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Abstract

Bean leaf beetle (BLB), *Cerotoma trifurcata* (Forster; Coleoptera: Chrysomelidae), exhibits considerable color variation but little is known about the underlying genetic structure and gene flow among color phenotypes. Genetic and morphological variation among four color phenotypes—green with spots (G+S), green without spots (G-S), red with spots (R+S) and red without spots (R-S)—were analyzed using amplified fragment length polymorphisms (AFLP) and morphometrics, respectively. AFLP generated 175 markers that showed ≥80% polymorphism. Analysis of molecular variance (AMOVA) indicated that genetic variation was greatest within phenotypes (82.6–84.0%); gene flow among the four phenotypes was relatively high (N_m = 3.82). The dendrogram and STRUCTURE analysis indicated some population divergence of G-S from the other phenotypes. Morphological parameters were similar among phenotypes except that R+S showed significant differences in weight and body-length. Canonical variables 1 and 2, based on average morphometric characters, accounted for 98% of the total variation; some divergence was indicated between G+S and R+S from each other and from the G-S/R-S BLB color morphs. The pattern of genetic variation indicated potential divergence of G-S and G+S from each other and from R-S and R+S. Although these results indicate that the four different color morphs are not genetically or reproductively isolated, there is some genetic differentiation/structure and morphological dissimilarity suggesting weak/incomplete isolation.

Key words: amplified fragment length polymorphism, morphometrics, genetic variation, phenotype

Bean leaf beetle (BLB), *Cerotoma trifurcata* (Forster; Coleoptera: Chrysomelidae), is native to the United States, has a large host range and its biology is well described (Johnson et al. 2008, Hadi et al. 2012). It was once considered a minor pest of soybeans (*Glycine max* (L.) Merr.) in the Midwest United States because incidence was low and plants could compensate for feeding damage caused by BLB (Kogan et al. 1980). Increased incidence of BLB, in recent years, has resulted in increased economic damage to soybeans, its preferred host (Lam and Pedigo 2004, Bradshaw et al. 2008, Hadi et al. 2012). Damage caused by BLB is both direct and indirect in many soybean production areas of the United States (Hadi et al. 2012). Direct damage from BLB adults feeding on pods or larvae feeding on plant roots and root hairs can be severe (Riedell et al. 2005). Indirect damage results because BLB is an efficient vector of some viruses, such as

the bean pod mottle virus and soybean mosaic virus, that negatively impact soybean plants (Giesler et al. 2002, Hadi et al. 2012).

BLB adults exhibit an array of color and marking patterns with little sexual dimorphism (Chittenden 1897, Horn 1893, Hadi et al. 2012). Herzog (1968) reported color pattern polymorphisms (color morphs) by sex and geographic origin of BLB samples. They are typically dark yellow with a distinct, black, triangular area involving the scutellum plus the anterior of the elytra and two black spots on each elytrum (Fig. 1A and B). However, the body color can vary to some hue between yellow, orange, and red and may be devoid of spots on the elytra (Fig. 1C and D); the distinct, black, triangular area at the anterior of the elytra is a consistent feature (Herzog 1968, Smelser and Pedigo 1991). Herzog (1973) postulated that the inheritance of variation in color and marking patterns may be sex linked. Yet there is no documentation on these
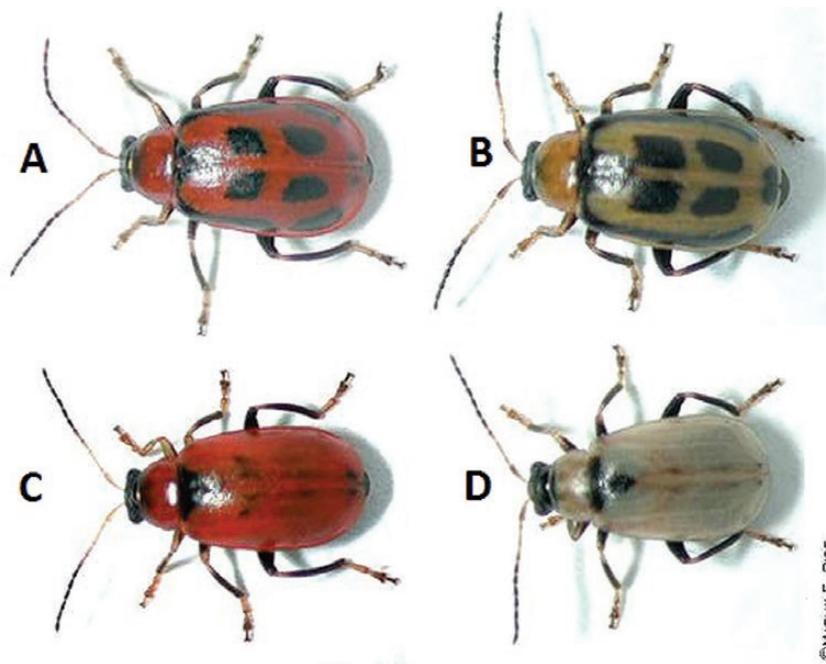
Fig. 1. Examples of bean leaf beetle phenotypes in this study: (A) red with spots; (B) green with spots; (C) red without spots; and (D) green without spots (photo found on the web at https://www.bing.com/images/search?view=detailV2&ccid=B1AZHJvT&id=C83FC6441B4418CB1973DBA6EEADA8F20541B594&thid=OIP. B1AZHJvTh7UIEy1L4gC3jAHaJZ&mediaurl=https%3a%2f%2fwww.vegedge.umn.edu%2fsites%2fvegedge.umn.edu%2ffiles%2fstyles%2fpanopoly_ image_original%2fpublic%2fmorphbg.jpg%3fitok%3d3pgIrAIQ&exph=548&expw=432&q=bean+leaf+beetle+pictures&simid=608015028718276108& selectedIndex=25&qpvt=bean+leaf+beetle+pictures).

