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Field Introgression of *Diabrotica barberi* and *Diabrotica longicornis* (Coleoptera: Chrysomelidae) Based on Genetic and Morphological Characters

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Abstract

Diabrotica barberi Smith & Lawrence and *Diabrotica longicornis* (Say) (Coleoptera: Chrysomelidae) are considered to be sister species, and it has been proposed that the two species may hybridize under field conditions. The objective of this study was to examine genetic and morphological characters of *D. barberi* and *D. longicornis* for evidence of field introgression. Both species were collected from sympatric and allopatric areas. Amplified fragment length polymorphisms and morphological characters (color and head capsule width) were used to examine variation within and among populations of *D. barberi* and *D. longicornis*. Relatively little of the overall genetic variation was explained by the putative species designation, and most of the genetic variation, both between and within species, was found within populations. In addition, genetic differences were not correlated with geographic location. Beetle color did differ significantly between putative species, with the darkest individuals occurring at the most distal portions of the ranges. Head capsule width varied significantly among populations, but not among species. Both genetic and morphological data support the hypothesis that introgression is occurring between species in the area of sympatry.

Keywords: hybridization, northern corn rootworm, amplified fragment length polymorphism

Diabrotica barberi Smith & Lawrence, the northern corn rootworm, and *Diabrotica longicornis* (Say) (Coleoptera: Chrysomelidae) are closely related chrysomelid beetles and are considered to be sister species. The two species

are very similar morphologically, in terms of color and size (Krysan et al. 1983, Golden 1990), male genitalia, female spermathecae (Krysan and Smith 1987), and egg chorion sculpturing (Krysan 1987). Allozyme (McDonald et al. 1982, Krysan et al. 1989) and molecular (Clark et al. 2001a,b) data also have supported a close relationship between *D. barberi* and *D. longicornis*. Both taxa were originally considered to be the same species, *D. longicornis* (Say 1824, Webster 1913). Two subspecies, i.e., *D. l. longicornis* and *D. l. barberi,* were subsequently named by Smith and Lawrence (1967) and later were elevated to species rank (Krysan et al. 1983).

Despite their similarities, the two species exhibit some distinct differences. The range of *D. barberi* and *D. longicornis* overlap primarily in the eastern half of Nebraska and Kansas (Krysan et al. 1983), but the range of *D. barberi* extends to the east coast and north to Ontario and Quebec, Canada, whereas the range of *D. longicornis* extends west and south to Arizona and Chihuahua, Mexico (Krysan et al. 1983). *D. barberi* and *D. longicornis* also seem to differ in habitat preference. *D. barberi* larvae and adults commonly occur in corn, *Zea mays* L., and the larvae are an important pest of corn (Krysan et al. 1983). However, *D. barberi* larvae can survive on multiple prairie grass species in the laboratory, which suggests that there may be some production of adults outside of corn (Oyediran et al. 2008). *D. longicornis* adults are found infrequently in cornfields (Krysan et al. 1983, Krysan and Smith 1987). The larval host of *D. longicornis* is unknown but most likely includes native prairie grasses (Krysan and Smith 1987), although they can use corn as a larval host in the laboratory (Krysan et al. 1983, Golden and Meinke 1991). *D. barberi* and *D. longicornis* also differ in pheromone responses. Each species responds to a different stereoisomer of the sex pheromone 8-methyl- 2-decyl propanoate (Guss et al. 1985, Krysan et al. 1986). *D. longicornis* has darker tibiae, tarsi, clypeus, and antennae than *D. barberi* (Krysan et al. 1983), although these structures are darker in both species in the more distal parts of their ranges (Krysan et al. 1983, Krysan and Smith 1987).

D. barberi and *D. longicornis* have the potential to hybridize under field conditions, although no previous study has documented the occurrence of field hybridization. The two species can hybridize under laboratory conditions, but the cross is unidirectional; crosses of a *D. longicornis* female and *D. barberi* male are much more viable than the opposite cross (Krysan et al. 1983, Golden 1990, Campbell and Meinke 2010). *D. barberi* and *D. longicornis* from allopatric populations have distinct cuticular hydrocarbon profiles (Golden et al. 1992), but individuals from some sympatric populations have intermediate cuticular hydrocarbon scores, similar to laboratory-created hybrids (Golden 1990). Allozyme allele frequencies from *D. barberi* populations in the area of sympatry are most different from other *D. barberi* populations, which may have resulted from range overlap with *D. longicornis* (McDonald et al. 1985).

The extent to which *D. barberi* and *D. longicornis* interact in the field is unknown. Krysan et al. (1983) hypothesized that, because of the differences in habitat, the area of sympatry was simply an area where the ranges of the two species overlapped. However, given the evidence that hybridization may occur under field conditions, the area of sympatry could be a hybrid zone, where genetically distinct populations can produce some offspring of mixed ancestry (Barton and Hewitt 1985). Hybridization could potentially impact the evolution of the two taxa and the pest status of *D. barberi* in the sympatric area. Therefore, as part of a larger effort to evaluate the pest potential of *D. barberi* in Nebraska and to more clearly understand the evolutionary relationship of *D. barberi* and *D. longicornis,* we conducted this study to examine field populations of both species for evidence of introgression under natural conditions. This objective was accomplished by analyzing genetic (amplified fragment length polymorphism [AFLP]) and morphological (color and head capsule width) data sets that included allopatric and sympatric populations of each species.

Materials and Methods

Collections of Genetic Material

Collections of *D. barberi* and *D. longicornis* were made in 1998–1999 from 22 locations in Illinois, Iowa, Minnesota, South Dakota, Nebraska, and Kansas; putative species was identified based on the morphological criteria provided by Krysan et al. (1983). Populations were defined by separate collection locations and putative species. Populations in the eastern halves of Nebraska and Kansas were considered sympatric, based on the work of Krysan et al. (1983), Golden (1990), and previous observations by the authors. Collections outside eastern Nebraska and Kansas were considered to be allopatric. At two locations (Lancaster Co. and Webster Co., NE), individuals identified to both species were collected. In total, 212 individuals (36 *D. longicornis,* 176 *D. barberi*) were collected from various hosts (Table 1). Beetles were preserved in 95% ethanol or frozen at –80°C.

