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Keywords
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Genetic structuring of boll weevil populations in the US based on RAPD markers

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Abstract

Randomly amplified polymorphic DNA (RAPD) analysis was performed to infer the magnitude and pattern of genetic differentiation among boll weevil populations from eighteen locations across eight US states and north-east Mexico. Sixty-seven reproducible bands from six random primers were analysed for genetic variation within and between weevil populations. Genetic and geographical distances among all populations were positively correlated, reflecting a pattern of isolation by distance within a larger metapopulation. Gene flow between south-central, western and eastern regions is limited, but migration between locations within regions appears to be relatively frequent up to distances of ~300–400 km. However, estimates of effective migration were much lower than those estimated from mtDNA-RFLP data reported previously.

Keywords: RAPD, genetic diversity, gene flow, boll weevil, *Anthonomus grandis*.

Introduction

The boll weevil (*Anthonomus grandis* Boheman) has been the most destructive insect pest of cotton in the United States since its invasion about a century ago. After undergoing a host shift in Southern Mexico to cultivated cotton sometime in the 19th century, this insect first entered the US through natural dispersal in 1892 via the southern tip of Texas (Coad *et al*., 1922; Burke *et al*., 1986). It rapidly dispersed across the central and eastern Cotton Belt of the United States during the early 20th century, expanding its range by 64–193 km per year until it reached the Atlantic Coast in the early 1920s (Coad *et al*., 1922; Hunter & Coad, 1923; Burke *et al*., 1986; Culin *et al*., 1990). A slower, secondary range expansion of the weevil into the High Plains of Texas and New Mexico began in the late 1950s (Bottrell *et al*., 1972), presumably from established populations in central and southern Texas.

The boll weevil has been eradicated from much of the US, but most of the central Cotton Belt, ~60% of total US acreage, is still infested (Smith, 1998; Grefenstette & El-Lissy, 2003). Many areas in this region are in different stages of eradication, and dispersal or human-mediated transport of weevils from infested areas into eradicated or suppressed zones is of great concern. It has been difficult to judge the likelihood of natural dispersal from one region to another, because little is known about boll weevil dispersal behaviour and capacity. Although the ability of boll weevils to disperse long distances has been documented through mark-recapture studies (Guerra, 1988; Lukefahr *et al*., 1994), the frequency and patterns of long-range movement remain largely unknown.

Estimates of gene flow derived from molecular markers can serve as an index of dispersal rates (Krafsur *et al*., 2001). In addition, studies on genetic variation among populations of invasive species can provide insights into the geographical origin of colonizing populations (e.g. Williams *et al*., 1994; Sheppard & Smith, 2000), which would be of great value in identifying sources of boll weevil reintroductions to eradication zones. Previous population genetics studies of the boll weevil have relied mainly on isozyme analyses (Biggers & Bancroft, 1977; Bartlett, 1981; Bartlett *et al*., 1983; Terranova *et al*., 1990, 1991; Biggers *et al*., 1996). However, proteins are not necessarily selectively neutral (Biggers *et al*., 1996), and the amount of polymorphism in US populations of boll weevils revealed in these studies seems not to be great enough to elucidate gene flow on a fine enough geographical scale to be useful in answering the questions that we are attempting to address.

DNA markers are especially effective tools in making inferences about movement between insect populations,

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because they represent selectively neutral characters (Black *et al*., 2001). Different kinds of molecular markers can reveal different levels of genetic variation, making population genetics studies possible on a wide range of geographical scales. There are a few studies reporting the use of DNA markers to characterize boll weevil populations (Roehrdanz & North, 1992; Roehrdanz, 1995, 2001; Scataglini *et al*., 2000; Kim & Sappington, 2004). Roehrdanz (2001) showed that restriction fragment length polymorphisms (RFLP) of mitochondrial DNA (mtDNA) can distinguish 'thurberia' boll weevils, a subspecies or race which reproduces on wild cotton in the mountains of Arizona, from weevils reproducing in cultivated cotton further east. Scataglini *et al*. (2000) used random amplification of polymorphic DNA (RAPD) markers to analyse genetic structuring and gene flow among populations of boll weevils in South America collected from native plants and cultivated cotton. Their analyses suggest that despite the apparent range expansion in South America beginning in the early 1980s, populations were already present on native hosts before the introduction of cultivated cotton.

Roehrdanz & Degrugillier (1998) published a number of primer sequences with which they successfully amplified portions of boll weevil mtDNA. Recently, we used two of those primers to amplify long PCR fragments of mtDNA for RFLP analysis of weevils sampled from many locations throughout the south-central US (Kim & Sappington, 2004). The results suggested limited gene flow between eastern and western regions, and that migration between locations separated by < 300 km is fairly frequent. Phylogenetic reconstructions of the sampled populations based on the RFLP data were consistent with historic range expansions of the weevil out of Mexico and through the central and south-eastern Cotton Belt, with some interesting patterns of dispersal revealed on smaller scales. The amount of variation revealed by the RFLPs was not great, and the number of markers diagnostic (alone or in combination) of specific locations were too few to be of much use as fingerprints for tracking the origins of individuals captured in eradication zones. Consequently, we were interested in examining gene flow using a marker with greater genetic resolution.

The RAPD technique (Williams *et al*., 1990) has been widely used to elucidate the geographical origin of, and gene flow among, insect populations (e.g. Vandewoestijne & Baguette, 2002; Ayres *et al*., 2003), including curculionid weevils (Williams *et al*., 1994; Armstrong & Wratten, 1996; Taberner *et al*., 1997; Bas *et al*., 2000; Scataglini *et al*., 2000). It has proved to be a very efficient and sensitive method for obtaining genetic markers for many different kinds of organisms, demanding no prior information about genomic organization. The limitations associated with RAPD markers are well known, including variable reproducibility unless reaction conditions are stringently controlled, and a dominant mode of inheritance (Black, 1993; Lynch & Milligan, 1994; Loxdale *et al*., 1996). Nevertheless, properly performed RAPD analysis is a useful and reliable tool for studying the ecology and genetic structuring of populations (Armstrong & Wratten, 1996; Brown *et al*., 1997; Vaughn & Antolin, 1998; Pearson *et al*., 2002).

