Utility of Morphological and Molecular Techniques for Determination of Paternity in Two Subspecies of *Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae)[1]

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Abstract An experiment was conducted to determine the paternity of F_1 progeny using morphological and molecular methods in *Diabrotica* (Coleoptera: Chrysomelidae) subspecies: *Diabrotica undecimpunctata howardi* Barber, also known as spotted cucumber beetle, and *D. undecimpunctata undecimpunctata* Mannerheim, also known as western cucumber spotted beetle. Results from crosses that involved the females of *D. u. howardi* and the males of *D. u. undecimpunctata* had all F_1 progeny with phenotypes as the male parent. Similarly, in all the crosses that involved the females of *D. u. undecimpunctata* and the males of the *D. u. howardi*, all the F_1 progeny had phenotypes as the male parent. DNA from females and males were amplified using two primers to confirm the paternity of F_1 progeny. The study on the inheritance of body color in these two subspecies appeared to be governed by one dominant gene (monogenic), and it is the male that determines the body color of progeny in both subspecies.

Key Words *Diabrotica* subspecies, body color, marker, inheritance

Diabrotica undecimpunctata is taxonomically divided into a complex of at least 4 described subspecies (Smith and Lawrence 1967, Krysan 1986) with each being separated by geographical range and morphological characters (i.e., color). For example, coloration and patterns on the elytra of southern corn rootworm, *D. u.* subsp. *howardi,* and western spotted cucumber beetle, *D. u. undecimpunctata*, are the same whereas the former has a black abdomen and latter has a yellow abdomen. While such phenotypic observations are common for many insect species (and subspecies), they can have important utility if their modes of inheritance are known making it possible to study topics such as mating patterns, mating frequency, mate choice, and paternity. For example, Lu and Logan (1994) used body color as a marker to study color inheritance in *Leptinotarsa decemlineata* Say.

In addition to phenotypic characters, molecular techniques such as allozyme and DNA markers have proven valuable in genotypic studies of paternity determination; Huettel et al. (1976) studied paternity in plum curculio, *Conotrechelus nenuphar* Herbst (Coleoptera: Curculionidae), using allozyme markers. DNA methods, such as

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random amplified polymorphic DNA (RAPD) markers that consist of relatively short DNA fragments, also have proven useful in paternity inheritance studies (Huettel et al. 1976). An advantage of the RAPD technique is that it requires no foreknowledge or any specific gene in a target taxon, and it is random with respect to the genome (Hoy 1990). Hadrys et al. (1993) first demonstrated the usefulness of RAPD for analyzing paternity in the dragonfly, *Anax parthenope* Sélys (Odonata: Aeshnidae). Also, Achmann et al. (1992) and Hooper and Siva-Jothy (1996) used RAPD-PCR for determining paternity in bush cricket, *Poecillimon veluchianus* Fieber (Orthoptera: Tettigonidae).

In the current study, we used body color a morphological trait and molecular techniques to determine paternity in two subspecies of *D. u. undecimpunctata*. The mode of inheritance of the body color also was studied.

Materials and Methods

Insect cultures. *Diabrotica u. howardi* colony originated from beetles collected from the University of Nebraska-Lincoln, Agricultural Research Development Center farm near Ithaca, NE (Saunders Co). The *D. u. undecimpunctata* colony was provided by Syngenta Corp. (formerly Zeneca Corp.) with beetles originating near Richmond, CA (Alameda Co). Beetles (both subspecies) were maintained in environmental growth chambers at 27°C and at a relative humidity between 30-60%, in cages measuring 28 × 28 cm, at 16:8 [L:D]. Standard *Diabrotica* rearing techniques were used throughout the study (Cuthbert et al.1968, Branson et al. 1988, Tallamy and Pesek 1996); larvae were reared on seedlings of maize and beetles on lettuce, *Lactuca sativa* L., and fresh sweet maize ears. To reduce disease incidence, insect cages were washed every other day with detergent and a 1% sodium hypochlorite solution.

