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Spatial and Temporal Genetic Analyses Reveal High Gene Flow Among European Corn Borer (Lepidoptera: Crambidae) Populations Across the Central U.S. Corn Belt

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ABSTRACT

European corn borer, *Ostrinia nubilalis* (Hübner), adults were sampled at 13 sites along two perpendicular 720-km transects intersecting in central Iowa, and for the following two generations at four of the same sites separated by 240-km in the cardinal directions. More than 50 moths from each sample location and time were genotyped at eight microsatellite loci. Spatial analyses indicated that there is no spatial genetic structuring between European corn borer populations sampled 720 km apart at the extremes of the transects, and no pattern of genetic isolation by distance at that geographic scale. Although these results suggest high gene flow over the spatial scale tested, it is possible that populations have not had time to diverge since the central Corn Belt was invaded by this insect about 60 years ago. However, temporal analyses of genetic changes in single locations over time suggest that the rate of migration is indeed very high. The results of this study suggest that the geographic dimensions of European corn borer populations are quite large, indicating that monitoring for resistance to transgenic *Bt* corn at widely separated distances is justified, at least in the central Corn Belt. High gene flow further implies that resistance to *Bt* corn may be slow to evolve, but once it does develop it may spread geographically with such speed that mitigation strategies will have to be implemented quickly to be effective.

KEYWORDS: *Ostrinia nubilalis*, population genetics, gene flow, dispersal, microsatellites.

The European corn borer, *Ostrinia nubilalis* (Hübner), is a chronic pest of corn (*Zea mays*) in Europe, North Africa, parts of Asia, and the eastern two-thirds of North America. It is an invasive pest in North America, having been introduced at least twice into the eastern US from Europe in the early 20th century (Caffrey and Worthley 1927, Brindley and Dicke 1963). It spread westward across the Corn Belt, reaching Iowa in the 1940s. In much of the US the European corn borer has two generations, or "flights", per year with full grown larvae of the second generation entering diapause to overwinter in corn stubble (Showers et al. 1975). Moths lay their eggs on leaves, and larvae tunnel into the stalk. European corn borer is the main target of transgenic *Bt* corn expressing the Cry1Ab toxin from *Bacillus thuringiensis*, and until its commercialization in 1996 (Rice and Pilcher 1998), this insect was responsible for over one billion dollars in yield and control costs annually in the U.S. (Mason et al. 1996).

Bt corn has been widely adopted by American farmers because of its excellent control of European corn borer, but with that wide adoption has come concern that prolonged and strong selection pressure will lead to the evolution of resistance in this pest (Tabashnik et al. 2003, 2008; Qiao et al. 2008; Tyutyunov et al. 2008). Substantial efforts are being made to delay the development of resistance in natural populations as long as possible through insect resistance management (IRM) strategies (Bourguet et al. 2005, Sivasupramaniam et al. 2007). Currently, preventative IRM tactics for European corn borer are implemented at the local scale, and are based on the high-dose/refuge strategy (Alstad and Andow 1995, Gould 1998, Bourguet et al. 2005). There are two basic components to this strategy, under the assumption of a single recessive resistance allele: 1) the use of a high dose of *Bt* toxin to render heterozygous resistant individuals

functionally susceptible, and 2) the placement of non-*Bt* corn refuges within 800 m of *Bt* corn to serve as nurseries for production of homozygous susceptible moths to mate with any resistant survivors in nearby *Bt* fields. Together, these tactics are expected to delay the production of homozygous resistant individuals and the subsequent increase in resistance allele frequency (Caprio 2001, Ives and Andow 2002, Tyutyunov et al. 2008).

A key element of the ongoing European corn borer IRM strategy is to monitor resistance development in local populations (EPA 2001, Sivasupramaniam et al. 2007), but this is one of its weakest aspects (Bourguet et al. 2005). A major difficulty in monitoring has been in determining the appropriate geographic scale at which sampling should be performed (ILSI 1998, Andow and Ives 2002). Monitoring is expensive, so it is highly desirable to be as efficient as possible. However, it is essential that monitoring efforts be geographically thorough enough that developing resistance in any population is detected before it increases to the point of control failure and spreads to other populations. Designing a sampling strategy that maximizes efficiency under the constraint of adequate spatial sensitivity relies critically on knowing the genetic structuring within and among European corn borer populations and the amount of gene flow that can be expected across different geographic distances (Caprio and Tabashnik 1992, Roderick 1996, Andow 2002). Without this knowledge, the area over which a monitoring site can be expected to detect a resistance allele or a change in allele frequency is undefined (ILSI 1998) and rates of resistance evolution and spread cannot be modeled accurately (Sisterson et al. 2004).

The geographic dimensions of a population are defined by per-generation gene flow, which is determined in large part by dispersal distances. Several mark-release studies have suggested that long distance dispersal by European corn borer likely occurs, but

most have been limited to recapture distances of < 1 km (Hunt et al. 2001, Qureshi et al. 2005, Dalecky et al. 2006b, Reardon et al. 2006a, Bailey et al. 2007, Reardon and Sappington 2007). An exception is a study by Showers et al. (2001), where marked adults were recaptured 23-49 km from the release site, but no traps were monitored beyond 49 km. Flight mill studies by Dorhout et al. (2008) indicate that 1-d old unmated females engage in obligate migratory behavior, with maximum distances of > 20 km observed for both males and females. Range and ecotype expansion data (Chiang 1972, Showers 1979, Showers et al. 1995), and circumstantial sampling data (Caffrey and Worthley 1927, Colenutt 1995, Bretherton and Chalmers-Hunt 1989, Langmaid and Young 2006) suggest that European corn borer dispersal can occur up to at least 80 km, but the frequency of dispersal to these distances or beyond has not been determined.

