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Reviews

Molecular Genetic and Hybridization Studies of *Diorhabda* spp. Released for Biological Control of *Tamarix*

Dan W. Bean, David J. Kazmer, Kevin Gardner, David C. Thompson, Beth (Petersen) Reynolds, Julie C. Keller, and John F. Gaskin*

The genus *Diorhabda* (Coleoptera: Chrysomelidae) was recently revised, using morphological characters, into five tamarisk-feeding species, four of which have been used in the tamarisk (*Tamarix* spp.) biological control program in North America and are the subject of these studies. The taxonomic revision is here supported using molecular genetic and hybridization studies. Four *Diorhabda* species separated into five clades using cytochrome c oxidase subunit 1 sequence data with *Diorhabda elongata* separating into two clades. Amplified fragment length polymorphism (AFLP) analysis using genomic DNA revealed only four clades, which corresponded to the four morphospecies. Hybridization between the four species yielded viable eggs in F_1 crosses but viability was significantly lower than achieved with intraspecific crosses. Crosses involving *Diorhabda carinulata* and the other three species resulted in low F_2 egg viability, whereas crosses between *D. elongata*, *Diorhabda sublineata* and *Diorhabda carinata* resulted in $> 40\%$ F_2 egg viability. Crosses between *D. carinulata* and the other three species resulted in high mortality of *D. carinulata* females due to genital mismatch. AFLP patterns combined with principal coordinates analysis enabled effective separation between *D. elongata* and *D. sublineata*, providing a method to measure genetic introgression in the field.

Nomenclature: Northern tamarisk beetle, *Diorhabda carinulata* Desbrochers; Mediterranean tamarisk beetle, *Diorhabda elongata* Brullé; subtropical tamarisk beetle, *Diorhabda sublineata* Lucas; larger tamarisk beetle, *Diorhabda carinata* Faldermann; tamarisk species: *Tamarix chinensis* Lour.; *Tamarix parviflora* DC.; *Tamarix ramosissima* Ledeb.

Key words: Genital lock and key, AFLP, PCOA, hybrid male sterility.

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Tamarisks (*Tamarix* spp., Tamaricaceae), also known as saltcedars, are exotic weeds that have invaded riparian areas across western North America causing extensive economic and ecological damage (Shafroth et al. 2005). Five species of tamarisks have become invasive in North America and there are several known instances of hybridization, with hybrids of *Tamarix ramosissima* Ledeb. and *Tamarix chinensis* Lour. comprising a large share of the tamarisk invasion in western North America (Gaskin and Kazmer 2009; Gaskin and Schaal 2002). The severity and widespread nature of the tamarisk invasion led to development of a biological control program, which in 2001 resulted in the first open field releases of a tamarisk feeding leaf beetle *Diorhabda elongata* Brullé (Chrysomelidae). Beetles were initially released at two locations in Nevada and one location in each of four states: Wyoming,

Management Implications

Tamarisks (aka saltcedar) are invasive shrubs that have become a widespread problem in riparian and wetland areas in the western United States. Biological control utilizing tamarisk beetles (*Diorhabda* spp.) is an effective management option, but it has become clear that we need to know more about the beetles to use them effectively for control in the diverse ecological settings that tamarisks have invaded. In this study we use molecular methods to complement the taxonomic revision of the tamarisk-feeding *Diorhabda*. The four closely related species used in the North American tamarisk biological control program were separable using molecular methods. We performed interspecific crosses between these four species and found that hybrids could be formed and that genetic exchange between species is possible, particularly between the Mediterranean tamarisk beetle and the subtropical tamarisk beetle. We developed a molecular method for detection of interspecific hybrids and show that under some circumstances there has been interbreeding and introgression in populations of the Mediterranean tamarisk beetle and the subtropical tamarisk beetle used in the North American biological control program. This work will help determine the potential geographic distributions of these species and their hybrids.

Utah, California, and Colorado (DeLoach et al. 2004). Since 2001 the tamarisk beetles have become widely established and they have provided control over large areas of the tamarisk infestation (Carruthers et al. 2008). Even so, the beetles failed to establish or did poorly in some areas, especially in the southern portion of the invaded range of tamarisks (Texas, New Mexico, Southern California) and in coastal California, where the primary invader is *Tamarix parviflora* DC. (Carruthers et al. 2008; Dalin et al. 2009; Lewis et al. 2003).

At the outset of the tamarisk biological control program the leaf beetles were classified as a single species, *D. elongata*, with a range across North Africa and Eurasia into China and Mongolia (Tracy and Robbins 2009), and in all regions they were known to be specialists on the genus *Tamarix.* The initial collections of *D. elongata* sensu lato for tamarisk biological control were from the interior of central Asia: one near the town of Chilik in Kazakhstan, and the other near the city of Fukang in the Xinjiang Province of northwestern China (DeLoach et al. 2003). These populations did well against *T. ramosissima* and *T. chinensis* and their hybrids in the northern interior states of Nevada, Utah, Colorado, and Wyoming but failed to thrive in coastal California, Texas, or southern New Mexico (DeLoach et al. 2004). This was because of several factors, including mismatches in the photoperiodic requirements for reproduction and diapause at more southern latitudes, which restricted the normally multivoltine beetles to a univoltine life history (Bean et al. 2007a; Lewis et al. 2003). Another factor was the mismatch of target species in areas where *T. parviflora* was dominant because it appears to be a suboptimal host for central Asian *Diorhabda* (Dalin et al. 2009). The presence of predators, especially ants, was also shown to inhibit establishment of tamarisk beetles (Herrera 2003). Recognizing these problems, additional ecotypes of *D. elongata* were imported for use in the *Tamarix* biological control program, beginning in 2002 (DeLoach et al. 2004; Milbrath and DeLoach 2006a; Tracy and Robins 2009). Because *D. elongata* is widely distributed in the native range, multiple ecotypes could be found to match the diverse ecological conditions encountered in North America, including host plant species, climate, and latitude.

New ecotypes were collected from sites in the Mediterranean basin, Uzbekistan, and China (DeLoach et al. 2004). Beetles from new collections were considered to be novel ecotypes and were initially held under quarantine conditions, tested, and treated in much the same way as if they were distinct species. Host range testing was done for each ecotype (Milbrath and DeLoach 2006a, b; Herr et al. 2009) and they were found to have the same or similar host range properties as the original ecotypes; that is they were all tamarisk specialists. In addition to safety testing, the new ecotypes were screened for traits that might make them more effective in areas where the original releases had been ineffective. New ecotypes were found to be better adapted to southern *Tamarix* phenology, as well as to photoperiod regimes found in the southernmost ranges of *Tamarix* in North America (Dalin et al. 2010; Dudley et al. 2006; Milbrath et al. 2007). The ecotype collected from Crete, Greece, was shown to establish on *T. parviflora* whereas the Fukang, China, ecotype had failed (Carruthers et al. 2008; Thomas et al. 2009). The new ecotypes displayed an array of traits that were extremely useful for the *Tamarix* biological control program but it became clear that genetic and taxonomic relationships among ecotypes needed further evaluation. Characterization of the *Diorhabda* ecotypes was initiated and coordinated under the auspices of the Saltcedar Consortium, a group of scientists, weed managers, government agencies, and other stakeholders with an interest in tamarisk biological control (DeLoach et al. 2004). Characterization included the molecular genetic and hybridization investigations described in this study as well as a taxonomic revision of the tamarisk-feeding leaf beetles based on morphology (Tracy and Robbins 2009).