Our overall goal is to characterize gene flow and dispersal patterns among boll weevil populations in the south-central Cotton Belt of the US and north-east Mexico. In this study, we surveyed genetic variation among populations of boll weevils by RAPD analyses of 292 individuals from eighteen widely separated locations. We chose RAPD markers because of their potential for revealing greater genetic diversity, and thus for providing improved genetic resolution over what we observed with mtDNA PCR-RFLP data (Kim & Sappington, 2004). Because of its lack of recombination, the entire mtDNA is the equivalent of a single locus with many alleles (Dowling *et al*., 1990). Thus, RAPD markers provide an important complement to the mtDNA-RFLPs, because they represent multiple loci sampled across the nuclear genome. It is important to remember, however, that there are four assumptions implicit in the statistical analyses of RAPD markers: (1) RAPD alleles show Mendelian inheritance; (2) bands of equal electrophoretic mobility are directly homologous; (3) RAPD alleles that encode an amplifiable band segregate as dominant markers; and (4) alleles that are not amplified segregate as recessive markers.

Results

Of twenty primers tested, six that produced clearly discernable bands were selected and applied to all samples (Table 1). For the selected primers, the same bands were always amplified in subsequent PCR analyses. Most bands were between 300 and 2000 bp (Fig. 1). An average of 16 (± 2.5 SD) bands were amplified per primer. However, ambiguous and indistinguishable bands were excluded, so that a total of 67 bands were used in this study, with an average of 11.2 (± 1.9 SD) bands per primer (Table 1). Boll weevils sampled from eighteen locations in the US and Mexico (Table 2, Fig. 2) were scored at the 67 RAPD loci. The six primers exhibited somewhat different degrees of heterozygosity among the 292 weevils tested. The percentage

Table 1. RAPD primers, percentage of polymorphic loci (*P*), and Nei's unbiased heterozygosity (H_E) calculated for the total sampled boll weevils

Primer	Sequence (5′–3′)	No. bands studied	*P*	H_E (SE)
OPE-11	GAGTCTCAGG	11	54.6	0.204 (0.053)
OPE-12	TTATCGCCCC	14	64.3	0.252 (0.055)
OPE-14	TGCGGCTGAG	11	36.4	0.171 (0.057)
OPE-15	ACGCACAACC	12	58.3	0.188 (0.046)
OPE-18	GGACTGCAGA	11	54.5	0.170 (0.053)
OPE-19	CTGGGGACTT	8	62.5	0.231 (0.066)

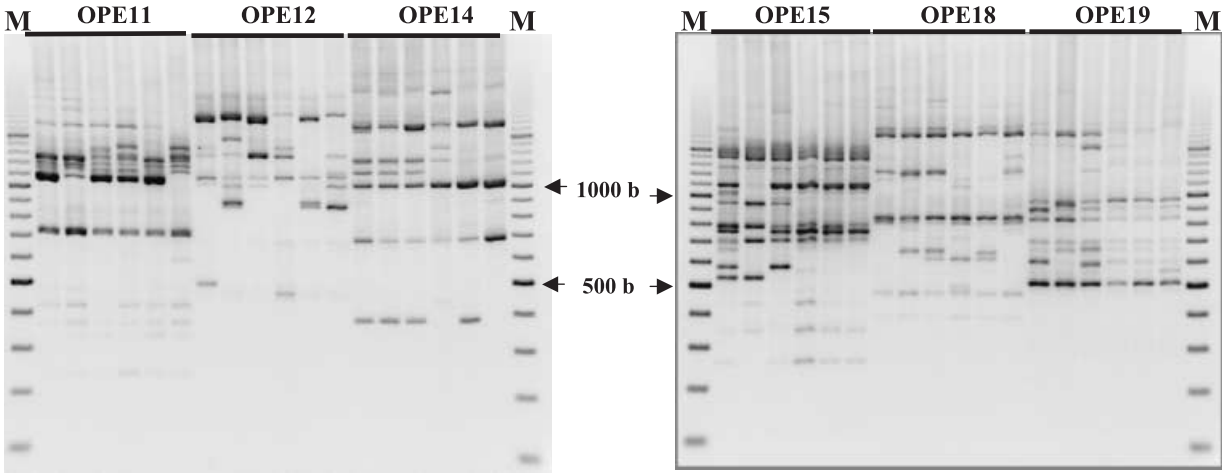

Figure 1. PCR profiles after amplification by RAPD primers. Each lane contains amplification products for an individual boll weevil. M, Molecular size marker (100 bp ladder).

Table 2. Locations of boll weevil collections and corresponding abbreviations, sample sizes, collection dates, and collectors