Molecular Genetic Techniques

DNA was extracted from the thorax and legs of individual beetles by using a modification of the hexadecyltrimethylammonium bromide extraction protocol of Black and DuTeau (1997). The AFLP-polymerase chain reaction (PCR) procedure followed Vos et al. (1995), with some modification. Infrared-labeled (IRD-700, LI-COR Biosciences, Lincoln, NE) EcoRI primers were used in the polymerase chain reaction. Restriction digestion and adapter-ligation were conducted by incubating the DNA with restriction endonucleases EcoRI and MseI (New England Biolabs, Ipswich, MA). Restriction digestion

involved incubation of 7 μl of genomic DNA (_160 ng) for 2.5 h at 37°C, in a total volume of 12.5 μl. This mix included 1.25 μl of 10× One-Phor-All buffer (GE Healthcare Biosciences Corp., Piscataway, NJ), 0.125 μl of 10 U/μl MseI enzyme (1.25 U per reaction), 0.0625μl of 20U/μl of EcoRI enzyme (1.25 U per reaction), 0.125 μl of 10 mg/ml bovine serum albumin (New England Biolabs), and autoclaved nanopure water. Adapter-ligation involved incubating the restriction fragments for 11.5 h at 25°C with 5 μl of ligation mixture (0.15 μl of T4 DNA ligase, 0.5 μl of 10_T4 ligase buffer [New England Biolabs], 0.5 μl of 5 pmol/μl EcoRI adapter, 0.5 μl of 5 pmol/μl MseI adapter [Eurofins MWG Operon Technologies, Huntsville, AL], and 3.35 μl of water). Each ligation mixture was diluted with 1:10 with water. Preselective and selective primers were based on primer core sequences EcoRI 5′-GACTGCGTACCAATTC-3′ and MseI 5′-GATGAGTCCTGAGTAA-3′. Standard preselective PCR conditions were used (Clark et al. 2007). Preselective amplifications were run with 1.25 μl of diluted restriction-ligation product, 10 μl of preamp primer mix II (Eurofins MWG Operon Technologies), and 1.25 μl of 10_ PCR buffer containing 15 mMMgCl2 and 0.25 μl of 5 U/μl Ampli*Taq*DNA polymerase (1.25 U per reaction) (Applied Biosystems, Foster City, CA). The oligonucleotide primers in the preamp primer mix II were complementary to the adapter/restriction site, with the MseI primer containing one selective nucleotide (cytosine) and the EcoRI primer containing no selective nucleotide. Each preamplification product was diluted 1:20 with water.

Selective PCR amplifications were run with 2.0 μl of preamplified template, 4.74 μl of water, 1.2 μl of 10× PCR buffer containing 15 mM MgCl2 and 0.06 μl of 5 U/μl Ampli*Taq* polymerase (Applied Biosystems), 2.0 μl of MseI primer (Eurofins MWG Operon Technologies), and 0.5 μl of 1.0 pmol/μl IRD-700–labeled EcoRI primer (LI-COR Biosciences). EcoRI-selective and MseI-selective amplification primers had three extra nucleotides at the 3_ ends; three EcoRI and MseI primer pairs were used for amplification (EcoRI-AGG and MseI-CAT, EcoRI-ACT and MseI-CTA, and EcoRI-ACA and MseI-CTC). Standard selective PCR conditions were used, and samples were denatured as describe previously (Clark et al. 2007). One microliter of the sample was electrophoresed through KB^{plus} 6.5% ready-to-use gel matrix (LI-COR Biosciences), and the infrared fluorescent bands were detected by a Read IR 4200 sequencer (LI-COR Biosciences). AFLP bands were evaluated using an IRD-700–labeled 50–700-bp marker as a reference and scored using SAGA Generation 2 software, version 3.2 (LICOR Biosciences). The data set consisted of 1's and 0's (presence and absence, respectively) as analyzed from each AFLP gel; 99 loci were scored.

Population Structure Analyses

Hierarchical analysis of molecular variance (AMOVA) was conducted to assess the genetic structure and genetic variance between the two species,

between populations within species, and within populations (Excoffier et al. 1992). AMOVA was conducted in Arlequin, version 3.1 (Excoffier et al. 2005) as haplotypic, codominant data, with 10,000 permutations. Arlequin also was used to obtain expected heterozygosity estimates and pairwise differences between populations. The number of migrants per generation (*Nm*) was calculated as follows:

$$Nm = \tfrac{1}{2}[(1/F_{ST}) - 1]$$

where the one-half multiplier adjusts the calculation for haplotypic data (McDermott and McDonald 1993). Mantel tests, examining the correlation of genetic divergence (F_{ST}) and geographic distance (to the nearest 10 km) between populations, were also conducted in Arlequin (Mantel 1967, Smouse et al. 1986) with 10,000 permutations. Average gene diversity, the probability that two randomly chosen haplotypes are different in the sample (Nei 1987), and the proportion of polymorphic loci for each population, also were calculated using Arlequin. Population differentiation was tested in Arlequin with pairwise multilocus F_{ST} estimates; this procedure tested the null hypothesis of random distribution of individuals between pairs of populations (panmixia) (Goudet et al. 1996, Excoffier et al. 2005). The population differentiation tested was conducted with a 100,000-step Markov chain, with significant differences at $P<0.05$. Pairwise differences for populations from Arlequin were used to construct an unrooted neighbor-joining tree in PAUP* version b10 (Swofford 2001). The neighbor-joining tree was visualized using FigTree, version 1.3.1 (Rambaut 2006). AMOVA, calculations of *Nm,* and Mantel tests also were conducted for each of the putative species.

The program Structure (Pritchard 2007) was used to assign genotypes to *K* population clusters with a Markov chain Monte Carlo (MCMC) algorithm (Pritchard et al. 2000). The program uses a model that attempts to find population groups by minimizing linkage disequilibrium within populations (Pritchard et al. 2000). Structure was run with an admixture model and correlated allele frequencies between populations (Falush et al. 2003), with modifications for dominant markers (Falush et al. 2007). Structure was used to test values of *K* from 1 to 15, with ten replications at each value of *K*. Each replication at each level of *K* was run with a burn-in of 10,000 and a run length of 10,000 replications; longer burn-in or MCMC lengths did not change the results. The number of *K* populations was determined by the value of *K* with the highest log probability scores.

Field Collections for Morphological Data

Collections of 478 *D. barberi* and *D. longicornis* were made from 33 counties (50 sites) in nine states (Table 2). Individuals were identified to putative species as described above. At two locations (Lincoln Co., NE; Riley Co., KS),

individuals identified to both species were treated as separate populations. Most of the beetles were collected during 2004–2006 (Table 2) and were stored in 70 or 95% ethanol.

Morphological Characters

Field-collected beetles were color-scored to examine the variation of various morphological characters between species and among populations (Krysan et al. 1983, Krysan and Smith 1987). The color of antennae, clypeus, episternites, scutellum, and tibiae were scored on a 1–4 scale, with 1 representing yellow/testaceous (1.5 representing dark yellow), 2 representing light brown, 3 representing dark brown, and 4 representing piceous (Krysan et al. 1983). All color-scoring was conducted under a dissecting microscope (Wild Heerbrugg) at 12× with a bright fiber optic light to minimize differences due to lighting.

