organism. Supporters of the morphological approaches (Kraus 1998, Wagele et al. 1999, Bitsch et al. 2004) argued that the use of arthropod morphology and development produce more plausible relationships than those based on a few loci and often for few taxa in molecular phylogenetics. Morphological data have some advantages over molecular data.

Morphology may yield a large number of observable characters for analysis, can be used for fossil taxa, and many genes are involved in the expression of a particular morphological character. Hillis and Wiens (2000) noted that dense taxon sampling is the greatest advantage of morphological data.

There are three general approaches in morphometric study: 1) classical distance measurements (dimensions of body parts), 2) landmark-based study, and 3) outline-based study. Elliptical Fourier analysis (EFA) is a commonly used method utilizing the outline-based approach. The EFA was developed by Kuhl and Giardina (1982) and has been used for analysis of complex forms (Le Minor and Schmittbuhl 1999). The mathematical details of the elliptical Fourier descriptor calculation function can be found elsewhere (Kuhl and Giardina 1982, Tort 2003, Daegling and Jungers 2000, Rohlf and Archie 1984). Among different variants of Fourier analysis, elliptical Fourier analysis is the most promising approach according to Rohlf and Archie (1984). Fourier analysis of shape was first used in systematics by Kaesler and Water (1972) and since that time many people have been using this approach for various taxa and in different fields of biology (Tort 2003, Monti et al. 2001, Tracey et al. 2006). One of the advantages of fitting Fourier functions is that they can be applied directly to the boundary outline without the need for homologous landmarks, do not require equally spaced points along outline, can be used for complex curved forms, and can be normalized to size, position, orientation and starting points (Crampton 1995).

Evaluation of ontogenic shape change, allometry and heterochrony is important for understanding the real biological differences between closely related species. During the process of evolution and speciation, changes might take placed early during the developmental process which can significantly contribute to differences in the adult stage. Ontogenic morphometric studies have not been done with *Lygus* species, so the objective of this study was to compare the ontogenic shape changes between *L. hesperus* and *L. lineolaris*, the two most dominant species of *Lygus* in Texas. The null hypothesis is that there are no shape differences between and within species during different developmental stages.

Materials and Methods

Eggs of *Lygus hesperus* and *Lygus lineolaris* were collected from a laboratory colony established at the Cotton Entomology Laboratory, Texas Agricultural Experimental Station, Lubbock, Texas. The eggs were transferred individually into small plastic petri dishes (6-cm dia.) with filter paper and a fresh green bean. Both species of *Lygus* were reared on green beans in a growth chamber programmed for 25± 2 ^{o}C temperature, 50-70% relative humidity and 14-h photoperiod. First instar nymphs were photographed on the first day after egg hatch while the four subsequent instars were photographed immediately after each molt. Digital images were taken using a Cannon Powershot-G™ camera mounted on a Meiji™ stereo microscope. The camera setting was standardized and controlled automatically using PC and Zoom Browser software but the magnification setting and light conditions were adjusted manually. The color and distance measurement system was calibrated using standard color checker and ocular and stage micrometers.

Based upon focal clarity, digital images were selected from all 5 instars of 20 *Lygus* (10 specimens per species). These 100 dorsal images were digitized around the dorsal body (no antennae or legs) using tpsDig-2 software version 2.10 (Rohlf, 2006). Around 100 points (x, y coordinates) were digitized along the margin of the body of *Lygus* from each image. The common biologically homologous point (i.e., base of right antennae) was used as the starting point for outline digitization. The x, y coordinates of the digital points along the boundaries was normalized by converting the polygon into unit centroid size and rotating the polygon around the centroid to match the major axis. This way, the size, location and orientation effect on the boundary data was removed from the analysis.