different color morphs indicating their similarities and/or compatibility. Unrecognized differences, both morphological and genetic, within a species can be a major concern, especially for insect pest management, because this can imply different responses to control measures.

The variation in morphological traits provides the basis for taxonomic and bio-geographic studies and for the development and testing of hypotheses in evolutionary biology. This variation arises from the combined effects of mutation and migration, drift, selection, and historical factors, including founder events, population bottlenecks, and rate of population expansion following a bottleneck or founding event (Cwynar and MacDonald 1987). Although phenotypic differences between sub-populations have a genetic basis, these differences (i.e., body color, body length, elytra length and weight) do not necessarily imply genetic differentiation at a level that would result in distinct BLB groupings (such as ecotypes). This indicates usefulness in studies that combine morphometry, the measurement and analysis of morphological form (Sorensen and Foottit 1992, Dujardin et al. 1999, Dujardin et al. 2000), with molecular genetic analysis to detect potential genetic differences (Crouau-Roy 1989, Gonzalez-Rodriguez et al. 2000).

The use of molecular techniques in taxonomic studies, often in conjunction with more traditional morphological approaches, may provide increased taxonomic clarity. Despite their importance as pest species, the significance of BLB color morphs is poorly understood. To date Tiroesele et al. (2014) showed no substantial genetic differences in BLB populations sampled from across the Midwest United States. But no molecular genetic studies have been used to investigate the relationships or differences within BLB subpopulations that exhibit color differences. The goals of this study, done in conjunction with but separate from previously reported work (Tiroesele et al. 2014), were to quantify morphological variation in four BLB color morphs, decipher genetic relationships among four different color phenotypes of BLB using genetic markers generated by amplified fragment length polymorphisms (AFLP), and investigate whether

phenotypic differences between body color of the four BLB color morphs are reflected in genetic structure. The hypotheses of interest were: 1) outside of color, these four BLB color morphs are not morphometrically different and 2) these four BLB color morphs are not genetically differentiated.

Materials and Methods

Sample Collection
Collection of BLB adults was on 11 August 2010 at Pemiscot County, MO in the Midwest United States (N 36° 23′ 43.98″; W 89° 36′ 48.74″). Sampling was done between 08:00 a.m. and 11:00 a.m., using a sweep net in a single soybean field, regardless of body color. The color morphs collected included green with spots (G+S), green without spots (G-S), red without spots (R-S) and the red with spots (R+S). The adults were stored in 4 ml glass bottles with 95% ethanol soon after collection. The alcohol was changed twice after collecting the samples, by decanting and then adding fresh ethanol, to avoid alcohol dilution by fluids from sampled adults that can lead to DNA degradation. These were kept in a freezer (–20°C) prior to sending to the Genetics Laboratory in the Department of Entomology at the University of Nebraska - Lincoln where they were kept in –80°C freezer until they were used.

Morphological Analysis
A total of 139 specimens were measured for morphological comparative analysis. The number of specimens per color morph varied considerably due to natural abundance, G+S (n = 43), G-S (n = 40), R+S (n = 33) and R-S (n = 23). Sexes were not separated as there is little dimorphism (Chittenden 1897, Horn 1893, Hadi et al. 2012). Twelve morphological parameters were measured for all specimens of each color morph: weight, elytron length, elytron width, scutellum length, pronotum length, pronotum width, head width, total

body length, abdomen length, hind femur length, hind tibia length, and antennae length. Linear measurements were made using a fluorescent magnifying lamp and standard electronic vernier caliper standardized to two decimal places. The weight of each beetle was determined, using a top loading electronic balance (±0.1 mg), subsequent to air drying for 45 min on a paper towel; three weight measurements of each beetle were taken and the average was reported.