Direct estimates of gene flow and inferred dispersal rates can be derived from analyses of neutral genetic markers (Roderick 1996, Krafsur et al. 2001, Lowe et al. 2004). Several studies have examined genetic differentiation among European corn borer populations using allozyme or DNA markers, but in North America most of these have been concerned with gene flow between partially isolated "Z" and "E" pheromone races and between voltinism races (Harrison and Vawter 1977, Cianchi et al. 1980, Glover et al. 1991, Pornkulwat et al. 1998, Willet and Harrison 1999, Coates and Hellmich 2003). Allozyme polymorphisms in northern France revealed restricted gene flow between populations of *O. nubilalis* from corn and populations of what is now thought to be *O. scapulalis* (Frolov et al. 2007, Malausa et al. 2007) from mugwort and hops (Bourguet et al. 2000b, Martel et al. 2003, Malausa et al. 2005, Leniaud et al. 2006).

Estimates of gene flow within races are more limited, especially in North America.

Marçon et al. (1999) reported a lack of variation among widely separated North American populations of European corn borer in a 500-bp sequence of the nuclear ribosomal internal transcribed spacer 1 region and in restriction fragment length polymorphism (RFLP) patterns of four short PCR-amplified fragments of mtDNA, but the markers were all monomorphic so no conclusions about genetic structuring can be drawn. Coates et al. (2004) examined variation in mtDNA RFLP haplotypes from cytochrome oxidase I and II genes in wild populations from eight U.S. states, but haplotype variation was very low. Although the analyses were confounded by pheromone and voltinism races, the data suggest that a bivoltine, Z-pheromone race from Maine may be genetically differentiated from bivoltine, Z-pheromone races from Indiana westward. Recently, Krumm et al. (2008) used amplified fragment length polymorphism (AFLP) markers to examine genetic structuring and gene flow among populations of European corn borer in nine states, mostly in the western part of its U.S. range. Although voltinism races were confounded with distance over the total area of the study and pairwise differentiation between populations was not reported, a surprisingly high average G_{ST} (0.17) was estimated among far western populations, which presumably are all bivoltine. The calculated average rate of migration per generation (Nm) was likewise moderate to low (2.41) over the geographic scale sampled in this region. Despite the evidence for genetic structuring, no significant isolation by distance pattern was observed and the authors concluded that gene flow was high over large distances. Population genetics studies in France suggest that high gene flow can occur over long distances, although nearby populations are sometimes differentiated from one another (Bourguet et al. 2000a; Martel et al. 2003; Leniaud et al. 2006; Malausa et al. 2007).

Here, we examined genetic variability in European corn borer at 8 microsatellite DNA loci to measure gene flow spatially along two 720-km transects through the central Corn Belt of the US and temporally between years at a subset of locations. Spatial analyses are crucial to understanding parameters like geographic population size that have an inherent spatial element (Wilson 2004). Temporal analyses provide a way of measuring real-time migration regardless of population history, and of identifying individuals in a sample as probable immigrants (Cornuet et al. 1999, Wilson and Rannala 2003, Paetkau et al. 2004). They also provide the most robust estimates possible of effective population size and migration rate (Wang and Whitlock 2003).

MATERIALS AND METHODS

Sampling. For spatial analyses, adult male European corn borers of the second flight of 2005 were sampled in early August with pheromone traps from a total of 13 sites along two perpendicular 720-km transects running from Minnesota to Missouri, and from Nebraska to Illinois, intersecting in Ames, Iowa (Fig. 1). Collections were made at 120 and 360 km from Ames on all four arms of the transects. Five additional samples were taken at 16-km intervals from 40 to 104 km west of Ames. For temporal analyses, the four locations 120 km from Ames in the cardinal directions were resampled during the first and second flights of 2006 (Fig. 1). Locations are coded as cardinal direction followed immediately by distance from Ames in km, followed by year (after a hyphen), and finally whether 1st or 2nd flight. For example, E120-05-2nd refers to the population sampled 120 km east of Ames during the second flight of 2005.

Each location was sampled with five pheromone cone-style traps of three different designs, including the Hartstack wiremesh, 75-cm diameter cone trap (Hartstack et al. 1979), a modified Hartstack wiremesh, 35-cm-diameter cone trap, and the nylon-mesh Heliothis, 35-cm-diameter cone trap (Gemplers, Madison, WI), as described in Reardon et al. (2006b), with each of four traps placed approximately 2 km from a central trap. Traps were placed in grassy sites (Mason et al. 1997; Reardon et al. 2006a), where adults aggregate for mating and daytime resting (Showers et al. 1976; Sappington and Showers 1983). Collections from the five traps were pooled until at least 50 individuals were accumulated for that location. Collected moths were stored at -20^{o}C until processing for DNA isolation.

Genotyping. Eight European corn borer microsatellite loci were selected for inferring population genetic structure, with previous data existing for seven of them showing no deviation from Hardy-Weinberg Equilibrium (HWE), adequate polymorphism, and ease of scoring. These included On-T2, On-T3, and On-T4 from Kim et al. (2008), and D63, D65, D145 and T81 from Dalecky et al. (2006a). The eighth locus (On-D1), containing a GA dinucleotide repeat motif, was recently developed by the ARS lab (Forward: CACAAGGGATACACGAGCGA, Reverse: CTCGTACTCTCCCCGCACTT), which met the same criteria (unpublished data).

DNA was extracted from individual European corn borer adults using BioRad's Aqua Pure isolation kit (BioRad, Hercules, CA), according to the manufacturer's protocol. Seven of the eight microsatellites were amplified by polymerase chain reaction (PCR) in two separate multiplex reactions for each sample: Multiplex 1: D63, D65, and T81;