Data Analysis

Radius functions were fitted to the digitized x, y coordinates of the boundary of each nymphal stages and the average form for each species was estimated by averaging the radius function for the visualization of the average body shape of *Lygus* at different developmental stages. The boundary of each insect form was decomposed by elliptical Fourier analysis (EFA). The EFA was conducted by using MATLAB™ 6.5 software, which converted our spatial points to the frequency domain. From this harmonic analysis, we were able to produce mathematical descriptors of the shapes in the form of sine and cosine terms. Sixty mathematical variables, harmonic coefficients (x-sine, x-cosine, y-sine and y-cosine terms for 15 harmonics), were produced for each individual image. Those Fourier coefficients were normalized to size and orientation as described by Rohlf and Archie (1984). Then principal components analysis (PCA) was run using those elliptical Fourier coefficients based upon a correlation matrix of all

60 coefficients. The structure of data was determined by PCA and the data set was reduced for further multivariate analysis. Principal components that accounted for the two highest percentage variations were selected. These two PCs were then used to determine the variables with highest loadings (contributing to majority of variation in data). Two-way multiple analysis of variance (MANOVA) was performed using statistical analysis system (SAS Institute, 2003.) to test the significance of differences in shape between two species and among different stages within each species. Discriminant function analysis (DFA) of the PCA scores was performed using MATLAB function to optimize the discrimination between the two species. Canonical variate analysis (CVA) was used to discriminate the different nymphal stages. Mahalonobis distances (D^2) were calculated for all the groups (species and developmental stages) to quantify the differences in shape of different groups of *Lygus* and these distances were used to generate the unweighted paired group method with arithmetic means (UPGMA) tree.

Result and Discussion

Lygus is a hemimetabolous insect which has 7 developmental stages (i.e., egg, 5 nymphal instars and adult) (Fig.1), but we compared the dorsal body shape among only the 5 nymphal stages of two species of *Lygus*. Because *Lygus* nymphs are very small and morphological characters are not apparent, it is difficult to identify them visually, especially in the early stages, and discriminating the species is a challenging task. Color and size of different *Lygus* body parts have been used to discriminate insect growth stages and species, but these two characters are not very reliable. The color pattern may not often have fully developed, may have faded out or the color may vary depending on temperature and growing conditions. Body size also varies depending on age, diet and climate. Body shape is a more reliable inherent character of a species so we decided to analyze shape variation between *L. hesperus* and *L. lineolaris*.

The average of radial functions fitted along the boundary (Fig. 2) of the images of each stage gave subtle visual differences in shapes among 5 developmental nymphal stages and between the species, but detailed analysis of shape is necessary to determine the differences statistically. Elliptical Fourier Analysis (EFA) orthogonally decomposes the x, y boundary coordinates into different harmonic functions. Each harmonic function has 4 coefficients (x-sine, x-cosine, y-sine and y-cosine) and we generated 15 harmonic functions, thus each form had 60 Fourier coefficients which strongly represent the shape of the particular insect. The coefficients can be directly used in multivariate analysis. Reconstruction of the shape of insect with different harmonic elliptical Fourier coefficients was done to visualize and determine how many harmonic coefficients effectively represent the true shape of the *Lygus* nymph. Forms were reconstructed using 3, 6, 9, 12, 15 and 50 harmonic coefficients (Fig. 3). The 15^{th} harmonic coefficients were found to be sufficient enough to reconstruct the shape of *Lygus,* very close to the original shape so we have used the Fourier coefficient up to 15^{th} harmonics for further multivariate analysis.

Thirty-nine PC axes were needed to account for 99% of the data variation; the first 10 PC axes collectively accounted 76% of the variation. The remaining PC axes each accounted for <3% variation. Therefore, only 10 PCs were presented in a scree plot (Fig. 4). The first two PC axes accounted for 32 % of the data variation. PC scores of individual *Lygus* samples projected on PC1 and PC2 were plotted (Fig. 5) which showed obvious clusters of shape data. When we placed the convexhull boundary over the data from *L. lineolaris* and *L. hesperus,* the two clusters had some overlapping. Because we already know the groups of *Lygus* (i.e. two broad groups of two species and within each group, 5 sub-groups of different nymphal stages), the DFA was applied to optimize the discrimination between these groups. The EFA coefficient data were reduced from 60 to 26 variables, depending on the contribution of each harmonic coefficient on the first two PC as indicated by the PC-loadings. Then DFA analysis of the 26 selected harmonic Fourier coefficients for all individuals was done. The DFA showed *L. lineolaris* and *L. hesperus* shapes are two distinct clusters with little overlap in their DF1 scores (Fig. 6). The vector plot (Fig. 6) clearly showed that *L. lineolaris* had higher values for 4, 11, 13, 14, 21, 23, and 25 EF-coefficients (i.e., x-sine 5^{th}, x-cosine 10^{th}, y-sine 4^{th}, y-sine 6^{th}, y-cosine 7^{th}, y-cosine 9^{th}, and y-cosine 11^{th} harmonic coefficients) as compared to *L. hesperus*. *Lygus hesperus* had a higher value for the 2, 5, 6, 7, 24, and 26 EF coefficients (i.e. x-sine 3^{rd}, x-sine 4^{th}, x-sine 8^{th}, x-sine 10^{th} and y-cosine of 10^{th} harmonic coefficients) as compared to *L. lineolaris.* Because the first component of DF accounted for 100% of the data variations, the DF scores from the DF1 were used in the univariate ANOVA. The ANOVA revealed that DF1 scores of *L. lineolaris* and *L. hesperus* were significantly different (df=1, F=105.2 and P=0.0001).