Analysis of variance (ANOVA), using SAS 9.1 (SAS Institute 2006), was used to detect differences between morphological parameters between the four BLB color morphs. When statistical differences were detected, means for each of the color morphs were compared using least significant difference. Stepwise discriminant analysis (SAS STEPDISC; SAS Institute 2006) was used to determine the best combination of variables that would separate four BLB color morphs. Canonical discriminant analysis (SAS Proc DISCRIM; SAS Institute 2006) was then used. This dimension reduction technique, related to stepwise discriminant analysis, derives canonical variables (linear combinations of the variables) that summarize between class variations and examines the differences between two or more groups with respect to several variables simultaneously (Ye and Robbins 2004). The analysis produced pooled, within-class, standardized, canonical coefficients that were pooled within canonical structure coefficients. The pooled within canonical structure and pooled within class standardized canonical coefficients were used to determine each variable's contribution to the discriminant function. The standardized coefficient, a measure of a variable's relative contribution to the overall classification, and the structure coefficient, the correlation between the variables and the discriminant function, together provide a relative measure of the specific variable's ability to discriminate between the two groups. These were then used to determine total sample standardized canonical coefficients (SAS 2006).

Molecular Genetic Analysis

DNA was extracted from the thorax of 22 randomly selected BLB adults of each phenotype using the Black and Duteau (1997) CTAB extraction protocol as modified by Clark et al. (2007). The procedures for extraction included homogenization of the thorax, addition and incubation of Proteinase K and RNase A, and centrifugation followed by supernatant transfer and cold storage. Quantification of DNA from individuals for each sample location using a spectrophotometer

and visualization on an agarose gel were the same as previously reported (Tiroesele et al. 2014). Diluted DNA samples were then kept at –20°C until they were used after which the samples were kept at –80°C as vouchers in the Genetics Laboratory of the Entomology Department at the University of Nebraska - Lincoln.

A modified AFLP protocol (Vos et al. 1995) was used to assess the genetic variability within and among BLB subpopulations. DNA extracted from 15 to 20 individuals per color morph of BLB was used. All steps of AFLP-PCR were completed following the same procedure as in Tiroesele et al. (2014) and included: 1) restriction of the DNA with MseI and EcoRI restriction enzymes; 2) adapter ligation with MseI and EcoRI adapters; 3) pre-amplification (Table 1); and 4) selective amplification with 3 combinations of MseI and EcoRI primers (Table 2). Afterwards, 1.5 µl of each sample with specific primer combinations was electrophoresed on KBplus 6.5% polyacrylamide gel in the GeneReadIR 4200 DNA analyzer (LI-COR, Lincoln, NE) for 2 h to separate the DNA markers. The gel image was saved for scoring (developing a binary matrix of 1's = band present and 0's = band absent) and further analysis.

Each primer combination was used with subsamples of DNA from 24 randomly selected individuals for assessing the genotyping error as reported previously (Tiroesele et al. 2014). The error rate, calculated as the ratio of the total number of mismatches (presence or absence of a band) at a locus to the number of the replicated individuals, should never exceed 10% to better ensure reliability and reproducibility of results (Bonin et al. 2004, Pompanon et al. 2005).

Bootstrap analysis was used as a way of testing the robustness of the dataset for further analysis by the creation of a pseudo-replicate which re-sampled the data 10,000 times using BOOD-P software ver. 3.1 (Coelho 2001). DBOOT (Coelho 2001) was used to evaluate the correlation between the coefficient of variation and the number of molecular markers observed, thus providing an estimate of the robustness of the data (Hoelzel 1995). Genetic similarity was estimated using the Jaccard index through the SIMQUAL procedure using NTSYSpc (Rohlf 2000). Dendrograms were constructed to illustrate genetic similarity, following the methodology described by Sneath and Sokal (1973).