Region	Location	Abbrev.	Sample size	Collection date	Collector[a]
South-central	Tampico, Mexico	MEX	19	07-Apr-99	Greenberg, ARS-IFNRRU
	Weslaco, TX	WTX	20	05-Jun-00	Sappington, ARS-IFNRRU
	Kingsville, TX	KTX	16	07-Oct-02	Montgomery, TBWEF
	El Campo, TX	ETX	16	01-Aug-02	Mote, TBWEF
	College Station, TX	CSTX	15	30-May-00	Spurgeon, ARS-APMRU
	Waxahachie, TX	WATX	18	16-Sep-02	Knutson, Texas A & M
Western	Hobart, OK	HOK	15	11-Dec-01	Massey, OBWEO
	Stamford, TX	STX	14	14-Aug-01	Cleveland, TBWEF
	Childress, TX	CHTX	17	01-Aug-01	Isbell, TBWEF
	Plainview, TX	PTX	16	11-Sep-01	Jones, TBWEF
	Big Spring, TX	BTX	15	14-Aug-01	Melendez, TBWEF
	Artesia, NM	ANM	16	16-Oct-01	Norman, PVCBWCC
Eastern	Winnsboro, LA	WLA	16	06-Jul-01	Pylant, LDAF
	Little Rock, AR	LAR	15	17-Jul-01	Kiser, ABWEF
	Cleveland, MS	CMS	17	24-Sep-01	Sprouse, SEBWEP
	Yazoo City, MS	YMS	16	11-Oct-01	Keene, SEBWEF
	Malden, MO	MMO	15	30-Jan-02	Smith, SEBWEF
	Brownsville, TN	BTN	15	21-Jun-01	Seward, SEBWEF

[a]ARS, Agricultural Research Service; IFNRRU, Integrated Farming and Natural Resources Research Unit; TBWEF, Texas Boll Weevil Eradication Foundation; APMRU, Areawide Pest Management Research Unit; OBWEO, Oklahoma Boll Weevil Eradication Organization; PVCBWCC, Pecos Valley Cotton Boll Weevil Control Committee; LDAF, Louisiana Department of Agriculture and Food; ABWEF, Arkansas Boll Weevil Eradication Foundation; SEBWEF, South-Eastern Boll Weevil Eradication Foundation.

of RAPD loci that were polymorphic ranged between 36.4 and 64.3%, and calculated heterozygosity ranged from 0.170 to 0.252 (Table 1).

A 600-bp band generated by OPE14 was observed almost exclusively in the sample from Plainview, TX (PTX) weevils, and a band of 650 bp from the same primer was fixed in the Big Spring, TX (BTX) population, but was very rare in other populations. A band of about 890 bp amplified by OPE12 was predominant in western populations but was rare in eastern, whereas a 910-bp band from OPE12 was predominant in eastern populations but rare in western. the presence of the 890- and 910-bp bands, however, was variable in south-central populations.

Genetic diversity within populations

Altogether, 67 discernable bands were employed to estimate genetic diversity within weevil populations. The percentage of polymorphic loci and Nei's (1978) unbiased mean heterozygosity revealed a considerable amount of variation among weevil populations from the eighteen sampled locations (Table 3). The total number of polymorphic bands within locations varied from 12 in BTX to 55 in the sample

Figure 2. Geographic location of boll weevils sampled. Location abbreviations as in Table 2.

Table 3. Sample size, total number of polymorphic bands, percentage of polymorphic loci (*P*), and Nei's unbiased mean heterozygosity (H_E) per RAPD locus

Location	Sample size	Total no. polymorphic bands	*P*	H_E (SE)
MEX	19	55	76.6	0.293 (0.058)
WTX	20	49	64.3	0.239 (0.063)
KTX	16	38	48.7	0.161 (0.052)
ETX	16	35	39.9	0.148 (0.058)
CSTX	15	27	33.5	0.117 (0.054)
WATX	19	29	41.4	0.144 (0.059)
HOK	15	33	43.7	0.165 (0.062)
STX	14	23	28.6	0.110 (0.047)
CHTX	17	43	52.5	0.182 (0.062)
PTX	16	32	45.4	0.178 (0.064)
BTX	15	12	17.0	0.077 (0.050)
ANM	16	24	32.8	0.131 (0.058)
WLA	16	29	30.3	0.109 (0.053)
LAR	15	14	13.6	0.060 (0.038)
CMS	17	26	27.9	0.105 (0.052)
YMS	16	20	20.0	0.071 (0.044)
MMO	15	29	31.8	0.108 (0.049)
BTN	15	14	18.0	0.076 (0.050)
Mean	16.2	29.6	37.0	0.137

from Tampico, Mexico (MEX), with a median of 29. Percentage of polymorphic loci (*P*) ranged from 13.6% in the sample of Little Rock, AR (LAR) to 76.6% in MEX, and mean heterozygosity ranged from 0.060 in LAR to 0.293 in MEX, with a median of 0.124 (Table 3). The sample from Mexico showed the highest degree of genetic diversity (P = 76.6%, H_E = 0.293), as would be expected of the population closest to the geographical origin of this colonizing species. LAR displayed the lowest diversity (P = 13.6%, H_E = 0.060) (Table 3).

Kruskal–Wallis (KW) tests (Daniel, 1990) indicated that region (Table 2) had a significant effect on genetic diversity as measured by both *P* (KW statistic = 8.92; p = 0.012) and He (KW statistic = 10.05; p = 0.007). *P* and H_E were significantly greater (p = 0.05; critical rank value = 7.38) in south-central populations (median P = 45.1%; median H_E = 0.155) than in eastern populations (median P = 24.0%; median H_E = 0.091). These measures of genetic diversity were intermediate in the western region (median P = 38.3%; median H_E = 0.148), and did not differ significantly from those in either of the other two regions. In general, there was higher genetic variation among western than eastern populations. A higher genetic diversity variance (s^2) was observed among weevil populations from western (s^2 = 0.0017) than eastern (s^2 = 0.0005) regions, and estimates of heterozygosity varied over a wider range in western (from 0.178 in PTX to 0.077 in BTX) than eastern (from 0.109 in WLA to 0.060 in LAR) populations (Table 3).

