

## Genetic Structure of *Tribolium castaneum* (Coleoptera: Tenebrionidae) Populations in Mills

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**ABSTRACT** The red flour beetle, *Tribolium castaneum* (Herbst), is primarily found associated with human structures such as wheat and rice mills. Such structures are predicted to be spatially isolated resource patches with frequent population bottlenecks that should influence their genetic structure. Genetic diversity and differentiation among nine populations of *T. castaneum* collected from wheat and rice mills (ranging from <1–5,700 km apart) were investigated using eight polymorphic loci (microsatellites and other insertion–deletion polymorphisms, each with 3–14 alleles). Seventy-two locus-by-population combinations were evaluated, of which 31 deviated significantly from Hardy–Weinberg equilibrium, all because of a deficiency of heterozygotes. AMOVA analysis indicated significant differences among populations, with 8.3% of the variation in allele frequency resulting from comparisons among populations, and commodity type and geographic region not significant factors. Although there were significant differences in genetic differentiation among populations ( $F_{ST}$  values = 0.018–0.149), genetic distance was not significantly correlated with geographic distance. Correct assignment to the source population was successful for only 56% of individuals collected. Further analyses confirmed the occurrence of recent genetic bottlenecks in five out of nine populations. These results provide evidence that populations of *T. castaneum* collected from mills show spatial genetic structure, but the poor ability to assign individuals to source populations and lack of isolation by distance suggest greater levels of gene flow than predicted originally.

**KEY WORDS** *Tribolium castaneum*, genetic structure,  $F_{ST}$ , genetic fingerprinting, bottleneck

Genetic variation at the intra and interpopulation level has been widely used as a discriminating criterion for estimating levels of isolation and gene flow among populations. Ecology (e.g., mating system, social structure, dispersal, and spatial distribution); genetics (e.g., population bottlenecks, rate of mutation, genetic drift, and natural selection); and environment (e.g., landscape fragmentation, and physical barriers) are major factors that can prevent panmixia and lead to the genetic differentiation of populations. Stored-product insects typically occur in food processing facilities such as mills and consequently occupy habitat patches that are considered spatially isolated and discontinuous (Campbell 2005), which may facilitate the

development of genetic differentiation among facilities or even unique genetic fingerprints for a given facility. However, this tendency toward genetic isolation and differentiation may be countered by immigration of new individuals through long-range flight and passive movement by transport of infested food materials. The potential for exchange of individuals among populations is likely impacted by the type of facility, commodity processed, geographic location, and insect species studied. Genetic structure in a food facility also is likely to be impacted by frequent severe population reductions or extinctions because of periodic treatments with fumigants or heat (Fields and White 2002). Population recovery after such treatments can be through rebound of survivors, immigration, or both (Campbell and Arbogast 2004; Campbell et al. 2010a, 2010b), both of which can impact population structure. Although the population genetic structure of stored product pests has received some attention (e.g., Dowdy and McCaughey 1996, Fleurat-Lessard and Pronier 2006, Ryne and Bensch 2008, Drury et al. 2009, Ridley et al. 2011), the population structure of pests in food facilities and the processes that generate this structure are not well understood. Population genetics information could provide insight that would help improve the implementation and interpretation of monitoring and pest management programs, as well as potentially generate management tools such as fingerprinting tech-

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nologies so that infestation sources in food distribution channels could be identified.

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is a major pest in wheat and rice mills worldwide (Sokoloff 1974). The global distribution of this insect and its long association with stored food suggest that passive movement via human dispersal and commerce has been an important determinant of its distribution and population structure. *T. castaneum* does readily initiate flight under certain conditions (Perez-Mendoza et al. 2011) and has been captured away from human-made grain storages (Sinclair and Haddrell 1985, Daglish et al. 2010, Ridley et al. 2011), but is not frequently observed flying at commercial food facilities. It has been hypothesized that *T. castaneum* populations in flour mills are likely to be relatively self contained within the mills relative to other stored-product species that occur there and therefore more likely to be genetically isolated (Campbell and Arbogast 2004; Toews et al. 2006; Campbell et al. 2010a, 2010b). Grain mills may have a lower probability of human-aided transport of *T. castaneum* into the facility compared with other food facilities such as processing plants and warehouses because limited processed material is transported onto a site. However, beetles could be transported to the mill site in the raw grain and then move into the mill by using behavioral mechanisms either directly from the grain storage areas or during cleaning processes. Recently, Ridley et al. (2011) demonstrated that mated females dispersed from grain stores and beetles captured more than a kilometer from grain bins were genetically related with those captured near bins.

*T. castaneum* has a long history as a model organism for population biology and as the only beetle with a completely sequenced genome, it also has become an important model for studying molecular and developmental genetics (Lorenzen et al. 2005, Wang et al. 2006, Denell 2008, Tribolium Genome Sequencing Consortium 2008). The genetic characterization of this species has facilitated the identification of unique and polymorphic molecular markers, including an abundance of microsatellite loci, and has triggered recent studies leading to possible use of such markers for assessment of the genetic structure of populations (Pai et al. 2003, Demuth et al. 2007, Drury et al. 2009, Ridley et al. 2011).

The current study focuses on understanding the genetic structure of *T. castaneum* populations found in commercial mills. The long-term objectives of this research were to understand how populations within mills are initiated and maintained, identify importance of different mechanisms for dispersal, and develop DNA fingerprinting tools that could be used to identify or eliminate specific mills as sources of subsequent infestations. Unlike previous studies (Pai et al. 2003, Demuth et al. 2007, Drury et al. 2009) that relied on laboratory colonies, we collected beetles directly from wheat and rice mills (within both the continental United States and Puerto Rico). The geographic distance between mills ranged from <1 km to thousands of kilometers. Moreover, to control for temporal variation, beetles were collected as much as possible at an

equivalent time point. By focusing on mills that should have more limited importation of infested material directly into the structures, and including mills that process disparate commodities (wheat and rice) that see little exchange of material and mills in different geographic regions that should see limited exchange of grain, the potential for genetic differentiation among populations should be maximized. Specifically, this research sought to determine the 1) genetic structure of these populations, 2) correlation between levels of differentiation and geographic separation of populations and commodity type, and 3) ability to accurately assign individuals to their source population.