POPGENE version 1.32 (Yeh and Boyle 1997) was used to determine the degree of genetic polymorphisms both within and between color morphs of BLB. Genetic differentiation between color morphs samples was assessed in POPGENE version 1.32

Table 1. The sequences of oligonucleotide adapters and primers used for AFLP analysis

Primer ID	Primer Type	Sequence (5′-3′)	AFLP STEP
*Eco*RI-F	Forward adapter	CTCGTAGACTGCGTACC	Adapter Ligation
*Eco*RI-R	Reverse adapter	AATTGGTACGCAGTCTAC	Adapter Ligation
*Mse*I-F	Forward adapter	GACGATGAGTCCTGAG	Adapter Ligation
*Mse*I-R	Reverse adapter	TACTCAGGACTCAT	Adapter Ligation
E(N+0)	*Eco*RI Preamp primer	GACTGCGTACCAATTC	Preamplification
M(N+1)	*Mse*I Preamp primer	GATGAGTCCTGAGTAA C	Preamplification

Table 2. Selective AFLP primer combinations, number of markers, fragment size and percentage mismatch for the primer pair combinations used with bean leaf beetle color morphs

Primer combination[a]	Number of markers	Fragment size range (bp)	% Mismatch
M-AA × E-ACA	74	50–400	6.17
M-AG × E-AAC	47	50–400	5.50
M-AG × E-ACA	54	50–370	5.89
Total	175		
Average			5.85

[a]M, *Mse*I preamplification primer plus 2 bases added to 3′ end; E, *Eco*RI preamplification primer plus 3 bases added to 3′ end.

(Yeh and Boyle 1997) using Nei's gene diversity index (G_{ST}); gene flow (N_m) between color morphs was estimated from G_{ST}, expressed as (N_m) = (1–G_{ST})/$4G_{ST}$ (Culley et al. 2002, Bonin et al. 2007, Ryman and Leimar 2009). Fall armyworm larvae, *Spodoptera frugiperda* (J.E. Smith; Lepidoptera: Noctuidae), were used as the out-group to test the robustness of the dendrogram developed in the POPGENE analysis. The potential that the samples exhibited population structure was investigated using the program STRUCTURE (Falush et al. 2007). One to four potential populations ($K = 1$ to $K = 4$) were evaluated (burn-in = 10,000; replicates = 10,000); 15 iterations were done for each K value. Results from STRUCTURE were further evaluated using STRUCTURE HARVESTER (Earl and Vonholdt 2012). Analysis of molecular variance (AMOVA) was used to evaluate genetic variability among groups and within groups (color morphs) using the software package Arlequin version 3.5 (Excoffier et al. 2005). AMOVA were calculated with color morphs based on results from STRUCTURE (considered as three sub-populations of G+S, G-S, and R+S with R-S) and, for comparison, an AMOVA was calculated for all color morphs belonging to one population. ARLEQUIN was also used for pairwise comparisons to test genetic divergence (F_{ST}-Wright's inbreeding coefficient) (Wright 1978, Slatkin 1995).

Results

Morphological Analysis

The morphological data revealed no statistical differences among the four color morphs of BLB for head size ($F_{3,135} = 4.06$, $P = 0.3690$), elytra width ($F_{3,135} = 3.82$, $P = 0.150$), scutellum length ($F_{3,135} = 4.25$, $P = 0.2932$), abdomen length ($F_{3,135} = 6.58$, $P = 0.1974$), antennae length ($F_{3,135} = 5.83$, $P = 0.1448$), tibia ($F_{3,135} = 3.82$, $P = 0.4825$), femur ($F_{3,135} = 4.92$, $P = 0.1301$), pronotum length ($F_{3,135} = 3.76$,

$P = 0.5182$) and pronotum width ($F_{3,135} = 1.34$, $P = 0.2645$) (Table 3). However, R+S beetles were significantly different from the other morphs for weight (heavier; $F_{3,135} = 4.47$, $P = 0.005$) and body length (longer; $F_{3,135} = 10.64$, $P = 0.0001$). The elytra length of the G+S and R+S did not differ from each other but were found to be longer ($F_{3,135} = 24.74$, $P = 0.0001$) than those of the G-S and R-S (which also did not differ from each other) (Table 3).

Of the three canonical variables generated, Eigen values 1 and 2 accounted for ~98% of the total variation (Table 4) and were used to illustrate how the BLB color morphs are grouped and separated, providing an indication of the most useful morphometric characters for distinguishing BLB. Resultant canonical variables 1 and 2 grouped and separated the BLB color morphs (Fig. 2). Separation of the R+S (represented by number 3 on Fig. 2) from the other morphs was nearly complete and was most influenced by canonical variable 1. The G+S morphs (number 1 on Fig. 2) were also mostly separated from the other morphs; again canonical variable 1 contributed more discrimination but there was influence by canonical variable 2. The G-S and R-S BLB (numbers 2 and 4, respectively, on Fig. 2) closely grouped together and were intermixed; neither canonical variable was discriminating.