Genetic differentiation and gene flow among populations

F_{ST} estimates calculated by Weir and Cockerham's method ranged from 0.036 (WLA vs. CMS) to 0.459 (BTX vs. YMS) (Table 4). Most paired comparisons of populations revealed significant genetic differentiation. However, F_{ST} values for two comparisons (WLA vs. CMS, and LAR vs. CMS) within the eastern region were not significantly different from 0, indicating little genetic differentiation between those populations. Region had a significant effect on within-region F_{ST} values (KW statistic = 10.31; p = 0.006). They were significantly higher (p = 0.05; critical rank value = 11.48) among populations from Western (median F_{ST} = 0.183) than Eastern (median F_{ST} = 0.088) regions. Within-region F_{ST} values were intermediate among south-central populations (median F_{ST} = 0.138), and did not differ significantly from those in either of the other two regions.

Genetic distance inferred from $F_{ST}/(1-F_{ST})$ was positively correlated with geographical distance between populations (r = 0.475; p = 0.0002) (Fig. 3A), indicating that individuals from proximal locations exchange genes more frequently than those from more distant locations. When the three major regions were considered separately, there was a significant correlation between genetic and geographical distance (r = 0.854; p = 0.004) within the south-central region (Fig. 3B). However, correlations were not significant within the Eastern (r = 0.017; p = 0.496) (Fig. 3C) or Western regions (r = –0.257; p = 0.270) (Fig. 3D), suggesting frequent gene flow among populations within these respective regions.

Indirect estimates of gene flow (*Nm* values) ranged from 0.3 (BTX vs. LAR) to 6.7 (WLA vs. CMS). The values indicate that at least one migrant per generation was exchanged between populations separated by ~400–500 km or less,

Table 4. Pair-wise F_{ST} (Weir & Cockerham, 1984) (above diagonal) and effective migrants per generation (*Nm*) (below diagonal) among boll weevil populations. Abbreviations as in Table 2

	MEX	WTX	KTX	ETX	CSTX	WATX	HOK	STX	CHTX	PTX	BTX	ANM	WLA	LAR	CMS	YMS	MMO	BTN
MEX		0.126**	0.221***	0.243***	0.283***	0.269***	0.228***	0.319***	0.195***	0.235***	0.390***	0.290***	0.321***	0.386***	0.320***	0.384***	0.330***	0.369***
WTX	1.7		0.136**	0.138**	0.181***	0.136**	0.170***	0.202***	0.148**	0.200***	0.331***	0.169***	0.239***	0.280***	0.223***	0.265***	0.206**	0.229***
KTX	0.9	1.6		0.147**	0.128**	0.153**	0.071*	0.216***	0.130**	0.231***	0.322***	0.138**	0.208***	0.237***	0.191***	0.241***	0.172**	0.217***
ETX	0.8	1.6	1.5		0.122**	0.090*	0.143**	0.249**	0.134***	0.178**	0.377***	0.209***	0.130**	0.182***	0.126**	0.244***	0.144**	0.160**
CSTX	0.6	1.1	1.7	1.8		0.092*	0.115**	0.185***	0.153**	0.225***	0.324***	0.165**	0.097*	0.103*	0.070*	0.180***	0.086*	0.127**
WATX	0.7	1.6	1.4	2.5	2.5		0.145**	0.112**	0.135**	0.153**	0.317***	0.111**	0.143**	0.191***	0.118**	0.166***	0.092*	0.121**
HOK	0.8	1.2	3.3	1.5	1.9	1.5		0.162**	0.084*	0.158**	0.242***	0.088*	0.165**	0.194***	0.144**	0.233***	0.114**	0.202***
STX	0.5	1.0	0.9	0.8	1.1	2.0	1.3		0.183***	0.217***	0.348***	0.089*	0.288***	0.285***	0.210***	0.253***	0.161**	0.264***
CHTX	1.0	1.4	1.7	1.6	1.4	1.6	2.7	1.1		0.091*	0.251***	0.143**	0.197***	0.270***	0.179***	0.306***	0.178***	0.248***
PTX	0.8	1.0	0.8	1.2	0.9	1.4	1.3	0.9	2.5		0.312***	0.220***	0.253***	0.341***	0.243***	0.328***	0.217***	0.303***
BTX	0.4	0.5	0.5	0.4	0.5	0.5	0.8	0.5	0.7	0.6		0.296***	0.358***	0.446***	0.360***	0.459***	0.307***	0.451***
ANM	0.6	1.2	1.6	0.9	1.3	2.0	2.6	2.6	1.5	0.9	0.6		0.236***	0.245***	0.187***	0.245***	0.142**	0.226***
WLA	0.5	0.8	1.0	1.7	2.3	1.5	1.3	0.6	1.0	0.7	0.4	0.8		0.070*	0.036^{NS}	0.222***	0.080*	0.126**
LAR	0.4	0.6	0.8	1.1	2.2	1.1	1.0	0.6	0.7	0.5	0.3	0.8	3.3		0.048^{NS}	0.183***	0.080*	0.088*
CMS	0.5	0.9	1.1	1.7	3.3	1.9	1.5	0.9	1.1	0.8	0.4	1.1	6.7	4.9		0.131**	0.080*	0.112**
YMS	0.4	0.7	0.8	0.8	1.1	1.3	0.8	0.7	0.6	0.5	0.3	0.8	0.9	1.1	1.7		0.178**	0.146**
MMO	0.5	1.0	1.2	1.5	2.7	2.5	1.9	1.3	1.2	0.9	0.6	1.5	2.9	2.9	2.9	1.2		0.077*
BTN	0.4	0.8	0.9	1.3	1.7	1.8	1.0	0.7	0.8	0.6	0.3	0.9	1.7	2.6	2.0	1.5	3.0	

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ^{NS} not significant.

with some exceptions. For example, the BTX population consistently showed very limited gene exchange with all other populations, even those relatively nearby (Table 4). Average *Nm* values among populations within the three major regions indicate relatively frequent gene flow among eastern populations (2.6 effective migrants per generation) compared with south-central (*Nm* = 1.5) and western (*Nm* = 1.4) populations. There was also relatively limited gene flow (*Nm* = 0.83) between western and eastern regions compared with that between south-central and eastern (*Nm* = 1.24) or between south-central and western (*Nm* = 1.16) regions.