Head capsule widths were measured for all beetles, and adult gender was determined using the method of White (1977). Head capsule widths of lab-reared beetles have previously been shown to vary between *D. barberi* and *D. longicornis* (Campbell and Meinke 2010). Head capsule widths were measured at the widest point of the head, at the outside edge of the eyes, with a dissecting microscope (Wild Heerbrugg, Heerbrugg, Switzerland) using an ocular lens of 20× and an objective lens at 12×.

Morphological Data Analysis

All morphological data were analyzed in SAS, version 9.1.3 (SAS Institute 2003). Color scores for each individual morphological character (antennae, clypeus, scutellum, three episternites, and tibiae) were summed for population analysis. The effect of population on total color was analyzed with one-way analysis of variance (ANOVA) using PROC MIXED. The effects of population and gender on head capsule width also were analyzed with a factorial ANOVA. A significance level of $P<0.05$ was used in all analyses. For ANOVA, means were separated using Fisher's protected least significant difference (LSD) test. Means and SEs were obtained from the LSMEANS statement in the PROC MIXED procedure (Littell et al. 2006).

Principal component (PC) analysis was conducted with PROC PRINCOMP for head capsule width and for color of the individual morphological characters. Components with eigenvalues greater than the average eigenvalue were considered to be important for each eigenvector (SAS Institute 2003). Linear discriminant analysis of color scores and head capsule width was conducted using PROC DISCRIM. The discriminant analysis classified the field-collected beetles into assigned groups on the basis of color score and head capsule width. The species designation was calibrated with beetles from allopatric populations, which was then used to discriminate beetles from sympatric populations. Allopatric populations included 119 *D. barberi* and 57 *D. longicornis* (Table 2).

Results

Genetic Data

Ninety-nine total AFLP loci were scored for each of the 212 individuals, and each of the 212 individuals had a unique haplotype. Genetic diversity within populations was generally high, with a large proportion of polymorphic loci in each population (*D. barberi* population means, 0.48–0.92; *D. longicornis* population means, 0.43–0.64), and 67.9 ± 11.6 (SD) mean polymorphic loci per population (Table 3). Mean expected heterozygosity of both species was 0.29 ± 0.22, and total heterozygosity was 0.36 ± 0.15 (mean ± SD). The Scott, KS, population had the lowest overall average gene diversity (0.23 ± 0.12), whereas the Martin, MN, population had the highest (0.36 ± 0.20).

Relatively little (5.33%) of the overall genetic variation could be explained by putative species designation (Table 3). Differences among populations within species accounted for 16.35% of the total variation, and 78.33% of the variation occurred within populations (Table 3). None of the 276 pairwise tests for genetic differentiation of populations, with the null hypothesis of panmixia, were significantly different (P=1.00 for each pairwise test). The overall level of genetic differentiation was moderate, with an F_{ST} of 0.22. Migrants per generation between populations, *Nm,* for *D. barberi* and *D. longicornis* combined was calculated as 1.81. Genetic differentiation of the populations showed no correlation with geographic distance (Mantel test, r^2 = 0.01, P = 0.14, mean geographic distance: 440.1 km; range, 0–1,160 km; mean pairwise F_{ST}: 0.17; range, –0.02–0.38).

Within putative *D. barberi* only, differences among populations accounted for 18.26% of the total variation, and 81.74% of the variation was explained by variation within populations (Table 3). *Nm* for *D. barberi* was calculated as 2.24 migrants per generation between populations. Genetic differentiation of the populations showed no correlation with geographic distance (Mantel test, r^2 = 0.00006, P = 0.43; mean geographic distance: 423.2 km; range, 10–890 km; mean pairwise F_{ST}: 0.17; range, –0.02–0.38). Within *D. longicornis* only, differences among populations accounted for only 8.48% of the total variation, and 91.52% of the variation was explained by variation within populations (Table 3). *Nm* for *D. longicornis* was calculated as 5.40 migrants per generation among populations. Genetic differentiation of the populations showed no correlation with geographic distance (Mantel test, r^2 = 0.009, P = 0.59; mean geographic distance: 256.0 km; range, 50–440 km; mean pairwise F_{ST}: 0.07; range, –0.02–0.19).

The results obtained from Structure further confirmed a lack of correlation between genetic differences and geography (Fig. 1). Individuals from the 24 populations were assigned to six genetic clusters, with most putative *D. barberi* assigned to five clusters, whereas most putative *D. longicornis* were assigned to one cluster. However, putative *D. barberi* from Jefferson

County, NE, which is in the area of sympatry, and from Brookings 2, SD, which is outside the area of sympatry, primarily grouped with *D. longicornis*. Even within putative *D. barberi,* populations grouped to the same genetic cluster often were geographically distant, as in the case of *D. barberi* from Champaign and Peoria, IL, and Lancaster, NE.

The neighbor-joining tree (Fig. 2) showed a similar pattern to the results from Structure. Putative *D. longicornis* formed a single cluster, but *D. barberi* populations from Jefferson and Brookings 2 grouped again with *D. longicornis*.

Morphological Data

Total color score varied significantly among *D. barberi* and *D. longicornis* populations (Table 4). Most putative *D. barberi* did not have significantly different mean color scores among populations. Mean color scores of putative *D. barberi* and *D. longicornis* exhibited little overlap; mean total color scores for *D. barberi* ranged between 10.9 and 12.1, excluding the Tompkins Co. population. Mean total color of *D. barberi* from Tompkins Co., NY, was significantly darker than all other *D. barberi* populations (Table 4). Excluding the one allopatric collection of *D. longicornis* from Scott Co., KS, putative *D. longicornis* had color scores ranging between 16.3 and 19.8 (Table 4). Although mean color scores for populations did not overlap much between putative species, the ranges for individual color scores did. *D. barberi* individuals had total color scores ranging from 9.0 to 17.0 (mean = 11.8 0.1; N = 369), and *D. longicornis* individuals had total color scores ranging from 13.0 to 20.5 (mean = 17.0 ± 0.2; N = 109). Each putative species was darkest at the distal portions of its range (Table 4).

Head capsule width was not significantly affected by putative species (F = 2.55; df = 1, 476; P = 0.11). However, head capsule widths did vary significantly among *D. barberi* and *D. longicornis* populations (F = 2.22; df = 32, 427; P = 0.0002) but did not seem to be associated with geographic range (Table 4). Head capsule widths ranged between 0.96 and 1.29mm,with the largest mean head capsule widths in the Santa Cruz Co., AZ, population of *D. longicornis* and the Gage Co., NE, population of *D. barberi* (Table 4). Head capsule width also was significantly affected by gender (F = 8.79; df = 1, 427; P = 0.003), but the interaction of population and gender was not significant (F = 0.67; df = 17, 427; P = 0.84). Male head capsule widths (1.13 ± 0.01 mm; N = 210) were significantly larger than female head capsule widths (1.09 0.01 mm; N = 268).