Canonical variable 1 had the highest correlation with pronotum length (0.5999) followed by tibia length (0.2499) and body length (0.2031) (Table 5). Canonical variable 2 had the greatest correlation with elytra length (0.6617) followed by pronotum width (0.5560), head width (0.5038) and elytra width (0.4995). Both canonical variable 1 and 2 had high discriminant power because their axes showed similar dispersion of values between BLB phenotypes (Fig. 2).

Molecular Analysis

No loci were eliminated from this work due to genotyping error; the average error rate found here was 5.85% (Table 2) and, therefore, was

Table 3. Morphological variables and mean (±SE) measurements for the comparison of the four bean leaf beetle color morphs

	Mean ± SE[a]			
Variables (mm)	G+S[b] ($n = 43$)	G-S[b] ($n = 40$)	R+S[b] ($n = 33$)	R-S[b] ($n = 23$)
Weight (g)	0.015 ± 0.001ab	0.013 ± 0.001b	0.018 ± 0.001a	0.011 ± 0.002b
Head size	1.16 ± 0.01	1.15 ± 0.01	1.16 ± 0.01	1.14 ± 0.01
Body length	5.41 ± 0.03b	5.27 ± 0.04b	5.53 ± 0.04a	5.25 ± 0.05b
Elytra length	4.15 ± 0.02a	3.93 ± 0.02b	4.20 ± 0.03a	4.01 ± 0.03b
Elytra width	1.71 ± 0.01	1.70 ± 0.01	1.73 ± 0.01	1.69 ± 0.02
Scutellum length	1.12 ± 0.01	1.09 ± 0.01	1.08 ± 0.02	1.11 ± 0.02
Abdomen length	3.06 ± 0.02	3.07 ± 0.02	3.11 ± 0.02	3.10 ± 0.03
Antennae length	3.25 ± 0.02	3.21 ± 0.03	3.27 ± 0.02	3.20 ± 0.02
Tibia length	1.90 ± 0.01	1.87 ± 0.02	1.88 ± 0.02	1.90 ± 0.02
Femur length	2.00 ± 0.01	1.97 ± 0.01	1.99 ± 0.02	1.96 ± 0.02
Pronotum length	0.99 ± 0.01	0.98 ± 0.01	1.00 ± 0.01	0.98 ± 0.01
Pronotum width	1.61 ± 0.01	1.60 ± 0.01	1.62 ± 0.01	1.59 ± 0.01

[a]Means within a row followed by different letters were significantly different ($P \leq 0.05$; ANOVA; Least Significant Difference).

[b]G+S = green adult beetles with black spots on elytra, G-S = green adult beetles with no black spots on elytra, R+S = red adult beetles with black spots on elytra), R-S = red adult beetles with no black spots on elytra.

Table 4. Results from Canonical discriminant analysis (SAS Institute 2006) used with the morphological measurements on bean leaf beetle color morphs

Canonical variable	Canonical correlation	Adjusted canonical correlation	Eigen value	Proportion	Cumulative	F value (P value)
1	0.304109	0.25934	3.9897	0.6934	0.6934	15.2 (<0.001)
2	0.313505	0.25732	1.6300	0.2833	0.9767	8.3 (<0.001)
3			0.1339	0.0233	1	1.7 (0.10)

within the acceptable level to ensure at least 90% reproducibility. About 94.15% of the genetic variability was accounted for, using bootstrap analysis in BOOD-P software ver. 3.1, indicating that there was a sufficient number of markers for further robust analysis (Coelho 2001). The number of polymorphic loci ranged between 140 and 164 with an average of 143.76 loci (Table 6). More than 80% of the 175 loci observed were polymorphic: average polymorphism was 82.1% and ranged from 80% to 93.7% (Table 6). Fragment length ranged from 50–400 bp in each primer combination. Average gene flow (N_m) among

Fig. 2. Plot of the canonical variables 1 and 2 of four bean leaf beetle color morphs from Pemiscot County, MO. 1 = Green with spots; 2 = Green without spots; 3 = Red with spots; 4 = Red without spots.

the four BLB color morphs was 3.82 and Nei's coefficient of gene variation (G_{ST}) was moderate (0.1156) among the four color morph samples (Table 6). Heterozygosity (H_S) within BLB color morphs ranged from 0.348 to 0.423 and overall heterozygosity (H_T) was 0.397 (Table 6).

Analyses of the potential structure of the samples (Fig. 3) indicated that: 1) G-S BLB were divergent from the other phenotypes (Fig. 3B and C); 2) G+S BLB may be diverging from the other phenotypes (Fig. 3C); and 3) R+S and R-S BLB were not diverging from each other. Results support that three populations (K) are developing (Mean ± SD of LnP(K) = –6650.11 ± 9.27, Delta K = 41.1 (for K = 3) compared to LnP(K) = –7342.83 ± 1.89, Delta K = 0.00 (for K = 1); LnP(K) = –7013.36 ± 36.09, Delta K = 0.90 (for K = 2) and LnP(K) = –6667.87 ± 1112.43, Delta K = 0.10 (for K = 4).