Genetic relationships among populations

To help resolve genetic relationships among populations, neighbour-joining and FITCH phylogenetic trees were reconstructed based on Nei's unbiased genetic distances (Fig. 4). The two phylogenetic trees showed only weak bootstrap support for most clades. However, the separation of eastern and western populations into different clades is supported by bootstrap values of 58 and 77%. In addition, two western populations, ANM and STX, form a single clade with ~85% support in both trees. All but one of the populations from the south-central region are grouped among western populations, with MEX and WTX clearly occupying a distinct clade (95% support). The exception is CSTX, which was included in the eastern clade.

Principal component (PC) analysis was performed on RAPD loci frequency data to obtain further insights into the genetic relationships among weevil populations. Mean factor scores for the eighteen populations were plotted along the first two PC axes, which together accounted for 51% of the total variance in the covariance matrix (Fig. 5). The distinct spatial distribution of populations in the eastern and western populations along the PC axes is striking. The far south populations (MEX and WTX) are quite distinct, while the other south-central populations cluster as a group close to the western populations along PC1, and the eastern populations along PC2.

Discussion

We are currently applying several types of molecular genetic markers to infer the genetic structure of, and gene flow among, boll weevil populations in the central US Cotton Belt. In the present study, RAPD markers generated from six random primers revealed sufficient polymorphism to characterize genetic variation within and between boll weevil populations. The results are generally consistent with the conclusions drawn from RFLP analysis of long PCR fragments of weevil mtDNA (Kim & Sappington, 2004), but with some important exceptions. Both sets of data indicate that the highest genetic diversity is present in the population sampled from Mexico, whereas low levels of diversity

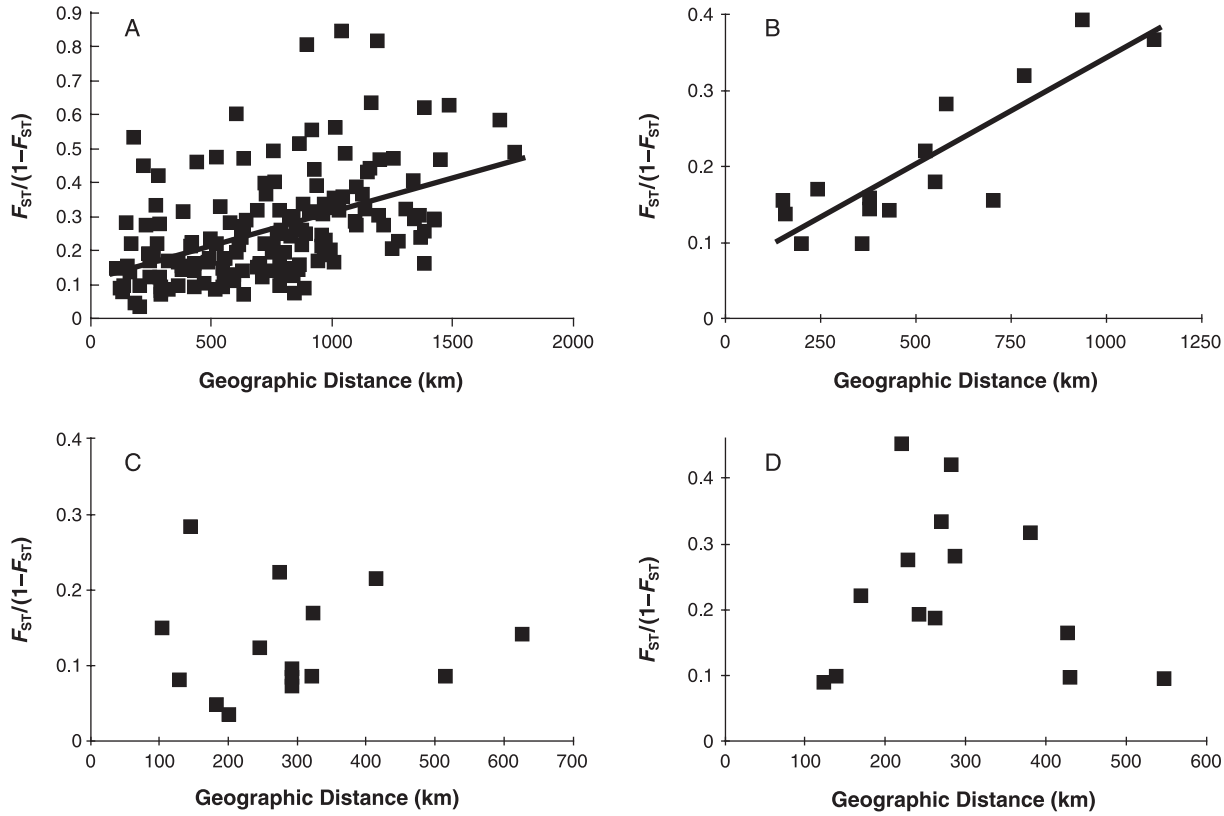

Figure 3. Relationship of $F_{ST}/(1-F_{ST})$ to geographical distance among boll weevil populations. (A) All populations ($r = 0.475$; $p = 0.0002$). (B) South-central populations only ($r = 0.854$; $p = 0.004$). (C) Eastern populations only ($r = 0.017$; $p = 0.496$). (D) Western populations only ($r = -0.257$; $p = 0.270$).

characterize populations in the more recently colonized areas of the northern Cotton Belt. The genetic variation in the colonizing founders of an invasive species generally represents only a fraction of that present in the source population, so our results appear to reflect the historical boll weevil range expansion into the southern US from Mexico, and subsequently across the south-eastern Cotton Belt and into the High Plains. This suggests that region-wide genetic structuring among boll weevil populations is not yet at equilibrium after the colonizing events of a century and of a half-century ago, respectively. Results of earlier studies are consistent with this conclusion (Terranova *et al*., 1990; Roehrdanz, 2001).