The principal components analysis resulted in the first two components explaining 66.1% of the data. The first principal component axis explained 51.1% of the variation and was determined by the color score of the antennae, clypeus, scutellum, third episternite, and tibiae (Table 5; Fig. 3). Darker color scores for the various morphological characters resulted in greater PC1

values. The second principal component axis explained 14.9% of the variation and was composed of the head capsule width and the first and second episternites (Table 5; Fig. 3). Larger head capsule widths and lighter color scores for the first and second episternites resulted in greater PC2 values. Although most of putative *D. barberi* and *D. longicornis* fell into two distinct clusters, many individuals had intermediate or overlapping scores (Fig. 3).

Using the calibration of allopatric individuals for individuals in sympatry, the analysis classified 2.4% of putative *D. barberi* as *D. longicornis* and 11.5% of putative *D. longicornis* as *D. barberi* (Table 6). Most beetles initially identified as *D. longicornis* that the discriminant analysis classified as *D. barberi* were from Riley and Ottawa Counties, KS, whereas beetles initially identified as *D. barberi* and classified as *D. longicornis* by the discriminant analysis were collected in Lancaster and Saunders Counties, NE, from cucurbits. The total error rate of species classification was 7.0% (Table 6).

Discussion

Morphological and Genetic Variation

Color for *D. barberi* and *D. longicornis* has been reported to follow a bimodal pattern, with few intermediate individuals (Krysan et al. 1983, Krysan and Smith 1987). In this study, a similar bimodality also was observed between the putative species (Fig. 3; Table 4), but some individuals could not be distinguished solely on the basis of color (Fig. 3; Table 6). Discriminant analysis of color demonstrated that some individuals were misclassified (Table 6). *D. barberi* did exhibit a geographic cline in color (Table 4), as observed by Krysan et al. (1983), but *D. longicornis* did not (Table 4). Color may be correlated with real genetic differences between taxa. Individuals for genetic analysis were assigned to different putative species by color, and these were mostly assigned to the same putative species cluster by Structure (Fig. 1).

Head capsule width has been shown to be different between *D. barberi* and *D. longicornis* reared in the laboratory (Campbell and Meinke 2010), but this result was not found in field-collected beetles (Table 4). Head capsule widths for *D. barberi* and *D. longicornis* are probably influenced by local conditions in larval habitats (Branson and Sutter 1985, Naranjo 1991, Woodson and Jackson 1996, Ellsbury et al. 2005) and most likely vary from year-to-year within populations. However, differences in head capsule width by gender were consistent with laboratory studies, with male beetles having wider head capsules than females (Woodson and Jackson 1996, Campbell and Meinke 2010).

The genetic data presented here do not reliably distinguish *D. barberi* and *D. longicornis,* which is consistent with results from other genetic studies. *D. longicornis* males have been shown to have a unique esterase not found in *D. barberi* males, but no other allozymes could be used to reliably discriminate

the species (McDonald et al. 1982). Clark et al. (2001a) could find no differences between *D. barberi* and *D. longicornis* in restriction fragment length polymorphism profiles of mitochondrial cytochrome oxidase subunit I.

The AMOVA results indicate that there is little distinction between putative species (Table 3), and values within the individual species and among all populations indicate that migration; therefore, gene flow is occurring between populations. The relatively high levels of genetic diversity and within-population variation in *D. barberi* have been documented in other studies. Using seven polymorphic allozyme loci from *D. barberi* collected across the Corn Belt, McDonald et al. (1985) found a similarly high percentage of the total variation to be within populations (90.2% in their study). Krafsur et al. (1993) found greater variation to be among populations (52.2%) than within populations using 27 polymorphic allozyme loci, but sampling for that study was restricted to four counties in northwestern Iowa. Populations in northwest Iowa exhibited even greater rates of migration (12.4 beetles per generation) than found in this study, but that may simply be because the populations sampled by Krafsur et al. (1993) were geographically close, compared with the mean distance of 423 km among sites in this study. Roehrdanz et al. (2003) found that *D. barberi* populations west of central Illinois exhibited more variability in the ITS1 gene and in mitochondrial haplotypes than populations east of central Illinois. In general, our study supports previous work that has documented substantial within-population genetic variation of *D. barberi* in the western Corn Belt (west of central Illinois).

At the population level, *D. barberi* was not strongly differentiated, either morphologically or genetically over the geographic area included in this study. *D. barberi* beetles typically only take short, trivial flights (Naranjo 1990), although they are highly motile and frequently move outside of corn habitats (Cinereski and Chiang 1968, Naranjo and Sawyer 1988). McDonald et al. (1985) and Krafsur et al. (1993) have suggested that *D. barberi* may consist of partially isolated breeding populations because of limited flight ability. Examinations of aldrin susceptibility in *D. barberi* also indicated that variation in *D. barberi* could be highly localized (Ball and Weekman 1963, Krysan and Sutter 1986). In our study, populations of *D. barberi* exhibited limited isolation. The lack of population differentiation in *D. barberi* could be due to frequent short-range movement by individual beetles, causing large-scale mixing of populations over the landscape.

There was some separation of putative *D. barberi* populations into separate clusters that were not geographically related (Figs. 1 and 2). Roehrdanz et al. (2003) examined variation in ITS1 and mitochondrial DNA and also found differentiation within *D. barberi* unrelated to geography. The neighbor-joining tree and results from Structure produced similar groupings; these groups may reflect real biological differences (Figs. 1 and 2), even when they have no relationship to geography or putative species designation.

Populations and individuals of *D. barberi* that are in proximity geographically may be genetically distant. It is currently unclear why this pattern exists; this warrants additional study in the future.

Within *D. longicornis,* populations also were not strongly differentiated morphologically or genetically (Fig. 1; Tables 4 and 5). Based on cuticular hydrocarbon analysis, Golden (1990) suggested that gene flow might be common among Nebraska populations, which would most likely extend into the Kansas and eastern Colorado *D. longicornis* populations studied here. Populations in the southwestern United States were more isolated based on cuticular hydrocarbon profiles (Golden 1990), although the color scores from the one Arizona population in our study were not significantly different from populations in Nebraska (Table 4). The results of our study strongly support the idea that gene flow occurs among Midwestern populations (Fig. 1; Table 3). Movement of *D. longicornis* among habitats has not been studied. However, our results, in conjunction with Golden (1990), suggest that *D. longicornis* beetles are at least as motile as *D. barberi*.