Shape differences among the different stages within each species were analyzed with two-way MANOVA. The two-way MANOVA using the first 10 PC scores from PCA revealed that the difference in PC scores between two

species was highly significant (*Wilks' Lambda*=0.594, *F*=5.53, *ndf*=10, *ddf*=81, and *P* <0.0001). Likewise, the PC-scores between different growth stages were also significantly different (*Wilks' Lambda*=0.457, *F*=1.77, *ndf*=40, *ddf*=309 and *P*= 0.004) but there were no significant interactions between species and growth stages *(Wilks' Lambda*=0.587*, F*=1.17, *ndf*=40*, ddf*= 309*,* and *P*= 0.235*).* The canonical variate analysis (CVA) of elliptical Fourier coefficients of different growth stages of each *Lygus* species showed that CV1 and CV2 clearly differentiated all nymphal instars of *L. hesperus* (Fig. 7) with some overlap in their CV-scores. In the case of *L. lineolaris,* (Fig. 7), the CV function could not clearly differentiate first, second and third instars. The fourth and fifth instars had clearly distinguishable scores as compared with the first, second and third instars. The MANOVA of the first 10 CV scores of *L. lineolaris* showed no significant differences among the five nymphal stages (*Wilk's ambda* = 0.339, *F* =1.14, *P* =0.27, *df* = 40 and 138) but the CV scores of *L. hesperus* were significantly different among all nymphal instars (*Wilk's ambda* = 0.197, *F* = 1.84, *P* = 0.008, *df* = 40 and 138).

The UPGMA tree of the mahalonobis distance (Fig. 8) showed *L. hesperus* and *L. lineolaris* as two distinct clades, except that the fifth instars were at the closest distance and were in a single clade. In *L. hesperus,* first and second instars were similar and clearly separated from the third and fourth instars of the same species and the fifth instars were clearly in a different clade. It was interesting that the fourth and second instars of *L. lineolaris* were closely similar, as compared to the first and third instars of the same species based on shape analysis.

Conclusion and Future Research

Elliptical Fourier analysis of body shape is an effective approach for discriminating forms such as immature insects, especially when they are very small in size and do not have clear landmarks or when only a low resolution image acquisition facility is available. To determine the ontogenic growth trajectories, a landmark-based shape and size (allometry) analysis is necessary, which requires homologous landmarks. Because early-instar Since *Lygus* are very small in size and do not have clear homologous landmark points, this may be possible only where a high resolution imaging facility is available such as a scanning electron microscope. The elliptical Fourier analysis of boundaries can only explain the shape differences along the boundary but not the local deformations or the change in particular body parts. Therefore, our future research aims to evaluate the ontogenic shape changes along with their ontogenic allometry.

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A. *Lygus hesperus* (egg, 5 nymphal stages and adult)

B. *Lygus lineolaris* (egg, 5 nymphal stages and adult)

Figure 1. Different developmental stages of *Lygus hesperus* and *Lygus lineolaris*. Note: the pictures may not be true to the size of insect due to different magnifications.