186 Multiplex 2: On-T2, OnT-3, OnT-4 and On-D1. Microsatellite locus D145 was amplified
187 by itself. The loci were amplified from 57-60 individuals per population using the
188 QIAGEN Multiplex Kit (QIAGEN) according to the protocol described by Dalecky et al.
189 (2006a). The PCR fragments were analyzed by capillary gel electrophoresis on an ABI
190 3730XL (Applied Biosystems, Foster City, CA), or a Beckman-Coulter CEQ 8000
191 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA). Genotypes were
192 determined using Genemarker v1.60 software (SoftGenetics LLC, State College, PA) for
193 data from the ABI sequencers, and using CEQ 8000 Software, version 5.0 for data from
194 the Beckman-Coulter CEQ 8000. About 10% of the genotypes from each sequencer were
195 cross checked to verify repeatability. Individual loci within a multiplex panel that yielded
196 ambiguous genotypes for a particular sample (~5% of moths) were reamplified with
197 single primer pairs and reanalyzed.
198
199 **Data Analysis.**
200 *Genetic Structure of European corn borer.* Within-population genetic variability was
201 assessed with three estimates of genetic diversity: the mean number of alleles per locus,
202 observed heterozygosity (H_O), and unbiased estimates of expected heterozygosity (H_E)
203 (Nei 1987) under Hardy-Weinberg assumptions using the Microsatellite Toolkit (Park
204 2001). Linkage disequilibrium between pairs of loci and deviation from Hardy-Weinberg
205 equilibrium (HWE) for each locus and population were tested using the exact probability
206 test approach (Guo and Thomson 1992), as implemented in the program GENEPOP4.0.6
207 (Raymond and Rousset 1995). GENEPOP4.0.6 was used to test the null hypothesis of no
208 differences in spatial and temporal variation of allelic and genotypic frequencies between

209 each pair of samples and over all samples. *F*-statistics (Weir and Cockerham 1984) for
210 each locus and pairwise F_{ST} estimates were calculated for both spatial and temporal
211 samples using the program FSTAT v. 2.9.3 (Goudet 1995); significance values were
212 calculated using a permutation approach. The sequential Bonferroni correction was
213 applied in deriving significance levels in cases of multiple comparisons (Rice 1989).
214 Kruskal-Wallis (KW) statistics tested for differences in central tendencies of allele
215 frequency distributions between generations at the same site, and between locations
216 within the same generation using Statistix 8 software (Analytical Software 2000).
217 The potential occurrence of null alleles was tested using the program MICRO-
218 CHECKER (Van Oosterhout et al. 2004). Null alleles are suspected for a given locus when
219 MICRO-CHECKER rejects HWE and if excess homozygotes are evenly distributed among
220 allelic size classes. Because all of the loci appeared to harbor a low frequency of null
221 alleles (see Results), corrected pairwise F_{ST}'s were calculated for all populations by
222 applying the ENA correction in the FREENA package (Chapuis and Estoup 2007). All
223 values of F_{ST} reported in this study are corrected values except where noted.
224 Isolation by distance (IBD) (Wright 1943) was inferred from the relationship
225 between $F_{ST}/(1-F_{ST})$ and the $\log_{10}$ geographic distance between populations sampled
226 during the second flight of 2005. The relationship was calculated from 5000 resamplings
227 and normalized by the Mantel statistic Z option using the MXCOMP program in
228 NTSYSPC, version 1.70 (Rohlf 1992).
229 The program STRUCTURE 2.0 (Pritchard et al. 2000) was used to test for the
230 existence of population structuring among spatial and temporal European corn borer
231 samples by estimating the number of distinct populations (*K*) present in the set of samples

using a Bayesian clustering approach. The posterior probability of K, Pr($X|K$), is the probability of the observed set of genotypes (X), conditioned on a given K between 1 and 10. The program was run using an initial burn-in of 100,000 iterations followed by 1,000,000 iterations, an admixture model of individual ancestry, and correlated allele frequencies among populations. Five runs were performed independently for each value of K to verify consistency of estimates of Pr($X|K$) between runs.

Effective population size (N_e) and the migration rate (m). We employed the method of Wang and Whitlock (2003), which uses the computer program MLNE, to estimate m and N_e simultaneously via a maximum-likelihood strategy. This method uses a temporal approach that compares allele frequencies from at least two generations. Simulation studies show that it performs better than other methods (Wang and Whitlock, 2003). N_e and m were calculated simultaneously for four sampling sites (S120, N120, E120 and W120) between two years (2005 2nd flight and 2006 2nd flight) located 120 km from Ames, Iowa in the cardinal directions, and thus separated by 170 to 240 km. Since there is no apparent geographic barrier to European corn borer dispersal at this spatial scale in central Iowa, we assumed any of these populations could be a potential source of migrants to any other. Thus, a pooled sample from the other three sites of the 2006 2nd-flight was used to estimate allele frequencies from a potential source population. Values for m indicate the proportion of the sample from that location estimated to be immigrants from potential source populations. The maximum possible *Ne* value was set to 10000.

Population Bottleneck Tests. Genetic bottlenecks were likely associated with the original invasion of North America, and it is possible that human control practices, including widespread adoption of transgenic *Bt* corn, have caused bottlenecks in more recent years. Three different measures – heterozygosity, stability of allele frequencies, and the mean ratio of the number of alleles to the allele size range (*M*) – were used to detect signatures of population decline and recovery over different time scales. The first two parameters were assessed using the program BOTTLENECK 1.2 (Cornuet and Luikart 1996). Significance ($\alpha = 0.05$) of observed heterozygosity excess or heterozygosity deficiency relative to that expected at drift-mutation equilibrium was tested by the Wilcoxon sign-rank test (Luikart et al. 1998a, Luikart and Cornuet 1998). Both a strict stepwise mutation model (Kimura and Ohta 1978), and a two-phase model (Di Rienzo et al. 1994) were employed with 1000 iterations each. For the two-phase model, generalized stepwise mutation was assumed, in which a proportion of the stepwise mutation model was set to 0 with a variance in mutation lengths of 0.36 (Estoup et al. 2001). A mode-shift in allele frequency distribution was used as a qualitative indicator of population bottlenecks (Luikart et al. 1998b). As an alternative test to detect reductions in population size over a much longer time frame, Garza and Williamson's (2001) *M* value and its variance across loci were calculated using the program AGARST (Harley, 2001). The *M* ratio is expected to have a long recovery time after a decline in population size, e.g. >100 generations, and thus allows one to distinguish recent population reductions from those occurring a long time ago (Garza and Williamson 2001).