Introgression

The genetic data presented here indicate that introgression occurs between the two putative species. Putative *D. barberi* do not cluster into one group (Figs. 1 and 2), and, as reported by the population differentiation tests, *D. barberi* and *D. longicornis* together are acting as one panmictic group. This also may help to explain why western populations of *D. barberi,* i.e., those from South Dakota, Nebraska, and Kansas, have exhibited different genetic patterns than eastern populations (McDonald et al. 1985, Roehrdanz et al. 2003). The morphological data also provide support for introgression between species but are less conclusive because they are not directly linked to the genetic data. Although the genetic and morphological data collectively support the hypothesis that introgression is occurring in the area of sympatry, the data do not elucidate much about the nature of the area of sympatry. It is unknown how long the two species have been in contact. It is also unclear whether the two species originated in the Great Plains and ranges diverged away from the current area of sympatry, or if ranges only recently converged. The widespread cultivation of corn in the western Corn Belt may have expanded the range and increased the population density of *D. barberi,* which may have facilitated more contact between the species. Much of the area of sympatry includes areas that are frequently affected by drought (CPC 2009). Ranges of either species may contract in dry years and expand in wetter years, which would then affect the opportunities for introgression. Therefore, the area of sympatry may be a patchy network of contact zones where introgression sporadically occurs.

The unified species concept (de Queiroz 2007) provides a useful framework for analysis of available biological knowledge and evaluation of the

relationship of closely related taxa like *D. barberi* and *D. longicornis*. Under the unified species concept, the only characteristic of a species is a separately evolving metapopulation lineage. Other species concepts serve as lines of evidence (secondary species properties) for recognizing lineage separation (de Queiroz 2007). *D. barberi* and *D. longicornis* exhibit differences in many characteristics, but some differences may not be strong enough to classify the taxa as separately evolving metapopulation lineages.

The evidence for intrinsic reproductive isolation, the traditional biological species concept (Mayr 1963, de Queiroz 2007), is equivocal. Unidirectional hybridization readily occurs under laboratory conditions (McDonald et al. 1982, Golden 1990, Campbell and Meinke 2010), and *D. longicornis* female × *D. barberi* male hybrids and their offspring are at least as fit as the parental species in terms of larval development time, longevity, and fecundity (Campbell and Meinke 2010). This successful cross might be favored by natural selection and potentially contributes much of the gene flow recorded between species in this study. Selection would operate strongly against hybridization in the opposite direction (Krysan et al. 1983, Campbell and Meinke 2010); therefore, the evolutionary effects of hybridization in the area of sympatry would be mediated by the unidirectionality of hybridization.

There is some evidence for mate recognition (Paterson 1985, de Queiroz 2007). Under field conditions in eastern Kansas, *D. barberi* and *D. longicornis* each strongly preferred their own pheromone (Krysan et al. 1983). However, in lab behavioral assays, *D. barberi* and *D. longicornis* males' response to their own pheromones was not as strong (Campbell 2009). Male *D. barberi* also seemed to be more persistent in courting heterospecific and conspecific females, compared with *D. longicornis* males (Campbell 2009).

Under phylogenetic properties (de Queiroz 2007), the available evidence does not support lineage separation. Whereas *D. barberi* and *D. longicornis* do exhibit limited differentiation, they show little genetic separation (Table 3; Figs. 1 and 2). Previous genetic work also revealed no strong genetic divergence between *D. barberi* and *D. longicornis* (McDonald et al. 1982, Clark et al. 2001b).

The level of ecological isolation (de Queiroz 2007) between the two species is not clearly understood. Habitat fidelity of both described species could affect ecological isolation in important ways: ovipositional choices made by females, contact between males and virgin females during the mating period, and host utilization. Female ovipositional selection is critical to larval survival and would determine the host plants available to larvae, because larvae cannot move long distances in the soil (Krysan 1999). *D. barberi* frequently leave corn, but many females return to oviposit in cornfields (Cinereski and Chiang 1968, Naranjo and Sawyer 1988), whereas *D. longicornis* are rarely found in cornfields and have been presumed to oviposit in habitats other than cornfields (Krysan et al. 1983). *D. barberi* and *D. longicornis*

could be diverging if females demonstrate distinct ovipositional choices. Host plant preference or use also could contribute to divergence or development of taxa specific host plant-related strains. Contact between the two species during mating would depend on adult movement among habitats. *D. barberi* and *D. longicornis* beetles often occur in neighboring habitats and can be collected from the same adult hosts when mating could occur (L.J.M., unpublished data, Tables 1 and 2; Golden 1990, Clark et al. 2001b), but relatively little is known about behavior and host use of adult *D. barberi* outside corn and of adult *D. longicornis* in general.

Data are inconclusive when evaluating the evolutionary relationship of *D. barberi* and *D. longicornis*. Field pheromone response coupled with limited habitat preference observations provides evidence that mate recognition and ecological niche differences may partially isolate the taxa. These criteria were major reasons why the taxa were elevated from subspecies to species status although no genetic information was available to the authors when the change in taxonomic status was made (Krysan et al. 1983). Some secondary species properties (i.e., limited intrinsic reproductive isolation, excellent fitness of hybrids, and little genetic divergence) do not provide much support for the hypothesis that the taxa clearly are evolving separately (de Queiroz 2007). Additional work on the ecology of both taxa and examination of the genetic and morphological variation in *D. barberi* and *D. longicornis* throughout their entire ranges within the context of host plant use would help to determine whether the putative species are actually separately evolving metapopulation lineages.

