

Genetic Differentiation of Southeastern Boll Weevil and Thurberia Weevil Populations of *Anthonomus grandis* (Coleoptera: Curculionidae) Using Mitochondrial DNA

RICHARD L. ROEHRDANZ¹

Biosciences Research Laboratory, Red River Valley Agricultural Research Center, USDA-ARS, Fargo, ND 58105

Ann. Entomol. Soc. Am. 94(6): 928-935 (2001)

ABSTRACT The southeastern boll weevil, the Mexican boll weevil, and the thurberia weevil are considered to be morphologically similar but behaviorally different variants of the same species, *Anthonomus grandis* Boheman. A polymerase chain reaction (PCR)-amplified 9.2-kb section of the mitochondrial DNA was cleaved with restriction enzymes. RFLPs of weevils from three cotton growing locations in Texas and one in northeastern Mexico were compared with thurberia weevil from three sites in Arizona. Six haplotypes were observed in the Texas/Mexico collections and 12 haplotypes were found among the thurberia weevil. There were no shared haplotypes between these two groups. Polymorphism was observed within the weevil types. The three thurberia weevil locations exhibit some geographic isolation and exhibit differences in both the haplotypes present and the relative frequencies of the haplotypes. Only one haplotype was recovered at all three Arizona sites. The Texas/Mexico samples showed less genetic variability with the northern most site having the lowest polymorphism. 52/53 of these weevils appear to be genetically southeastern boll weevil. Two haplotypes were shared by all four of these populations and comprised 72% of the insects examined. The range of genetic distances between haplotypes was <0.001–0.022. The Mexican boll weevil was not explicitly examined; however, three individuals were discovered that appear to represent a genetically distinct third population. One was from Mexico and the other two were from a thurberia weevil site. These three individuals may represent the Mexican boll weevil. The results include apparent diagnostic restriction fragment differences between the thurberia weevil and the southeastern boll weevil that could be used to help determine whether future weevils found in Arizona or California cotton are thurberia weevil, southeastern boll weevil, or another population of weevils.

KEY WORDS boll weevil, thurberia weevil, Mexican boll weevil, mtDNA, PCR-RFLP, cotton

THE BOLL WEEVIL, *Anthonomus grandis* Boheman, is a serious pest of domestic cotton, causing fruit loss and boll damage. The insect is native to Mexico and Central America. According to the history of boll weevil dispersal by Burke et al. (1986), the first reports of the boll weevil north of the Rio Grande came from Texas as early as 1892. In subsequent years it spread eastward across the Cotton Belt to the Carolinas. The boll weevil in the United States north and east of the Rio Grande is a variant of *A. grandis* known as the southeastern boll weevil. Southeastern boll weevil may also extend into northeastern Mexico (Warner 1966, Burke et al. 1986). The boll weevil became a problem in the Arizona/California region in the 1960s and again in the 1980s. Both outbreaks were associated with the practice of growing stub cotton. In 1985 the Southwest Boll Weevil Eradication Program was established to coordinate eradication of boll weevil from southern California, western Arizona, and northwest Mexico. The number of boll weevils caught in pheromone traps in Arizona dropped from >4 million in 1988 to officially zero in 1992 (ACRPC 2000). The control program was

extended to the cotton-growing region of the Mexican state of Sonora lying directly south of Arizona. Constant monitoring is maintained to guard against reinfestation. Similar aggressive measures have also effectively eliminated the boll weevil from much of the southeastern United States (National Cotton Council 2000, USDA APHIS 2000).

The thurberia weevil is a variant of *A. grandis* that is native to the mountains of southern Arizona and parts of northwestern Mexico and uses wild thurberia cotton (*Gossypium thurberi* Todaro) as a host plant. It overwinters in dried thurberia cotton fruit (Fye 1968, Burke et al. 1986). Some physical characters have been used to try to distinguish southeastern boll weevil from thurberia weevil. However, it was subsequently discovered that weevils collected from thurberia bolls and subsequently reared in the laboratory lose some of the morphological characters associated with thurberia weevil (Warner 1966, Burke et al. 1986). Characters that vary with diet do not seem to be reliable characters for defining a particular population. The thurberia weevil was accorded subspecific status in some of the older literature (*Anthonomus grandis thurberiae* Pierce), but a cogent argument has been presented

¹ E-mail: roehrdar@fargo.ars.usda.gov

that subspecies status may not be warranted (Burke et al. 1986). Primarily because of its behavioral differences, thurberia weevil is not considered as economically threatening. Historically there have been reports of thurberia weevil being collected in cotton fields, especially late in the season and when stands of thurberia cotton were in proximity to cultivated cotton (Fye 1968, Burke et al. 1986). Some weevils continue to be caught in traps in eradicated areas of Arizona, but these have been assumed to be thurberia weevils.

A third form of *A. grandis*, the Mexican boll weevil, has also been postulated. This group is considered to be distinct from both the southeastern boll weevil and thurberia weevil. Warner (1966) settled on three external morphological characters that she felt clearly differentiated an Arizona thurberia weevil collection from an Alabama southeastern boll weevil collection. When she examined many insects, especially from Mexico and Central America, she found many individuals that had one or more of the characters with properties intermediate between the original Alabama and Arizona samples. A sample of weevils from west Texas had 30% intermediate characters; a sample of weevils from thurberia bolls collected just south of Arizona had nearly 40% intermediate characters. A weevil with all three thurberia weevil characters was found in east central Mexico. Warner's "intermediate" forms are what are now known as the Mexican boll weevil. According to Burke et al. (1986), the Mexican boll weevil range is centered in Mexico and Central America. However, the Mexican boll weevil range extends up the west coast of Mexico where it is (or was, depending on the location and the extent of specific eradication programs) sympatric with the thurberia weevil. Likewise there is postulated to be considerable range overlap of southeastern boll weevil and Mexican boll weevil in northeastern Mexico. Mexican boll weevil is a threat to cultivated cotton and is probably the boll weevil variety responsible for the previous outbreaks in Arizona.