Insect crosses. Five crosses, of each of the following categories were done:

(1) WSCB♀ × WSCB♂; (2) WSCB♀ × SCB♂;

(3) SCB♀ × SCB♂; (4) SCB♀ × WSCB♂.

Each male and each female were placed in a small plastic oviposition box (Gray Plastic Packing Co, Bronx, NY), a modification of the Boetel and Fuller (1997) technique, at 16:8 [L:D] photoperiod and 27°C with moistened silt loam soil that had been sieved through a #60 mesh sieve and autoclaved at 250°C for 30 min. The soil was moistened to near saturation and scarified to serve as an oviposition substrate. After oviposition, eggs were removed from the soil once a week by washing (in water at room temperature) the mixture into a 60-mesh sieve and then washing onto moistened filter paper that was then placed in a Petri dish (14 × 2 cm). A 2% benomyl solution was sprinkled over the eggs to inhibit fungal contaminations.

DNA extraction and RAPD-PCR amplification. DNA was isolated from parents and 20 F_1 individuals that resulted from the crosses listed. DNA was extracted from the thorax of individual beetles using a modified protocol of Black and DuTeau (1997) CTAB (hexadecyltrimethylammonium bromide) extraction as described by Clark et al. (2001).

Reagents used for polymerase chain reaction, except primers, were obtained from Applied Biosystems (Foster City, CA). Five samples of the two *Diabrotica* subspecies,

randomly selected from the colony, were used for the initial screening of 20 arbitrary primers (Operon Technologies, Inc., Alameda, CA) (Table 4). Optimization of reaction was achieved by following the methods described by Pornkulwat et al. (1998) with the optimized protocol being reported here in. Individual RAPD-PCR reactions were conducted in 25-µL volumes (for each primer) with the following reagents: 12.2 µL sterile distilled water, 2.5 µL 10X Stoffel buffer, 3 µL of 10 mM dNTPs (2.5 mM of each dATP, dCTP, dGTP, dTTP), 1 µL of the appropriate 10-mer primer, 1 µL of 1% Nonidet P-40, 0.3 µL of AmpliTaq®DNA Polymerase Stoffel fragment (Applied Biosystems, Foster City, CA), 4 µL of 25 mM $MgCl_2$ and 1 µL of DNA template. For DNA polymerase chain reaction, a negative control that consisted of water was included to check for potential contamination. The polymerase chain reaction was conducted using the following temperature profile: an initial denaturing at 95°C for 5 min, then 10 cycles at 94°C for 1 min, 36°C for 30 sec, and 72°C for 1 min, followed by 30 cycles of 94°C for 10 sec, 35°C for 30 sec, and 72°C for 30 s; and a final extension step of 72°C for 5 min. All RAPD-PCRs were performed using a Perkin-Elmer Gene Amp® System 2400 (Perkin-Elmer, Branchburg, NJ). Upon completion of the PCR reaction, 8 µL of the PCR product and 2 µL dye (bromophenol) (including a negative control) was loaded on 2% Ultrapure Agarose Gel (Gibco-BRL, Gaithersburg, MD), DNA molecular size standards (1 kb ladder and 100 ladder bp), were added to the outside two wells of each gel, and electrophoresed at 60 V for 4 h on a Horizon 20-25 electrophoresis chamber. After electrophoresis, each gel was stained in ethidium bromide (10ng/ml) visualized over a UV transilluminator, and scanned into the Advanced Quantifier gel documentation program (Genomic Solutions, Ann Arbor, MI) and, thus, estimates of size relative to known size standards were determined. Primers that generated 6 or more bands with complex banding patterns were eliminated. Of the 20 arbitrary primers screened, only 2 arbitrary primers (OPH 11 and OPH 17 primer sets, Table 4) produced clear, consistent, discrete and reproducible 4 RAPD markers. These were selected for further examination. When the screening process was repeated, the same banding patterns were produced by 2 arbitrary primers. The 2 primers were