AMOVA, hypothesizing three BLB populations (K = 3) based on results from STRUCTURE, showed that 82.62% of the total variation was within the color morphs of *C. trifurcata*, 12.64% of the total genetic variation was among groups of color morphs and 4.74% of total genetic variation was among color morphs; the F_{ST} of 0.174 indicates moderate to high genetic differentiation (Table 7). AMOVA calculated for the BLB representing one population showed that 84.04% of the total variation was from within the color morphs of *C. trifurcata* and 15.97% of the total genetic variation was from among color morphs; the F_{ST} was moderate to high at 0.159 (data not shown). The dendrogram for these data modestly supported relatedness (74% bootstrap support for the BLB branch) among the four color morphs similarly to and supporting of results from STRUCTURE (Fig. 4).
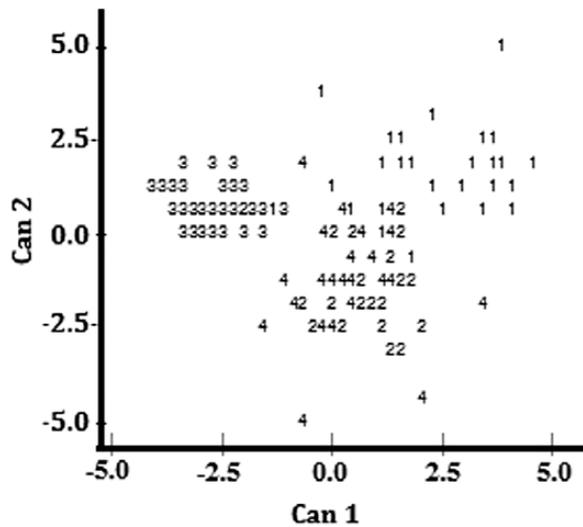
Discussion

Except for the obvious color difference, analyses of morphological characters showed that these BLB color morphs are generally similar

Table 5. Canonical structure and standardized canonical coefficients of canonical variables 1 (Can1) and 2 (Can 2) for morphological measurements on bean leaf beetle color morphs

Variable	Pooled within canonical structure		Pooled within-class standardized canonical coefficients	
	Can 1	Can 2	Can 1	Can 2
Weight	0.162137	0.271978	0.277355	0.23652
Body length	0.203074	0.344169	0.271995	–0.04973
Head width	–0.200912	0.503752	–0.38350	0.37180
Scutellum length	–0.141496	0.032343	–0.39387	–0.26737
Pronotum length	0.599914	0.078336	0.734047	–0.08939
Pronotum width	–0.098524	0.555989	–0.02995	0.25443
Elytra length	0.151265	0.661698	0.018214	0.56472
Elytra width	0.168595	0.499501	0.298572	0.28117
Femur length	–0.363390	0.336532	–0.39370	0.20046
Tibia length	0.249865	0.253615	–0.17966	0.07445
Abdomen length	0.104168	–0.074302	–0.05800	–0.54377
Antennae length	–0.356031	–0.039952	–0.21514	0.03005

Table 6. Genetic diversity estimates for the four color morph samples of bean leaf beetle from Pemiscot-MO: Number of polymorphic loci, percent loci polymorphism, heterozygosity (genetic variation) for a single color morph (H_S), heterozygosity for all the BLB color morph samples (H_T), gene flow among color morph samples (N_m), and genetic variation between color morph samples (G_{ST})