When characters vary over a continuum it becomes a much more subjective and difficult task to look at an unknown weevil and assign it to one of the three groups. In addition, characters that vary with diet do not seem to be reliable characters for defining a particular population. Although the southeastern boll weevil, thurberia weevil, and Mexican boll weevil cannot be reliably distinguished based on their morphology, they exhibit some behavioral differences, most notably the host plant preference and accompanying seasonal distribution of thurberia weevil. Because the overlapping physical traits confound identification, the discovery of any weevils in monitor traps in Arizona, where the boll weevil has been eliminated as a crop pest, raises the question: Are the newly collected weevils indicative of reintroduction of a pest boll weevil population or are they thurberia weevil and, therefore, not of major concern?

Although the boll weevil has been a major pest for over a century and is a continuing object of intense interest, not much attention has been given to its genetics and there has been little application of mo-

Table 1. Boll weevil and thurberia weevil collection details

Type	Site	Date	Location notes
TW	K	20 Mar 1996	Southwest of Tucson, AZ, along AZ highway 386 near Kitt Peak Observatory.
TW	M	4 Apr 1996	Northeast of Tucson, AZ in the Molino Basin of the Santa Catalina Mountains, Coronado National Forest
TW	R	4 Mar 1996	Southeast of Tucson, AZ, in the Santa Rita Mountains along the road to the Fred Whipple Observatory
BW	W	10 Dec 1998	Rio Grande Valley near Weslaco, TX
BW	B	27 Jul 1999	Near College Station, TX, in Burleson County along the Brazos River
BW	C	13 Sep 1999	Northwest of Tampico, near Cuauhtemoc, Tamaulipas, Mexico
BW	L	2 Oct 2000	Near Lubbock, TX

TW, thurberia boll weevil. BW, boll weevil.

lecular genetic techniques to population identification or biosystematics of the boll weevil and related taxa. A few genetic eye and body color mutations were described over 30 yr ago (Bartlett 1967). A number of allozyme studies compared natural populations and laboratory colonies of the boll weevil and a closely related species (Terranova 1980; Bartlett 1981; Bartlett et al. 1983; Terranova and North 1984, 1985; Bartlett and Leggett 1987; Terranova et al. 1990, 1991). The allozyme studies have demonstrated different levels of polymorphism in various populations but have not provided a definitive method for distinguishing the various forms of the boll weevil.

Boll weevil mitochondrial DNA (mtDNA) at 18–19 kb is somewhat larger than is typical for insects (Boyce et al. 1989, Roehrdanz and North 1992). MtDNA RFLP differences have previously been noted among four laboratory colonies of weevils that had different geographical and host plant origins (Roehrdanz and North 1992). One of those colonies was a thurberia weevil colony. Here I describe some additional results indicating that DNA genetic markers could be useful for identifying weevil populations. In this approach long PCR and restriction enzyme digestion was used to examine mtDNA. The weevils tested here represent two varieties, thurberia weevil as described above originating from *G. thurberi* in Arizona and boll weevils from domesticated cotton in Texas and northeastern Mexico. Although the presumption is that the latter group is mostly or entirely southeastern boll weevil, the presence of some Mexican boll weevil in those samples cannot be ruled out. Therefore, the broader designation has been applied to those insects.

Materials and Methods

In Table 1 and Fig. 1, I describe the geographic origins of the population samples. Two collection methods were used. Pheromone trap-caught boll weevils associated with cultivated cotton were obtained from three locations in Texas and one site in

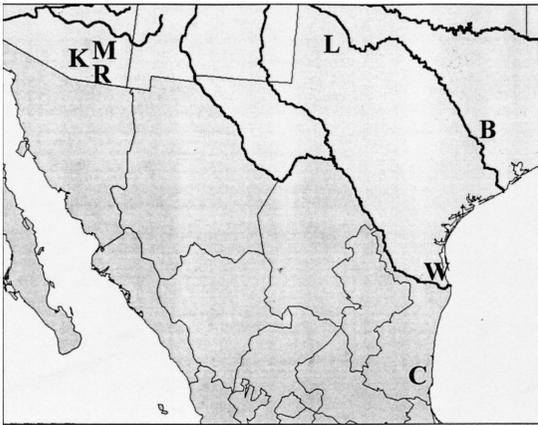


Fig. 1. Boll weevil and thurberia weevil collections sites. Site abbreviations as in Table 1.

Tamaulipas, Mexico, in 1999 and 2000. The insects were frozen and shipped to the USDA-ARS Biosciences Research Laboratory, Fargo, ND, on dry ice. Thurberia weevils were obtained from three Arizona locations in 1996. Fruits from thurberia cotton were collected and sent to Fargo where they were opened to release the insects, which were then frozen until used.