Table 1. Color of some adult morphological characters of *D. undecimpunctata undecimpunctata* and *D. undecimpunctata howardi*

	D. u. undecimpunctata	*D. u. howardi*
Antennae	First three segments tan remaining nine segments black	First three segments white remaining nine segments
Pronotum		
dorsal	Yellow	Yellow
ventral	Black	Yellow
Abdomen	Black	Yellow
Legs		
coxa	Black	Greenish-yellow
trochanter	Black	Greenish-yellow
femur	Black	Half greenish-yellow and half black
tibia	Black	Black
tarsus	Black	Black

used for determining body color of the F_1 progenies of the *Diabrotica* subspecies and run on the same gel.

Body color analysis. Body color of individuals for each cross was established from RAPD fingerprinting profiles of (OPH 11 and OPH 17 primer sets, Table 4) by comparing shared RAPD markers between F_1 progeny (20 individuals), and the male parents. Bands of identical molecular weight were considered as the same allele at that locus between individuals. The absence of a band represents the recessive genotype at the locus and also was scored in the analysis of genetic similarity (Black 1993). The proportion of matches, M (the shared presence/ absence of a band) was estimated from: $M = N_{ab}/N_T$ where N_{ab} is the total number of matches in individuals a and b and N_T is the total number of bands scored in the study (Black 1993). Individuals are identical (have identical banding patterns) when $M = 1$, and are completely dissimilar, share no bands, when $M = 0$. Amplified bands were scored directly from digitized images of gels using the Advanced Quantifier program (Genomic Solutions, Ann Arbor, MI).

Inheritance of body color. Five crosses of the following (i.e., individual crosses between 5 females and 5 males) were made: (1) *D. u. undecimpunctata* ♀ × *D. u. howardi* ♂ (2) *D. u. howardi* ♀ × *D. u. undecimpunctata* ♂ (Fig. 1). Females were placed with the respective males for 48 h. Each female was then placed in a small oviposition box as described in the insect culture section (Boetel and Fuller 1997), and beetles were fed as described in the insect culture section. The goodness of fit of segregation ratios were measured with chi-square (X^2) analysis of the resulting ratios was compared with a 3:1 Mendelian ratio.

Results

Body color and morphological characters as markers. Copulation was observed for all crosses. Based on abdomen and leg color patterns, all of the F_1 progeny had the color of the male parent in all the crosses (Tables 2, 3, Fig. 2).

In crosses between *D. u. undecimpunctata* females and males, the abdominal color of all the F_1 progeny was black ($n = 20$), whereas in the crosses between spotted cucumber females and spotted cucumber males, the abdominal color of all the progeny was yellow ($n = 20$). However, when the females of *D. u. undecimpunctata* mated with the males of *D. u. howardi,* the abdominal color of all the progeny was yellow. When the females of *D. u. howardi* mated with *D. u. undecimpunctata* males, the abdominal color of all the progeny was black ($n = 20$) (Table 3). In all the crosses, F_1 progenies had leg colors identical to their male parents (Table 3).

Initial RAPD primer screening and body color analysis. The two arbitrary primers OPH-11 and OPH-17 (Table 4) that produced clear, consistent, discrete and reproducible RAPD markers were used for determining paternity in the two subspecies of *Diabrotica*.

Polymerase chain reactions with RAPD primers OPH-11and OPH-17 yielded a series of repeatable discrete bands that were used to determine the paternity of the 20 F_1 progeny from each cross. In all the crosses, the male determined the body color of all the progeny. The bands generated by primers OPH-11 and OPH-17 in all the parents and F_1 progeny ranged from 150-700 bp (Tables 5, 6). Within a cross, parental RAPD profiles matched corresponding progeny profiles (Tables 5, 6). For example, using primer OPH-11, the presence of ~550, and ~450, bp products were used to determine body color of the progeny (Table 5). Similarly, using primer OPH-