## Materials and Methods

**Sample Collection.** *T. castaneum* were collected from rice and wheat mills across the United States and the territory of Puerto Rico by using the STORGARD Dome traps (Trécé Inc., Adair, OK), baited with *Tribolium* spp. pheromone lure and kairomone oil. Samples from each location ( $\geq 30$  beetles) were obtained from multiple traps placed on the ground and distributed throughout each facility over a  $\approx 2$ -wk monitoring period. Beetles were collected between July and August 2007, except for one sample that was collected in March 2005 (KS2). Collection sites were located on the west coast (California = CA1 and CA2); midwest (Nebraska = NE1 and NE2, Kansas = KS1 and KS2); and southeast (Louisiana = LA1, Florida = FL1, and Puerto Rico = PR1) regions of the United States and its territories. Three locations processed rice (LA1, CA1, and CA2) and the others processed wheat. After collection, but before DNA extraction, individual beetles were placed in 1.5-ml centrifuge tubes with 75% ethanol and frozen at  $-80^{\circ}\text{C}$ .

**DNA Extraction and Fragment Analysis.** Before DNA extraction, beetles were rinsed with Histo-Clear (National Diagnostics, Atlanta, GA) for 5 min and washed with double distilled water to remove contaminating oils (i.e., food oil derived from the traps). Genomic DNA from individual beetles was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Because of collection conditions (beetles dead for different periods of time and exposure to high temperatures), we modified the polymerase chain reaction (PCR) protocol of Lorenzen et al. (2005) to improve DNA amplification. The modified procedure was as follows: PCRs were conducted in a final volume of 12  $\mu\text{l}$  containing 0.5- $\mu\text{l}$  genomic DNA ( $\approx 8 \text{ ng}/\mu\text{l}$ ); 0.12- $\mu\text{l}$  (5 U/ $\mu\text{l}$ ) *Taq* DNA polymerase (Promega, Madison, WI); 0.12- $\mu\text{l}$  dNTP (25 mM); 1.68- $\mu\text{l}$   $\text{MgCl}_2$  (25 mM); 2.4- $\mu\text{l}$  5x PCR buffer; 0.12- $\mu\text{l}$  forward tailed primer (100 nM); 0.24- $\mu\text{l}$  reverse primer (200 nM); and 0.12- $\mu\text{l}$  M13 labeled primer (100 nM) (primers from IDT, Coralville, IA). The cycling program included  $95^{\circ}\text{C}$ , 5 min for initial denaturation;  $95^{\circ}\text{C}$ , 45 s;  $58^{\circ}\text{C}$ , 1 min;  $72^{\circ}\text{C}$ , 1 min for 15 cycles;  $95^{\circ}\text{C}$ , 45 s;  $50^{\circ}\text{C}$ , 2 min;  $72^{\circ}\text{C}$ , 1 min for 24 cycles; and a final extension at  $72^{\circ}\text{C}$  for 5 min.

Molecular markers were identified by screening the *T. castaneum* genome for microsatellite repeats, com-

**Table 1.** Marker code, linkage group (LG), motif, primer sequences, and locus-specific  $F_{ST}$  values for each of eight molecular markers used for evaluation of *Tribolium castaneum* population structure

Code	LG (cM)	Motif	Primer sequences (5'-3') <sup>a</sup>	Locus-specific $F_{ST}$ values	GeneBank no.
MS1	2 (2.4)	GTT(10)	CGACGACGAGAAGGGAGGTA GCAAGGAGGCCATGAATAAAA	0.074	NW_001093459
ID1	3 (0.0)	-	AACITTTAAACCCATCTCACTCAA ATCATACTTTCAGACCCAGACAC	0.132	NW_001092787
MS2	3 (42.6)	ATAA(5)	GTAACACAGGAGGACAGGCTAAAAGTG CATCGAACGAGGCTGTGAATAAAC	0.025	NW_001093437
MS3	3 (52.0)	AAT(7)	TATCCGAAATTTTATCTACTCAT AGGACCCTTTTACTTTTTCAG	0.052	NW_001093371
ID2	3 (100)	-	CCGCTTTCGTCTCRCAGITGC CTAWYGTAAAGACTTATTAGGCACGTTT	0.119	NW_001092815
MS4	9 (21.7)	AAT(3)-AAT(5)-AAT(8)	ACCCGCAACAAAGTAAGCAA TTCTGACTACCACCGACAGATTT	0.079	NW_001092888
MS5	9 (40.6)	TAA(16)	AAGTGCTGCTGATTTTTATT TCAGACTCGGTATCCTTTATT	0.105	NW_001093602
MS6	10 (15.8)	AAT(19)	AAATTCTGGGCTTTTTGGGT GAGCTGGCGGTTATATTGGA	0.070	NW_001092762

In the reverse primer for ID2, W and Y are used as nomenclature for incompletely specified bases (W is used to specify adenine or thymine use in that location and Y is for thymine or cytosine).

<sup>a</sup> Primer sequences for MS6 (Tca-10.1) were obtained, and primer sequences for MS4 (Tca-9.1) were modified, from Demuth et al. (2007).

paring sequences from different strains to identify variation, or from microsatellites identified in Demuth et al. (2007). In total, 31 markers were analyzed initially using a subset of the populations, but most were ultimately excluded because of poor quality of amplified fragments or because sufficiently specific primers could not be designed. Markers were assessed for uniqueness by similarity searches of the *T. castaneum* genome (<http://www.beetlebase.org>) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997). Markers also were selected so they were as widely distributed within the genome as feasible (Table 1). Ultimately, eight unique polymorphic loci were considered suitable, including six microsatellites (MS1 through MS6) and two insertion-deletion polymorphisms (ID1 and ID2) that consisted of regions with multiple short microsatellites within them (Table 1).