DNA extraction of individual weevils was carried out using a high salt method (Cheung et al. 1993). Total genomic DNA served as the template for the long PCR of mitochondrial DNA (mtDNA). The details of the long PCR amplification reaction were described elsewhere (Roehrdanz 1995, Roehrdanz and Degrugillier 1998). Two primers were used for mtDNA amplification: 16S2 (LR-N-12945) 5'-GC-GACCTCGATGTTGGATTAA-3' and C2R (C2-J-3684) 5'-GGTCAATGTTACAAATTTGTGG-3'. PCR primers were located in the 16S rRNA gene and in the cytochrome oxidase II gene. The primers belong to a category of conserved or universal insect primers that have been used to amplify mtDNA from a wide variety of insect species. A more complete description of the insect mitochondrial genome organization, universal insect primers and utilization of these primers in long PCR can be found in other sources (Simon et al. 1994, Roehrdanz and Degrugillier 1998, Boore 1999). Amplified DNA was cleaved with the restriction endonucleases *AluI*, *AseI*, *DraI*, *SspI*, and *MseI*, according to the suppliers' recommendations. The restriction enzymes were selected for their A+T recognition sequences which increases the number of restriction sites that can be sampled in insect mtDNA (Roehrdanz et al. 1994, Roehrdanz and Degrugillier 1998). Restriction fragments were separated on agarose gels containing ethidium bromide. Agarose varied from 1 to 2% depending on the number and size of the fragments to be resolved. 3% Metaphor agarose was used for *MseI* digestions. Gel photos are negative images of the ethidium fluorescence. Different restriction patterns were assigned a letter as they were observed. Each individual insect was assigned a composite haplotype based on RFLP patterns. Genetic distances for each pairwise combination of composite haplotypes were cal-

culated and unweighted pair-group method with arithmetic average and neighbor-joining trees were drawn using the program Restsite (Miller 1991).

Results

The amplified mtDNA fragment (16S2-C2R) contains ≈ 9.2 kb or about half of the boll weevil mitochondrial genome. Cleavage of the PCR product with the five restriction endonucleases produced 31 different fragment patterns (Table 2 and Fig. 2). A total of 105 restriction fragments was scored. In most cases fragments smaller than ≈ 140 bp were excluded. Thirty-two of the restriction fragments were observed in all of the weevils examined. It is likely that the *AluI* H pattern found in a single individual contains three of the four smallest fragments found in all other *AluI* patterns, but the bands in that portion of that gel were not sufficiently resolved to allow a certain identification. The remaining fragments were present in some, but not all, of the insects examined. A *DraI* C pattern was originally identified, but upon follow-up examination that pattern was merged into one of the others. Composite fragment pattern haplotypes were assigned to each individual and 18 haplotypes were recognized. The analysis was based on 53 boll weevils and 61 thurberia weevils.

In Table 3 I show the distribution of the 18 haplotypes among the seven collection sites. The most significant observation is that all of the haplotypes identified are restricted to one of the two groups. No haplotypes were found in both the thurberia weevil and boll weevil populations. Several haplotypes were found only in single individuals, five from the thurberia group (#2, #5, #13, #14, #17) and two from the boll weevils (#12, #18). Along with the greater number of rare haplotypes in thurberia weevil, there also appears to be greater diversity between collection sites than is evident for the boll weevil. Haplotype #1 predominates at the Santa Rita site (15/18) and was found nowhere else. Haplotype #3 is the only one found at all three thurberia weevil sites and is most frequent at Kitt Peak (13/17). Haplotypes #1 and #3 differ by seven restriction fragments. Only one other haplotype, #4, was recovered from more than one thurberia weevil location. The Molino Basin collection exhibited the greatest diversity with eight haplotypes recovered, six of them unique to the location. By contrast in the boll weevil, only three restriction fragment differences separate haplotypes #8, #9, #10, and #11, which constitute 51/53 boll weevils tested.

The genetic distance between haplotype pairs ranged from $<0.1\%$ to 2.2% (Table 4). For the purpose of this calculation the *MseI* fragments were excluded. The *MseI* recognition sequence (TTAA) is contained internally in both the *AseI* and *DraI* recognition sequences. Some of the *MseI* restriction fragments could result from cleavage at what are also *DraI* or *AseI* sites and would thus be counted twice in the calculations. Eliminating the *MseI* fragments also serves to merge boll weevil haplotypes #9, #10, and #11, which differ only in *MseI* patterns. Unweighted pair-group method

Table 2. Restriction fragment patterns from boll/thurberia weevil 16S2-C2R mtDNA PCR product