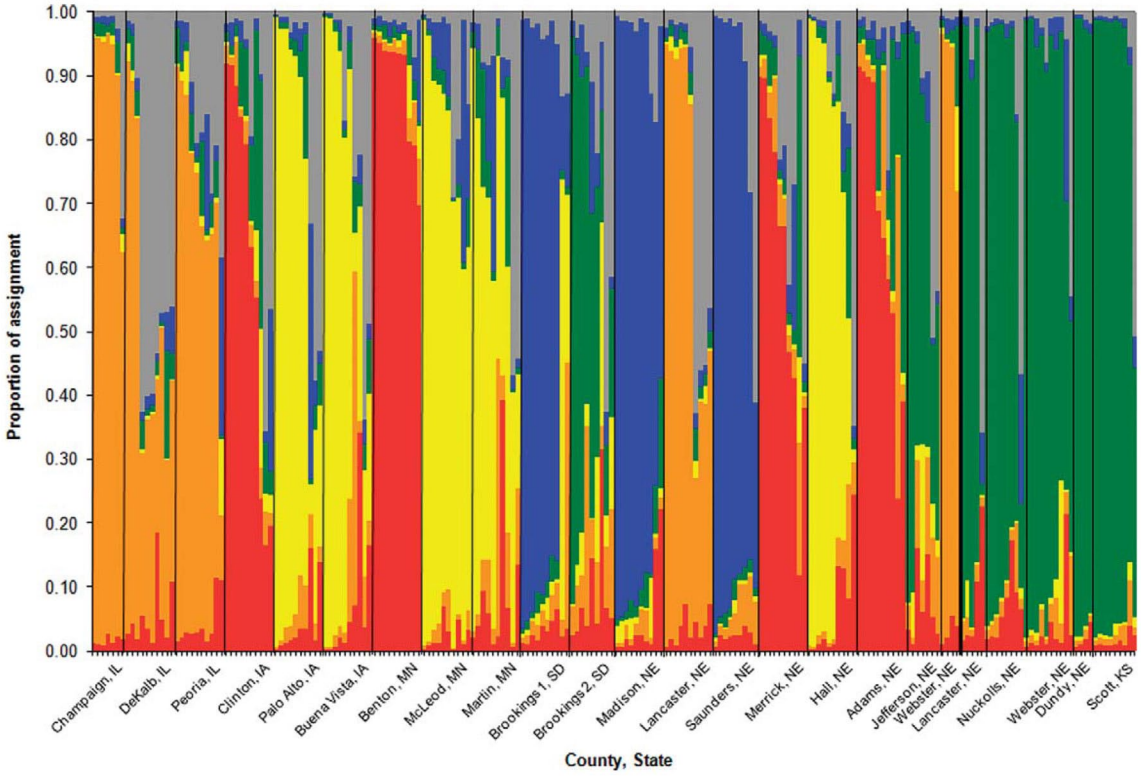

Fig. 1. Population assignment of *D. barberi* and *D. longicornis* to six clusters from Structure. Each color represents a different inferred ancestral population cluster, based on the genetic data. Each vertical bar represents one individual and the individual's estimated proportion of membership in each of the clusters. Black lines separate the geographic populations; populations to the left of the heavy black line represent putative *D. barberi*, and populations to the right of the heavy black line represent putative *D. longicornis*.

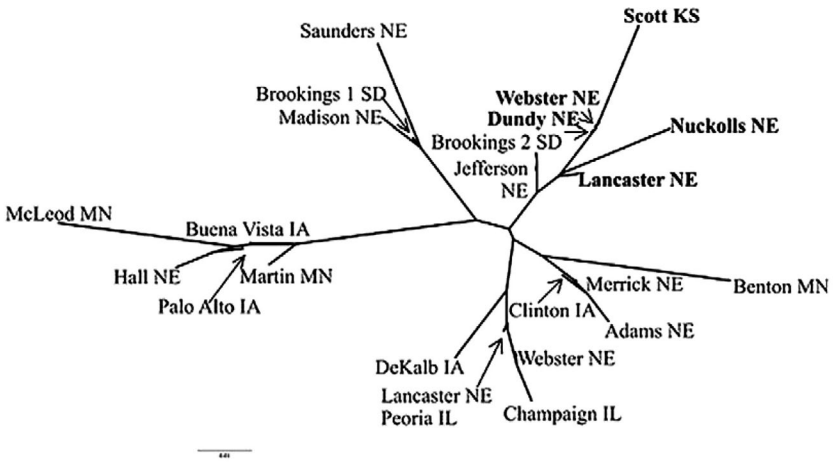

Fig. 2. Relationships between collection locations of *D. barberi* and *D. longicornis*. The tree is an unrooted neighbor-joining tree constructed from pairwise differences. *D. barberi* are marked in standard font; *D. longicornis* are marked in bold. Branch length corresponds to genetic distance.

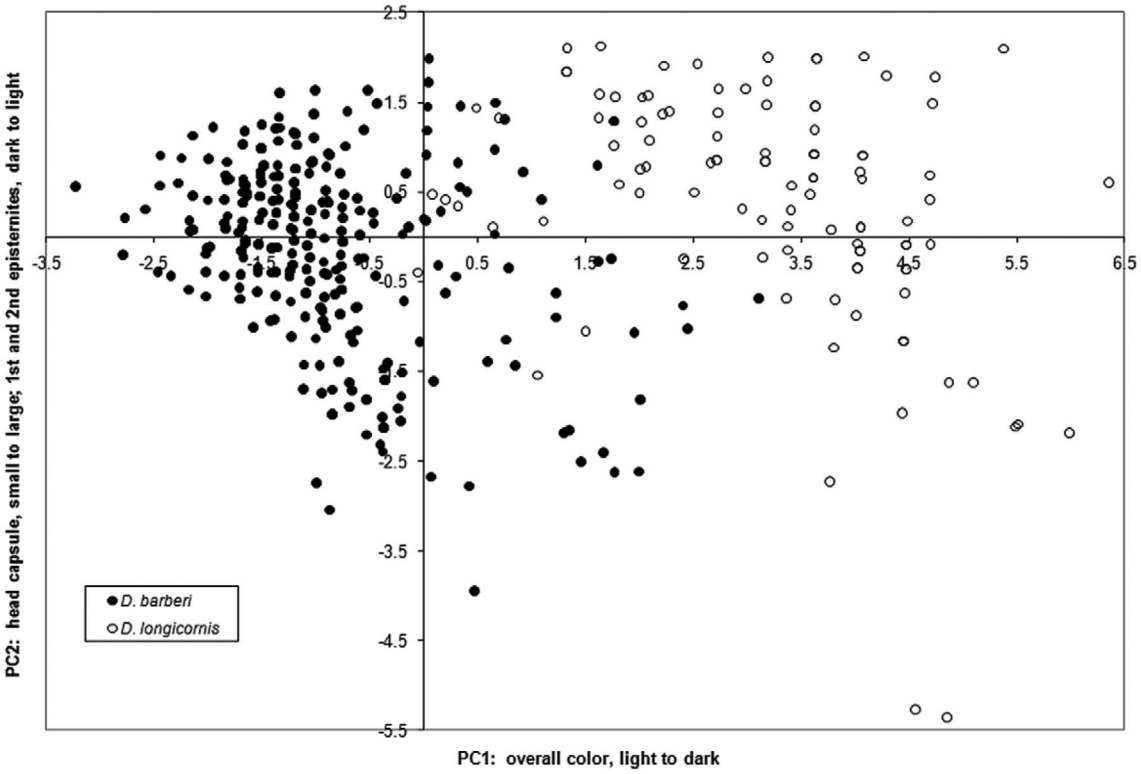

Fig. 3. Principal components analysis plot for *D. barberi* and *D. longicornis* from color-scoring and head capsule width (millimeters) data. Filled circles represent putative *D. barberi*, and open circles represent putative *D. longicornis*. PC1 and PC2 combined explained 66.1% of the total variation. See Table 5 for eigenvectors for principal components.