RESULTS

Allele Frequency and Within-population Diversity. A total of 70 alleles across eight microsatellite loci were observed for 1244 European corn borer individuals from 21samples over space and time in the central Corn Belt of the US (Table 2, Fig. 1). The number of alleles per locus ranged from 3 in On-D1 to 14 in T81, with an average of 8.8. Seven of 70 alleles were unique to one location, but they occurred at very low frequency <0.01. There was no evidence of significant genotypic linkage disequilibrium for any locus pair in any population, nor across all samples after correction for multiple testing (data not shown), confirming that all microsatellite loci used in this study effectively segregate independently. Exact tests for deviations from HWE across all loci revealed that 14 of 21 populations were significantly out of equilibrium, but only five populations showed significant deviation after correction for multiple testing. Null alleles were probably present at more than one locus in all but two populations. Notably, all populations deviating from HWE were estimated to have had at least one locus containing a null allele (Table 1). The T81 locus appeared to have null alleles present in the largest number of populations (13 of 21 populations), while the On-D1 and On-T2 loci showed evidence of null alleles in only one population each. Mean frequency of null alleles estimated at each locus ranged from 0.014 in On-D1 to 0.066 in T81 (Table 2).

The average numbers of alleles per locus, expected heterozygosity, and observed heterozygosity, all indicate high levels of genetic diversity across all populations (Table 1). Average numbers of alleles per locus were similar across all populations, ranging from 6.0 (E120-05-2nd and N120-05-2nd) to 7.0 (W120-06-1st). H_E ranged from 0.581 (N120-06-1st) to 0.642 (W56-05-2nd), averaging 0.623. There were no significant

differences in genetic diversity across locations (KW statistic = 2.0389, $P = 1.0$ for allelic difference, KW statistic = 1.7355, $P = 1.0$ for H_E).

Genetic Structure Within and Among Populations. Across all populations, F_{IS} estimates of individual loci ranged from -0.028 to 0.140 among spatial samples and 0.035 to 0.148 among temporal samples, with slightly higher multilocus F_{IS} estimates among temporal than spatial samples (Table 2). F_{IS} estimates for each locus were significantly correlated with the frequency of null alleles calculated by FREENA (Spearman Rank Correlation = 0.9701, p = 0.0007 for spatial samples, Spearman Rank Correlation = 0.9222, p = 0.0042 for temporal samples), indicating that null alleles are the most probable reason for high and significant F_{IS} values rather than non-random mating.

Global estimates of F_{ST} across all loci and all populations were very low for both spatial and temporal samples. When corrected by ENA for the presence of null alleles, the *F*st value was about twice as high among temporal populations (ENA corrected F_{ST} = 0.0039) as among spatial populations (ENA corrected $F_{ST} = 0.0017$) (Table 2). Corrected pairwise F_{ST} estimates across all loci ranged from -0.0051 to 0.0140 for spatial samples (Table 3) and from -0.0037 to 0.0156 for temporal samples (Table 4). Only one of the 78 pairwise comparisons showed significant allelic differentiation among spatial samples (Table 3), and only two of the 40 comparisons were significant among temporal samples (Table 4), indicating stable allele frequencies over space and time. No spatial or temporal pairwise comparisons showed significant genotypic differentiation after correction for multiple testing (data not shown). There was no significant relationship between genetic distance and geographic distance ($R^2 = 0.00257$, p = 0.395).

323 The Bayesian estimation of the number of populations within both spatial and
324 temporal datasets did not provide evidence of any population structure. The STRUCTURE
325 analyses indicated a single panmictic population is represented by the spatial and
326 temporal samples, where the posterior probability for $K = 1$ was >0.999 for each case.
327
328 **Effective Population Size (*Ne*) and Migration Rate (*m*).** Maximum likelihood
329 (ML) and moment (MT) estimations of *Ne* varied depending on location, but was quite
330 high, ranging from 188.6 at E120 to 4931.8 at S120 for ML. However, the distributions
331 of *Ne* and *m* estimates had long tails for each sample, so the true value may be much
332 larger or smaller than the estimates. For example, the upper 95% confidence interval of
333 *Ne* was > 10000 for all four sites. MT estimates of *Ne* were infinite for three of the sites
334 and 645 for N120 (Table 5). ML and MT estimates of *m* also were generally large across
335 sites, ranging from 0.0378 to 0.5367 for ML, and from 0.1691 to 0.5094 for MT (Table 5).
336
337 **Population Bottlenecks.** No evidence of a recent population decline in European
338 corn borer was detected from the central Corn Belt, although the *M* ratios provide
339 somewhat equivocal evidence for a bottleneck in the past (Table 6). Wilcoxon sign-rank
340 tests did not detect a significant excess of observed heterozygosity relative to the
341 expected equilibrium heterozygosity under drift-mutation equilibrium. However, three
342 populations under the TPM, and 17 populations under the SMM showed significant
343 heterozygote deficiency, providing some evidence for past population expansion or
344 introduction of exotic alleles by immigration (Luikart and Cornuet 1998). The mode shift
345 test did not detect deviation in any of the populations from the typical L-shaped allele

346 frequency distribution expected of a large, stable, non-bottlenecked population. The *M*
347 ratios ranged from 0.685 to 0.835 (Table 6).
348
349
350 **DISCUSSION**
351 The nearly complete lack of spatial genetic structuring among samples of European
352 corn borer across such a large geographic scale (720 km) was unexpected for this species.
353 Although allozyme studies in France have consistently indicated high gene flow (F_{ST} or θ
354 values typically about 0.01) even over distances of about 600 km (Bourguet et al. 2000a;
355 Martel et al. 2003; Leniaud et al. 2006), significant structuring was detected in these
356 same studies in a number of pairwise comparisons, generally unrelated to distance
357 between samples. Analyses of mtDNA haplotypes indicated much higher (F_{ST} = 0.039)
358 and significant differentiation among populations in northern France (Martel et al. 2003).
359 Recently, Malausa et al. (2007) examined gene flow among 13 populations of the
360 European corn borer in France at different spatial scales using microsatellite markers.
361 Though again suggesting high gene flow globally, 33% of pairwise comparisons
362 indicated significant genetic differentiation, even among nearby populations. In our study,
363 the only significant pairwise F_{ST} was quite low (0.0125; Table 3) and transient,
364 disappearing in the following two generations (Table 4).
365 A lack of a signficant isolation by distance (IBD) pattern can occur for several
366 reasons. If pairwise F_{ST} values are high and significant, a flat IBD regression line
367 indicates that the spatial scale between samples was too large. In other words, gene flow
368 is so restricted, that population pairs separated by the minimum distance tested are as