Restriction enzyme	Pattern	Fragment size in base pairs
<i>Ase I</i>	A	2000, 1400, 900, 850, 560, 500, 380, 300, 290, 200, 180
	B	1400, 1250, 1050, 900, 850, 560, 500, 450, 380, 300, 290, 200, 170
	C	1400, 1050, 900, 850, 560, 500, 450, 380, 300, 290, 200, 180
	D	1400, 1250, 1050, 850, 750, 560, 500, 450, 380, 300, 290, 235, 180
	E	1400, 1250, 1050, 850, 750, 560, 500, 380, 300, 290, 235
	F	1400, 1250, 1200, 1050, 900, 560, 500, 450, 380, 300, 290, 200, 180
	G	2000, 900, 850, 560, 500, (450), 380, 300, 290, 200, 180
<i>Alu I</i>	A	1000, 850, 800, 650, 550, 530, 500, 400, 360, 350, 210, 200, 175, 160, 150
	B	1000, 800, 650, 590, 550, 495, 400, 360, 350, 270, 230, 210, 200, 175, 160, 150
	C	1000, 850, 800, 650, 550, 500, 495, 400, 360, 350, 270, 210, 200, 175, 160, 150
	D	1000, 850, 840, 650, 550, 500, 410, 360, 320, 270, 263, 250, 210, 200, 160, 150
	E	1000, 850, 800, 650, 590, 550, 495, 400, 360, 350, 270, 210, 200, 175, 160, 150
	F	1000, 850, 800, 650, 590, 550, 495, 400, 360, 350, 270, 230, 210, 200, 175, 160, 150
	G	1000, 850, 840, 650, 645, 590, 550, 500, 495, 360, 270, 250, 230, 210, 200, 175, 160, 150
	H	850, 800, 650, 645?, 590, 550, 500?, 495, 400, 370, 360, 350, 270, . . . ?
<i>Dra I</i>	A	1750, 1300, 1000, 970, 600, 560, 510, 370, 310, 285, 260, 240, 150
	B	1300, 1050, 1000, 970, 700, 600, 560, 530, 510, 370, 330, 310, 260, 240, 150
	D	1300, 1050, 1000, 970, 715, 700, 685, 635, 560, 550, 530, 510, 465, 260, 240, 150
	E	1750, 1300, 1000, 970, 600, 560, 510, 465, 310, 285, 265, 245, 150?
	F	1300, 1050, 970, 750, 700, 640, 560, 530, 510, 456, 260, 240, . . . ?
	G	1800, 1000, 970, 840, 750, 410, 375, 365, 235, 200, 175, 170, 150, 140
<i>Ssp I</i>	A	1800, 1000, 970, 840, 750, 410, 375, 365, 235, 200, 175, 170, 150, 140
	B	1800, 1000, 970, 840, 750, 410, 375, 365, 300, 235, 200, 175, 170, 150, 140
	C	1800, 1000, 970, 840, 750, 410, 375, 365, 300, 270, 235, 200, 175, 170, 150, 140
	D	1800, 1000, 970, 850, 840, 410, 375, 365, 235, 200, 175, 170, 150, 140
	E	2500, 1000, 970, 840, 410, 375, 365, 300, 235, 200, 175, 170, 150, 140
<i>Mse I</i>	A	360, 290, 250, 240, 205, 200, 195, 190, 175, 155, 135
	B	360, 290, 250, 240, 200, 195, 190, 175, 155, 135?
	C	500, 360, 290, 250, 240, 200, 190, 180, 175, 155, 135?
	D	360, 290, 250, 248, 195, 190, 188?, 175, 165, 163?, 155, 138, 135
	E	360, 290, 250, 248, 195, 190, 188?, 175, 165, 163?, 155, 142, 138, 135
	F	500, 360, 195, 180, 175, 155, 138, 135
	G	360, 290, 250, 248, 195, 190, 188?, 175, 165, 155, 138, 135, 130

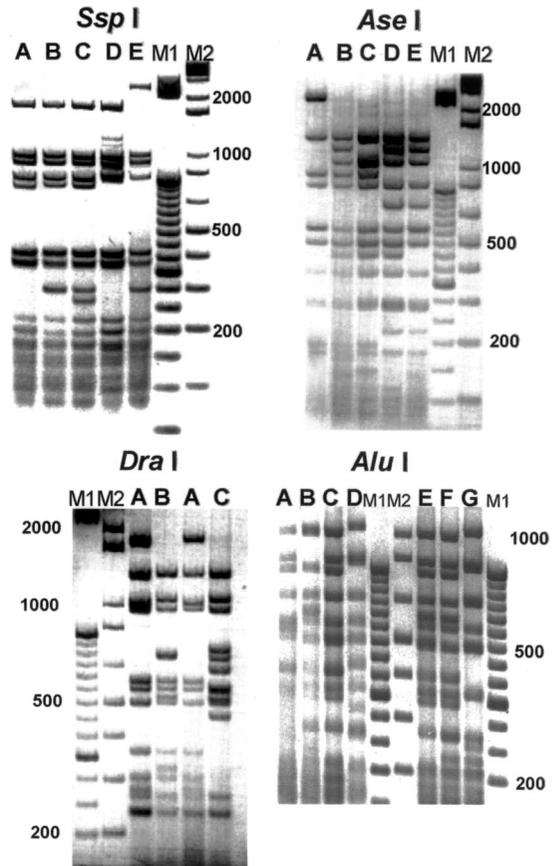


Fig. 2. Agarose gels showing some of the restriction pattern differences for four restriction enzymes. Images are computer-generated negatives of photos of ethidium bromide stained gels. Letters for the RFLP patterns correspond to those used in Table 2. M1 is a 50-bp DNA marker ladder. M2 is a 1-kb Plus marker ladder. Marker numbers are in base pairs.

from the southernmost collection near Cuauhtemoc (haplotype #12) is quite divergent from the others and clusters with two thurberia weevil individuals from Kitt Peak. The majority of the Kitt Peak thurberia weevil samples are more closely related to the Molino Basin collection. Most of the Santa Rita thurberia weevils are in a small cluster of their own separate from all but one of the Molino insects.