The *Diorhabda elongata* species complex was defined and described using morphological characteristics, primarily of the genitalia, in a study that also included extensive biogeographical information (Tracy and Robbins 2009). Tracy and Robbins divided the tamarisk-feeding *Diorhabda* into five species, including four species that had been considered ecotypes within the tamarisk biological control program. Central Asian ecotypes formerly known as *Diorhabda elongata deserticola*, became *Diorhabda carinulata* Desbrochers, with the common name of northern tamarisk beetle. Western Asian ecotypes, including beetles

collected in Uzbekistan, became *Diorhabda carinata* Faldermann or the larger tamarisk beetle. Ecotypes from the northeastern Mediterranean basin, including beetles collected in Crete and Possidi, Greece, remained *D. elongata*, now known as Mediterranean tamarisk beetle whereas the North African and western Mediterranean ecotypes became *Diorhabda sublineata* Lucas or the subtropical tamarisk beetle. The fifth species, not currently used in the biological control program, was classified as *Diorhabda meridionalis* Berti and Rapilly with a range centered in southern and western Iran. There are a few subtle differences in outward appearance between the newly designated species but the characteristics that define them are found in the hidden sclerites of the endophallus and to a lesser extent in sclerites and palpi of the female reproductive system (Tracy and Robbins 2009), making these essentially cryptic species.

This study presents the molecular genetic component of the ecotype characterization project in order to determine the relationships among the *Tamarix*-feeding members of the *Diorhabda elongata* sensu lato species complex and examines concordance of molecular genetic traits with morphological, behavioral, and ecological traits. Data on interspecific hybridization within the species complex and within species is also presented, along with molecular genetic assays to assess hybridization and genetic introgression between species and ecotypes. This information will benefit the tamarisk biological control program by enabling better identification of *Diorhabda* species and by providing precise methods for measuring gene flow between ecotypes in the field or under laboratory conditions.

Materials and Methods

The insects used in this study were originally collected on *Tamarix* in Eurasia and North Africa (Figure 1). Cultures were maintained at four facilities in North America: the U.S. Department of Agriculture Agricultural Research Service (USDA ARS) Western Regional Research Center, Exotic and Invasive Weeds quarantine facility in Albany, CA (USDA Albany); the USDA ARS Grassland Soil and Water Research Laboratory quarantine facility, Temple, TX (USDA Temple); the New Mexico State University Entomology, Plant Pathology and Weed Science quarantine facility, Las Cruces, NM (NMSU Las Cruces); and the Palisade Insectary, Biological Pest Control Program, Colorado Department of Agriculture, Palisade, CO (CDA Palisade). Beetles were cultured on live cuttings of *Tamarix,* including *T. ramosissima*, *T. chinensis*, and their hybrids (Gaskin and Schaal 2002), which served as the primary culturing material at USDA Temple, NMSU Las Cruces, and CDA Palisade. *Tamarix parviflora* was the primary species used to culture insects at USDA Albany. Beetles were reared in well-ventilated plastic containers under day lengths of at least 16 h to prevent diapause induction. More detailed culturing methods are described elsewhere (Bean et al. 2007b, Petersen 2007). Cultures of *D. carinulata* were derived from beetles collected near the cities of Fukang, China (44.17°N, 87.98°E; elevation 552 m [1,811 ft]); Turpan, China (42.86°N, 89.22°E; elevation 70 m below sea level); and Chilik, Kazakhstan (43.6°N 78.25°E; elevation 662 m). They are referred to as the Fukang, Turpan, and Chilik ecotypes of *D. carinulata*. The Chilik ecotype was released near the town of Delta, UT, and cultures used in this study were derived from beetles collected there in 2003. Cultures of *D. carinata* used in this study originated from collections made in 2002 near Karshi (Qarshi), Uzbekistan (38.86°N, 65.72°E; elevation 350 m). Cultures of *D. elongata* originated from near Possidi, Greece (39.96°N, 23.36°E, elevation 5 m) and from near Sfakaki, Crete, Greece (35.83°N, 24.6°E; elevation 7 m) and were called the Possidi and Crete ecotypes, respectively. Cultures of *D. sublineata* originated near the town of Sfax, Tunisia (34.66°N, 10.67°E; elevation 10 m). Beetles from all sites were originally identified as *D. elongata* (DeLoach et al. 2004; Milbrath and DeLoach 2006 a, b).

Beetles used for DNA analysis originated from the sites listed above or were collected from four additional sites, including Bukhara, Uzbekistan (39.82°N, 64.39°E); Ashgabat, Turkmenistan (37.95°N, 58.67°E); Kyparissia, Greece (37.26N° 22.65°E); and Astros, Greece (37.44°N, 22.75°E). Collection sites are described in more detail elsewhere (Tracy and Robbins 2009).

DNA Extraction and Mitochondrial Cytochrome c Oxidase Subunit 1 (COI) Sequence Analysis. DNA was extracted from head, femur, or abdomen tissue of beetle specimens using a Chelex extraction (Kazmer et al. 1995) or DNeasy blood and tissue kit (Qiagen Corp, Valencia, CA) following standard protocols.

We sequenced COI mtDNA fragments from 48 *Diorhabda* and 2 *Galerucella birmanica* specimens. The *G. birmanica* specimens were collected in China and provided by B. Blossey (Cornell University, Ithaca, NY). Sequence template was amplified using primer pairs C1-J-1718 (forward; Simon et al. 1994)–C1-N-2616 (reverse; 5′-TGCTATAATTGCAAATACTGCTCCT-3′, designed by DJK) and C1-J-2195 (forward)–TL2-N-3014 (reverse), both from Simon et al. (1994). The thermal cycling program was as follows: 180 s at 94 C (201.2 F); 25 cycles of 30 s at 94 C, 60 s at 50 C or 54 C (depending on primers used), 120 s at 72 C; and a final 300 s at 72 C. Each 30-µl reaction contained 3 µl of genomic DNA, 3 µl 10× NH_4 polymerase chain reaction (PCR) buffer (Bioline USA Inc., Tuaton, MA), 1.5 µl 50 mM $MgCl_2$, 0.24 µl 100 mM dNTP mix, 3 µl 2 µM of each primer, and 0.015 µl (0.75 units) of Biolase DNA Polymerase (Bioline).

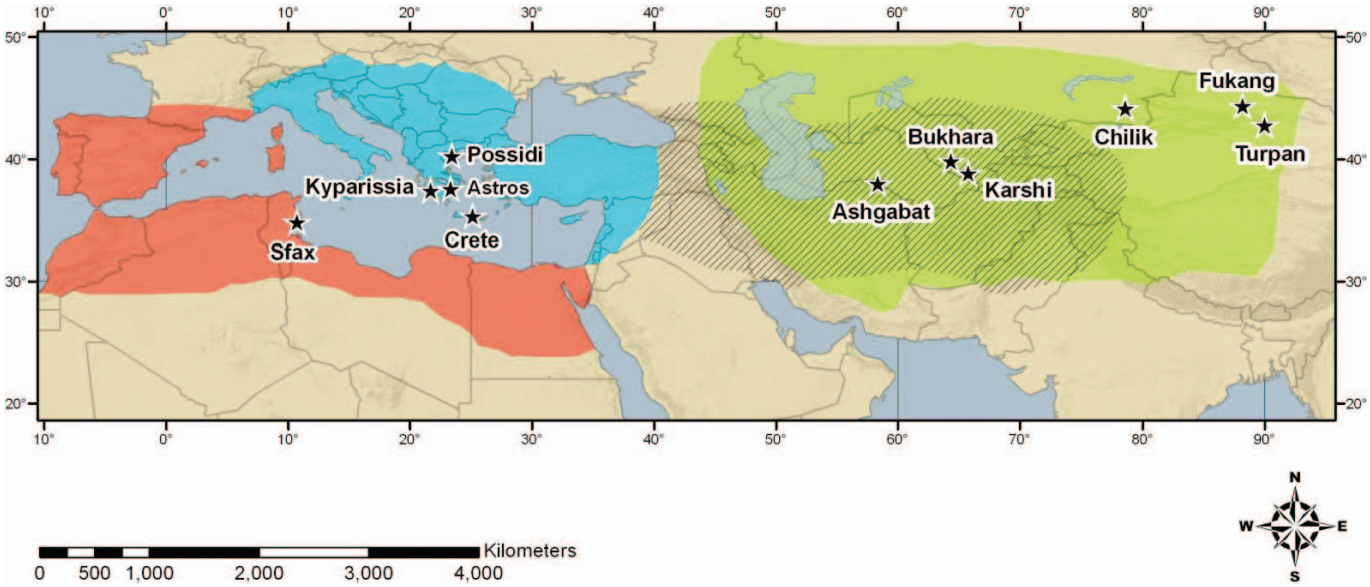

Figure 1. Origin of cultures and samples. Stars represent locations of towns and cities nearest collection sites for samples used in this study. Shading represents approximate ranges of *Diorhabda* species (taken from Tracy and Robbins 2009). Black = *Diorhabda sublineata*, gray stippled = *Diorhabda elongata,* hatching = *Diorhabda carinata,* and gray = *Diorhabda carinulata*. (Color for this figure is available in the online version of this paper.)