369 isolated as pairs separated by greater distances. In our case, uniformly low and
370 nonsignificant pairwise F_{ST} estimates at distances ranging from 16 – 720 km resulted in a
371 nonsignificant IBD. Usually this is an indication that the spatial scale of sampling was
372 too small and that gene flow is unrestricted over even the greatest distances tested.
373 However, the European corn borer is an invasive insect in North America. The size of a
374 founding population may have recovered rapidly before the westward range expansion,
375 so we must consider the possibility that the observed lack of genetic differentiation is an
376 after-effect of that expansion, given large population size and limited time for genetic
377 drift to create differences. We found no evidence for a recent population bottleneck.
378 However, the intermediate *M* values observed in this study hint that the genetic effects of
379 founder events accompanying the original invasion may not be completely erased. All
380 values were higher than the critical value of 0.68 expected for apparent bottlenecked
381 populations (Garza and Williamson 2001). On the other hand, the *M* values for many
382 samples are below those expected from historically stable populations (0.82) (Garza and
383 Williamson 2001). Furthermore, cases of heterozygote deficiency in some populations
384 suggest a lingering signal from the population expansion that accompanied the invasion
385 of North America (Luikart and Cornuet 1998).

386 Distinguishing between the two possibilities of high gene flow or post-invasion
387 non-equilibrium is difficult. We approached this question by conducting a temporal
388 analysis of gene flow at four locations each separated from the other by 170 or 240 km
389 (Fig. 1). Support for unrestricted gene flow comes from estimates of migration rates
390 which were very high, indicating that ~25-50% of the N120 and E120 moths of the
391 second flight in 2006 were immigrants. The lower immigration rates of ~4-6% estimated

392 by the maximum-likelihood method for the W120 and S120 locations are consistent with
393 prevailing wind direction in late spring and summer out of the west and southwest. As
394 with most flying insects, the direction and distance of European corn borer adult dispersal
395 is likely strongly influenced by wind (Mikkola 1986; Showers et al. 1995, 2001).
396 The region of the Corn Belt traversed by our transects is distinguished by very high
397 corn production with no obvious topographical barriers to European corn borer dispersal.
398 It is possible that movement and gene flow in this species may be more restricted in the
399 eastern U.S. where corn hectarage is much lower, agricultural land use is more diverse,
400 and the landscape is characterized by higher topographic relief. The lack of IBD in the
401 French studies of gene flow (Bourguet et al. 2000a; Martel et al. 2003; Malausa et al.
402 2007) seems to be the result of variable rates of gene flow among populations, with some
403 genetic differentiation evident but unrelated to geographic distance. The reasons for
404 differential gene flow in France are unknown, but may reflect landscape-level factors
405 affecting movement. It will be important to examine gene flow in areas of the U.S. such
406 as the northeast where local populations may be more isolated. Restricted gene flow
407 could increase the chance of *Bt* resistance developing in local areas (Taylor et al. 1983,
408 Caprio and Tabashnik 1992, Lenormand and Raymond 1998) compared to the Corn Belt
409 where gene flow seems to be occurring over great distances.
410 In any population genetics study employing microsatellites, the potential for null
411 alleles must be addressed (Pemberton et al. 1995, Girard and Angers 2008). A null allele
412 is caused when nucleotide variation in the flanking region of the microsatellite locus
413 prevents primer binding and PCR amplification, making the locus appear homozygous
414 for the one allele that does amplify (de Sousa et al. 2005). This functionally recessive

behavior leads to a decrease in genotyping accuracy, which in turn can result in a number of artifacts including heterozygote deficiency, inaccurate allele frequency estimates, and inflated F_{IS}, F_{ST}, and genetic distance estimates (de Sousa et al. 2005, Chapuis and Estoup 2007, Girard and Angers 2008). Incidence of null alleles is particularly high in Lepidoptera (Meglécz et al. 2004, 2007, Zhang 2004, Van't Hof et al. 2007), and European corn borer is no exception (Coates et al. 2005, Dalecky et al. 2006a, Malausa et al. 2007, Kim et al. 2008). At the population level, most loci deviating from HWE were estimated by MICRO-CHECKER to segregate for null alleles (Table 1). Therefore, the heterozygote deficiencies detected in populations that significantly deviated from HWE are most likely the result of null alleles, as was concluded for European corn borer microsatellites in French populations (Malausa et al. 2007). However, a possible Wahlund effect caused by transient genetic structure within samples cannot be ruled out. Despite all loci showing evidence of a null allele in at least one population in our study, the frequencies were relatively low and any potential biases they introduced in F_{ST} estimates were mitigated by the ENA correction method of Chapuis and Estoup (2007).

Together, our data strongly suggest that European corn borer gene flow, and therefore dispersal, occurs over much greater distances in the central U.S. Corn Belt than previously suspected. In general, high gene flow should help impede evolution of resistance to *Bt* corn (Peck et al. 1999, Ives and Andow 2002), but very high gene flow paradoxically can reduce the efficacy of refuges and accelerate resistance evolution (Tyutyunov et al. 2008). Once it does develop, migration of resistant insects can spread the trait to susceptible populations (Peck et al. 1999, Morjan and Rieseberg 2004). Our data imply that unless other factors, such as cost of resistance (Lenormand and Raymond

1998, Gassmann et al. 2009), are more important than migration, a resistance phenotype could spread geographically with such speed that mitigation strategies will have to be implemented quickly and at a large enough spatial scale to be effective. High gene flow also implies that sites for *Bt* resistance monitoring in the Corn Belt can be widely spaced, because the geographic dimensions of a population are very large. Thus, increasing statistical power by pooling of F_2 screen data to detect resistance alleles from sample sites 300-400 km apart, as done by Bourguet et al. (2003) and Stodola et al. (2006), may well be justified.

Our conclusion that high gene flow is the primary reason for lack of structuring across long distances in North America rather than it being an effect of the range expansion is being further tested by substantially increasing the spatial scale of sampling. If spatial structuring can be detected at greater distances, then estimates of high gene flow at the smaller scales in the current study will be supported. Results of that study are near completion and will be reported elsewhere.