For high-throughput genotyping, fluorescently-labeled PCR fragments were produced using a M13 oligonucleotide adaptor sequence attached to the 5' end of the forward primers that allowed for the incorporation of the fluorescent dye into the amplicons (Schuelke 2000). PCR products were analyzed using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) (Sequencing and Genotyping Facility, Plant Pathology, Kansas State University) and allele sizes scored using GeneMarker v.1.85 (SoftGenetics, State College, PA).

**Population Analysis.** For each population-by-locus combination, the expected and observed heterozygosities were calculated using the Genetic Data Analysis program GDA v.1.1 (Lewis and Zaykin 2001). Linkage disequilibrium and deviation from Hardy-Weinberg equilibrium (HWE) were tested using GENEPOP v.4.0 (Raymond and Rousset 1995), and for multiple comparisons, the sequential Bonferroni procedure was applied to determine the significance level (Rice 1989). Although the DNA amplification procedure was modified to handle varying levels of DNA degradation, thus reducing the possibility of allele

dropout, other factors such as null alleles still could affect the results. Therefore Micro-Checker (van Oosterhout et al. 2004) was used to determine if deviations from HWE were because of the presence of null alleles or genotyping errors. Presence of null alleles and failure to account for their presence can underestimate the within-population genetic diversity and overestimate differentiation among populations (Dakin and Avise 2004). The software FreeNA was used to estimate corrected  $F_{ST}$  values (Chapuis and Estoup 2007).

Analysis of Molecular Variance (AMOVA) by using Arlequin v.3.11 software (Schneider et al. 2000) was conducted to evaluate the genetic structure of population and groups of populations (i.e., commodity processed [rice or wheat] and geographic region [west coast, midwest, or southeast]). Isolation by distance was investigated with the Mantel test using semimetrics of genetic distance ( $F_{ST}/1-F_{ST}$ ) and of geographic distance (natural log [km]) as implemented in GENEPOP. An assignment test was used to evaluate the likelihood of correctly assigning individuals to their population of origin by using the software GENECLASS 2 (Piry et al. 2004).

Bottlenecks were inferred for each population under the assumption of mutation-drift equilibrium by using the BOTTLENECK program v.1.2.02 (Cornuet and Luikart 1996) and the infinite-alleles (IAM) and two-phase (TPM) models. The BOTTLENECK program measures the temporary excess of heterozygosity that results from a reduced population size (Cornuet and Luikart 1996; Luikart et al. 1998a,b). The significance ( $\alpha = 0.05$ ) of observed excesses or deficiencies in heterozygosity relative to that expected at mutation-drift equilibrium was tested using the Wilcoxon sign-rank test (Luikart et al. 1998a, Luikart and Cornuet 1998). One-thousand iterations each were performed using IAM or TPM.

## Results

**Genetic Diversity Within Populations.** No significant linkage disequilibrium was observed in any of the pairwise comparisons using GENEPOP ( $P > 0.05$ ), indicating that all loci included in this study assorted independently or recombined freely. The mean number of alleles per locus varied from 4.5 to 7.6, and within a population the number of alleles per locus varied from 3 to 14 (Fig. 1; Table 2). The population from Puerto Rico (PR1) had the greatest diversity of alleles when considering all loci. Allele frequencies, regardless of allele size or locus, were highly variable among populations: ranging from 0.00 to 0.87. Most alleles were not unique to one population, but at least one private allele was present in each population. The definition of private allele used was an allele unique to a single population and occurring in more than one individual (Neel 1973). Private allele frequencies within populations ranged from 0.02 to 0.39, with PR1 having the most private alleles (total of eight). Null alleles were estimated to be present in all loci tested, but were not common and frequencies were in line with other studies (Dakin and Avise 2004, Ridley et al. 2011), mean frequency in each population ranged from 0.08 to 0.16.

There was considerable individual genetic variability within populations for most loci, with observed heterozygosity ranging from 0.06 to 0.84. In many cases observed heterozygosity was lower than the expected (Table 2), which has been reported previously for *T. castaneum* (Demuth et al. 2007, Drury et al. 2009) and other beetle species (Brouat et al. 2003, Schrey et al. 2008). Expected heterozygosities varied among populations, with values ranging from 0.22 to 0.86 (Table 2). Almost half of the locus-by-population combinations (31 of 72) showed significant deviation from HWE after Bonferroni correction ( $P < 0.01$ ), manifested by a deficit in heterozygotes (Table 2).

**Genetic Differentiation Among Populations.** Analysis of molecular variance showed significant variation both among and within populations, with 8.32% of total genetic variability among populations, but neither commodity type nor geographic region were significant factors (Table 3). After correcting for the presence of null alleles,  $F_{ST}$  values ranged from 0.018 to 0.149 (Table 4, upper diagonal), with the global pairwise  $F_{ST}$  for all loci and population pairs being 0.082. Average  $F_{ST}$  value for the rice and wheat mills was 0.063 and 0.091, respectively, whereas the average genetic differentiation between the two groups was 0.082. Average  $F_{ST}$  value for midwest and southeast mills was 0.097 and 0.047, respectively, whereas the average genetic differentiation between these two groups was 0.087. In all cases the variation within commodity or region grouping was equal to or greater than that between groups.

Level of differentiation is predicted to increase with the distance between sources, but there was no significant correlation between geographic distance between mills (Table 4, lower diagonal) and genetic distance ( $F_{ST}/1-F_{ST}$ ) based on the Mantel test ( $P = 0.61$ ) (Fig. 2). For example, the  $F_{ST}$  value for the pairwise comparisons between NE1 and KS1 (dis-

tance = 247 km), LA1 and FL1 (distance = 1072 km), and CA1 and NE1 (distance = 2148 km) were similar; 0.069, 0.064, and 0.059, respectively.