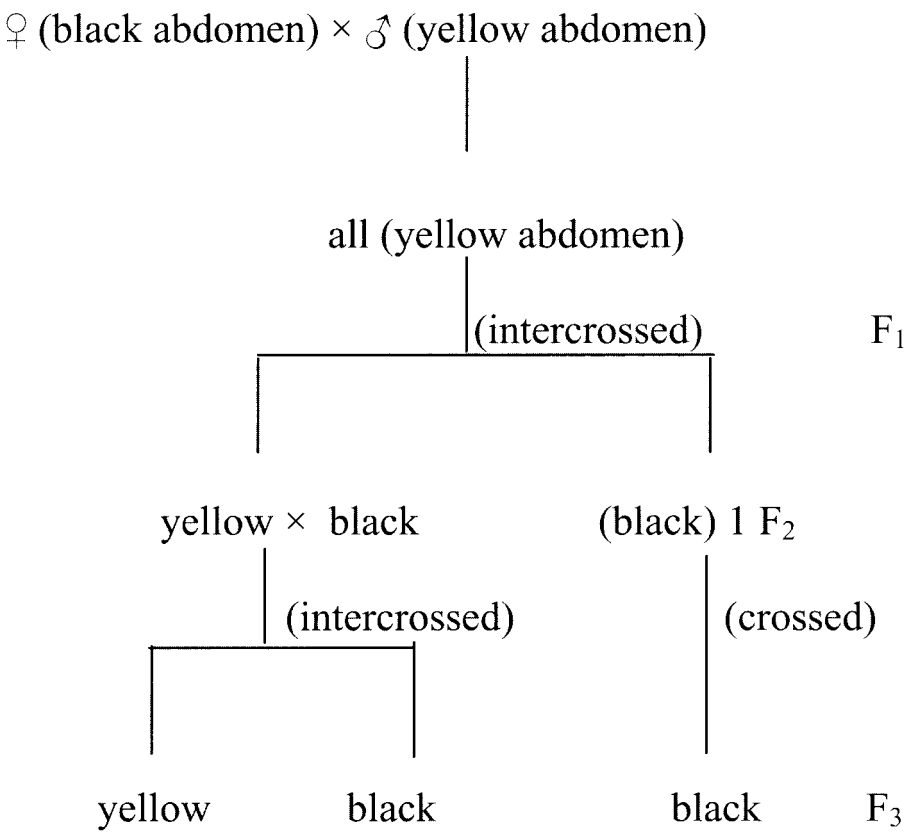

Fig. 1. Mating scheme used for studying inheritance of body color in two *Diabrotica* subspecies. (black = *D. u. undecimpunctata*, yellow = *D. u. howardi*).

17, presence of ~700, and ~570, bp products were used for determining body color of the progeny (Table 6). Analysis of band matches between fathers and the resultant F_1 progeny revealed $M = 1$ in matches between the male and the F_1 progeny. This indicated that the male determines the body color of progeny in crosses.

Inheritance of body color. The cross between *D. u. undecimpunctata* females and *D. u. howardi* males resulted in the following F_1 progeny ratio: 75 beetles with the color of selected morphological characters as in spotted cucumber to 0 beetles with color of selected morphological characters as in *D. u. undecimpunctata* (Table 4). The cross between the spotted cucumber females and western cucumber males resulted in the following F_1 progeny: 55 beetles with color of selected morphological characters as in *D. u. undecimpunctata* to 0 beetles with color of selected morphological characters as in *D. u. howardi* (Table 4).