Thus, the recent colonization of the US by the boll weevil may influence estimates of genetic structuring and gene flow if, as appears to be the case, migration and genetic drift have not yet reached equilibrium (Felsenstein, 1982; Neigel, 1997; Avise, 2000). However, in such situations it is still possible to detect patterns of gene flow and isolation by distance, against a lingering genetic backdrop of an original range expansion, by using markers with a high mutation rate (Vandewoestijne & Baguette, 2002) and/or sampling at least ten polymorphic loci (Slatkin, 1993). RAPDs are thought to be characterized by relatively high mutation rates, perhaps in the range of 10^{-4}–10^{-6} per locus per generation (Lougheed *et al*., 2000; Vandewoestijne & Baguette, 2002). In addition, we sampled 67 polymorphic RAPD loci. Thus, we expected our estimates of gene flow to be fairly robust, but with a possible bias in the direction of overestimation (Avise, 2000; Ayres *et al*., 2003). Indeed, we found a significant positive correlation between genetic distance and geographical distance across all populations sampled, and for populations within the south-central region, indicating a general pattern of isolation by distance (Fig. 3A,B), although the correlations were not significant for populations within the western and eastern regions (Fig. 3C,D). Using RAPD markers, Scataglini *et al*. (2000) were unable to demonstrate significant genetic isolation by distance among neighbouring populations of boll weevils in southern Brazil, Paraguay, and northern Argentina, perhaps reflecting the recent range expansion from a common ancestral population out of Mexico, or extensive ongoing multidirectional gene flow among many locations.

Results of the RAPD analyses indicate that gene flow among populations between the three designated regions is limited, but that migration between locations within regions appears to be relatively frequent up to distances of ~300–400 km. An exception is the population from Big Spring, TX which appears quite isolated, although the reason for this is unclear. Pair-wise estimates of effective migration (*Nm*)

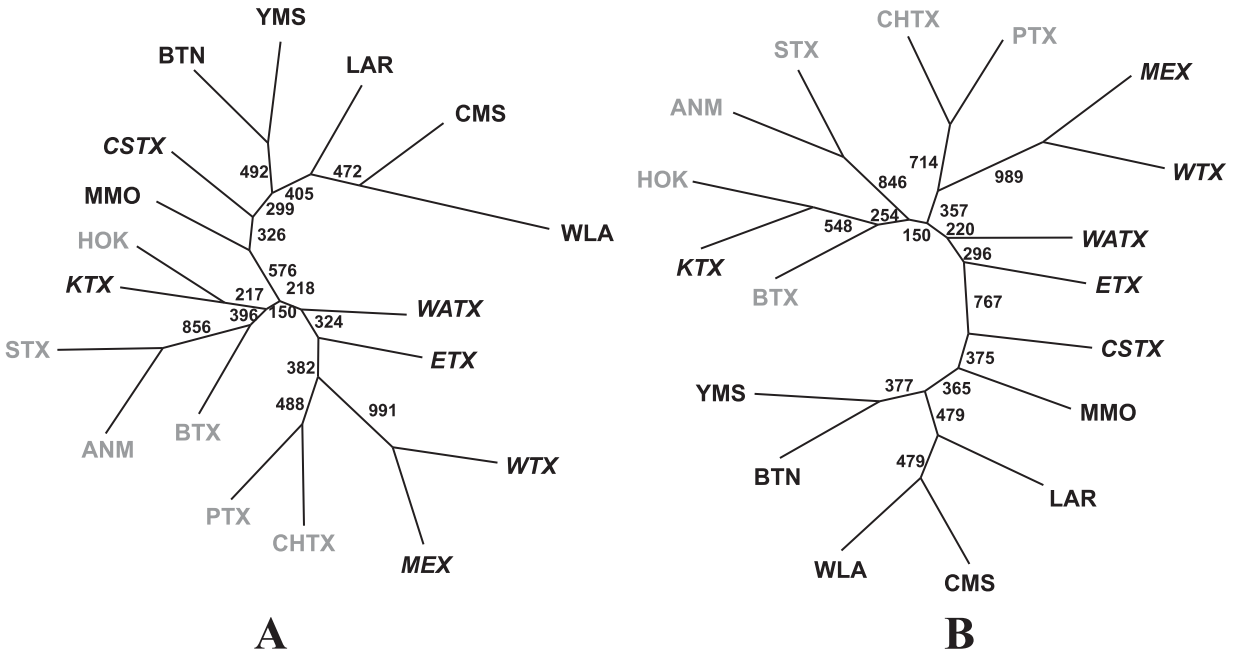

Figure 4. Unrooted phylogenetic trees showing genetic relationships among boll weevil populations based on RAPD data. A: Neighbour-joining tree; B: FITCH tree. Numbers indicate bootstrap support out of 1000 replications. Location abbreviations as in Table 2. Designated regions: italic font, south-central; grey font, western; black font, eastern.