**Assignment of Individuals to their Population of Origin.** Individual beetles could not be assigned to their population of origin with a high degree of accuracy by using GENECLASS-based assignment test. Overall, the Bayesian probability of correct assignment was only 56%, and all mill locations had similar assignment probabilities: LA1 ( $0.43 \pm 0.06$ ), KS2 ( $0.43 \pm 0.05$ ), NE1 ( $0.45 \pm 0.06$ ), PR1 ( $0.48 \pm 0.06$ ), KS1 ( $0.49 \pm 0.05$ ), NE2 ( $0.50 \pm 0.06$ ), CA1 ( $0.51 \pm 0.07$ ), CA2 ( $0.54 \pm 0.06$ ), and FL1 ( $0.60 \pm 0.06$ ).

**Population Bottlenecks.** Analysis revealed a significant excess of heterozygotes from that predicted under mutation-drift equilibrium, suggesting the existence of genetic bottlenecks in the recent past, for five out of nine populations. Wilcoxon rank tests under IAM and TPM generally gave similar results, so only results of the IAM are presented below. Mills with evidence of genetic bottlenecks were the wheat mills NE1 ( $P = 0.01$ ), NE2 ( $P = 0.01$ ), and FL1 ( $P = 0.01$ ), and the rice mills CA2 ( $P = 0.01$ ) and LA1 ( $P = 0.03$ ). No genetic bottleneck (no excess of heterozygotes) was detected for the wheat mills KS1 ( $P = 0.10$ ), PR1 ( $P = 0.37$ ) and KS2 ( $P = 0.16$ ), and the rice mill CA1 ( $P = 0.16$ ). However, these differences among mills do not appear to be related to either the frequency of fumigation at the mills (the most likely cause of genetic bottlenecks) based on information on fumigation frequency provided by personnel at the facilities, or with commodity type. Differences in fumigation efficacy and recolonization patterns may contribute to the poor relationship with fumigation activity. Interestingly, the two rice mills CA1 and CA2 that are very close to each other and receive fumigations at similar times, differed in the detection of genetic bottlenecks.

## Discussion

Isolation by distance (IBD) is predicted in subdivided populations where the rate of gene flow between subpopulations is limited by distance and within continuous populations when dispersal distance is limited. Although our results provide evidence of genetic differentiation among populations of *T. castaneum* in individual mills, they do not support IBD despite the obvious expectation given the distances between facilities evaluated. Another recently published study also has shown genetic differentiation among populations of *T. castaneum* with a concomitant lack of IBD (Drury et al. 2009). Although there were differences in the approaches used, both studies support the hypothesis of greater gene flow than predicted initially based on the model of active dispersal (by flight). Drury et al. (2009) analyzed microsatellites from laboratory colonies of *T. castaneum* (colonies established at different times before analysis and from locations both within the United States and from different continents) and found  $F_{ST}$  values that ranged from 0.03 to 0.35, with a global pairwise  $F_{ST}$  of 0.18. Even with the greater distances between population sources, Drury et al. (2009) saw no relationship

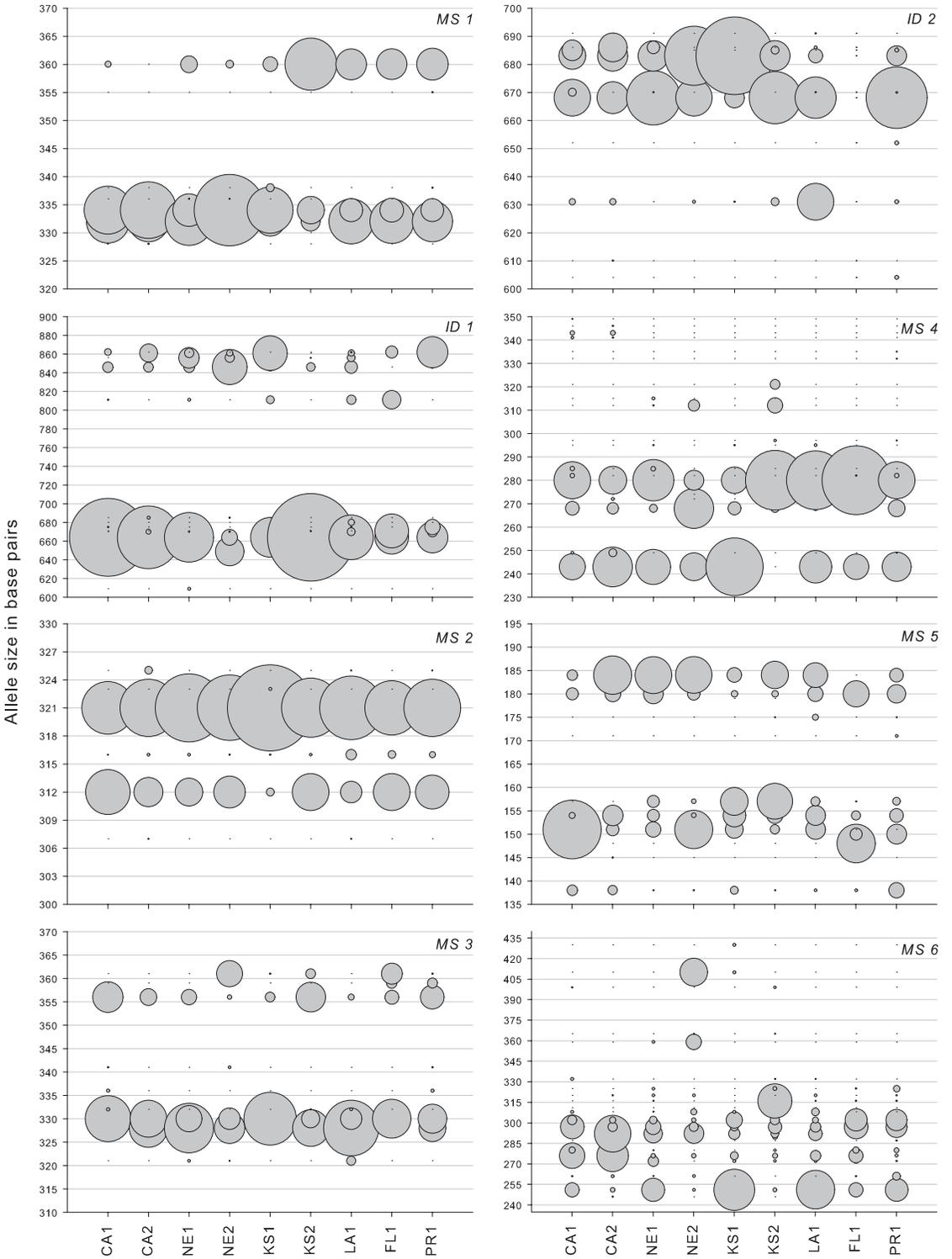


Fig. 1. Distribution of allele sizes and frequencies for eight molecular markers (MS1-MS6, and ID1-ID2) in nine populations of *T. castaneum* (x-axis). Y-axis values correspond to the allele size in base pairs and the diameter of circles corresponds to the relative frequency of the respective allele in each population.