The absence of overlapping haplotypes in the boll weevil and thurberia weevil samples permits the identification of restriction fragments that are diagnostic for one group. 52/53 boll weevils have the *AseI* pattern A or G, which contains a 2000-bp restriction fragment. Haplotype #12 (pattern E) is the only exception. None of the other *AseI* patterns contains restriction fragments larger than 1400 bp, and the 2000-bp band was not observed in any thurberia weevil. The 61 thurberia weevils exhibited four *AseI* patterns (B, C, D, and F). All of these patterns have a 450-bp fragment that was not observed in 52/53 boll weevils and was observed at a less than stoichiometric level in the

with arithmetic average and neighbor-joining trees indicate a tight cluster that contains 52/53 of the boll weevil individuals (Figs. 3 and 4). One boll weevil

Table 3. Distribution of composite boll/thurberia weevil mtDNA haplotypes

Hap #	Restriction pattern					Thurberia weevils ^a			Boll weevils ^a			
	<i>AseI</i>	<i>AluI</i>	<i>DraI</i>	<i>SspI</i>	<i>MseI</i>	K	M	R	B	L	W	C
1	B	A	A	A	A			15				
2	C	C	A	A	B				1			
3	B	B	A	A	A	13	6	2				
4	B	E	A	A	A	2	4					
5	D	G	A	E	C	1						
6	B	F	A	D	A		7					
7	B	C	A	A	A		2					
8	A	D	B	C	D					4	7	
9	A	D	B	B	E				1	2	3	2
10	A	D	B	B	D			9	15	2	4	
11	A	D	B	B	G							1
12	E	G	D	E	F							1
13	D	G	F	E	C/F	1						1
14	B	H	E	A	—		1					
15	B	F	A	A	A		3					
16	F	F	A	A	A		2					
17	B	E	A	D	—		1					
18	C	D	B	B	D					1		
Total						17	26	18	10	18	10	15

^a Number of individuals. *MseI* patterns not determined for haplotypes #14 and #17.

remaining boll weevils (pattern G). The presence/absence of these two fragments in the *AseI* RFLP patterns distinguishes the boll weevil from the thurberia weevil. Among the *AluI* fragments, the 410, 320, and 263 bp were recovered from all boll weevils except the haplotype #12 individual. The 800, 400, and 350-bp fragments were present in all thurberia weevils and absent from the boll weevils. Similarly, the *MseI* 248 and 165-bp fragments and the *DraI* 300-bp fragment were in all boll weevils except haplotype #12. The 240 and 200-bp *MseI* fragments were found in all thurberia weevils but no boll weevils. Another group of fragments distinguished the boll weevil from thurberia weevil, except for the anomalous haplotype #13 thurberia weevil individual from Kitt Peak. The *DraI* 1050, 700, 530, and the *SspI* 300-bp fragments were in all of the boll weevil but also in haplotype #13. Conversely, all of the thurberia weevils except haplotype #13 had the 1750 and 285-bp *DraI* fragments.

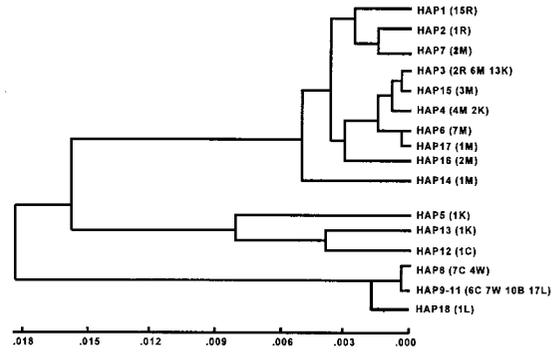


Fig. 3. Unweighted pair-group method with arithmetic average tree of the mtDNA haplotypes derived from the genetic distance data in Table 4. Parentheses indicate the number of individuals and the collection site.

Discussion

The extent of genetic differentiation and the nature of the genetic relationship among populations of *A. grandis* have been unresolved questions for some time. A previous examination of *A. grandis* mtDNA (Roehrdanz and North 1992) relied on laboratory-reared colonies of weevils to obtain large amounts of DNA for analysis, and the resulting sample was the equivalent of only four individuals from diverse geography and host plants. The geographic and host plant sources of the four colonies were described in that paper. They were as follows: (1) A long time boll weevil colony strain carrying the Ebony body color mutation. The precursors of this colony were probably originally collected from cotton in northeastern Mexico (see Terranova et al. 1991 for additional background on this colony). (2) A colony derived from weevils collected in Tabasco, Mexico, from a wild host plant, *Hampea nutricia* Fryxell. (3) A colony from cultivated cotton in El Salvador. (4) A colony from individuals collected from *G. thurberi* in the Molino Basin of the Santa Catalina Mountains in Arizona, the same location as one of the collections in this work. Despite the minimal sample size, those results indicated a genetic differentiation between boll weevil from cultivated

Table 4. Genetic distance between pairwise combinations of weevil mtDNA restriction fragment haplotypes

Hap #	1	12	13	14	15	16	17	18	2	3	4	5	6	7	8
12	0.021														
13	0.022	0.004													
14	0.006	0.017	0.018												
15	0.004	0.019	0.020	0.003											
16	0.006	0.021	0.020	0.006	0.002										
17	0.004	0.020	0.021	0.005	0.002	0.004									
18	0.016	0.018	0.017	0.020	0.017	0.017	0.018								
2	0.003	0.020	0.019	0.006	0.003	0.003	0.004	0.013							
3	0.004	0.020	0.021	0.004	0.001	0.003	0.002	0.018	0.004						
4	0.003	0.020	0.021	0.004	0.001	0.003	0.001	0.016	0.003	0.001					
5	0.013	0.008	0.008	0.012	0.011	0.011	0.012	0.015	0.010	0.012	0.012				
6	0.005	0.019	0.020	0.004	0.001	0.003	0.001	0.018	0.005	0.002	0.002	0.011			
7	0.002	0.019	0.020	0.004	0.002	0.004	0.002	0.014	0.002	0.002	0.001	0.010	0.003		
8	0.017	0.018	0.019	0.021	0.018	0.017	0.019	0.002	0.014	0.020	0.018	0.016	0.020	0.016	
9	0.016	0.017	0.018	0.021	0.018	0.017	0.019	0.002	0.013	0.019	0.017	0.016	0.019	0.015	0.001