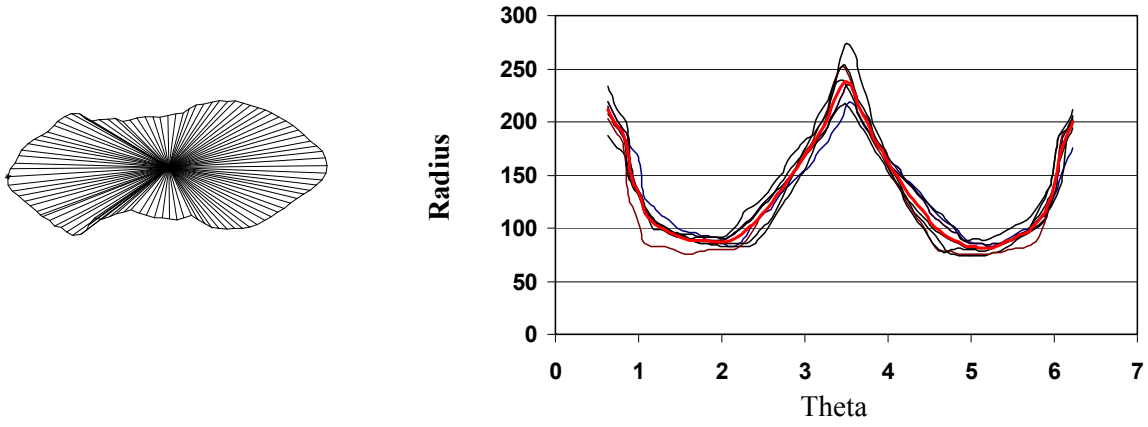

Figure 2. Construction of average shape of *Lygus* (dorsal outline of body without legs and antennae) by fitting radius function and averaging the radius over the theta angle around the centroid. A. Equally spaced radii from centroid of the *Lygus* boundary, B. Radial function plot of ten individual *Lygus* nymphs and the average form (orange line).

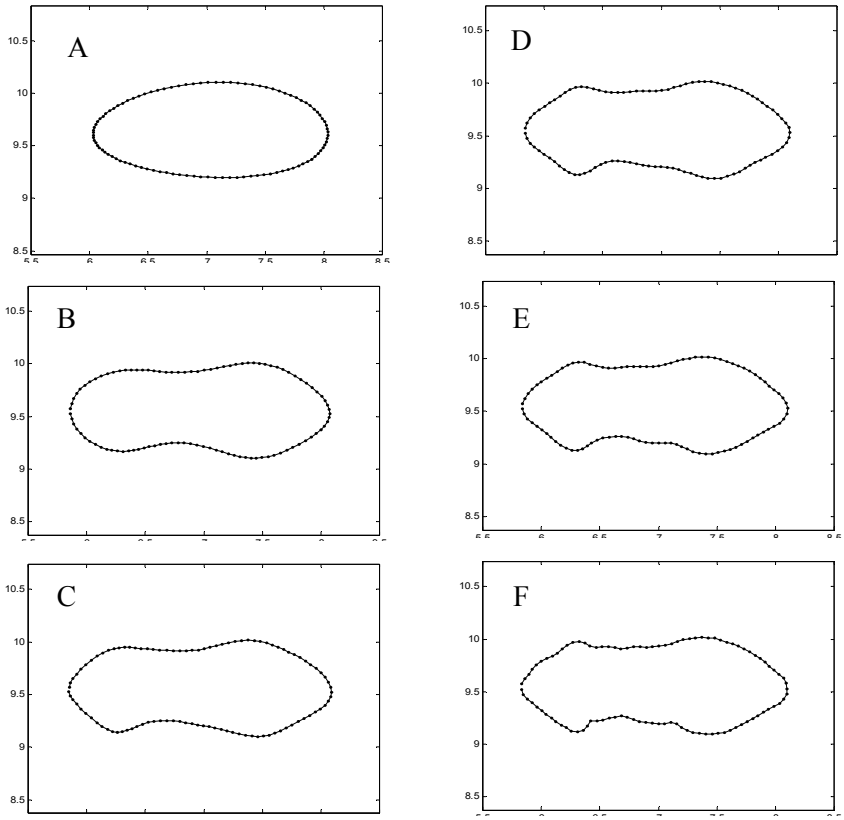

Figure 3. Average shape decomposition and reconstruction of *Lygus* nymphs using elliptical Fourier coefficients of different harmonics (A= first 3 harmonics; B= first 6 harmonics; C=9 harmonics; D=12 harmonics; E= 15 harmonics and F= 50 harmonics). Note the 15th and 50th harmonic gave similar models except for very minor details; thus, we decided to use the first 15 harmonic coefficients for further analysis of the shape difference.