Sequence reactions used the primers above as well as C1-N-2191 (reverse; Simon et al. 1994). PCR products were purified using QIAquick PCR Purification kit (Qiagen Corp.) prior to sequencing in a CEQ 2000XL automated sequencer (Beckman Coulter, Inc., Fullerton, CA) using standard protocols. DNA sequences are listed in GenBank as accessions JQ782459 to JQ782491.

Maximum parsimony (MP) analysis of the data set was performed using PAUP* v. 4.0b8 (Sinauer Associates; Sunderland, MA). The heuristic MP search employed 500 random taxon addition sequences and the tree-bisection-reconnection branch-swapping algorithm. All characters were weighed equally and there were no insertion/deletion events. A 5,000-replicate fast stepwise-addition bootstrap analysis was conducted to assess clade support. *Galerucella birmanica* and *Diabrotica undecimpunctata* (GenBank accession number AF 278555) were included in this analysis as outgroups. *Galerucella* and *Diorhabda* are members of the tribe Galerucini in the subfamily Galerucinae and *Diabrotica* is a member of the tribe Luperini in the same subfamily (Riley et al. 2003).

AFLP Analysis. The AFLP method followed Vos et al. (1995) as modified in Gaskin and Kazmer (2009). Four primer pair combinations (*Mse*I + CTC/*Eco*RI + AAG, *Mse*I + CTC/*Eco*RI + ACC, *Mse*I + CTC/*Eco*RI + ACT, and *Mse*I + CAC/*Eco*RI + ACC, where *Mse*I = GATGAGTCCTGAGTAA and *Eco*RI = GACTGCG-TACCAATTC) were used to produce fragments, which were first scored using the Fragment Analysis module of the Beckman Coulter CEQ Genetic Analysis System software (bin width of one nucleotide, accepted peak height = 10% of second highest peak). These bins were then manually screened and scored using Genographer (Montana State University, Bozeman, MT) to visualize fragments, making this a semiautomatic scoring method, as suggested by Papa et al. (2005). Gel images were normalized across total signal to avoid errors in scoring due to band intensity.

NTSYS-pc ver. 2.1 software, SIMQUAL program(Exeter Software; Setauket, NY) was used to calculate the Nei and Li (1979) coefficient: 2a/(2a + b + c) where a = number of bands present in both samples and b and c = number of bands present in only one or the other sample. To present a visual representation of genotype clustering, principal coordinates analysis (PCOA) was performed on similarity coefficients using the DCENTER and EIGEN programs of NTSYS. The unweighted pair group method with arithmetic mean dendrogram and fast stepwise-addition bootstrap values were created using Nei–Li distance measures in PAUP* v. 4.0b8.

Hybridization Studies. For hybridization studies all *Diorhabda* used were from laboratory colonies raised in either 1.0- or 2.8-L (1.06 or 2.96 qt) transparent containers with screen lids at 25 C under a photoperiod of 16 h light/8 h dark. Beetles were reared on fresh cuttings of *T. parviflora*, *T. chinensis*, *T. ramosissima*, or *T. chinensis/ramosissima* hybrids. Larvae of the parental generation were reared in either 1.0- or 2.8-L containers with cuttings of *Tamarix* sp. until they reached the prepupal stage; then they were transferred to small plastic containers with sand or provided with floral foam (Greenleaf Wholesale Florist Inc., Albuquerque, NM)

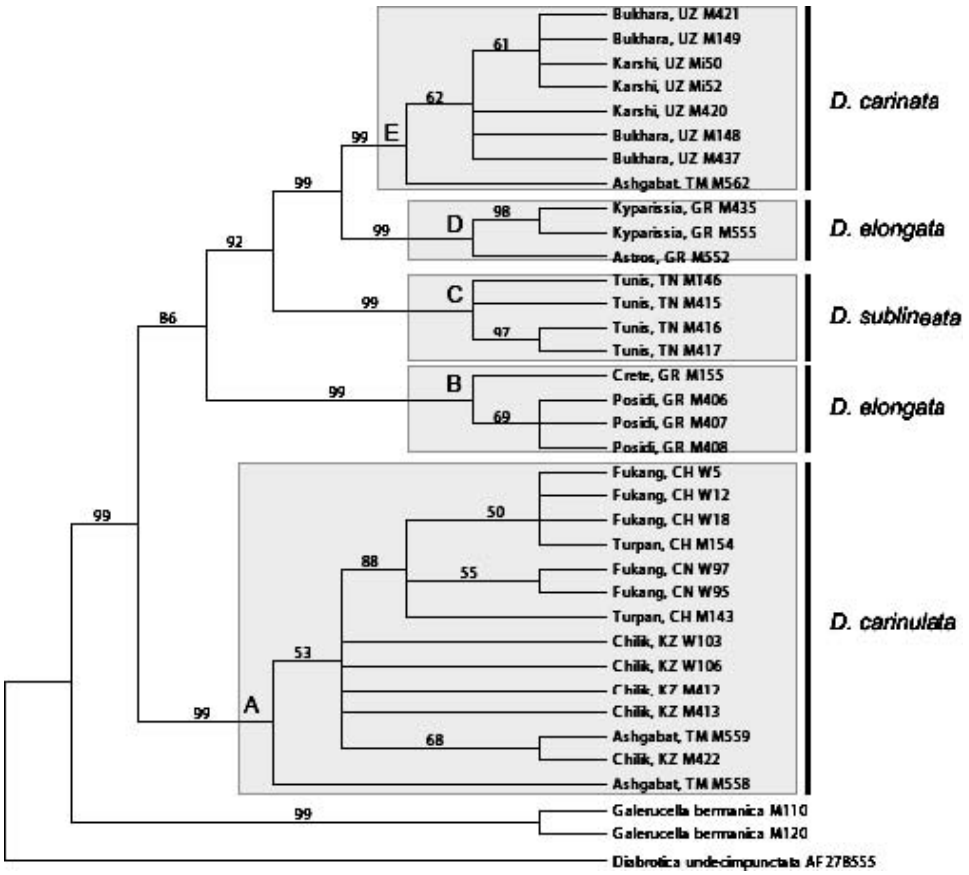

Figure 2. Maximum parsimony 50% majority consensus cladogram of 288 trees of cytochrome c oxidase subunit 1 mtDNA region for *Diorhabda* spp. and outgroups. Tree is 518 steps in length, representing 1,270 base pairs of aligned sequence data. Bootstrap values are shown above branches. Major clades are indicated by capitalized letters A to E and shaded regions. City, country, and accession number are at tips of tree. *Diobrotica* specimen is followed by GenBank accession number.

where they burrowed into the sand or foam, formed casings, and pupated, emerging as adults 12 to 14 d later.

To ensure unmated status, pupae were separated prior to adult emergence in some experiments. In other experiments, adult females were separated from adult males on the day of emergence because it was previously shown that reproductive development was not complete and mating did not occur until after day 3 following adult emergence (Bean et al. 2007b, Petersen 2007). Unmated males and females were paired and all eggs were collected and pooled for each pair or each group of pairs. In cases where single pairs were used, extra females and males were kept as substitutes in the event of a death in the experimental pairs.