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FOOTNOTES TO THE TEXT

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Table 1. Characteristics of transect samples of adult European corn borer: Sample size (N) and date, total alleles (average number of alleles per locus), observed (H_O) and expected (H_E) heterozygosity, F_{IS} per sample over all loci

Transect samples	Lon. °W Lat. °N	N	Sampling dates	Total alleles (mean / locus)	H_O	H_E	F_{IS} [a]	No. (identity[b]) of loci deviating from HWE	P [c]	No. (identity[b]) of loci with null allele[d]
W40-05-2nd	94.093 42.023	58	Jul 20,22,27-2005	51 (6.4)	0.552	0.606	0.091 NS	1 (8)	0.7246	0
W56-05-2nd	94.436 42.014	58	Jul 28, Aug 4-2005	54 (6.8)	0.588	0.642	0.084 NS	3 (4,6,8)	0.0079	2 (6,8)
W72-05-2nd	94.456 42.038	57	Jul 20,25,28, Aug 4-2005	50 (6.3)	0.621	0.628	0.012 NS	2 (2,7)	0.0029	1 (7)
W88-05-2nd	94.685 42.078	57	Jul 19,25,28, Aug 4-2005	52 (6.5)	0.564	0.633	0.110 NS	2 (4,8)	0.1120	1 (8)
W104-05-2nd	94.860 42.019	60	Jul 19,25,29, Aug 4-2005	50 (6.3)	0.565	0.638	0.115*	2 (4,5)	0.0011	3 (4,5,7)
W120-05-2nd	95.073 42.048	58	Jul 21,29, Aug 4-2005	52 (6.5)	0.575	0.616	0.066 NS	0	0.1022	1 (6)
W360-05-2nd	97.843 42.076	60	Jul 22,30,31-2005	50 (6.3)	0.538	0.601	0.106 NS	2 (4,8)	0.0003	3 (4,6,8)
S120-05-2nd	93.463 40.912	60	Jul 19,25, Aug 9,10-2005	55 (6.9)	0.479	0.637	0.250 *	4 (2,5,6,8)	<0.0001	5 (2,3,5,6,8)

S360-05-2nd	93.465 38.755	60	Jul 19,26, Aug 1,9,17-2005	53 (6.6)	0.571	0.620	0.079 ^{NS}	2 (3,8)	<0.0001	1 (8)
N120-05-2nd	93.626 43.119	60	Jul 19,20,25, Aug 2,8,18-2005	48 (6.0)	0.540	0.610	0.117 *	2 (3,7)	0.0240	3 (3,4,7)
N360-05-2nd	93.666 45.312	60	Jul 20,26, Aug 1,8,19-2005	53 (6.6)	0.619	0.630	0.018 ^{NS}	2 (5,8)	0.0432	1 (7,8)
E120-05-2nd	92.067 41.949	60	Jul 21,27, Aug 3-2005	48 (6.0)	0.567	0.611	0.074 ^{NS}	1 (3)	0.0901	2 (3,4)
E360-05-2nd	89.168 41.788	60	Jul 22,28, Aug 4-2005	52 (6.5)	0.598	0.639	0.065 ^{NS}	0	0.3004	0
W120-06-1st	95.062 42.050	60	May 30, Jun 7-2006	56 (7.0)	0.549	0.621	0.117 *	1 (6)	<0.0001	3 (4,6,8)
S120-06-1st	93.461 40.908	60	May 31-2006	52 (6.5)	0.572	0.617	0.073 ^{NS}	0	0.1234	2 (4,8)
N120-06-1st	93.626 43.127	60	Jun 1-2006	53 (6.6)	0.545	0.581	0.062 ^{NS}	1 (8)	<0.0001	1 (8)
E120-06-1st	92.070 41.949	60	May 24,25, Jun 2-2006	53 (6.6)	0.581	0.639	0.091 ^{NS}	1 (1)	0.0336	3 (1,3,8)
W120-06-2nd	95.062 42.050	60	Jul 26,28,31, Aug 3,10-2006	51 (6.4)	0.552	0.636	0.133 *	4 (3,4,5,8)	<0.0001	3 (3,4,8)
S120-06-2nd	93.461 40.908	59	Jul 24, Aug 1-2006	55 (6.9)	0.568	0.626	0.093 ^{NS}	2 (4,6)	0.0037	3 (4,6,8)

N120-06-2nd	93.626 43.127	58	Jul 26-2006	50 (6.3)	0.599	0.625	0.042 [NS]	1 (7)	0.6958	1 (7)
E120-06-2nd	92.070 41.949	59	Jul 24,28-2006	49 (6.1)	0.564	0.618	0.089 [NS]	3 (1,5,7)	0.0057	3 (5,7,8)

[a] P-value for F_{IS} within samples based on 3360 randomizations; Indicative adjusted nominal level (5%) is 0.00030

[b] Locus identity codes: 1 = On-D1; 2 = On-T2; 3 = On-T3; 4 = On-T4; 5 = D145; 6 = D65; 7 = D63; 8 = T81

[c] Probability that sample-wide deviation from HWE is by chance alone, based on Fisher's method

[d] Based on MICRO-CHECKER

Table 2. Characteristics of each microsatellite locus for 21 population samples, including total number of alleles, mean estimated frequency of null alleles, and estimates of F_{IS} and F_{ST} from both spatial and temporal analyses

Locus	Total alleles	Mean estimated frequency of null alleles [a]	F_{IS} [b]		Uncorrected F_{ST} [c] (F_{ST} corrected by ENA)	
			Spatial	Temporal	Spatial	Temporal
On-D1	3	0.014	-0.028 [NS]	0.035 [NS]	-0.0007 [NS] (-0.0011)	0.0032 [NS] (0.0065)
On-T2	6	0.022	0.011 [NS]	0.039 [NS]	0.0103* (0.0182)	0.0201*** (0.0284)
On-T3	7	0.046	0.108***	0.138***	-0.0009 [NS] (-0.0011)	-0.0009 [NS] (-0.0003)
On-T4	9	0.046	0.128***	0.127***	0.0022 [NS] (0.0026)	0.0005* (0.0009)
D145	8	0.034	0.069**	0.093**	-0.0014 [NS] (-0.0001)	0.0007 [NS] (0.0025)
D65	13	0.047	0.131***	0.107***	-0.0027 [NS] (-0.0015)	-0.0002 [NS] (0.0005)
D63	10	0.028	0.078***	0.074***	-0.0012 [NS] (-0.0008)	-0.0002 [NS] (0.0006)

T81	14	0.066	0.140***	0.148***	-0.0019 ^{NS} (-0.0003)	-0.0017* (-0.0001)
All loci	70	-	0.092***	0.102***	0.0002 ^{NS} (0.0017)	0.0020*** (0.0039)

[a] Null allele frequency for each locus was estimated for each of the 21 spatial and temporal samples using the EM algorithm (Dempster et al. 1977) and then averaged.