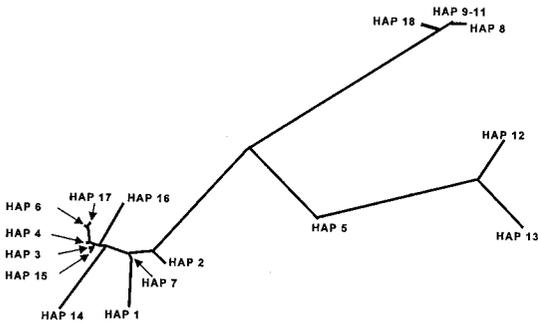


Fig. 4. Neighbor-joining tree of the mtDNA haplotypes derived from the genetic distance data in Table 4. The length of the connector lines is proportional to the genetic distance except that the smallest connectors have been enlarged sufficiently to make them visible. The number of individuals and collection sites for each haplotype are the same as in Fig. 3.

cotton and thurberia weevil. PCR technology has allowed revisitation of this question with emphasis on increasing the effective sample size.

With the exception of the single individual with haplotype #12, the combined weevil collections from Texas and Mexico display little genetic variability. Excluding haplotype #12, the mean genetic distance within these populations is <0.002 . There is a possibility that the small amount of genetic diversity observed decreases in a south to north gradient particularly if the *MseI* patterns are also considered. Only two haplotypes were observed from the Brazos and Lubbock sites, whereas five haplotypes were found in the Cuauhtemoc population including haplotype #12 which is the most divergent haplotype among the weevils from the Texas and Mexico sites. Terranova et al. (1990, 1991) examined allozyme polymorphism in what they defined as southeastern boll weevil populations collected from Texas to the Carolinas and found a similar pattern. The Rio Grande Valley population had the greatest allozyme variability and the variability decreased eastward across the southern United States. They noted some exceptions that occurred in the vicinity of research facilities where weevils had been imported from distant locations and released in conjunction with research projects. The limited mtDNA variability of the weevils from the Texas/Mexico locations observed here is not unexpected given the rapid and relentless expansion of the population from the Rio Grande River to the Atlantic Coast between 1890 and 1920. Based on the historical records and the minimal genetic diversity, I believe the Texas/Mexico haplotype cluster (everything except haplotype #12) can be reasonably labeled as southeastern boll weevil. Rapid population expansions are frequently associated with reduced genetic variability. A similar situation, rapid expansion associated with minimal genetic diversity, occurred in the western corn rootworm in the latter half of the 20th century when it spread from Colorado to the east coast of the United States (Szalanski et al. 1999).

In contrast to the weevils from the Texas/Mexico sites, the thurberia weevils display both greater variability at each location and evidence of geographic differentiation between sites. The three Arizona mountain collection sites are separated by stretches of desert that could be a barrier to frequent movement between locations. The Molino site had the most haplotypes with eight and the mean genetic distance between these haplotypes is 0.0026. The mean haplotype divergence at Santa Rita is 0.0037; however, 15/18 individuals are haplotype #1, which is unique to that site. Kitt Peak is more unusual. Haplotype #3 predominates and differs from haplotype #4 by <0.001 . Together #3 and #4 account for 15/17 Kitt Peak individuals. Haplotype #3 is the only one recovered at all three thurberia weevil locations and haplotype #4 was found at Molino as well as Kitt Peak. So the majority of Kitt Peak samples are genetically similar to those from Molino. The other two individuals from Kitt Peak represent haplotypes #5 and #13 and are very divergent.

Haplotype #5 shares the rare *AluI* G and *SspI* E restriction patterns with two other unusual haplotypes, #12 and #13. It is most similar to haplotypes #12 and #13 but is closer to the other two clusters of haplotypes than either #12 or #13, as evidenced by the neighbor-joining tree (Fig. 4). The mean genetic distance of #5 to the primary Kitt Peak group is 0.012, which is essentially the same as the mean distance to the whole thurberia weevil collection (minus #13). The genetic distance of #5 to the Texas/Mexico cluster (minus #12) is 0.016. The genetic distance between #5 and the #12/#13 cluster is 0.008. The mean genetic distance from haplotype #13 to the main Kitt Peak group is 0.021 and 0.20 to thurberia weevils as a whole. The mtDNA results here are consistent with the previous mtDNA data in that the genetic distance between the thurberia weevil and the Ebony boll weevil was 0.0236 (Roehrdanz and North 1992). Here the genetic distance between the Texas/Mexico cluster and the most numerous thurberia weevil haplotype (#3) is similar, 0.0195.

Haplotypes #12 (boll weevil, Mexico) and #13 (thurberia weevil) clearly represent a distinct branch of the *Anthonomus* tree. The distance between them is only 0.004 but their mean distance to the main thurberia weevil cluster is 0.020 and to the main Texas/Mexico cluster is 0.18. It is not possible to determine if these two haplotypes along with haplotype #5 represent vestiges of an ancient mtDNA lineage that has survived at low levels in both thurberia weevil and Mexican populations or if they are recent travelers or outliers from another distinct population of *Anthonomus*. They could be haplotypes usually associated with the Mexican boll weevil. The Cuauhtemoc, Mexico site is within the region where southeastern boll weevil and Mexican boll weevil populations are said to overlap. The divergent haplotype is 1/15 individuals, which is less than the previously recorded frequency of Mexican boll weevil phenotypes in this part of northeastern Mexico. However, the current sample was collected from only a single site. Furthermore,

there is no reason to assume that the broad phenotypic boundary of the early 1960s was in the same place in 1999 nor that the phenotypic and mitochondrial genetic boundaries are coincident. In the western part of the range, the occasional use of *G. thurberi* as a host plant by Mexican boll weevil has not been ruled out (Burke et al. 1986). This would provide an opportunity for interaction and limited gene flow between Mexican boll weevil and thurberia weevil populations. The presence of haplotypes #5 and #13 in thurberia weevils would be consistent with such interaction. As pointed out in the introduction, Warner (1966) found a significant number of "intermediate" characters in some samples of insects collected from *G. thurberi*, which could also be taken as evidence of gene flow between thurberia weevil and Mexican boll weevil.