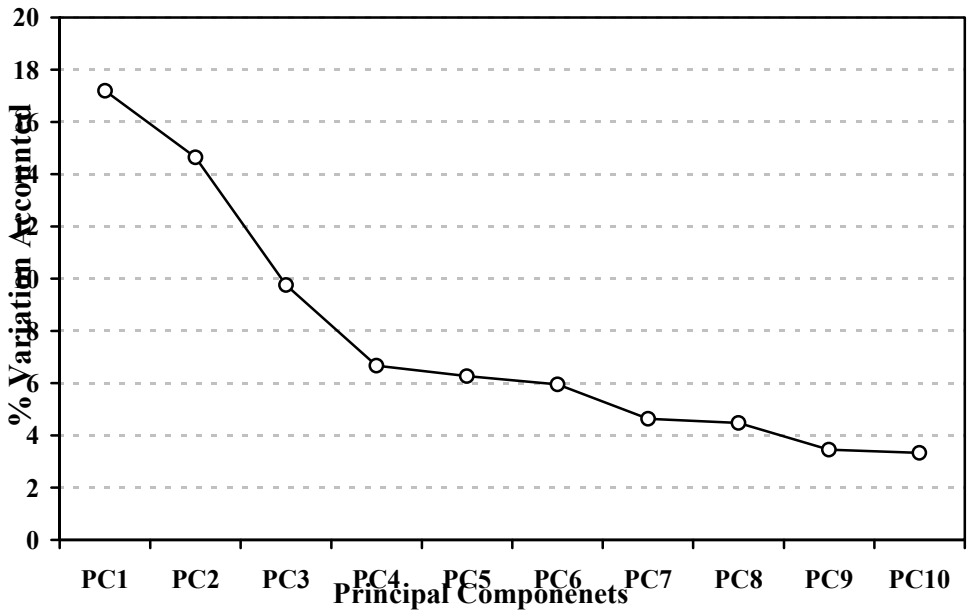

Figure 4. Scree plot showing the percent variation accounted by 10 major principal components of the elliptical Fourier coefficients of the two *Lygus* species.

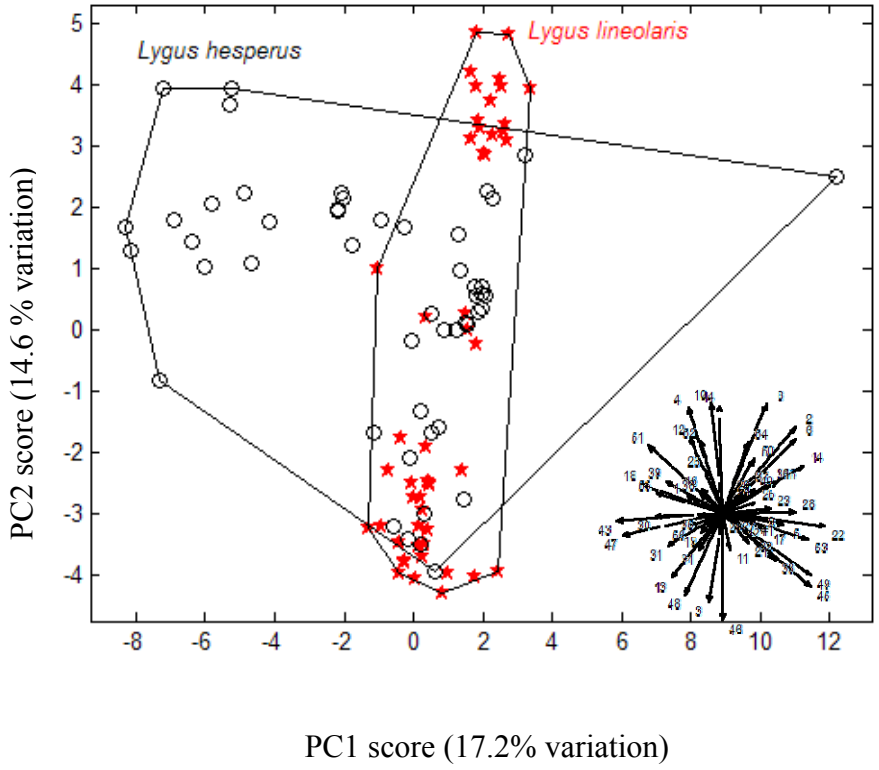

Figure 5. Principal component scores of individual *Lygus* as projected onto PC1 and PC2 and a vector diagram of PCA eigenvectors.