**Table 2.** Summary of genetic information for nine populations of *Tribolium castaneum* (columns 3–11) and eight molecular markers: number of individuals (*N*), number of alleles (*Na*), number of private alleles (*Npa*), observed heterozygosity ( $H_{obs}$ ), unbiased expected heterozygosity ( $H_{exp}$ ), and Hardy–Weinberg disequilibrium level (*HW*) at 5% adjusted by Bonferroni's correction ( $P < 0.0007$ ), with nonsignificance indicated by NS

Marker	Variable	CA1	CA2	NE1	NE2	KS1	KS2	LA1	FL1	PR1
MS1	<i>N</i>	32	28	32	32	30	36	32	29	28
	<i>Na</i>	4	3	4	5	4	3	3	3	5
	<i>Npa</i>	0	0	0	1	0	0	0	0	1
	$H_{obs}$	0.281	0.179	0.250	0.188	0.400	0.111	0.281	0.793	0.464
	$H_{exp}$	0.579	0.514	0.638	0.454	0.674	0.615	0.652	0.656	0.686
	<i>HW</i>	0.0006	0.0002	<0.0001	<0.0001	0.0016 <sup>NS</sup>	<0.0001	<0.0001	0.1340 <sup>NS</sup>	0.0132 <sup>NS</sup>
ID1	<i>N</i>	28	30	31	31	30	34	31	16	19
	<i>Na</i>	5	5	7	7	5	4	9	4	7
	<i>Npa</i>	0	0	1	1	0	0	1	0	0
	$H_{obs}$	0.143	0.367	0.484	0.355	0.433	0.147	0.387	0.063	0.158
	$H_{exp}$	0.372	0.561	0.693	0.762	0.708	0.216	0.760	0.736	0.781
	<i>HW</i>	<0.0001	0.00021	0.0285 <sup>NS</sup>	<0.0001	0.0028 <sup>NS</sup>	0.0486 <sup>NS</sup>	<0.0001	<0.0001	<0.0001
MS2	<i>N</i>	32	32	32	31	30	36	32	32	32
	<i>Na</i>	3	5	3	3	4	3	5	3	4
	<i>Npa</i>	0	0	0	0	1	0	0	0	0
	$H_{obs}$	0.531	0.688	0.500	0.484	0.200	0.444	0.625	0.563	0.594
	$H_{exp}$	0.520	0.579	0.454	0.466	0.245	0.509	0.538	0.563	0.552
	<i>HW</i>	0.7115 <sup>NS</sup>	0.7349 <sup>NS</sup>	0.8460 <sup>NS</sup>	0.956 <sup>NS</sup>	0.0643 <sup>NS</sup>	0.6571 <sup>NS</sup>	0.2441 <sup>NS</sup>	0.8280 <sup>NS</sup>	0.5251 <sup>NS</sup>
MS3	<i>N</i>	29	32	32	32	30	35	32	14	29
	<i>Na</i>	6	4	5	7	5	6	6	5	8
	<i>Npa</i>	1	1	1	0	0	1	0	0	2
	$H_{obs}$	0.448	0.313	0.438	0.500	0.633	0.314	0.313	0.071	0.379
	$H_{exp}$	0.677	0.684	0.662	0.781	0.651	0.737	0.631	0.775	0.780
	<i>HW</i>	0.0102 <sup>NS</sup>	<0.0001	0.0327 <sup>NS</sup>	0.0044 <sup>NS</sup>	0.2362 <sup>NS</sup>	<0.0001	<0.0001	<0.0001	<0.0001
ID2	<i>N</i>	31	31	31	31	30	36	31	31	25
	<i>Na</i>	5	5	4	3	3	4	6	0	7
	<i>Npa</i>	0	1	0	0	0	0	1	0	2
	$H_{obs}$	0.839	0.645	0.419	0.516	0.300	0.361	0.677	0.000	0.560
	$H_{exp}$	0.745	0.725	0.598	0.513	0.352	0.623	0.675	0.000	0.580
	<i>HW</i>	0.6003 <sup>NS</sup>	0.0047 <sup>NS</sup>	0.0087 <sup>NS</sup>	0.9510 <sup>NS</sup>	0.6440 <sup>NS</sup>	0.0002	0.2759 <sup>NS</sup>	na	0.2142 <sup>NS</sup>
MS4	<i>N</i>	32	30	31	30	30	35	32	31	32
	<i>Na</i>	9	8	8	4	4	6	4	4	9
	<i>Npa</i>	1	1	1	0	0	1	0	0	2
	$H_{obs}$	0.563	0.333	0.194	0.133	0.333	0.400	0.250	0.452	0.563
	$H_{exp}$	0.772	0.747	0.698	0.718	0.580	0.605	0.545	0.458	0.748
	<i>HW</i>	<0.0001	<0.0001	<0.0001	<0.0001	0.0005	0.0001	<0.0001	0.8758 <sup>NS</sup>	0.0358 <sup>NS</sup>
MS5	<i>N</i>	32	31	32	31	30	36	32	32	32
	<i>Na</i>	5	6	6	6	6	7	7	7	9
	<i>Npa</i>	0	1	0	0	0	0	0	1	1
	$H_{obs}$	0.594	0.516	0.375	0.484	0.833	0.417	0.625	0.750	0.563
	$H_{exp}$	0.614	0.766	0.774	0.702	0.811	0.761	0.830	0.757	0.864
	<i>HW</i>	0.1134 <sup>NS</sup>	<0.0001	<0.0001	0.0052 <sup>NS</sup>	0.2716 <sup>NS</sup>	0.0003	0.0300 <sup>NS</sup>	0.2717 <sup>NS</sup>	0.0176 <sup>NS</sup>
MS6	<i>N</i>	31	31	32	32	30	36	32	31	30
	<i>Na</i>	10	9	14	11	9	14	11	10	12
	<i>Npa</i>	0	0	0	0	1	2	0	0	0
	$H_{obs}$	0.839	0.742	0.719	0.563	0.567	0.611	0.781	0.774	0.433
	$H_{exp}$	0.842	0.752	0.874	0.848	0.780	0.842	0.804	0.848	0.849
	<i>HW</i>	0.7563 <sup>NS</sup>	0.6410 <sup>NS</sup>	0.0124 <sup>NS</sup>	<0.0001	0.0042 <sup>NS</sup>	0.0147 <sup>NS</sup>	0.7274 <sup>NS</sup>	0.1604 <sup>NS</sup>	<0.0001
All loci	<i>N</i>	32	32	32	32	30	36	32	32	32
	<i>Na</i>	47	45	51	46	40	47	51	36	61
	<i>Npa</i>	2	4	3	2	2	4	1	1	8
	$H_{obs}$	0.530	0.473	0.422	0.403	0.463	0.351	0.492	0.495	0.464
	$H_{exp}$	0.640	0.666	0.674	0.656	0.600	0.614	0.679	0.685	0.730