It seems highly improbable that the three divergent haplotypes represent a previously unknown species. Currently the genus *Anthonomus* is large while the *A. grandis* group within the genus contains just five known species, three of which have been described recently (Jones and Burke 1997, Jones 2001). All five species use plants from the genus *Hampea* for larval development in southern Mexico or Central America. Only *A. grandis* has demonstrated a host range extending to other Malvaceae from the genera *Gossypium*, *Cienfuegosia*, and *Thespesia*. The genetic distance of $\approx 2\%$ between the main haplotype groups is consistent with intraspecific variation.

The DNA genetic data available for other weevil species has dealt with some similar issues concerning the relationships and differentiation of populations ranging from morphotypes to putative species. Normark (1996) found up to 9% mtDNA sequence divergence between 12 mt lineages from six morphotypes of the South American broad-nosed weevil, *Aramigus tessellatus* species complex. The majority of these types represented parthenogenetic lineages that had diverged by 4–7% from their nearest sexual lineage. Parthenogenesis is a complicating factor that is not present in boll weevils. The North American bark weevil, *Pinus strobi* species complex, contains four currently recognized species that are extremely similar in morphology and allozymes and are capable of producing fertile hybrids. Despite the extensive similarities, both RFLP and sequence analysis of mtDNA have detected high levels of divergence in the complex. RFLP divergence ranges up to 16% and sequence divergence ranges to 7.5% between the species (Boyce et al. 1994, Langor and Sperling 1997). These values are much higher than what has been observed between the boll weevil populations. A third weevil that has been the object of mtDNA investigations is the Eurasian alfalfa weevil, *Hyper postica* (Gyllenhal). This weevil was introduced into the United States at three different times and locations in the 20th century. Erney et al. (1996) sequenced several small sections of the mtDNA from these three strains. Two of them were nearly identical and differed from the third by $\approx 5\%$, a level of divergence usually associated with distinct species. Despite their wide-spread geographic distribution these authors found little intrastrain vari-

ability which can be attributed to recent expansion from a small population just like the southeastern boll weevil. Unlike the boll weevil haplotypes #12 and #13, they did not find any mtDNA evidence to suggest an interaction between the two divergent strains.

The RFLP patterns described here present a fairly unambiguous method for distinguishing thurberia weevil from southeastern boll weevil. An important caveat is that additional weevils may appear that are not part of these two groups. RFLP identification should be beneficial if weevils are collected from traps put out in zones of eradication. Weevils could be assigned to one of these groups, which would help in making management decisions. This is of significance in Arizona where weevils appearing in the eradication zone could be thurberia weevil that have migrated from their natural montane habitat into cultivated cotton. Alternatively, weevils could be transported from cotton growing regions elsewhere in North America. Conventional wisdom insinuates that the thurberia weevil is a substantially less serious threat than a return of the boll weevil. Burke et al. (1986) reviewed the behavioral patterns that form the basis of this belief. The belief is reinforced by the fact that there have been no serious reinfestations of Arizona cotton in the past ten years (ACRPC 2000, National Cotton Council 2000, USDA APHIS 2000) despite the continued presence of thurberia weevil populations in the wild. Because it has been demonstrated that thurberia weevil can be adapted to carry out its life cycle on commercial cotton varieties, continued monitoring of the situation is imperative.

Future DNA analysis could include the other members of the *A. grandis* species group and examine the evolutionary relationships between this major pest insect and its nonpest closest relatives, complementing the existing phylogeny of physical characters and host plant associations (Jones and Burke 1997, Jones 2001). Additional molecular biosystematic analysis of *A. grandis* from Mexico and Central America would be needed to determine the extent of intraspecific diversity in *A. grandis*, whether the Mexican boll weevil exists as a genetically recognizable group, and if the observed haplotype cluster is characteristic of the Mexican boll weevil.

Acknowledgments

I thank S. Degrugillier, M. Degrugillier, P. Evenson, and P. Senechal Willis in Fargo, ND, for technical contributions. Assistance with weevil collections was provided by J. Leggett and A. Bartlett from ARS, Phoenix, AZ; T. Sappington and O. Zamora from ARS Weslaco, TX; and D. Spurgeon from ARS College Station, TX. Valuable comments on early versions of the manuscript were provided by A. Bartlett, H. Burke, and T. Sappington.

References Cited

- (ACRPC) Arizona Cotton Research and Protection Council. 2000. Southwest Boll Weevil Eradication Program (<http://www.azlink.com/~azcotton/swSBWep.htm>).