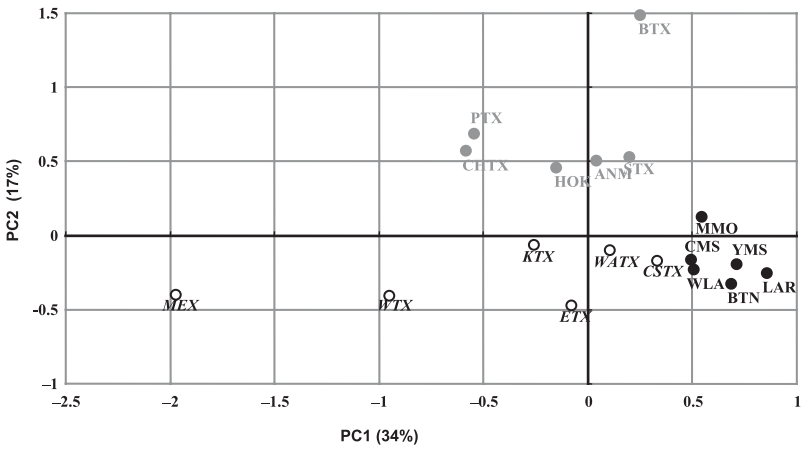

Figure 5. Mean factor scores from RAPD data for boll weevil populations from eighteen locations, plotted along the two major principal component axes. Location abbreviations as in Table 2. Designated regions: open circle and italic font, south-central; grey circle and grey font, western; black circle and black font, eastern.

based on RAPD data were much lower than those derived from mtDNA-RFLP data, where estimated *Nm*s above 100 and apparent panmixus were common (Kim & Sappington, 2004). In contrast, the highest *Nm* estimated from the RAPD data was only 6.7 for CMS–WLA, locations separated by 200 km. Given the heightened sensitivity of mtDNA to genetic bottlenecks and founder effects (Birky *et al*., 1983), and its functional equivalence to a single locus (Dowling *et al*., 1990), it is likely that the F_{ST} estimates and corresponding *Nm* estimates from the RAPD data are more reliable. If anything, RAPDs may underestimate F_{ST}, and therefore overestimate *Nm*, because independent mutations can occur in the RAPD primer annealing sites. In addition, the presence of numerous private RFLP alleles suggests that the high *Nm* values estimated from the RFLP markers are inflated. Nevertheless, the RAPD and RFLP analyses show similar patterns of genetic relationships among population pairs, with corresponding F_{ST} values from both analyses being positively correlated ($r = 0.700$, $p = 0.0002$).

The RAPD analyses revealed much more frequent gene flow among populations within the eastern region than within south-central and western regions, a trend also detected with the mtDNA-RFLP markers (Kim & Sappington, 2004). The mean (± SE) geographical distance between eastern populations (290 ± 36.8 km) is not significantly greater than that between western populations (278 ±

31.0 km), so the greater gene flow in the former is not an artefact of differential separation distances, although mean distances are greater among south-central populations (500 ± 74.4 km) (KW statistic = 6.78; $p = 0.03$). Thus, the data suggest that there is greater dispersal activity among eastern than western weevils. Why this should be so is unclear, though it is possible that the much windier conditions in the western region is a factor. Wind plays an important role in boll weevil flight behaviour, with moderate to high winds probably suppressing flight activity, and wind direction affecting dispersal patterns (Sappington & Spurgeon, 2000; Westbrook *et al.*, 2000; Sappington, 2002). Another possibility is that a more continuous boll weevil metapopulation in the east, where there are fewer and smaller gaps between areas of cotton cultivation, promotes gene flow.

Genetic differentiation between eastern and western populations is pronounced, with south-central populations being genetically intermediate. This is clearly supported by both phylogenetic and principal component analyses of the RAPD data (Figs 4 and 5). The same pattern was revealed by AMOVA and phylogenetic analyses of mtDNA-RFLP data (Kim & Sappington, 2004). In other respects, however, the two analyses revealed different patterns of relationships. Phylogenetic reconstructions from mtDNA-RFLP data exposed two major clades, one containing populations from the south-central and eastern regions, and the other clade containing populations from the western region. In contrast, phylogenetic reconstructions from the RAPD data placed the south-central populations in the clade with the western populations, with the exception of CSTX which clustered with eastern populations (Fig. 4). This relationship is supported by the principal component analysis (Fig. 5) in which south-central populations occupy an intermediate position between the eastern and western populations, but overlap extensively with the western populations along the more important PC1 axis. Both the eastern and presumably western populations were colonized originally from the south, but colonization of the western region has occurred only in more recent decades (Bottrell *et al.*, 1972). Thus, the pattern of genetic relationships revealed by the RAPD data may reflect this more recent colonization event. Alternatively, recurrent recolonizations of the west from the south may be more common than of the east from the south.

Genetic diversity is indexed by Nei's unbiased heterozygosity (H_E) for RAPD data, and by haplotype diversity (h) and nucleotide diversity (π) for mtDNA RFLP data. Both the RAPD (Table 3) and previous mtDNA-RFLP analyses (Kim & Sappington, 2004) produced consistently high values of these indices for south-central populations. However, the RAPD diversity index values for western populations were higher than those for eastern populations, while the opposite trend was indicated by the RFLP indices. Furthermore, correlations between genetic diversity indices for each population obtained by both analyses are not statistically significant. The RAPD data also revealed greater genetic diversity in the BTX population, which exhibited only one mtDNA-RFLP haplotype, suggesting that mtDNA haplotype distribution may not provide as realistic an estimation of genetic diversity as RAPDs in the case of boll weevils. This might be expected for mtDNA markers, which are highly sensitive to bottlenecks and founder events (Birky *et al.*, 1983), in the case of a pest like the boll weevil whose populations are often the target of insecticide applications. Nevertheless, both the RAPD and mtDNA-RFLP analyses showed similar trends in genetic differentiation among populations, indicating that neither is severely distorting estimates of population genetic structure.