between geographic distance and genetic distance. This global pairwise  $F_{ST}$  was more than twice that obtained in the current study; high versus moderate levels of differentiation (Balloux and Lugon-Moulin 2001). However, if only populations originating within the United States are considered, Drury and co-workers' (2009) global pairwise  $F_{ST}$  value would be 0.127, which is similar to that obtained in our study (both indicating a moderate level of differentiation). Ridley et al. (2011) reported a low global  $F_{ST}$  value of 0.024 for *T. castaneum* captured within an agricultural landscape, with potential for different locations to be connected by flight activity. In the cur-

rent study, two mills (CA1 and CA2) that were close enough for beetles to readily transverse the distance via flight had a lower pairwise  $F_{ST}$  value (0.051), but similar values also were obtained between mills in different geographic areas (i.e., regions which cannot be linked directly by flight). This lack of relationship could indicate that human aided dispersal is greater than hypothesized initially. However, it may also be because of using microsatellites as markers, because although widely used for these types of studies, interpopulation differentiation levels lower than hypothesized have been reported for other insect species (e.g., Lehmann et al. 1996, Paupy et

**Table 3.** AMOVA analyses for different hierarchical levels

Source of variation	d.f.	Sum of Squares	Variance component	Percentage of variation	P value
Among populations	8	88.36	0.15	8.32	<0.001
Within populations	571	921.26	1.61	91.68	<0.001
Among commodity groups	1	9.29	-0.01	-0.44	0.630
Among populations within commodities	7	79.07	0.15	8.56	<0.001
Within populations	571	921.26	1.61	91.88	<0.001
Among geographic regions	2	28.51	0.02	1.31	0.156
Among populations within regions	6	59.85	0.13	7.35	<0.001
Within populations	571	921.26	1.61	91.35	<0.001

First, analysis was used for comparison among all individual populations. Second, besides individual populations, analysis also compared group of commodities (rice and wheat). Finally, three different geographic region (western, midwest, and southeast) were compared. Significant differentiation ( $P < 0.001$ ) was based on permutation tests (10,000 permutations).

al. 2004, Roos and Markow 2006). Lehmann et al. (1996), for example, found that *Anopheles gambiae* (Diptera: Culicidae) showed low estimates of differentiation between populations that were as much as 6,000 km apart, despite mark-release-recapture experiments that suggested that active dispersal of this species was restricted to a few kilometers.

In addition to geographic distances, we had also predicted that commodity type processed would be associated with barriers to *T. castaneum* movement and gene flow. Our prediction was that rice and wheat mills would have the greatest genetic differentiation because there is a low potential for exchange of infested material between these two types of mills and little overlap in where the two grains are grown and transported to the mills. So rice and wheat mills should be more genetically isolated from each other based on both active dispersal and human transport in bulk grain. However, rice and wheat mills did not tend to have greater differentiation from each other than within a commodity type, nor were the California rice mills more differentiated from the rice mill in the southeast, even though rice for milling was not moved between these two locations.

Although population trends for *T. castaneum* compared with other species in flour mills supports that they are relatively self contained within a mill (Campbell and Arbogast 2004), immigration of individuals may still be occurring in sufficient levels to reduce genetic isolation. Results of this and other recent studies suggest that *T. castaneum* populations in wheat and rice mills may be less

isolated then originally hypothesized, which could be because of a combination of human aided transport resulting in long range dispersal (connecting the different geographic regions) coupled with greater connection of the mills with the surrounding environment through both active beetle dispersal behavior, human aided transport onto the sites were mills are located, or both. Mill sites contain a mixture of structures where bulk stored grain is stored, grain is milled, and where processed grain is stored and beetles being able to move among these structures and the surrounding environment may contribute to the limited genetic isolation of the populations within the mills. For example, beetles within the bulk grain storage structures could move from these areas into a mill in one of two ways: actively move between the two structures or being transported in the grain as it is moved into the mills for processing. *Tribolium castaneum* has been captured in traps placed between bulk storage areas and mill buildings (Campbell and Arbogast 2004, Semeao 2011) and found in grain residue spillage in elevators (Arthur et al. 2006) so they can potentially actively disperse into mill structure. As grain is prepared for milling, external insects are removed along with other extraneous material, but it is possible for beetles to escape from accumulated material before its disposal and contribute to the genetic composition of mill populations. Some evidence for and against these different mechanisms and their potential to facilitate gene flow are discussed below.