Four mating combinations were analyzed for each two-species or two-ecotype comparison: each species or ecotype crossed to itself; males from one species or ecotype crossed to females of the second species or ecotype; and the corresponding reciprocal cross. Isolated pairs were not used in experiments in which *D. carinata* were crossed with either *D. sublineata* or *D. elongata*, but rather multiple pairs (three to five pairs) were held together in the presence of fresh tamarisk cuttings and eggs were collected every other day. Each 2-d collection was observed for hatching and the number hatched was recorded for each collection. In the remainder of the experimental crosses, single pairs of adults were maintained in well-ventilated 237-ml (8 fl oz) containers in the presence of fresh tamarisk cuttings. Containers were checked every day or every other day for eggs. Approximately 50 eggs were collected from each container and the number of eggs that hatched was recorded. In a few cases beetles did not produce 50 eggs so as many as possible were collected. F_1 larvae were pooled, as were larvae from the parental crosses, and these were reared to adulthood following the same procedures used with parental beetles.

There were seven types of parental, F_1, and backcrosses: within parental strains, within the F_1 hybrids, two backcrosses of hybrid females into parental strains, and two backcrosses of hybrid males into the parental strains. Backcrosses were not conducted in *D. carinata* × *D. elongata* and *D. carinata* × *D. sublineata*. Eggs were collected, counted, and observed for hatching as with the parental crosses.

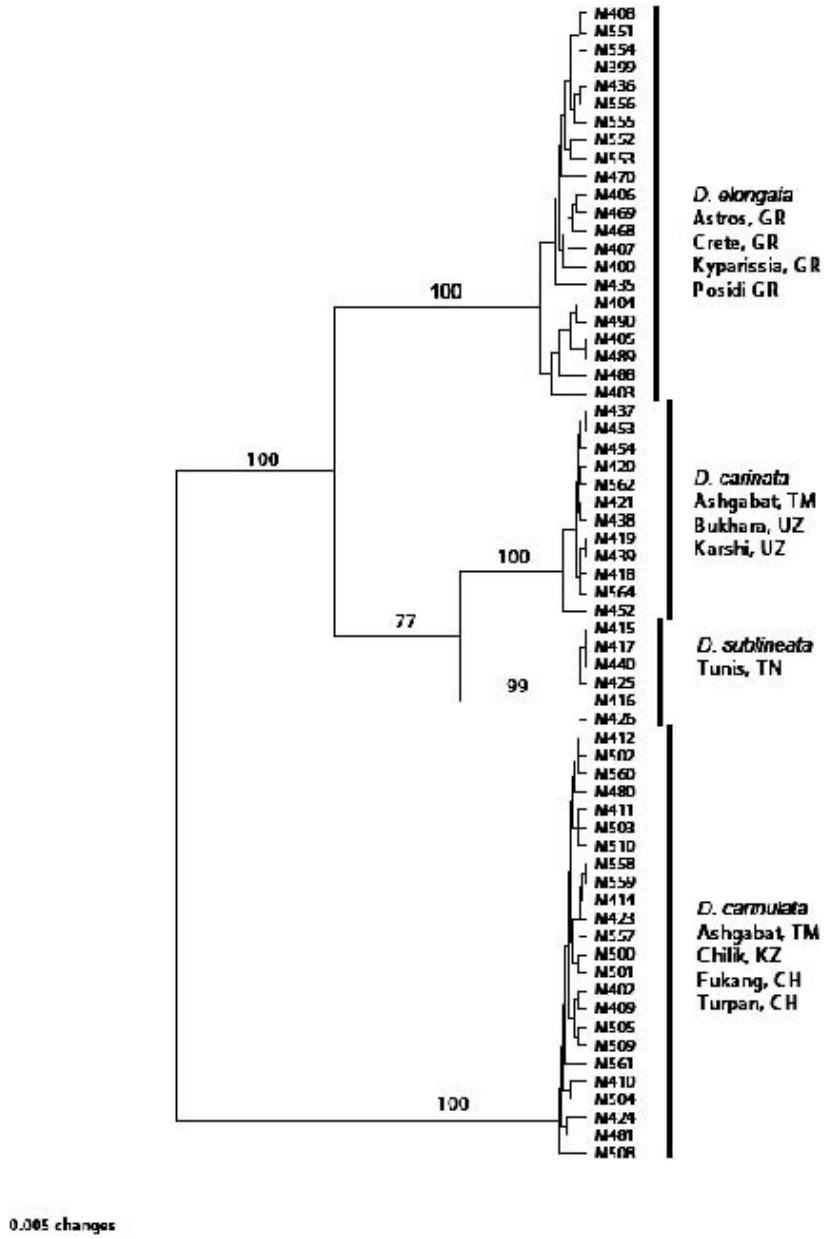

Figure 3. Unweighted pair group method with arithmetic mean dendrogram of 64 *Diorhabda* specimens from amplified fragment length polymorphism data. Bootstrap values (> 50%) shown above branches. Accession numbers, origins, and species designations are listed to right of dendrogram.

Egg counts were difficult, especially when clusters were larger than four or five eggs, so extra measures were taken to ensure accuracy of egg counts. Underestimates and overestimates of egg numbers occurred in 25 to 50% of the samples, necessitating careful follow-up after egg hatch. If the number of larvae observed exceeded the recorded number of eggs collected, the number of larvae was used as the actual number of eggs, clusters were checked for unhatched eggs, and the original count was disregarded as an underestimation. If egg number exceeded number of larvae hatched then egg clusters were recounted, and in all cases if there was doubt concerning the presence of unhatched eggs, clusters were crushed to see if yolk was present, indicating unhatched eggs.

The P values for comparisons of egg viabilities were obtained with the Tukey-Kramer test using either version 4 or 8 of JMP® software (SAS Institute, Cary, NC).

Results

Mitochondrial DNA Sequence Analysis. The mtDNA region flanked by primers C1-J-1718 and TL2-N-3014

Table 1. Egg viability following crosses between ecotypes[a] of *Diorhabda carinulata* and their F_1 hybrids[b] or first-generation backcrosses (BC_1).

Cross	Egg viability[c]	
♂ × ♀	F_1 or parental cross eggs	F_2, parental cross, or BC_1 eggs
	%	
F × F	86.23 ± 5.44	
C × C	95.44 ± 1.46	
F × F	86.23 ± 5.44	
F × C	94.05 ± 2.17	
C × F	88.47 ± 7.73	
C × C		93.73 ± 6.27
F × F		84.91 ± 10.13
FC × FC		90.90 ± 3.79
FC × C		95.41 ± 2.54
C × FC		88.60 ± 5.75
FC × F		100.00 ± 0.00
F × FC		96.17 ± 1.99
T × T	94.45 ± 1.88	
F × F	95.60 ± 1.99	
F × T	92.84 ± 3.02	
T × F	91.37 ± 3.35	
FT × FT		95.67 ± 3.95
FT × T		99.07 ± 0.64
T × FT		87.94 ± 9.92
FT × F		99.22 ± 0.48
F × FT		98.10 ± 1.10

[a] Three ecotypes of *D. carinulata* were used in these experiments and they were named according to town nearest to the collection site. The F ecotype originated from beetles collected near the town of Fukang, China; the C ecotype originated from beetles collected near the town of Chilik, Kazakhstan; and the T ecotype originated from beetles collected near the town of Turpan, China. For more details see Figure 1 and Material and Methods.

[b] Hybrids originating from both reciprocal crosses were pooled and treated as a single population.

[c] There were no significant differences between viabilities, within each of the four experimental groups ($P > 0.05$), as described in Results.

produced sequences 1,270 base pairs in length; 240 base positions were parsimony-informative. The maximum parsimony tree is shown in Figure 2. Three individuals from Bukhara, Uzbekistan, and two from Karshi, Uzbekistan,had identical sequences and are represented by accession M148. All four individuals from Crete, Greece, had identical sequences and are represented by accession M155. Three individuals from Fukang, China, with identical sequences are represented by accession W5. Four individuals from Turpan, China, with identical sequences are represented by accession M154. Three individuals from Ashgabat, Turkmenistan, are represented by accession M559.