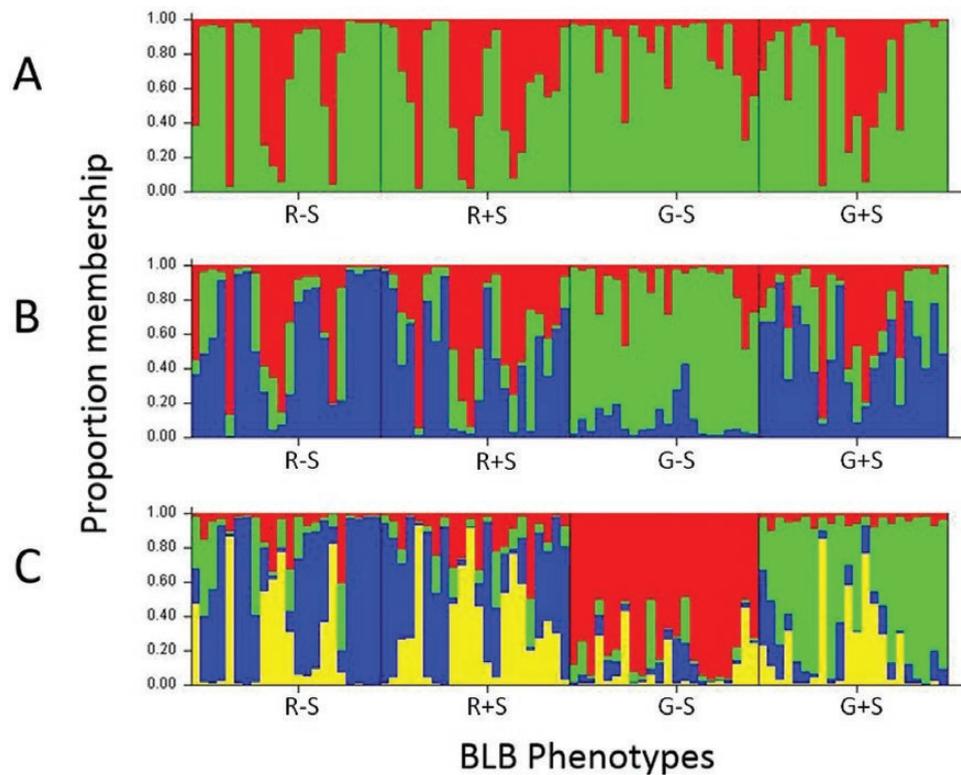| Color samples	No. of polymorphic loci	% Polymorphism	H_S	H_T	G_{ST}	N_m
G+S	163	93.14	0.4055 ± 0.1334			
G-S	140	80.00	0.3484 ± 0.1855			
R+S	162	92.57	0.4229 ± 0.1348			
R-S	164	93.71	0.4125 ± 0.1282			
Average	143.76	82.1488	0.4493 ± 0.0046	0.3973	0.1156	3.8244

Fig. 3. Graphic results from structure analyses exploring if samples of four phenotypes of bean leaf beetles (BLB) represent two populations (A), three populations (B) or four populations (C): the green without spots (G-S) BLB display considerable genetic divergence from the other phenotypes (panels B and C); the green with spots (G+S) may also be diverging (panel C); divergence is not detected within the two red (R-S and R+S) BLB morphs.

Table 7. Analysis of molecular variance for four different color morphs of bean leaf beetle sampled from Pemiscot, MO, based on results from the program STRUCTURE, with three groups; red (with and without spots), green with spots, and green without spots

Source of variation, $K = 3$	df	Sum of squares	Variance components	Percentage of variation
Among groups	2	245.39	3.322 Va	12.64
Among color samples	1	41.66	1.246 Vb	4.74
Within color samples	68	1476.70	21.716 Vc	82.62
Total	71	1763.75	26.284	
Fixation indices[a]				
F_{ST}	0.174	$P \leq 0.0000$		
F_{SC}	0.054	$P \geq 0.0010$		
F_{CT}	0.126	$P \geq 0.1584$		

[a]F_{ST}, calculated by permuting haplotypes among populations among groups, indicates the variance among subpopulations relative to total variance of the population; F_{SC}, calculated by permuting haplotypes within populations among groups, indicates the variance among subpopulations within groups; F_{CT}, calculated by permuting populations among groups, indicates the variance among groups relative to the total variance of the population.

with the exception that the R+S morphs were statistically heavier and longer; elytra also were longer in both spotted color morphs. It should also be noted here that the shape of the R+S and G+S individuals was observed to be dorsally concaved (data not shown), as compared to the G-S and the R-S, which could have affected some parameter measurements for these individuals.

Canonical discriminant analysis allowed an understanding of BLB color morph differences by considering the total variation between parameters jointly, which is biologically accepted and relevant, because many parameters measured may be naturally correlated (Sneath and Sokal 1973). The plot of the two canonical variables suggested that two of the four BLB color morphs (G+S and R+S) were nearly distinguishable with respect to canonical variable 1. The morphological variables that contributed most to canonical variable 1 were head width, pronotum length and width,

and femur length; body weight and length as well as elytra length, the only differences detected through ANOVA, were not included. This emphasizes the value of inclusion of multiple characteristics that are analyzed with dimension reduction techniques. By contrast, the samples of the two color morphs of BLB without spots were quite intermixed on both canonical variables. Although much more information is needed before conclusions may be made, such as the phenology and microhabitat preferences, these results suggest that that the G+S and R+S BLB may be diverging. Yet the results do not support rejection of the hypothesis that these four BLB color morphs are not morphometrically different.