F_2 progeny from the mating of F_1 beetles that resulted from parental crosses between *D. u. undecimpunctata* females and spotted cucumber males segregated into 48 beetles with color of selected morphological characters as in *D. u. howardi* and 14 beetles with morphological characters as in *D. u. undecimpunctata* (Table 7). This

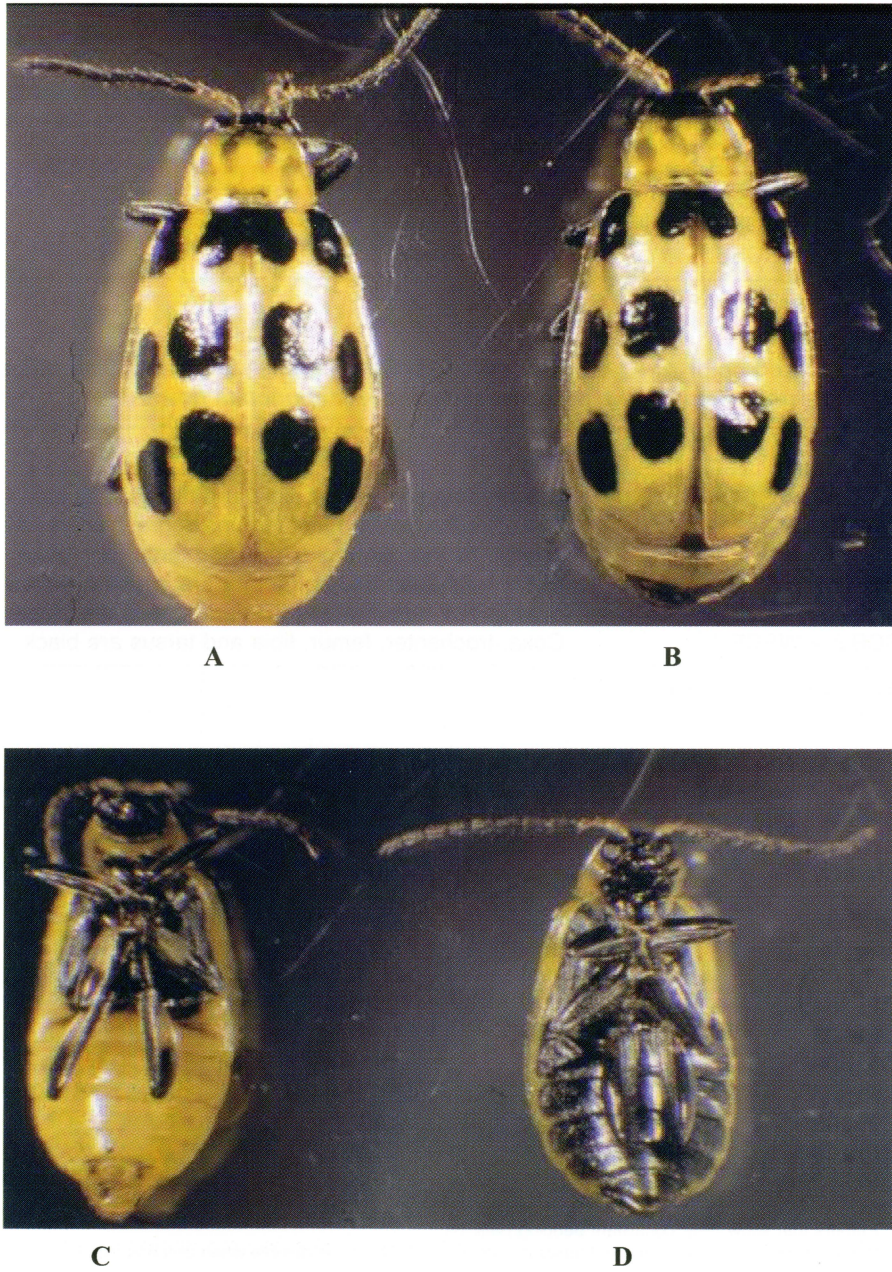

Fig. 2. (**A** and **B**) Adults, *D. u. howardi* (left), and *D. u. undecimpunctata* (right), showing the dorsal portion of the body. (C and D) Adults, *D. u. howardi* (left), and *D. u. undecimpunctata* (right), showing the ventral portion of the body (Photo courtesy of Pete L. Clark).