Five major clades are present within *Diorhabda* (Figure 2). Three of the clades—A, C, and E—are, respectively, *D. carinulata, D. sublineata*, and *D. carinata.* The remaining two clades are *D. elongata*.

AFLP Analysis. The four primer pairs produced 115 variable loci from the 64 insects included in the analysis, which resulted in the dendrogram shown in Figure 3. Four major groups, each corresponding to a morphospecies, are present. Unlike the mtDNA COI sequence results, the AFLP data assemble *D. elongata* sensu stricto specimens into a single group.

Egg Viability from Crosses within Species of *Diorhabda*. Crosses within *D. carinulata* between the Fukang ecotype and the Chilik ecotype yielded eggs with high viability (88 and 94%) with no significant viability differences between eggs from the F_1 (hybrid) crosses and eggs from the parental crosses ($P = 0.5392$, Table 1). Hybrid adults were either crossed with each other or backcrossed into the parental strains and eggs produced from these crosses had viabilities ranging from 89 to 100%. These viability values were not significantly different from those from parental ecotype crosses ($P = 0.4537$, Table 1). Eggs from crosses within *D. carinulata* and between the Fukang ecotype and the Turpan ecotype had viabilities $> 90\%$ and viability values were not significantly different from those from parental crosses ($P = 0.7212$, Table 1). Hybrid F_1 adults were crossed with each other and backcrossed into both parental ecotypes. Eggs from those crosses had viabilities ranging from 88 to 99% with no significant differences amongst the F_2 and first-generation backcrossed (BC_1) groups ($P = 0.5219$, Table 1). Parental ecotype crosses were not done in this experiment and so the hybrid crosses could not be directly compared with parental crosses. Crosses within *D. elongata*, between the Possidi ecotype and the Crete ecotype, yielded egg viabilities that were not significantly different from crosses within ecotypes ($P = 0.2304$ for F_1 egg viability and $P = 0.1161$ for F_2 egg viability).

F_2 and BC_1 Egg Viability from Hybrids between Species of *Diorhabda*. Crosses between *D. carinulata* (Fukang ecotype) and the other three species yielded hybrids that produced low-viability F_2 or BC_1 eggs (Figures 4–6). In all cases the viability of F_2 or BC_1 eggs was significantly lower ($P < 0.0001$) than egg viability within parental species crosses. In the case of *D. carinulata* × *D. elongata* (Possidi ecotype) there were no viable eggs produced when F_1 interspecific hybrid males were backcrossed into either parental strain or crossed with F_1 interspecific hybrid females (Figure 4). This pattern was nearly identical with *D. carinulata* × *D. elongata* (Crete ecotype) interspecific F_1 hybrids: the F_1 cross was sterile, backcrosses involving F_1 males also produced no viable eggs, and the F_1 females

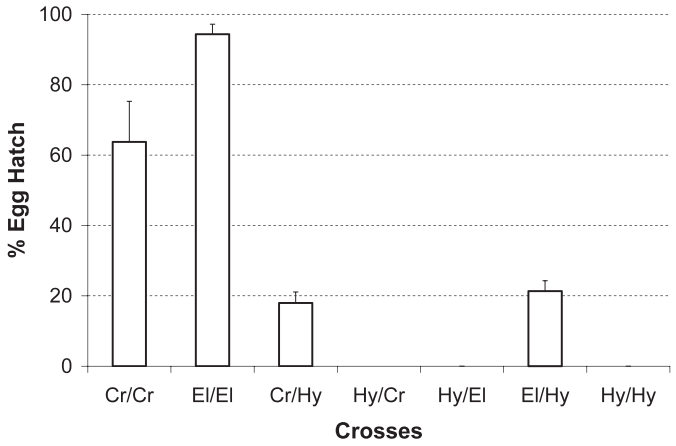

Figure 4. Egg hatch from crosses within *Diorhabda carinulata* (Cr) and *Diorhabda elongata* (El), reciprocal backcrosses of F_1 interspecific hybrids (Hy) to parental strains, and crosses of F_1 interspecific hybrids (Hy/Hy). Species of the male is indicated first, the female second. Error bars represent SE; $n = 7$ pairs for each group. Values for all crosses involving hybrids are significantly different from the parental species values ($P < 0.0001$). No viable eggs were produced when hybrid males were part of the cross. *Diorhabda carinulata* were the Fukang ecotype and *D. elongata* were the Possidi ecotype.

produced few viable eggs when backcrossed into the parental strains (data not shown). Overall the F_1 males from a *D. carinulata* × *D. elongata* were sterile and the F_1 females produced eggs of low viability (< 22%) when they

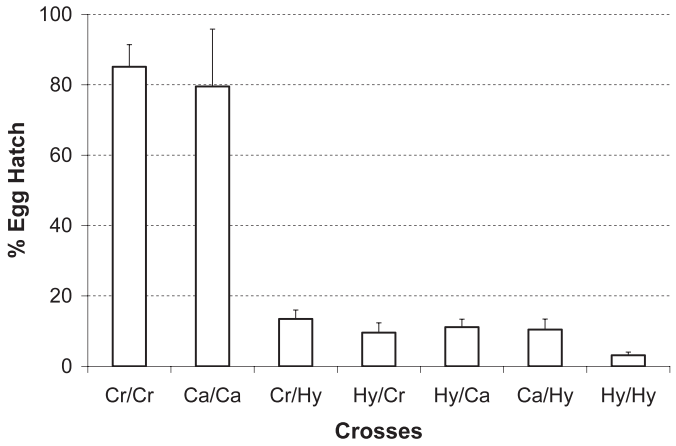

Figure 5. Egg hatch from crosses within *Diorhabda carinulata* (Cr) and *Diorhabda carinata* (Ca), reciprocal backcrosses of F_1 interspecific hybrids (Hy) to parental strains, and crosses of F_1 interspecific hybrids (Hy/Hy). Species of the male is indicated first, the female second. Error bars represent SE; $n = 7$ pairs for each group. Values for all crosses involving hybrids are significantly different from the parental cross values ($P < 0.0001$). *Diorhabda carinulata* were the Fukang ecotype.

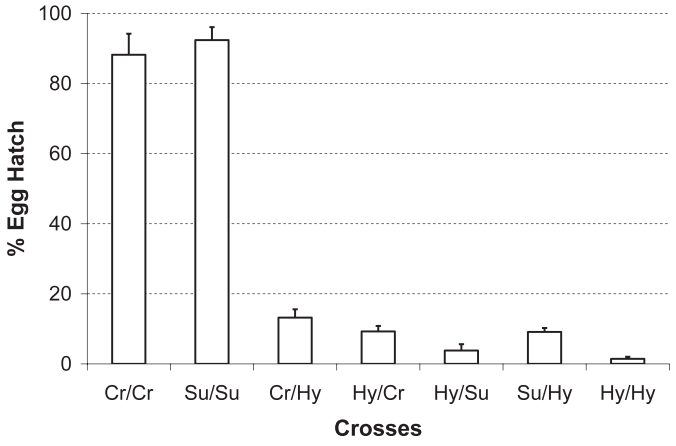

Figure 6. Egg hatch from crosses within *Diorhabda carinulata* (Cr) and *Diorhabda sublineata* (Su), reciprocal backcrosses of F_1 interspecific hybrids (Hy) to parental strains, and crosses of F_1 interspecific hybrids (Hy/Hy). Species of the male is indicated first, the female second. Error bars represent SE; $n = 10$ pairs except for Hy/Cr, which had 13 pairs. Values for all crosses involving hybrids are significantly different from the parental cross values ($P < 0.0001$). *Diorhabda carinulata* were the Fukang ecotype.

were backcrossed into the parental strains (Figure 4, and data not shown for the Crete ecotype).

Crosses between *D. carinulata* and *D. carinata* yielded F_1 interspecific hybrids that produced low-viability F_2 eggs (< 4%) or low-viability BC_1 eggs (< 15%) resulting from backcrosses into either parental species (Figure 5). All crosses involving F_1 interspecific hybrids had a significantly reduced egg viability compared to the parental crosses ($P < 0.0001$).