There is controversy about the usefulness of estimating gene flow from F_{ST} (Whitlock & McCauley, 1999). Gene flow in a population genetic context is inversely related to population divergence or differentiation. This measure is accurate only if a number of often unrealistic assumptions are met (Whitlock & McCauley, 1999). Slatkin & Barton (1989) demonstrated that for sample sizes typical of electrophoretic surveys of natural populations, estimates of *Nm* are accurate to within 20–50% if the population structure resembles an idealized structure. The demography and dispersal patterns of natural populations are unlikely to be stable. In particular, a metapopulation that is the product of a recent range expansion will reach demographic equilibrium only after migration and drift to have had sufficient time to reach equilibrium. *Nm*, derived from F_{ST}, implies the number of effective migrants, a subset of the total number of migrants. Because the effective population size is usually much less than the total number of individuals present, the total number of migrants into a deme usually will be much higher than that estimated by F_{ST} (Whitlock & McCauley, 1999). This suggests that migration between the locations sampled may be more frequent than our estimates of *Nm* imply. On the other hand, in an eradication context, only effective boll weevil migrants are of importance. Given the limitations just described, one should interpret these data with caution, including the linkage of migration rates and F_{ST} measures. Nevertheless, our estimates are still informative and provide the best understanding to date of the frequency of long-range migration of boll weevils, derived via our estimates of current or recent gene flow relative to genetic drift.

Although RAPD markers are neutral and polymorphic enough to distinguish closely related populations, even if they have diverged recently, their dominant mode of inheritance reduces their resolving power in molecular ecological studies. We have begun a study using microsatellites, which are codominant markers, to reconcile discrepancies in the RAPD and mtDNA-RFLP results, and to obtain better insights into gene flow and structuring of boll weevil populations.

Experimental procedures

Sample collection

Boll weevils of both sexes were collected from eighteen locations in New Mexico (NM), Texas (TX), Oklahoma (OK), Missouri (MO), Arkansas (AR), Louisiana (LA), Tennessee (TN), and Mississippi (MS) in the US, and in north-eastern Mexico (MX) from the cotton growing region just north of Tampico (Fig. 2, Table 2) using traps baited with aggregation pheromone. Weevils were collected in multiple traps at each location and frozen, and represent the same individuals analysed previously for mtDNA PCR-RFLP variation (Kim & Sappington, 2004). Locations were grouped into three main regions designated as south-central, western, and eastern as was done in a previous study (Kim & Sappington, 2004). Within these three regions, each location was separated by < 300 km from its nearest neighbour, except in the case of MEX located ~430 km south of WTX (Fig. 2).

PCR amplifications and electrophoresis

Total genomic DNA was extracted from individual boll weevils using Promega's Wizard isolation kit (Promega, Madison, WI), according to the manufacturer's protocol. A total of 20 decanucleotide primers were tested, and were obtained from Kit-E, Operon Technologies Inc. (Alameda, CA). Of these, six were selected based on the clarity and consistency of the resulting banding patterns. RAPD reactions were carried out according to Williams *et al*. (1990). Amplification reactions were performed in a total volume of 30 μl using 15–50 ng of genomic DNA, 2.0 mM $MgCl_2$, 200 μM of each dNTP, 0.3 μM of each primer, and 1.5 units of *Taq* DNA polymerase (Panvera, Madison, WI). Amplification was carried out in a GeneAmp PCR System 9700 thermocycler (Perkin Elmer, Norwalk, CT) programmed for 45 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C, with a final extension at 72 °C for 5 min. An aliquot (13 μl) of each amplification product was separated electrophoretically in a 1.7% agarose gel in 1 × TBE buffer (90 mM Tris-borate, 2 mM EDTA), followed by staining with ethidium bromide (0.2 μg/ml). Stained gels were digitally documented with a Chemi Doc imaging system (Bio-Rad Laboratories, Hercules, CA). A 100 bp DNA ladder (Fermentas Inc., Hanover, MD) provided molecular weight size standards. Two PCR amplifications per individual were carried out to evaluate the reproducibility of RAPD bands. All PCR reactions were performed after aliquoting from a 'Master mix' followed by adding individual genomic DNA. Thus we could expect equal concentrations of PCR reagents in each tube. A negative control without genomic DNA was performed for each series of PCR amplifications to ensure that there was no contamination. All PCR reactions were carried out in clean space using sterilized materials.

Data analysis

Each weevil was scored at each locus as 1 (present) or 0 (absent) across all polymorphic loci to create a binary matrix. A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95. Only distinct bands from gel to gel were used to generate the matrix data set. Bands with the same molecular weight were assumed to represent homologous loci.

Allele frequencies were estimated using the RAPDBIOS 2.0 program (Black, 1995), which employs the Lynch & Milligan (1994) correction for estimating allele frequencies. The genetic diversity within populations was quantified as the percentage of polymorphic loci estimated from allele frequencies and Nei's (1978) unbiased heterozygosity (H_E), calculated under the assumption of Hardy–Weinberg equilibrium. Genetic diversity was calculated using the program BIOSYS-1 (Swofford & Selander, 1981).

Genetic distances among populations were calculated based on Nei's (1978) unbiased genetic distance using the RAPDDIST program (Black, 1995), and were employed for reconstruction of phylogenetic trees using the FITCH and NEIGHBOUR programs from the PHYLIP 3.5c computer package (Felsenstein, 1993). Bootstrap resampling (n = 1000) was performed to test the robustness of dendrogram topology. A principal component (PC) analysis was performed on the molecular variation revealed by the RAPD data to visualize the geometric relationships among weevil populations. PC analysis was conducted with the XLSTAT program (Agresti, 1990; Saporta, 1991). The RAPDFST program (Black, 1995) was employed to estimate population differentiation and gene flow among populations from Weir & Cockerham's (1984) F_{ST}, corrected for small and unequal sample sizes.

The relationship between $F_{ST}/(1–F_{ST})$ and geographical distance between populations was calculated from 5000 replications and normalized by the Mantel statistic Z option using the MXCOMP program in NTSYSPC, version 1.70 (Rohlf, 1992). Multiple comparisons of polymorphic loci, heterozygosity, and F_{ST} across regions were performed with the non-parametric Kruskal–Wallis test corrected for experimentwise error rate (Daniel, 1990) using Statistix software (Analytical Software, 1998).

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