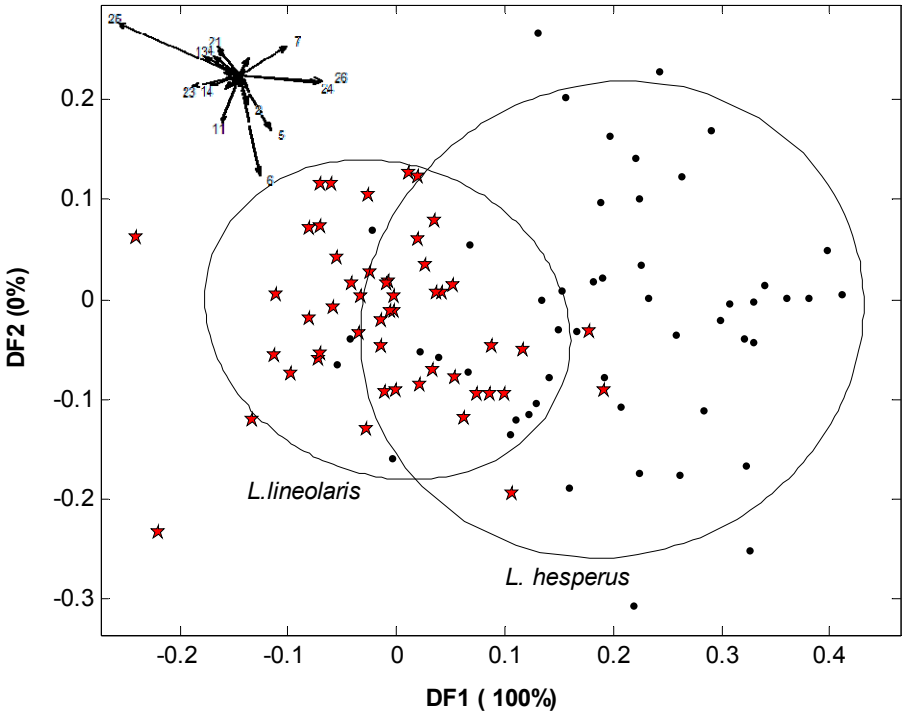

Figure 6. Discriminant function scores of individual *Lygus* as projected onto DF1 and DF2 and the 95% confidence ellipse around the scores and a vector plot of DFA eigenvectors.