Crosses between *D. carinulata* and *D. sublineata* yielded F_1 interspecific hybrids that produced low-viability F_2 or BC_1 eggs (< 15%) (Figure 6). F_2 and BC_1 egg viabilities were significantly reduced compared to those of the parental crosses ($P < 0.0001$).

Crosses between *D. elongata* (Crete ecotype) and *D. sublineata* yielded F_1 interspecific hybrids that produced high-viability F_2 eggs (> 85%) or high-viability BC_1 eggs in cases where the F_1 interspecific hybrid was female (Figure 7). Egg viability was reduced in the two backcrosses of F_1 interspecific hybrid males with females from either parental species (Figure 7). In these cases egg viability was significantly lower than with crosses within the parental strains ($P < 0.0001$). In another experiment, the *D. sublineata* × *D. elongata* hybrids were shown to produce stable cultures for three generations and these produced F_4 eggs with a viability of 75% (data not shown).

Hybrids resulting from *D. carinata* × *D. elongata* produced eggs with a mean viability of 67% ($n = 10$ egg collections, made every other day) whereas hybrids resulting from *D. carinata* × *D sublineata* produced eggs

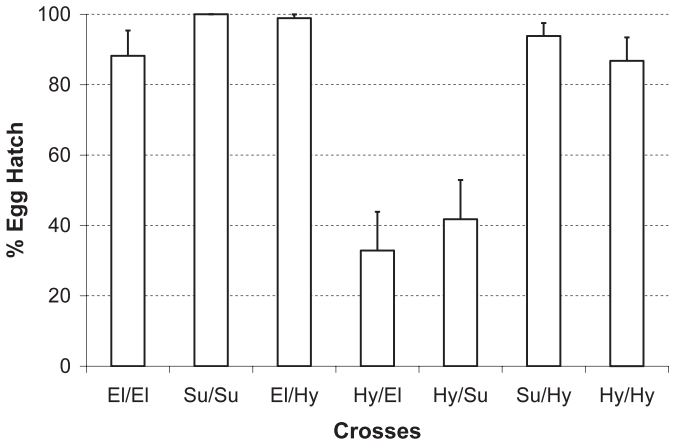

Figure 7. Egg hatch from crosses within *Diorhabda elongata* (El) and *Diorhabda sublineata* (Su), reciprocal backcrosses of F_1 interspecific hybrids (Hy) to parental strains, and crosses of F_1 interspecific hybrids (Hy/Hy). Species of the male is indicated first, the female second. Error bars represent SE; $n = 16$ pairs for each group. Values for crosses between male hybrids and the parental populations are significantly different from the parental cross values ($P < 0.0001$). *Diorhabda elongata* were the Crete ecotype.

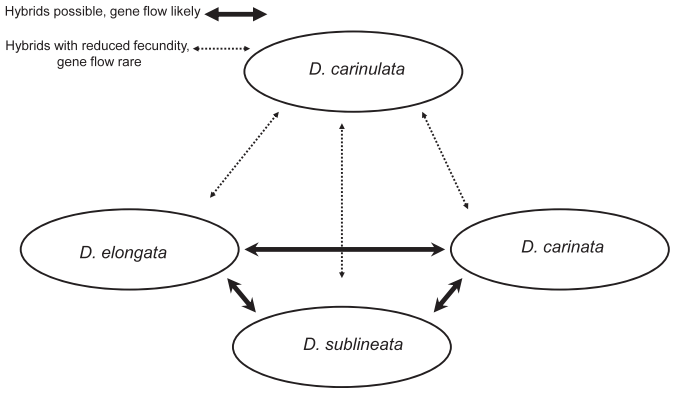

Figure 8. Relative likelihood of gene flow based on F_2 egg viability results.

with a mean viability of 42% ($n = 10$ egg collections, made every other day). These data are not directly comparable to data from other crosses because the pairs were pooled in these experiments and individual pairs were used in the other experiments. It is clear, though, that F_2 egg viability was higher in these crosses than from the three interspecific crosses involving *D. carinulata* where F_2 egg viability ranged from 0 to 3% (Figures 4–6).

The viability of F_2 and BC_1 eggs was used to estimate the likelihood of gene flow between species as they encounter each other in the field. F_2 egg viability of 40% or higher, as seen in crosses involving *D. carinata*, *D. sublineata*, and *D. elongata*, was seen as an indication that gene flow would be likely if these species encountered each other. In crosses involving *D. carinulata*, the F_2 and BC_1 egg viabilities were well below 30%, which would make gene flow less likely but still a distinct possibility. Some crosses involving *D. carinulata* also revealed male hybrid sterility and mortality due to genital mismatch. Therefore we considered the possibility of gene flow to be low in these crosses (Figure 8). These designations do not take into account the likelihood of prezygotic reproductive isolation, as described below in the Discussion.

Mortality in *D. carinulata* Crosses. Crosses between *D. carinulata* and the other three species resulted in high mortality in *D. carinulata* females (Table 2). In the most extreme example 100% female mortality was observed when *D. elongata* males were crossed with *D. carinulata* females. Sometimes pairs were unable to uncouple, indicating severe genital mismatch, which had been previously noted (Tracy and Robbins 2009). In most cases, uncoupling occurred but females ceased laying eggs and died, indicating internal damage.

Detection of Hybrids using AFLP PCOA. The four AFLP primer pairs produced 52 variable loci from the 57 insects included. The resulting PCOA is shown in Figure 9. PCOA axis 1 accounts for 58% of the variance and PCOA axis 2 accounts for 6% of the variance. This analysis provides clear separation of *D. elongata* (Crete ecotype), *D. sublineata*, and laboratory-generated F_1 interspecific hybrids between the two (Figure 9a). The F_1 interspecific hybrids have intermediate PCOA scores along PCOA axis 1. This result is expected as AFLP markers are dominant and therefore the F_1 hybrids exhibit AFLP markers of each parental type.

Additional PCOA results are shown in Figure 9b for specimens from several laboratory colonies that were suspected of containing hybrids, beginning in 2006. Three specimens from an indoor colony of *D. elongata* (Crete ecotype) at Temple, TX, have PCOA scores similar to that of the parental type and were not suspected to be hybrids based on morphology (J. Tracy, personal communication). All other specimens, thought to be *D. sublineata*, have PCOA scores intermediate to the known F_1 hybrids and the Crete ecotype of *D. elongata.* Six of the 10 specimens from the Temple, TX, outdoor colony of *D. sublineata* were suspected hybrids based on morphology (J. Tracy, personal communication). The NMSU Las Cruces colony of *D. sublineata* was founded using individuals from the Temple, TX, outdoor colony of *D. sublineata* and the CDA Palisade *D. sublineata* colony was subsequently founded using insects from the NMSU Las Cruces colony.

Additional PCOA results are shown for specimens from two field release sites, one near Kingsville, TX, and a second near Encino, TX (Figure 9c). The hybridization status of these populations was questioned after it was established that hybridization was occurring in some of the

Table 2. Adult survival during hybridization experiments[a] in which *Diorhabda carinulata* was crossed with the other three *Diorhabda* species.[b]

Species crossed[c]	Male survival [d]	Female survival[e]
♂ × ♀	% Survival (number alive/total)	% Survival (number alive/total)
D. carinulata × *D. carinulata*		100% (7/7)
D carinata × *D. carinata*		100% (7/7)
D. carinulata × *D. carinata*		71% (5/7)
D. carinata × *D. carinulata*		9% (1/11)
D. carinulata × *D. carinulata*		86% (6/7)
D. elongata × *D. elongata*		86% (6/7)
D. carinulata × *D. elongata*		100% (7/7)
D. elongata × *D. carinulata*		0% (0/16)
D. carinulata × *D. carinulata*	100% (10/10)	100% (10/10)
D. sublineata × *D. sublineata*	100% (10/10)	100% (10/10)
D. carinulata × *D. sublineata*	90% (9/10)	70% (7/10)
D. sublineata × *D. carinulata*	91% (10/11)	28% (8/29)

[a] Beetles were paired and eggs collected until 50 eggs were collected per pair. Beetles were scored as alive after 50 eggs had been laid per pair.