**Table 4.** Pairwise  $F_{ST}$  values (upper diagonal) and geographic distances in kilometers (lower diagonal) for populations of *Tribolium castaneum* with a global  $F_{ST}$  of 0.082

	CA1	CA2	NE1	NE2	KS1	KS2	LA1	FL1	PR1
CA1		0.051	0.059	0.097	0.106	0.094	0.078	0.107	0.073
CA2	<0.3		0.027	0.075	0.081	0.093	0.059	0.109	0.069
NE1	2,148	2,148		0.071	0.069	0.060	0.018 <sup>NS</sup>	0.078	0.025
NE2	2,195	2,195	50		0.089	0.145	0.107	0.149	0.094
KS1	2,153	2,153	247	236		0.149	0.091	0.118	0.096
KS2	19,901	1,990	413	422	217		0.075	0.116	0.073
LA1	2,747	2,747	1,277	1,250	1,043	1,007		0.064	0.038
FL1	3,787	3,787	1,967	1,922	1,807	1,880	1,072		0.040
PR1	5,763	5,763	3,852	3,804	3,732	3,834	3,030	1,976	

$F_{ST}$ s were corrected for the presence of null alleles by using Freena. Nonsignificant (NS)  $P$  value for the test of genotypic differentiation after Bonferroni's correction ( $P = 0.00139$ ).

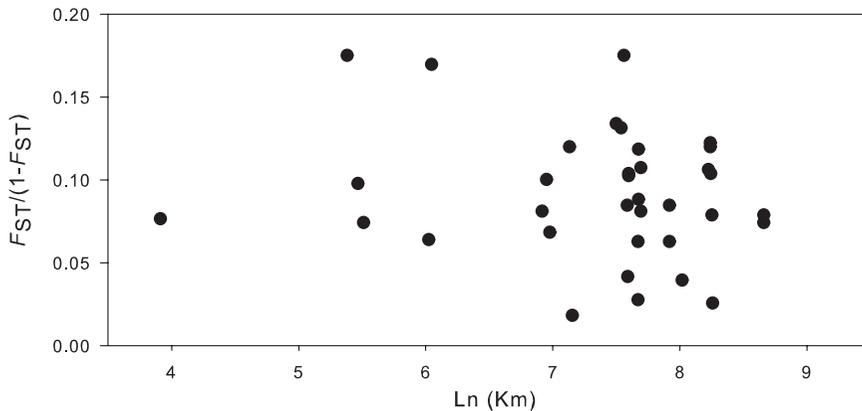


Fig. 2. Isolation by distance of *T. castaneum* populations based upon the relationship between genetic distance ( $F_{ST}/1-F_{ST}$ ) and the natural logarithm of geographic distances (Ln [Km]).

The role of active dispersal by *T. castaneum* flight while not supported in the genetic analysis, may be underestimated and play a role in reducing the genetic isolation of mills. Ridley et al. (2011) recently demonstrated that mated female *T. castaneum* were captured outside of grain storage facilities and that beetles captured in the surrounding landscape around grain storage facilities were genetically similar to those captured at grain storages. However, earlier population dynamics analysis of *T. castaneum* in two flour mills had not indicated a significant effect of season on activity inside mill or on recovery of beetles after fumigation treatment (Campbell et al. 2010a). So although there is not strong evidence that immigration is significant in the population dynamics of this species in flour mills, even low numbers of immigrants may have a significant impact the genetic structure. The recent research by Ridley et al. (2011) clearly demonstrates the potential for beetle movement from grain storage structures through active flight behavior, but the influence of this flight behavior on movement into the mills needs to be determined. Beetles also may be able to disperse by flight from sources in the surrounding area as well, whether they are grain storage sites or other potential resources such as retail, warehouse, processing, and residential areas. Analysis that includes beetles collected from grain storage locations and locations in the surrounding environment in addition to those in mills may be needed to resolve these questions.

Human transport of beetles in grain onto a mill location also may have contributed to the levels of gene flow observed among mill populations, however no information is available on the geographic area over which the grain is harvested and transported before the collection of the beetles used in this study. However, a qualitative prediction can be made and evaluated: larger geographic range over which grain is grown and shipped should be associated with greater genetic diversity and less geographic isolation. For the mills included in this study, smaller mills tended to process grain harvested over a more local area, southeast area flour mills that are not near wheat production areas will

process grain grown and transported over larger areas than those in the Midwest, and rice mills will tend to process grain produced more locally than wheat mills. However, the smallest flour mills (KS1 and KS2) had similar genetic diversity with larger mills; results from southeastern mills were variable with one having the greatest number of alleles and private alleles, whereas the other was similar to wheat mills in the central plains; and rice mills did not have lower genetic diversity or greater genetic isolation, with one rice mill having the second highest number of alleles. Although more detailed analysis of this mechanism is needed, results so far are not consistent with patterns of genetic isolation being related to the patterns of grain movement onto the mill site.

Another scenario for beetle movement that might explain the lack of IBD observed in this study and also fit with predictions about beetle populations in mills and the flight dispersal ability of this species, is that gene flow occurs because of a combination of human transport of beetles and active flight from the surrounding environment into the mill (i.e., stepping stone model). Within the U.S. food distribution system there is considerable potential for movement of infested material from a wide range of sources across large distances over relatively short periods of time, with the cumulative impact of a large potential of gene flow throughout the United States and more limited local population structure. Human-aided dispersal via transport of commodities such as flour has been shown to play an important role in mixing populations of stored-product species (Ryne and Bensch 2008). Although transport directly into a mill may be limited, beetles from the surrounding landscape could actively move either into stored grain that is then brought onto the mill site, into stored grain onsite at mill and then into the mill through mechanisms discussed above, or move directly into the mill from the surrounding environment. This stepping-stone model could limit the development of the more isolated populations predicted for rice and wheat mills and also be con-

sistent with new information on levels of active dispersal. Just about all of the mills included in this study were in or near urban areas, although their proximity and size varied. There is some fragmentary evidence that lends support to the stepping-stone model. *Tribolium castaneum* can be captured outside, both near and far from grain storage and processing facilities (Sinclair and Haddrell 1985, Dowdy and McGaughey 1994, Campbell and Arbogast 2004, Ridley et al. 2011) and diurnal patterns in flight activity (Boon and Ho 1988) and occurrence of emigration has been reported (Ridley et al. 2011), but other than Ridley et al. (2011) there is little information on long-range dispersal ability. Further evaluation of *T. castaneum* flight activity around mills, immigration and emigration rates, dispersal ability, and the impact of the surrounding environment such as level of urbanization is needed to address this question.