[b] Alleles randomized within samples, and testing for Hardy-Weinberg equilibrium within samples. Based on 1000 randomizations.

[c] Testing for population differentiation under assumption of random mating within samples. Statistic used is exact G-test. (Goudet et al. 1996)

Table 3. Corrected F_{ST} estimates (below diagonal) and significance of exact tests for allelic differentiation (above diagonal) across eight microsatellite loci in pairwise comparisons of European corn borer samples along transects through the central Corn Belt of the U.S. during the second flight of 2005 (NS = not significant; *** = p < 0.001).

	W40	W56	W72	W88	W104	W120	W360	S120	S360	N120	N360	E120	E360
W40		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
W56	0.0023		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
W72	-0.0023	-0.0012		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
W88	-0.0004	-0.0024	-0.0008		NS	NS	NS	NS	NS	NS	NS	NS	NS
W104	0.0003	0.0044	-0.0017	0.0018		NS	NS	NS	NS	NS	NS	NS	NS
W120	0.0072	0.0008	0.0067	0.0012	0.0065		NS	NS	NS	NS	NS	***	NS
W360	-0.0005	0.0036	0.0002	0.0037	0.0005	0.0024		NS	NS	NS	NS	NS	NS
S120	0.0021	0.0074	0.0036	0.0056	0.0059	0.0140	0.0081		NS	NS	NS	NS	NS
S360	-0.0015	0.0074	-0.0016	-0.0005	0.0019	0.0018	-0.0017	0.0046		NS	NS	NS	NS
N120	-0.0020	-0.0009	-0.0051	0.0016	-0.0010	0.0060	-0.0024	0.0059	-0.0025		NS	NS	NS
N360	-0.0027	0.0042	-0.0005	0.0018	-0.0007	0.0078	0.0009	0.0047	-0.0019	-0.0025		NS	NS
E120	-0.0018	0.0062	-0.0003	0.0009	0.0017	0.0125	0.0034	0.0042	0.0015	0.0025	0.0014		NS
E360	-0.0010	0.0015	-0.0023	-0.0013	-0.0028	0.0061	0.0014	0.0030	0.0002	0.0002	0.0007	-0.0022	

Table 4. Corrected F_{ST} estimates (below diagonal) and significance of exact tests for allelic differentiation (above diagonal) across eight microsatellite loci in pairwise comparisons of European corn borer samples at locations 120 km from Ames, IA in the cardinal directions. Within generations and between locations (bold), between generations and within locations (reverse phase, bold), and across generations and locations (plain). (NS = not significant; * = p < 0.05, ** = p < 0.01, *** = p < 0.001)

	S120-05-2nd	N120-05-2nd	E120-05-2nd	W120-05-2nd	S120-06-1st	N120-06-1st	E120-06-1st	W120-06-1st	S120-06-2nd	N120-06-2nd	E120-06-2nd	W120-06-2nd
S120-05-2nd	-	**NS**	**NS**	**NS**	**NS**	NS	NS	NS	**NS**	NS	NS	NS
N120-05-2nd	**0.0059**	**-**	**NS**	**NS**	NS	**NS**	NS	NS	NS	**NS**	NS	NS
E120-05-2nd	**0.0042**	**0.0025**	-	***	NS	**	**NS**	NS	NS	NS	**NS**	NS
W120-05-2nd	**0.0140**	**0.0060**	**0.0125**	-	NS	NS	NS	**NS**	NS	NS	*	**NS**
S120-06-1st	**0.0048**	-0.0037	0.0015	0.0080	-	**NS**	**NS**	**NS**	**NS**	NS	NS	NS
N120-06-1st	0.0156	**0.0037**	0.0162	0.0007	**0.0072**	-	**NS**	**NS**	NS	**NS**	NS	NS
E120-06-1st	0.0069	0.0005	**0.0026**	0.0047	**0.0007**	**0.0096**	-	**NS**	NS	NS	**NS**	NS
W120-06-1st	0.0056	-0.0019	0.0032	**0.0030**	**-0.0013**	**0.0053**	**0.0007**	-	NS	NS	NS	**NS**
S120-06-2nd	**0.0008**	-0.0008	-0.0002	0.0084	**-0.0027**	0.0095	0.0016	-0.0007	-	**NS**	**NS**	**NS**
N120-06-2nd	0.0045	**0.0016**	0.0005	0.0076	0.0026	**0.0098**	0.0001	0.0004	**0.0029**	-	**NS**	**NS**

E120-06-2nd	0.0027	-0.0023	**0.0010**	0.0140	0.0005	0.0135	**-0.0002**	0.0025	**0.0021**	**0.0002**	-	**NS**
W120-06-2nd	0.0077	0.0026	0.0062	**0.0013**	0.0035	0.0075	-0.0014	**0.0004**	**0.0036**	**0.0010**	**0.0061**	-

Table 5. Maximum-likelihood (ML) and moment (MT) estimates of effective population size (*Ne*) and migration rate (*m*) for temporal European corn borer samples from four locations 120 km from Ames, IA in the cardinal directions.