[b] Each set of crosses was a separate experiment; seven pairs were used in crosses with *D. carinata* and *D. elongata*, 10 pairs were used in crosses with *D. sublineata*

[c] All *D. elongata* in this experiment were the Possidi ecotype; *D. carinulata* were the Fukang ecotype.

[d] Male survival was not scored except in the crosses with *D. sublineata.*

[e] Additional females were added to the experiments if more eggs were needed in order to produce hybrids in the F_1 generation. This is why the total number of females is higher in the test groups where high mortality was scored.

laboratory colonies. The PCOA scores for the Kingsville population are generally within the PCOA score range of the Crete ecotype of *D. elongata.* Four specimens from the Kingsville population were identified as *D. elongata* based on morphology (J. Tracy, personal communication). Two specimens identified morphologically as *D. elongata* had PCOA axis 2 scores lower than other Crete ecotype specimens but in general there is little evidence of hybridization in the Kingsville population. PCOA scores for the specimens from the Encino population are closer to those of known F_1 hybrid specimens than to either *D. sublineata* or *D. elongata*.

Discussion

Members of the *Diorhabda* species complex currently used in the North American *Tamarix* biological control program separate into four distinct clades based on AFLP analysis of genomic DNA (Figure 3). These results support the conclusions of Tracy and Robbins (2009) who have divided the *Diorhabda* species complex into five related species, four of which are used in the *Tamarix* biological control program. Mitochondrial COI sequence analysis yielded five clades instead of four, with *D. elongata* separating into two groups: one from eastern Greece and Crete and the other from southern and western Greece (Figure 2). Incongruence between nuclear- and mitochondrial-based DNA phylogenies is not unusual (e.g., Sota and Vogler 2001) and can be traced to differences in DNA metabolism and inheritance patterns between the two genomes (Ballard and Whitlock 2004; Moore 1995; Rubinoff and Holland 2005). Because the incongruence between mitochondrial and genomic DNA is relatively minor (see Sota and Vogler 2001 for an example of a major incongruence in a beetle), we conclude that the molecular genetic data strongly support the morphological data.

There are no apparent genetic barriers between ecotypes of *D. carinulata* (Table 1) or between ecotypes of *D. elongata*, and molecular genetic analysis did not provide delineation of these ecotypes within species. This is evidence that the Chilik ecotype of *D. carinulata* will be compatible with the Fukang ecotype of that species as they meet across wide boundaries in the western United States. This is the only case in which two ecotypes of the same species are currently being used in the tamarisk biological control program, although the Crete and Possidi ecotypes of *D. elongata* have both been released in the field. The Crete ecotype is well established but the Possidi ecotype apparently failed to establish, although beetles overwintered and a small population persisted for 3 yr following release (Tracy and Robbins 2009).

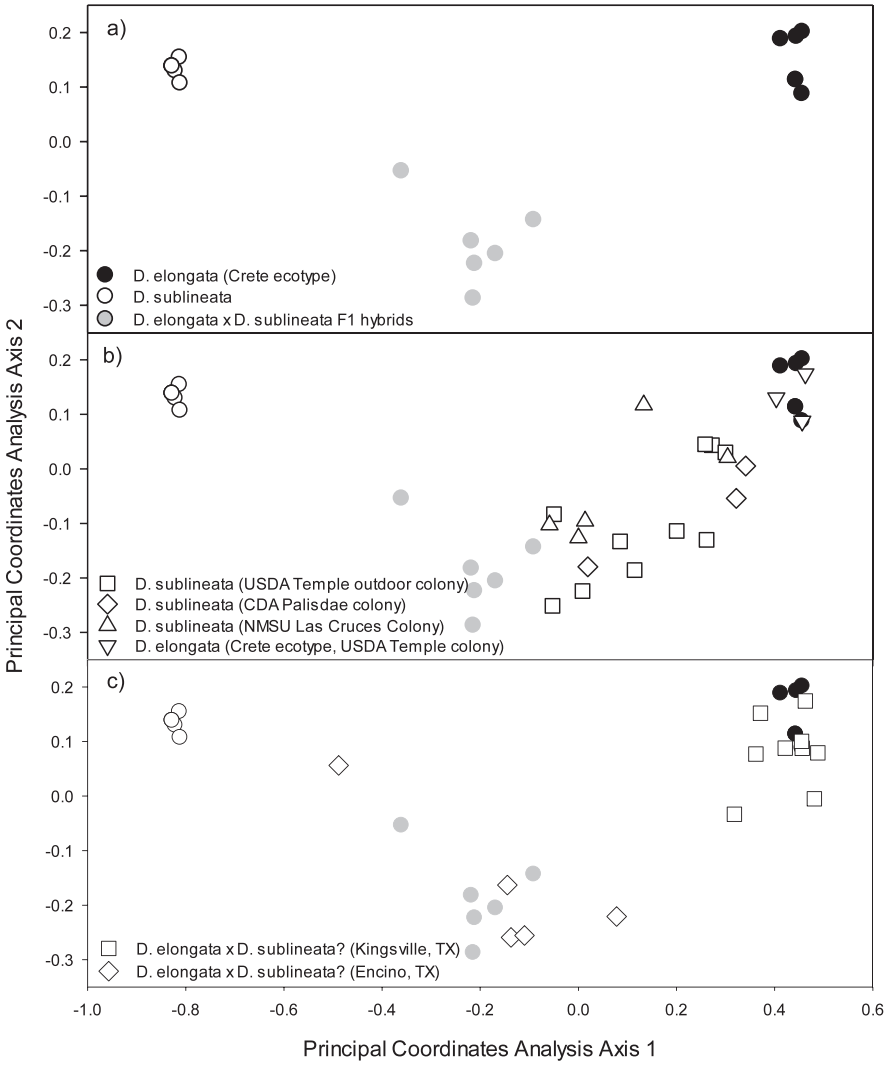

Figure 9. Principal coordinates analysis of amplified fragment length polymorphism data from 57 *Diorhabda* specimens including the following: (a) *Diorhabda elongata* (Crete ecotype), *Diorhabda sublineata*, and known *D. elongata* × *D. sublineata* F_1 hybrids; (b) inclusion of specimens from laboratory colonies including *D. elongata* (Crete ecotype) from the U.S. Department of Agriculture Agricultural Research Service Grassland Soil and Water Research Laboratory quarantine facility, Temple, TX (USDA Temple) and *D. sublineata* from the USDA Temple facility, the Colorado Department of Agriculture's Palisade Insectary,, and the New Mexico State University Entomology, Plant Pathology and Weed Science quarantine facility in Las Cruces, NM; and (c) inclusion of specimens collected in the field from release sites near the towns of Encino and Kingsville in southern Texas that were suspected to be *D. elongata* × *D. sublineata* F_1 hybrids.

Hybridization studies revealed the potential for gene flow between the four *Diorhabda* species, although in combinations involving *D. carinulata* there are substantial barriers to hybridization including F_1 male sterility (Figure 4), lethal mismatches in genitalia (Table 2; lock-and-key incompatibility, see Shapiro and Porter 1989), and low F_2 and BC_1 egg viability (Figures 4–6). Crosses between *D. carinulata* and *D. elongata* resulted in male sterility in the F_1 interspecific hybrids (Figure 4; data not shown for the Crete ecotype of *D. elongata*). Male sterility is a strong but not absolute barrier to hybridization in the field as hybrid females were capable of producing a low percentage of viable eggs when backcrossed into the parental strains (Figure 4) and it has been shown in other beetle species that male hybrid sterility does not completely eliminate hybridization and interspecific genetic introgression in natural populations (Brouat et al. 2006; Streiff et al. 2005).