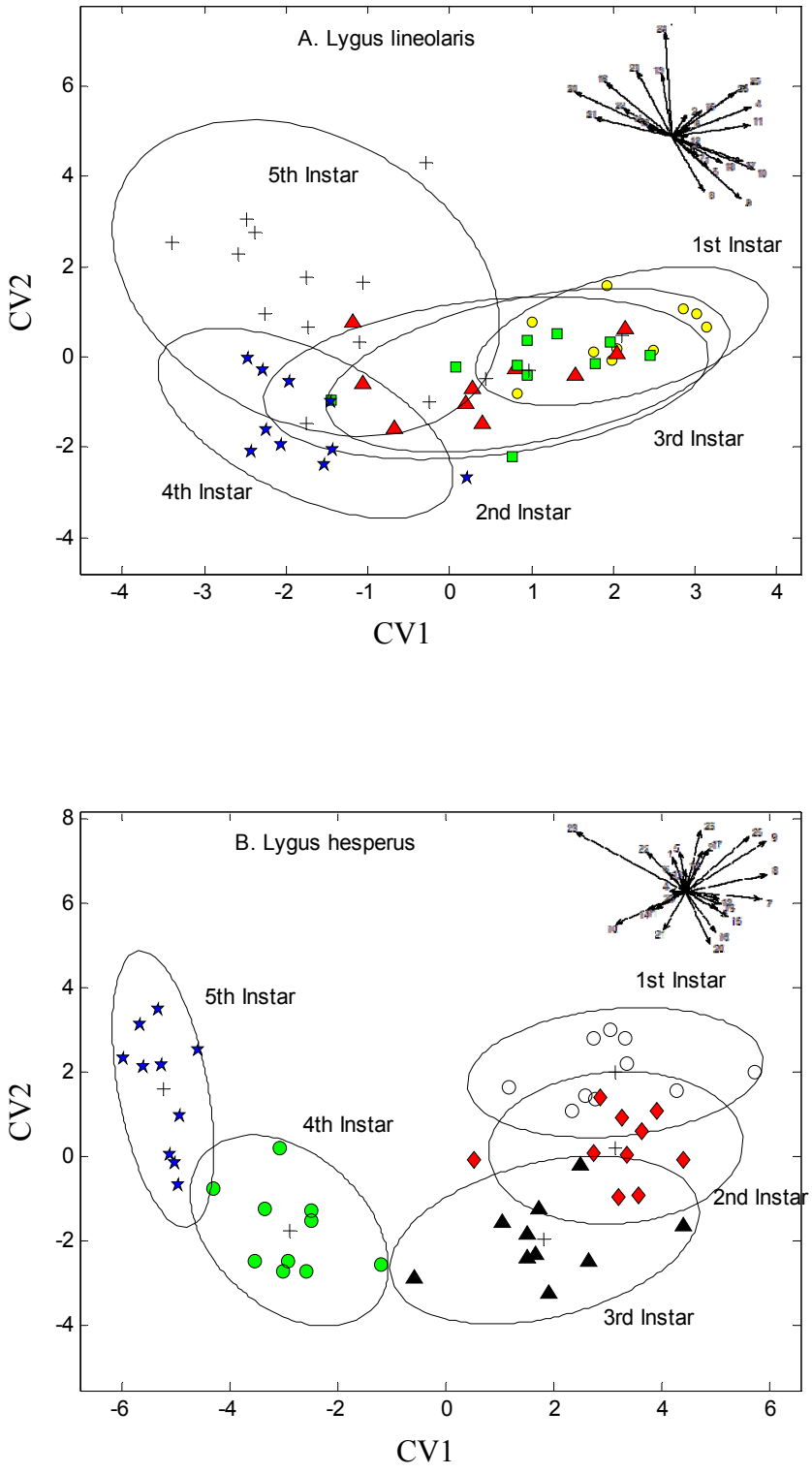

Figure 7. Canonical variate scores of different nymphal stages of *Lygus* as projected onto CV1 and CV2 and the 95% confidence ellipse around the scores and a vector diagram of CV eigenvectors.

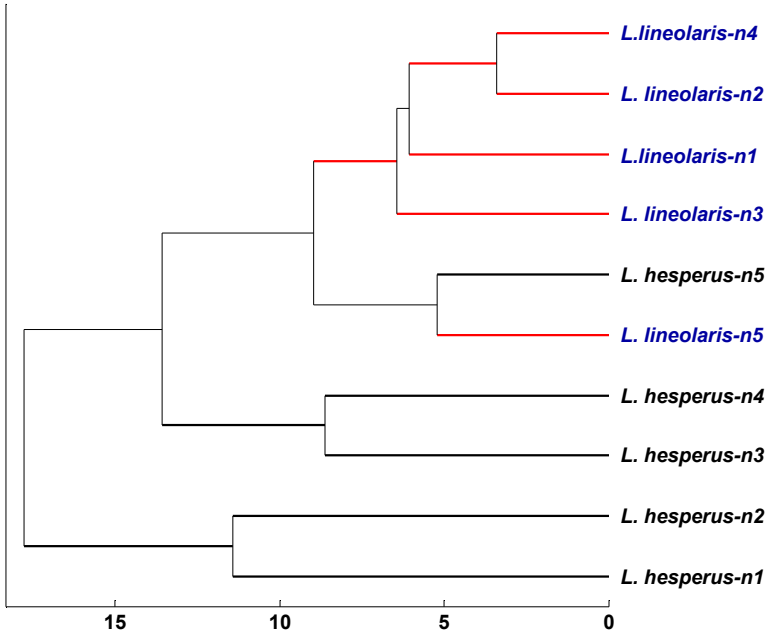

Figure 8. The UPGMA phenogram based on mahalonobis distances among the groups.