Table 1. Collection sites, dates, and hosts for *D. barberi* and *D. longicornis* used for genetic analysis

Putative species[a]	County	State	*N*	Date	Host(s)[b]
D. barberi	Champaign	IL	7	8/2/99	Corn
D. barberi	DeKalb	IL	10	8/22/99	Soybean
D. barberi	Peoria	IL	10	8/22/99	Soybean
D. barberi	Clinton	IA	10	7/27/98	Corn
D. barberi	Palo Alto	IA	10	8/31/99	Sunflower
D. barberi	Buena Vista	IA	10	8/31/99	Sunflower
D. barberi	Benton	MN	10	8/30/99	Sunflower
D. barberi	McLeod	MN	10	8/31/99	Sunflower
D. barberi	Martin	MN	10	8/31/99	Sunflower
D. barberi	Brookings	SD	10	9/15/99	Sunflower
D. barberi	Brookings (2)	SD	9	9/14/98	Sunflower
D. barberi	Madison	NE	10	9/16/98	Corn
D. barberi	Lancaster	NE	10	7/14/98, 8/18/98	Lights, amaranth
D. barberi	Saunders	NE	9	7/21/98	Corn
D. barberi	Merrick	NE	10	9/10/99	Sunflower
D. barberi	Hall	NE	10	9/10/99	Sunflower
D. barberi	Adams	NE	10		Corn
D. barberi	Jefferson	NE	7	8/25/99	Corn
D. barberi	Webster	NE	4	7/28/98, 8/25/99	Buffalo gourd
D. longicornis	Lancaster	NE	5	7/14/98	Pumpkin
D. longicornis	Nuckolls	NE	8	7/14/98	Buffalo gourd
D. longicornis	Webster	NE	10	7/28/98	Buffalo gourd
D. longicornis	Dundy	NE	4	8/13/98	Buffalo gourd
D. longicornis	Scott	KS	9	7/26/98	Buffalo gourd

a. Species determined by morphology and collection location.
b. Host species are: sunflower (*Helianthus annuus* L., Asteraceae), corn (*Zea mays* L., Poaceae), soybean (*Glycine max* (L.), Fabaceae), amaranth (*Amaranthus* sp., Amaranthaceae), buffalo gourd (*Cucurbita foetidissima* HBK, Cucurbitaceae), and pumpkin (*Cucurbita pepo* L., Cucurbitaceae).

Table 2. Collection sites, dates, and hosts for *D. barberi* and *D. longicornis* used for morphological analysis

Putative species[a]	*County*	*State*	*Region*[b]	*Latitude (°N)*	*Longitude (°W)*	*N*	*No. sites*	*Date*	*Host(s)*[c]
D. barberi	Tompkins	NY	Allopatry	42.44	76.53	10	1	9/16/05	Corn
D. barberi	Clark	OH	Allopatry	39.87	83.67	26	1	9/7/06	Corn
D. barberi	Lafayette	MO	Allopatry	39.00	93.70	21	1	Summer 2005	Corn
D. barberi	Nodaway	MO	Allopatry	40.44	95.08	20	1	8/6/05	Corn
D. barberi	Pipestone	MN	Allopatry	44.07	96.30	19	1	8/12/04	Corn
D. barberi	Traill	ND	Allopatry	47.35	97.33	23	1	8/21/06	Corn
D. barberi	Dodge	NE	Sympatry	41.63	96.66	11	1	8/16/05	Corn
D. barberi	Saunders	NE	Sympatry	41.11	96.68	64	4	7/13/88, 8/16/05, 9/12/06	Thistle, Rosinweed, corn, sunflower, goldenrod, buffalo gourd
D. barberi	Lancaster	NE	Sympatry	40.79	96.76	47	4	7/20/85, 8/9/05, 8/24/05, 9/12/06	Corn, thistle, sunflower, tall thistle, bur cucumber
D. barberi	Gage	NE	Sympatry	40.28	96.82	1	1	8/9/05	Corn
D. barberi	Dixon	NE	Sympatry	42.35	96.86	15	1	8/17/05	Corn
D. barberi	Polk	NE	Sympatry	41.18	97.77	5	1	8/11/05	Corn
D. barberi	Howard	NE	Sympatry	41.19	98.31	6	1	8/11/05	Corn
D. barberi	Hall	NE	Sympatry	40.80	98.47	17	1	8/11/05	Curlycup gumweed, horseweed, goldenrod, sunflower
D. barberi	Sherman	NE	Sympatry	41.27	98.86	15	2	8/11/05, 9/13/06	Corn, sunflower
D. barberi	Custer	NE	Sympatry	41.28	99.37	12	1	8/11/05	Corn, western ironweed, thistle
D. barberi	Buffalo	NE	Sympatry	40.79	99.39	13	1	8/11/05	Corn, sunflower
D. barberi	Dawson	NE	Sympatry	40.87	100.16	3	1	8/26/05	Sunflower
D. barberi	Frontier	NE	Sympatry	40.60	100.50	15	1	8/27/05	Sunflower
D. barberi	Lincoln	NE	Sympatry	41.09	100.78	2	1	8/26/05	Pumpkin, sunflower
D. barberi	Riley	KS	Sympatry	39.10	96.61	24	5	8/4/05–8/5/05	Buffalo gourd, rosinweed, corn
D. longicornis	Nuckolls	NE	Sympatry	40.07	98.07	2	1	8/9/05	Buffalo gourd
D. longicornis	Webster	NE	Sympatry	40.09	98.65	7	1	8/2/04	Buffalo gourd
D. longicornis	Lincoln	NE	Sympatry	41.09	100.78	1	1	8/26/05	Pumpkin, sunflower
D. longicornis	Dundy	NE	Allopatry	40.04	101.72	3	1	8/6/06	Buffalo gourd
D. longicornis	Keith	NE	Allopatry	41.26	101.96	3	1	7/22/07	Buffalo gourd
D. longicornis	Garden	NE	Allopatry	41.30	102.11	12	1	7/23/05	Buffalo gourd
D. longicornis	Riley	KS	Sympatry	39.11	96.60	20	4	8/4/05	Buffalo gourd, rosinweed
D. longicornis	Ottawa	KS	Sympatry	39.08	97.89	22	1	8/5/05	Buffalo gourd
D. longicornis	Finney	KS	Allopatry	38.07	100.56	4	1	7/29/05	Buffalo gourd
D. longicornis	Scott	KS	Allopatry	38.66	100.91	1	1	7/29/05	Buffalo gourd
D. longicornis	Pueblo	CO	Allopatry	38.23	104.27	32	3	7/28/05	Buffalo gourd
D. longicornis	Santa Cruz	AZ	Allopatry	31.62	110.70	2	2	7/25/05	Buffalo gourd

a. Species determined by morphology and collection location.

b. Region is allopatry or sympatry.

c. Host species are sunflower (*Helianthus annuus* L., Asteraceae), rosinweed (*Silphium* sp., Asteraceae), curlycup gumweed (*Grindelia squarrosa* (Pursh) Dunal, Asteraceae), horseweed (*Conyza canadensis* (L.) Cronquist), goldenrod (*Solidago* sp., Asteraceae), western ironweed (*Vernonia fasiculata* Michx., Asteraceae), thistle (*Cirsium* sp., Asteraceae), tall thistle (*Cirsium altissimum* (L.) Hill, Asteraceae), corn (*Zea mays* L., Poaceae), soybean (*Glycine max* (L.), Fabaceae), amaranth (*Amaranthus* sp., Amaranthaceae), buffalo gourd (*Cucurbita foetidissima* HBK, Cucurbitaceae), bur cucumber (*Sicyos angulatus* L.), and pumpkin (*Cucurbita pepo* L., Cucurbitaceae).