The genital mismatches noted between *D. carinulata* and the other three species resulted in high mortality in females (Table 2). These results are consistent with the genital lock-and-key hypothesis, which states that rapidly evolving

genital structures enforce genetic isolation between closely related species. The hypothesis is compelling because rapid evolutionary divergence is often observed in genital structures (Shapiro and Porter 1989), including within the *Diorhabda* species complex (Tracy and Robbins 2009). Nonetheless, examples of lock-and-key mismatch are rare (Gröning and Hochkirch 2008; Shapiro and Porter 1989;). One example was documented in the beetle genus *Ohomopterus* (Nagata et al. 2007; Sota and Kubota 1998) and *Diorhabda* may provide a second example in a beetle. It has been noted that uncoupling of mating pairs may not occur when *D. carinulata* are crossed with the other species (Tracy and Robbins 2009) but the nature of the physical damage has yet to be shown, as it was in *Ohomopterus* (Sota and Kubota 1998).

Crosses between *D. carinulata* and the other three species resulted in significantly decreased F_2 and BC_1 egg viability ($P < 0.0001$). This, as well as male hybrid sterility and genital incompatibility, will limit gene flow between *D. carinulata* and the other three species. The importance of these observations is magnified by the widespread distribution of *D. carinulata* in North America and the high likelihood that this species will eventually come into contact with *D. elongata* and *D. sublineata* in the field.

In the three crosses involving *D. sublineata*, *D. elongata*, and *D. carinata* we found no evidence of male sterility or genital mismatch, and these crosses yielded higher-viability F_2 eggs, and BC_1 eggs in the case of *D. sublineata* and *D. elongata* (Figure 7), than in the three crosses involving *D. carinulata* (Figures 4–6). These results point to a greater likelihood of hybridization in the field for these species combinations (Figure 8). *Diorhabda sublineata* × *D. elongata* showed a significantly reduced BC_1 egg hatch ($P < 0.0001$) when hybrid males were backcrossed into the parental stains (Figure 7). In spite of this, stable hybrid cultures of *D. sublineata* × *D. elongata* were established and maintained for four generations under laboratory conditions (data not shown) and genetic introgression occurred under the artificial conditions experienced in the laboratory and in field cages, with interesting patterns revealed by AFLP analysis (Figure 9). For instance, the PCOA scores of the apparent hybrids in Figure 9b are intermediate to the known F_1 hybrids and the Crete ecotype of *D. elongata*, suggesting two things. First, hybridization may have been ongoing for two or more generations. Second, the Crete ecotype has the more "dominant" genome. This could occur through selection in hybrid colonies for traits of the Crete ecotype that favor its persistence in colonies, more frequent invasion of the colonies by the Crete ecotype, or a higher rate of backcrossing of hybrids to the Crete ecotype rather than *D. sublineata*.

These data indicated that the colonies of *D. sublineata* were a mixture of *D. sublineata* and *D. elongata*. Subsequent to these studies all laboratory and field colonies of *D. sublineata* were destroyed and new collections were made from Sfax, Tunisia, in the spring of 2008. All *D. sublineata* used after 2008 for field releases and for laboratory studies were derived from beetles collected in 2008.

In the native range of *Diorhabda* there are regions where two or more species are sympatric or parapatric (Figure 1) yet intermediate forms have not been found, in spite of extensive examination of museum and freshly collected specimens (Tracy and Robbins 2009). In some cases where species are parapatric or narrowly sympatric there could be relatively small and undetected hybrid zones. In the case of *D. carinulata* and *D. carinata* there is a vast region in western Asia where they are sympatric and where both species have been collected in close proximity, even from the same plant (Tracy and Robbins 2009), yet no hybrids have been detected. The behavioral, mechanical, and genetic barriers appear to be sufficient to maintain species integrity.

Prezygotic isolation mechanisms may prevent hybridization in the native ranges of the *Diorhabda* species complex and these could also function to keep the species distinct in North America. *Diorhabda carinulata* utilizes a male-produced aggregation pheromone consisting of two components released in a ratio of approximately 1 : 1 (Cossé et al. 2005). The other three species have the same components, released in different ratios and amounts (R. Bartelt and A. Cossé, personal communication) which could result in species sorting during aggregation and mating (for a more detailed discussion of reproductive isolation via differences in aggregation pheromone see Tracy and Robbins 2009). In addition there are probably other chemical, visual, and tactile cues that bring about pair formation (Cossé et al. 2005) and could act as barriers between species in the field. Such barriers would probably not be detected in our experiments because artificial conditions are well known to interfere with prezygotic isolation mechanisms (Gröning and Hochkirch 2008).

Hybrids in general are considered to be less fit because the genetic combinations that result from hybridization have not been subject to natural selection and evolution (Barton 2001). However, in new ecological settings where parental species could be at a disadvantage, hybrids may provide a rich source of novel genetic combinations for natural selection (Seehausen 2004). The *Diorhabda* species complex and the new ecological settings they encounter in western North America may provide ideal starting material for hybridization, hybrid zones, and gene flow. Hybrid zones are known to form between beetle congeners (Nagata et al. 2007; Sota 2002), including chrysomelids (Gatto et al. 2008; Peterson et al. 2005), and these may (Sota 2002; Sota and Vogler 2001) or may not (Peterson et al. 2005) result in genetic introgression. In the case of the *Diorhabda*

species complex it is probable that there will be formation of hybrids in the field and that their fate will be a function of selection for new genetic combinations.

Hybridization and gene flow between *Diorhabda* species could result in new and stable genetic combinations in North America as has been documented with the host plant genus *Tamarix*. *Tamarix ramosissima* and *T. chinensis* don't hybridize in the native range, yet they form hybrids in North America (Gaskin and Schaal 2002, 2003) with introgression into the parental strains resulting in mixed genomes (Gaskin and Kazmer 2009). *Tamarix chinensis* contributes more heavily to the genetic composition of plants in the southern range of the North American distribution whereas *T. ramosissima* contributes more heavily to the genetic composition of plants in the northern range of the invasion (Gaskin and Kazmer 2009). This pattern may be an artifact of founding events, or may have arisen from differential natural selection across a latitudinal gradient (Friedman et al. 2008; Gaskin and Kazmer 2009), which could also happen with *Diorhabda* as the newly released species adapt to ecological settings very different from those in the native range.

The revision of the *Diorhabda* species complex based on morphological (Tracy and Robbins 2009) and now molecular traits has helped provide a biological basis for host specificity (Dalin et al. 2009; Herr et al. 2009) and other developmental and physiological differences among *Diorhabda* species used in the tamarisk biological control program (Dalin et al. 2010). Molecular methods will now be essential to track hybridization and introgression in the field. As shown in this study (Figure 9), AFLP analysis can be used to detect hybridization and introgression between *D. sublineata* and *D. elongata*, which will be important because both species have been introduced in Texas and will soon become sympatric in at least one area along the Rio Grande (C. Ritzi, personal communication). It will be instructive to see if they hybridize in the field, to what extent introgression occurs, and if beetles with new combinations of genes perform better in novel ecological settings. There is a need for molecular protocols that can be used to follow hybridization and new genetic combinations in the field within the *Diorhabda* species complex. Although *D. sublineata* × *D. elongata* was the most critical and timely of the possible crosses to characterize at the molecular level, the others may eventually become of equal or greater importance as populations expand and species meet in the field. Information on genetic composition and field performance within the *Diorhabda* species complex is a necessary component of the tamarisk biological control program.

Molecular analysis should have been incorporated early in the development of *Diorhabda* as a potential biological control agent for tamarisk. Clarification of the relationships between ecotypes, including the discovery of potential cryptic species, would have allowed a more targeted approach to agent development, including host range testing, and a more informed evaluation of other biological parameters. As it was, each ecotype was tested for host range (Herr et al. 2009; Milbrath and DeLoach 2006a, b) but this was done as a safety precaution and not with the knowledge that *Diorhabda elongata* was in fact a species complex. Molecular genetic analysis has become critical in decision-making within biological control programs (e.g., Madeira et al. 2001, 2006; Rauth et al. 2011) and this study is another example of the benefits that molecular genetic analysis can provide to biological control programs.

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