	Ne		*m*	
Location	ML	MT	ML	MT
S120	4931.82	∞	0.0378	0.1691
N120	1007.27	645.00	0.2530	0.5094
E120	188.60	∞	0.5367	0.3550
W120	886.31	∞	0.0552	0.3161

Table 6. Tests to detect a recent (SMM, TPM, Mode shift) or past (*M*) population reduction or expansion within *O. nubilalis* samples from the central Corn Belt of US

	Wilcoxon sign-rank tests [a]					
	SMM		TPM [b]			
Sample	Het excess	Het deficit	Het excess	Het deficit	Mode shift	*M*
W120-05-2nd	0.986	0.020	0.727	0.320	Normal	0.835 (0.063)
W104-05-2nd	0.994	0.010	0.473	0.578	Normal	0.740 (0.037)
W40-05-2nd	0.902	0.125	0.809	0.231	Normal	0.813 (0.048)
W56-05-2nd	0.963	0.098	0.809	0.231	Normal	0.753 (0.036)
W72-05-2nd	0.986	0.020	0.629	0.422	Normal	0.728 (0.048)
W88-05-2nd	0.980	0.027	0.770	0.273	Normal	0.820 (0.058)
W360-05-2nd	0.990	0.014	0.875	0.156	Normal	0.719 (0.036)
E360-05-2nd	0.986	0.020	0.727	0.320	Normal	0.695 (0.059)
N360-05-2nd	0.998	0.004	0.973	0.037	Normal	0.706 (0.052)
S360-05-2nd	0.994	0.010	0.875	0.156	Normal	0.759 (0.048)
E120-05-2nd	1.000	0.002	0.875	0.156	Normal	0.721 (0.070)
N120-05-2nd	0.844	0.191	0.680	0.371	Normal	0.731 (0.062)
S120-05-2nd	0.994	0.010	0.963	0.098	Normal	0.771 (0.052)
S120-06-1st	0.996	0.006	0.629	0.422	Normal	0.685 (0.035)
N120-06-1st	0.996	0.006	0.973	0.037	Normal	0.751 (0.062)
E120-06-1st	0.973	0.037	0.902	0.125	Normal	0.828 (0.075)
W120-06-1st	1.00	0.002	1.00	0.002	Normal	0.763 (0.053)
S120-06-2nd	0.998	0.004	0.875	0.156	Normal	0.771 (0.049)
N120-06-2nd	0.980	0.027	0.727	0.320	Normal	0.778 (0.069)
E120-06-2nd	0.963	0.098	0.727	0.320	Normal	0.696 (0.042)
W120-06-2nd	0.986	0.020	0.578	0.473	Normal	0.750 (0.057)

[a] One tail probability for excess or deficit of observed heterozygosity relative to the expected equilibrium heterozygosity, computed from the observed number of alleles under mutation-drift equilibrium. SMM, stepwise mutation model; TPM, two-phased model of mutation.

[b] The test was conducted assuming a generalized stepwise mutation model (GSM) with a variance of 0.36 in geometric distribution of mutation lengths (Estoup et al. 2001).

[c] *M* = mean ratio of the number of alleles to the range of allele size (Garza and Williamson 2001). Variance in parentheses.

Figure Caption

Fig. 1. European corn borer adult sample locations along two transects intersecting in Ames, IA. Distance from Ames in km is indicated after compass direction (N, S, E, W) from Ames. All locations were sampled during the second flight of European corn borer adults in 2005. Locations marked by squares also were sampled during first and second flights in 2006.

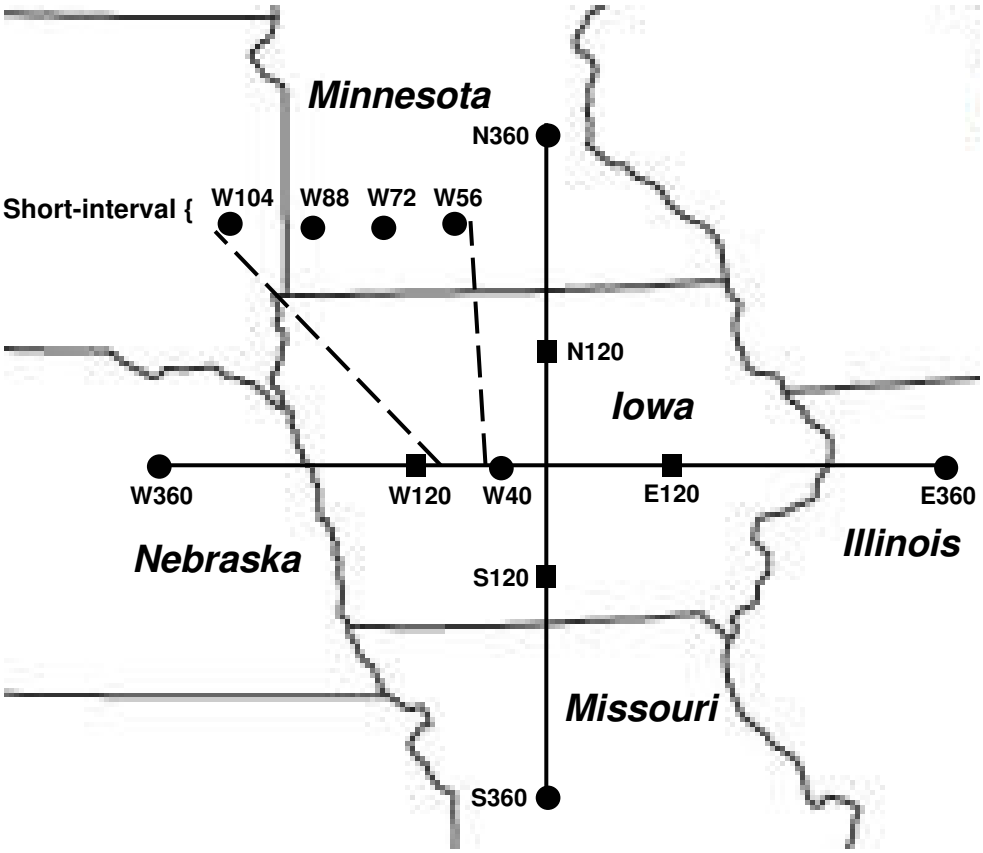
Minnesota
N360
Short-interval {
W104
W88
W72
W56
N120
Iowa
W360
W120
W40
E120
E360
Nebraska
Illinois
S120
Missouri
S360

Fig. 1