Table 3. Results from analysis of molecular variance for *D. barberi* and *D. longicornis* combined, and for each species separately

Source of variation	*df*	*Sum of squares*	*Variance components*	*% variation*	*Statistic*	*Value*	*P*
Among groups (species)	1	98.2	0.99	5.33	Φ_{CT}	0.05	0.01
Among population	22	907.2	3.03	16.35	Φ_{SC}	0.17	<0.0001
Within population	188	2724.4	14.49	78.33	Φ_{ST}	0.22	<0.0001
Total	212	3729.8	18.50				
D. barberi							
Among population	18	814.7	3.30	18.26			
Within population	157	2317.9	14.76	81.74	Φ_{ST}	0.18	<0.0001
Total	175		3132.6	18.06			
D. longicornis							
Among population	4	80.8	1.13	8.48			
Within population	31	379.6	12.24	91.52	Φ_{ST}	0.08	0.0001
Total	35	460.4	13.38				

Table 4. Mean total color scores (_SE) and mean head capsule width of beetle populations by county

Putative species	*County*	*State*	*Color ± SE*	*Head capsule width (mm) ± SE*
D. barberi	Tompkins	NY	14.5 ± 0.4c	1.08 ± 0.03bc
D. barberi	Clark	OH	12.1 ± 0.2d	1.11 ± 0.02bc
D. barberi	Lafayette	MO	11.3 ± 0.3d	1.14 ± 0.02b
D. barberi	Nodaway	MO	11.5 ± 0.3d	1.10 ± 0.02bc
D. barberi	Pipestone	MN	11.9 ± 0.3d	1.18 ± 0.02ab
D. barberi	Traill	ND	11.3 ± 0.2d	1.13 ± 0.02b
D. barberi	Dodge	NE	11.2 ± 0.3d	1.10 ± 0.03bc
D. barberi	Saunders	NE	12.0 ± 0.1d	1.08 ± 0.01bc
D. barberi	Lancaster	NE	12.3 ± 0.2d	1.08 ± 0.01c
D. barberi	Gage	NE	11.8 ± 1.1d	1.25 ± 0.09ab
D. barberi	Dixon	NE	11.5 ± 0.3d	1.08 ± 0.02bc
D. barberi	Polk	NE	11.9 ± 0.5d	1.12 ± 0.04bc
D. barberi	Howard	NE	11.2 ± 0.5d	1.04 ± 0.03c
D. barberi	Hall	NE	11.5 ± 0.3d	1.11 ± 0.02bc
D. barberi	Sherman	NE	11.4 ± 0.3d	1.10 ± 0.02bc
D. barberi	Custer	NE	11.3 ± 0.3d	1.07 ± 0.02c
D. barberi	Buffalo	NE	11.4 ± 0.3d	1.14 ± 0.02b
D. barberi	Dawson	NE	11.5 ± 0.7d	1.07 ± 0.05c
D. barberi	Frontier	NE	11.8 ± 0.3d	1.03 ± 0.02c
D. barberi	Lincoln	NE	10.6 ± 0.8d	1.10 ± 0.06bc
D. barberi	Riley	KS	10.9 ± 0.2d	1.11 ± 0.02bc
D. longicornis	Nuckolls	NE	17.8 ± 0.8ab	1.08 ± 0.06bc
D. longicornis	Webster	NE	16.9 ± 0.4b	1.14 ± 0.03b
D. longicornis	Lincoln	NE	18.5 ± 1.1ab	1.13 ± 0.09bc
D. longicornis	Dundy	NE	16.8 ± 0.7b	0.96 ± 0.05c
D. longicornis	Keith	NE	18.0 ± 0.7ab	1.14 ± 0.05b
D. longicornis	Garden	NE	17.5 ± 0.3b	1.14 ± 0.02b
D. longicornis	Riley	KS	16.3 ± 0.3b	1.13 ± 0.02b
D. longicornis	Ottawa	KS	16.7 ± 0.2b	1.12 ± 0.02bc
D. longicornis	Finney	KS	17.6 ± 0.6b	1.05 ± 0.04c
D. longicornis	Scott	KS	13.0 ± 1.1cd	1.08 ± 0.09bc
D. longicornis	Pueblo	CO	17.3 ± 0.2b	1.10 ± 0.02bc
D. longicornis	Santa Cruz	AZ	19.8 ± 0.8a	1.29 ± 0.06a

Total color score is the sum of the individual scores for antennae, clypeus, scutellum, three episternites, and tibiae. Greater values for color score were associated with greater degrees of piceousness for all morphological characters. Means presented are least-squares means (LSMEANS). Effect of population (putative species and county) was significant for total color score ($F = 62.18$; df = 32, 445; $P < 0.0001$) and for head capsule width ($F = 2.22$; df = 32, 427; $P = 0.0002$). Within columns, means followed by the same lower-case letter are not significantly different ($P > 0.05$).

Table 5. Eigenvectors for PC analysis

Morphological character[a]	*PC1*	*PC2*
Antennae	**0.41**	0.24
Clypeus	**0.41**	0.05
Scutellum	**0.40**	0.03
Episternite 1	0.31	**-0.38**
Episternite 2	0.20	**-0.64**
Episternite 3	**0.43**	0.043
Tibiae	**0.43**	0.21
Head capsule width (mm)	0.01	**0.57**

Values in bold were greater than the mean of 0.33 for PC1 and the mean of 0.27 for PC2. These components were considered important for explaining variation of each axis. The two principal components explain a total of 66.1% of the total variation.

a. Head capsule width was measured in mm. All other morphological characters were color-scored as described by Krysan et al. (1983) (see text).

Table 6. Classifications of sympatric putative *D. barberi* and *D. longicornis* by discriminant analysis, based on color scores and head capsule widths of allopatric beetles

Classified into	*Putative species*	
	D. barberi	*D. longicornis*
D. barberi	244 (97.6)	6 (11.5)
D. longicornis	6 (2.4)	46 (88.5)
Total	250 (100)	52 (100)

The first number represents the total beetles classified into a species by discriminant analysis; the number in parentheses represents the percentage of beetles classified into that species. Total represents the total number of beetles classified by discriminant analysis. Total error rate in putative species identification was 7.0%. Allopatric beetles were used for calibration of the discriminant analysis.

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