There are other factors that could have impeded the detection or development of population structure among mills such as the occurrence of null alleles, intrinsic characteristics of the markers, and presence of genetic bottlenecks caused by fumigations. Clear distinction among these factors is difficult, but it is possible to reduce the effects of some confounding factors. Although null alleles were present, it was possible to account for them and reduce their potential effects on the results. Nonetheless, it is important to note that there is some controversy regarding the presence of null alleles and their impact on population genetic structure studies: noticeably affecting F-statistics (Dakin and Avise 2004), although having less of an effect on Bayesian analysis (Orsini et al. 2008). Null alleles also may reduce the power of assignment tests, having a stronger effect if the total number of loci is low (Carlsson 2008). There are simulation studies suggesting that the bias introduced by null alleles is negligible when their frequency is  $<0.2$  (Dakin and Avise 2004), which was the case in the results reported here.

Some of the limited population differentiation could be because of the limitations of the microsatellite markers used. Microsatellites are known for rapid evolution, with mutation rates of  $\approx 10^{-3}$  per locus per generation (Weber and Wong 1993, Jarne and Lagoda 1996), which makes them useful for detecting differentiation among populations over relatively short periods of time. They also can show high within-population variance (Carbannelle et al. 2007), which can exaggerate the perceived genetic diversity within populations and reduce power to differentiate among populations. Microsatellites are more likely to be neutral than some other markers, but reduction in the levels of differentiation among populations can occur if there are constraints on microsatellite evolution, such as biased mutation rates (Garza et al. 1995) selection for certain allele sizes (Eppelen et al. 1993) even in populations that are geographically distant, or both. Differentiation indices across loci were not consistent; indicating

that heterogeneity among loci was large (Table 1). The use of indel-rich regions to evaluate variation at the subspecies level has been increasing, including the use of size variation (Beltrán et al. 2002, Steele et al. 2008, Väli et al. 2008, Ohshima and Yoshizawa 2011). Although indels have been reported to have lower heterozygosity than microsatellites, the heterozygosities of the two markers were correlated (Väli et al. 2008). Indel markers also have an advantage over microsatellites because identify by descent is more likely for similar sized indel products than with microsatellites (Väli et al. 2008). The two indels used in this study overall gave similar results to the microsatellites, and also contained short microsatellite regions that could have contributed to their polymorphism.

Processes occurring within each food processing facility also can impact *T. castaneum* population structure. Frequent structural fumigations (1–2 fumigations per year) cause large decreases in captures of beetles in traps, which likely is correlated with a large decrease in population size within a mill (Campbell et al. 2010a, 2010b). Fumigations could create genetic bottlenecks that reduce genetic variation or result in a nonrandom sample of the original population. Genetic bottlenecks were detected in five of the mill populations by using Wilcoxon rank test, which supports the hypothesis that frequent fumigations are an important influence on genetic structure. Populations were sampled in summer and most fumigations in these mills occurred in the previous fall at the earliest. Variation in the detection of bottlenecks in populations from different mills may be because of variation in how populations recover after these treatments as well as the timing of the fumigations. The population structure at mill site, including scale over which subpopulations are interconnected, are likely to impact the influence of fumigations on population structure. If each facility is considered as part of a metapopulation, then the genetic bottlenecks occurring in each subpopulation can be viewed as extinction and colonization events affecting individual demes and consequently the entire metapopulation. Colony formation can potentially follow two possible modes: a “migrant pool” mode in which genes are drawn individually and at random from all possible populations, or a “propagule pool” in which genes are drawn from just one of the possible source populations. If a “migrant pool” mode is occurring then smaller levels of differentiation may occur compared with the levels obtained when “propagule pool” is occurring (Slatkin 1977, McCauley et al. 1995).

Beetles used in this research were collected directly from mills, as opposed to other studies with *T. castaneum* that used populations that had been lab-cultured for varying periods of time. In general, the levels of heterozygosity in the populations collected directly from the field, were higher than those found in Drury et al. (2009) populations obtained from laboratory colonies, which might reflect some loss of

genetic diversity in the laboratory. The Drury et al. (2009) populations held the longest in the laboratory, in some cases >20 yr, tended to have relatively high pairwise  $F_{ST}$  values compared with other populations maintained for shorter periods of time in the laboratory. For some insect species, differences between laboratory colonies and field populations have been demonstrated (Norris et al. 2001, Gómez-Sucerquia et al. 2009). For example, long-term maintenance of laboratory colonies can result in a reduction of alleles per locus and overall heterozygosity levels.

The results obtained in this study and others dealing with *T. castaneum* populations (Beeman 2003, Drury et al. 2009) provide evidence of population structure within the United States. However, at this point there is insufficient information to explain the levels of differentiation found among some of the populations that, based on geography and other factors, would be predicted to show greater differentiation. Analysis suggests that greater gene flow is occurring among mill locations than predicted and not influenced by the hypothesized barriers to gene flow that included geographic area and commodity type. The fact that assignment of individuals to the source population was low, limits the potential of using these markers for the elucidation of source populations of individuals in infested food. Given the genetic information available for this species, a better understanding of population structure and gene flow should be possible using different types of markers and new approaches that will enable different mechanisms of gene flow to be assessed, which could lead to improved pest management programs.

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