Results from STRUCTURE analyses (indicating three populations) do not support the hypothesis that these four BLB color morphs are not genetically differentiated but support the conclusion of possible divergence between the BLB phenotypes even though all
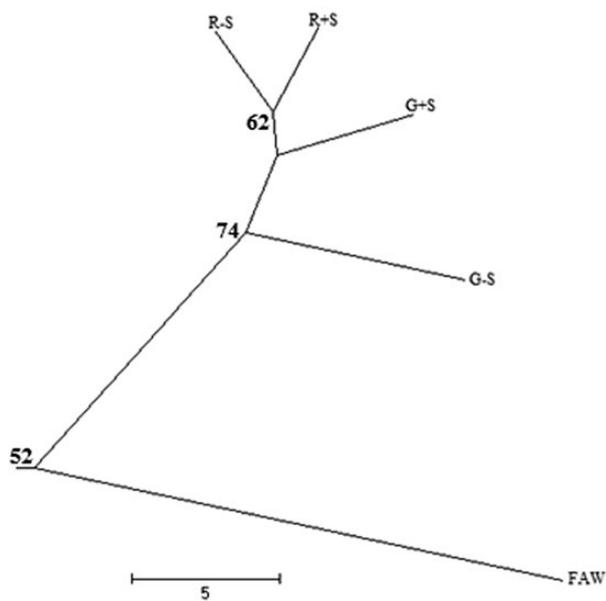
Fig. 4. UPGMA dendrogram illustrating the relationships among the four bean leaf beetle color morphs sampled from Pemiscot County, MO; fall armyworm (FAW) was the out-group. Bootstrap values are given at each of the forks (>50%, 1,000 replicates). R-S, Red without spots; R+S, Red with spots; G-S, Green without spots; G+S, Green with spots.

samples were collected from one site and on the same day. However, the indication that the BLB phenotype of G-S may be diverging contrasted with results from discriminant analysis indicating that G-S and R-S were closely related. There also was some indication from STRUCTURE results that the G+S phenotype is diverging; this does coincide with the discriminant analysis. The dendrogram helped to diagrammatically visualize the representation of the overall similarity or genetic relationships among a group of taxonomic units and further supports the suggestion that there is some genetic distinctiveness among the red (form a separate cluster) versus green (occupy two separate branches) BLB. Interestingly, the G+S BLB occupy a branch near the red BLB, further supporting the results from STRUCTURE.

The moderate percentage of variation among groups detected in the AMOVA calculations using three populations along with the consistent, moderately high F_{ST} support the results from the discriminant analysis suggesting some divergence. The differing outcomes with discriminant analysis (indicating that BLB without spots are similar while the BLB with spots are diverging along different paths) and STRUCTURE analysis (indicating the red BLB are similar and the green BLB may be diverging along different paths) emphasize that much more investigation is required. Future field studies should include phenology and microhabitat preferences. Laboratory studies on fitness, mating preferences, determining if one female produces offspring of different color forms, etc. should be considered and, as possible, future work should include morphological and genetic results.

The average value for gene flow (N_m) among the four beetle color morphs implies a level of interbreeding among these beetle color morphs. Nei's coefficient of gene variation (G_{ST}) was also moderate, implying modest genetic differentiation among the four color morph samples (Hartl and Clark 1997, Clark et al. 2007). The values of H_S and H_T, mean heterozygosity within subpopulations and in the entire population as sampled, respectively, further supports the AMOVA results of moderate variation within individuals in a BLB color morph sample.

Although the results indicate a moderate to high amount of genetic differentiation among the samples of four BLB color morphs, the level of gene flow recorded in this study, as well as in a recent, larger study (Tiroesele et al. 2014), indicates that there is still significant interbreeding. Patterns of geographic variation in phenotype or genotype have been found to provide evidence for natural selection (Endler 1977, Craig et al. 2007). But in this current study it is not clear what the source or function of variation in color is because the color morphs were obtained in the same geographic area (a single field), feeding on the same food (soybeans) and thus subjected to the same macro-environmental conditions. But there may be micro-environmental differences between these color morphs; future studies are needed to clarify this.

Generally, results presented here support the conclusion that these BLB represent an interbreeding population showing phenotypic differences in color that are yet to be explained. Yet, there exist significant support for some weak/incomplete population structure. This study provided baseline information which helps in understanding population structure, patterns of gene flow and morphological characters of BLB. Separating the confounding effects of phenotypic variation from relevant genetic differences will require laboratory studies on mating preferences and fitness of the BLB color morphs and more extensive field sampling to determine potential differences in phenology and preferred microhabitats through study of various BLB phenotypes from multiple, distinct, geographic areas over multiple sampling events.

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