Table 2. Abdomen color of F_1 progeny resulting from crosses involving *Diabrotica undecimpunctata undecimpunctata* and *Diabrotica undecimpunctata howardi*

Crosses	Abdomen color F_1 progeny	F_1 designation
WSCB♀ × WSCB♂	Black	WSCB
SCB♀ × SCB♂	Yellow	SCB
WSCB♀ × SCB♂	Yellow	SCB
SCB♀ × WSCB♂	Black	WSCB

N = 20 beetles for each cross

Table 3. Leg color of F_1 progeny resulting from crosses involving *Diabrotica undecimpunctata* and *Diabrotica undecimpunctata howardi*

Crosses	Leg color of F_1 progeny
WSCB♀ × WSCB♂	Coxa, trochanter, femur, tibia and tarsus are black
SCB♀ × SCB♂	Coxa, trochanter and half femur are greenish-yellow while half femur, entire tibia and tarsus are black
WSCB♀ × SCB♂	Coxa, trochanter and half femur are greenish-yellow while half femur, entire tibia and tarsus are black
SCB♀ × WSCB♂	Coxa, trochanter, femur, tibia and tarsus are black

N = 20 beetles for each cross

Table 4. The 20 arbitrary 10-mer primers used to determine genetic markers for body color of F_1 progenies from crosses of *Diabrotica undecimpunctata undecimpunctata* and *Diabrotica undecimpunctata howardi*

Primer	Sequence	Primer	Sequence
OPH-01	GGTCGGAGAA	OPH-11**	CTTCCGCAGT
OPH-02	TCGGACGTGA	OPH-12	ACGCGCATGT
OPH-03	AGACGTCCAC	OPH-13	GACGCCACAC
OPH-04	GGAAGTCGCC	OPH-14*	ACCAGGTTGG
OPH-05	ACTCGTCCCC	OPH-15	AATGGCGCAG
OPH-06	ACGCATCGTG	OPH-16	TCTCAGCTGG
OPH-07	CTGCATCGTG	OPH-17**	CACTCTCCTC
OPH-08	GAAACACCCC	OPH-18	GAATCGGCCA
OPH-09	TGTAGCTGGG	OPH-19*	CTGACCAGCC
OPH-10	CCTACGTCAG	OPH-20	GGGAGACATC

* Primers that show clear consistent banding patterns

** Primers that show clear consistent banding patterns that were reproducible when process repeated.

segregation fits the ratio 3:1 (X^2 = 0.193), indicating monogenic dominance of each gene for body color and the other selected morphological characters.

F_2 progeny from the F_1 (from parental crosses of spotted cucumber females and *D. u. undecimpunctata* males) segregated into 37 beetles with the color of selected

Table 5. The number of and size (bp) of RAPD bands using OPH-11, found in parents and twenty F_1 progenies crosses between *Diabrotica undecimpunctata undecimpunctata* and *Diabrotica undecimpunctata howardi*

RAPD marker (bp)[1]	Parents		F_1 progeny
	*black♀ × black♂		
550	0	550	550
350	350	0	0
150	150	150	150
	black♀ × **yellow♂		
450	0	450	450
350	350	0	0
150	150	150	150
	yellow♀ × yellow♂		
450	0	450	450
400	400	0	0
150	150	150	150
	yellow♀ × black♂		
550	0	550	550
400	400	0	0
150	150	150	150

[1] All bands used in paternity analysis.
* black = *D. u. undecimpunctata*
** yellow = *D. u. howardi*

morphological characters as in *D. u. undecimpunctata*, and 11 beetles with color of selected morphological characters as in *D. u. howardi* (Table 5). This segregation fits the ratio of 3:1 ($X^2 = 0.111$) indicating monogenic dominance of color of morphological characters as in *D. u. undecimpunctata* over the color and selected morphological characters as in *D. u. howardi* (Table 7). The F_2 progeny from the above parental crosses showed inheritance patterns typically expected of a dominant genetic trait.