- Bartlett, A. C. 1967. Genetic markers in the boll weevil. *J. Hered.* 58: 159–163.
- Bartlett, A. C. 1981. Isozyme polymorphisms in boll weevils and thurberia weevils from Arizona. *Ann. Entomol. Soc. Am.* 74: 9–13.
- Bartlett, A. C., and J. E. Leggett. 1987. A comparison of electromorphs of *Anthonomus grandis* and *Anthonomus peninsularis*. *Southwest. Entomol.* 12: 217–222.
- Bartlett, A. C., W. C. Randall, and J. E. May. 1983. Allozyme variation among populations of boll weevils in Arizona and Mexico. *Southwest. Entomol.* 8: 118–130.
- Boore, J. L. 1999. Animal mitochondrial genomes. *Nucleic Acid Res.* 27: 1767–1780.
- Boyce, T. M., M. E. Zwick, and C. F. Aquadro. 1989. Mitochondrial DNA in the pine weevil: size, structure and heterogeneity. *Genetics* 123: 825–836.
- Boyce, T. M., M. E. Zwick, and C. F. Aquadro. 1994. Mitochondrial DNA in the bark weevils: phylogeny and evolution in the *Pissodes strobi* species group (Coleoptera: Curculionidae). *Mol. Biol. Evol.* 11: 183–194.
- Burke, H. R., W. E. Clark, J. R. Cate, and P. A. Fryxell. 1986. Origin and dispersal of the boll weevil. *Bull. Entomol. Soc. Am.* 32: 228–238.
- Cheung, W. Y., N. Hubert, and B. Landry. 1993. A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and other PCR analysis. *PCR Methods Appl.* 3: 69–70.
- Erney, S. J., K. P. Preuss, S. D. Danielson, and T. O. Powers. 1996. Molecular differentiation of alfalfa weevil strains (Coleoptera: Curculionidae). *Ann. Entomol. Soc. Am.* 89: 804–811.
- Fye, R. E. 1968. The thurberia weevil in Arizona. *J. Econ. Entomol.* 61: 1264–1268.
- Jones, R. W. 2001. Evolution of the host plant associations of the *Anthonomus grandis* species group (Coleoptera: Curculionidae): phylogenetic tests of various hypotheses. *Ann. Entomol. Soc. Am.* 94: 51–58.
- Jones, R. W., and H. R. Burke. 1997. New species and host plants of the *Anthonomus grandis* species group (Coleoptera: Curculionidae). *Proc. Entomol. Soc. Wash.* 99: 705–719.
- Langor, D. W. and F.A.H. Sperling. 1997. Mitochondrial DNA sequence divergence in weevils of the *Pissodes strobi* species complex (Coleoptera: Curculionidae). *Insect Mol. Biol.* 6: 255–265.
- Miller, J. C. 1991. RESTSITE, version 1.2. Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park, PA.
- National Cotton Council. 2000. Boll weevil eradication program (<http://www.cotton.org/ncc/technical/weevil/eradication.htm>).
- Normark, B. B. 1996. Phylogeny and evolution of parthenogenetic weevils of the *Aramigus tessellatus* species complex (Coleoptera: Curculionidae: Naupactini): evidence from mitochondrial DNA sequences. *Evolution* 50: 734–745.
- Roehrdanz, R. L. 1995. Amplification of complete insect mitochondrial genome in two easy pieces. *Insect Mol. Biol.* 4: 169–172.
- Roehrdanz, R. L., and M. E. Degrugillier. 1998. Long sections of mitochondrial DNA amplified from fourteen orders of insects using conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* 91: 771–778.
- Roehrdanz, R. L., and D. T. North. 1992. Mitochondrial DNA restriction fragment variation and biosystematics of the boll weevil, *Anthonomus grandis*. *Southwest. Entomol.* 17: 101–108.
- Roehrdanz, R. L., J. D. Lopez, J. Loera, and D. E. Hendricks. 1994. Limited mitochondrial DNA polymorphism in North American populations of *Heliothis virescens* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 87: 856–866.
- Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* 87: 651–701.
- Szalanski, A. L., R. L. Roehrdanz, D. B. Taylor, and L. Chandler. 1999. Genetic variation in geographical populations of western and Mexican corn rootworm. *Insect Mol. Biol.* 8: 519–526.
- Terranova, A. C. 1980. Inheritance patterns of aldehyde oxidase, glutamate-oxaloacetate transaminase and phosphoglucomutase allozymes in the boll weevil. *Ann. Entomol. Soc. Am.* 73: 653–657.
- Terranova, A. C., and D. T. North. 1984. Genetics of glucose phosphate isomerase and glucose-6-phosphate dehydrogenase in *Anthonomus grandis grandis* and *A. grandis thurberiae*. *J. Agric. Entomol.* 1: 380–385.
- Terranova, A. C., and D. T. North. 1985. Inheritance of allozymes in the boll weevil (Coleoptera: Curculionidae). *Ann. Entomol. Soc. Am.* 78: 166–171.
- Terranova, A. C., R. G. Jones, and A. C. Bartlett. 1990. The southeastern boll weevil: an allozyme characterization of its population structure. *Southwest. Entomol.* 15: 481–495.
- Terranova, A. C., R. G. Jones, and A. C. Bartlett. 1991. An allozyme study of the laboratory boll weevil and its influence on some southeastern populations. *Southwest. Entomol.* 16: 1–11.
- USDA APHIS. 2000. Boll weevil eradication. (<http://www.aphis.usda.gov/ppq/weevil/>).
- Warner, R. E. 1966. Taxonomy of the subspecies of *Anthonomus grandis* (Coleoptera: Curculionidae). *Ann. Entomol. Soc. Am.* 59: 1073–1088.

Received for publication 19 January 2001; accepted 18 July 2001.