Discussion

Results from this study demonstrate that selected morphological characters such as body color can be used as paternal markers in *D. u. undecimpunctata* and *D. u. howardi* as all subspecific crosses resulted in progeny that had body color patterns that were identical to the male parent. When the F_1 progeny of the initial subspecific crosses were taken to the F_2 and F_3 color patterns in these 2 *Diabrotica* subspecies fit the Mendelian 3:1 ratio, implying that both yellow and black coloration is controlled by one major gene that is dominant. This was further evidenced by a lack of resulting progeny having an intermediate color pattern in any subsequent generation (F_1, F_2 or F_3). However, because the observed 3:1 Mendelian ratio in the F_3 population was different from the expected theoretical ratio of 4:1, we speculate that the inheritance of body color could be more complex as has been observed in other chrysomelid species. Lu and Logan (1994) reported that two loci with epistasis controlled larval body color. They also observed that the male in all the crosses determined the color

Table 6. The number of and size (bp) of RAPD bands using OPH-17, found in parents and twenty F_1 progenies in crosses *Diabrotica undecimpunctata undecimpunctata* and *Diabrotica undecimpunctata howardi*

RAPD marker (bp)[1]	Parents		F_1 Progeny
	*black♀ × black♂		
700	0	700	700
550	550	0	0
150	150	150	150
	black♀ × **yellow♂		
570	0	570	570
550	550	0	0
150	150	150	150
	yellow♀ × yellow♂		
570	0	570	570
450	450	0	0
160	150	150	150
	yellow♀ × black♂		
700	0	700	700
450	450	0	0
180	150	150	150

[1] All bands used determining color.
* black = *D. u. undecimpunctata*
** yellow = *D. u. howardi*

Table 7. Segregation ratio of body color in F_1 and F_2 progeny in crosses involving adults of *Diabrotica undecimpunctata undecimpunctata* and *Diabrotica undecimpunctata howardi*

Parents crosses	F_1		F_2 observed		F_2 expected			
♀ × ♂	B[1]	Y[2]	B	Y	B:Y	X^2	df	P[3]
black × yellow	0	75	14	48	(1:3)	0.193	1	0.66*
yellow × black	55	0	37	11	(3:1)	0.111	1	0.74*
Parents crosses	F_2		F_3 observed		F_3 expected			
♀ × ♂	B[1]	Y[2]	B	Y	B:Y	X^2	df	P[3]
black × yellow	14	48	11	35	(1:3)	0.029	1	0.86*
yellow × black	37	11	25	7	(3:1)	0.167	1	0.68*

[1] B (Black) *D. u. undecimpunctata*
[2] Y (Yellow) *D. u. howardi*
[3] *Cannot reject

of the larvae. These findings support our results that male determines the color of its progeny in these beetles.

Similar results have been observed in other insect species. For example, Boiteau (1988) used body color to document sperm precedence and ultimately paternity in *L.*

decemlineata, where F_1 progeny had similar body color as their second male parent, while Eady (1991) observed similar results for *C. maculatus* F.

Our examination of paternity using RAPD markers confirmed observations that the male determines body color in our subspecies crosses as has been reported in other studies (Hadrys et al. 1993, Hooper and Siva-Jothy 1996). Whereas other molecular methods (AFLP, Microsatellites, etc.) may be more appropriate for large population-based studies, the RAPD technique proved useful in our study where we examined controlled matings between the *D. undecimpunctata* subspecies. Similar studies on other *Diabrotica* species, such as economically important *D. v. virgifera*, could provide answers to biological questions such as mate choice, paternity, sperm precedence, and mating frequency.

Answers to these questions could prove important as *D. v. virgifera* is targeted for control by Bt transgenics and maintaining susceptibility to these transgenics is important for management of this technology. Therefore, a similar study using selected *D. v. virgifera* color morphs or populations could be initiated to examine the mating biology in these species where results could provide valuable insights into resistant questions.

In conclusion, we demonstrated that inheritance of color of selected morphological characters is paternally inherited in two subspecies of *D. undecimpunctata* and that this inheritance can be confirmed using molecular techniques such as RAPD. Similar investigations on other species, subspecies or populations may provide important insights into the mating of economically important pests.

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