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Geographic and voltinism differentiation among North American *Ostrinia nubilalis* (European corn borer) mitochondrial cytochrome *c* oxidase haplotypes

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Abstract

DNA sequence of European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), mitochondrial cytochrome *c* oxidase I (*cox1*) and II (*cox2*) genes were characterized and used for population genetic analysis. Twenty-six point mutations were identified from a 2,156 bp DNA sequence alignment. The frequency of polymorphic *cox1 Dde*I and *Hae*III, and *cox2 Sau*3AI and *Msp*I restriction sites were determined from 1,414 individuals by polymerase chain reaction restriction fragment length polymorphism. Ten haplotypes were observed. A single haplotype was present among 90% of individuals examined, and a *Hae*III haplotype was not present in samples from the Atlantic coast. Significant genetic differentiation existed between Atlantic coast and midwestern United States samples, and between sympatric uni- and bivoltine ecotypes. These genetic markers identify regional and ecotype differences in the North American *O. nubilalis* population.

Keywords: voltinism variation, geographic variation, Lepidoptera, Crambidae

Abbreviation:

D	genetic distance
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism

Introduction

Population structure and mating barriers affect rates of gene flow within a species (Mutebi *et al*. 2002). The European corn borer, *Ostrinia nubilalis* (Hübner), is an introduced insect pest of agricultural crops in North America (Mason *et al*. 1996). *O. nubilalis* is endemic to Europe and western Asia, and by 1917 it was observed in the eastern United States. It migrated westward, and reached North Dakota by 1950 (Chiang 1972; Showers 1993). The North American *O. nubilalis* population shows phenotypic variation in pheromone production and perception, and the number of generations per year (voltinism; Showers 1993; Mason *et al*. 1996).

The pheromone of female *O. nubilalis*, 11-tetradecenyl acetate, is produced in stereoisomeric forms (*E* and *Z*) and in varying blend ratios (Roelofs *et al*. 1972; Klun *et al*. 1973). Two ecotypes, the *E*-strain (New York type) and *Z*-strain (Iowa type), use 98:2 and 1:99 mixtures of *(E)*- and (*Z*)-11-tetradecenyl acetates, respectively (Glover *et al*. 1987). The *Z*-pheromone-emitting and responding populations are distributed in the central and eastern United States and southern Canada (Showers *et al*. 1974), whereas the *E*-strain inhabits the northeastern United States and Quebec (McLeod *et al*. 1979). The distribution of pheromone races overlap in the eastern United States and intermating of pheromone races has been documented (Roelofs *et al*. 1985; Durant *et al*. 1995).

The uni-, bi-, and multivoltine ecotypes of *O. nubilalis* differ in response to photoperiod and temperature for diapause induction, resulting in differences in the number of degree-days required for pupation (Showers 1979). Genes that determine voltinism are male sex-linked, and show temperature- and scotophase-dependent dominance (Showers *et al*. 1972; Showers 1981). Univoltine ecotypes may have a selective advantage in northern climates where short growing seasons favor a single generation, whereas in more temperate southern regions, extended larval dormancy may result in increased mortality (Showers 1993). Genetic and environmental factors determine voltinism phenotypes, resulting in north-south clines that correspond to barrier latitudes that restrict univoltine ecotypes to a northern distribution (Showers 1979; Showers 1993). Movement of bivoltine ecotypes into traditionally univoltine regions, without reciprocal migration, supports the concept of southern barrier latitudes for univoltine migration (Chiang *et al*. 1965; McEwen *et al*. 1968). Variation in post-diapause developmental time between voltinism ecotypes (Calvin and Song 1994; Hoard and Weiss 1995) may promote asynchrony in mating period that minimizes

genetic exchange (Roelofs *et al*. 1985). To date, no significant genetic differences have been detected between co-existing (sympatric) voltinism ecotypes.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) evaluation of four mitochondrial DNA fragments (Marcon *et al*. 1999), and variation at six allozyme markers (Bourguet *et al*. 2000) showed no genetic differences among voltinism ecotypes. PCR-random amplification of polymorphic DNA (RAPD) markers indicated that multivoltine ecotypes (>2 generations per year) were genetically different from both uni- and bivoltine ecotypes (Pornkulwat *et al*. 1998), but voltinism differences were confounded by geographic variance. Coates and Hellmich (2003) showed regional variation of northern (Minnesota) subpopulations from all other sample sites at two sex-linked microsatellite marker loci. In the current study polymorphism in *O. nubilalis cox1* and *cox2* genes was used to investigate contributions of voltinism, pheromone race, and geographic location to North American population structure.

Materials and Methods

Sample preparation

Second-flight *O. nubilalis* adults were obtained from 14 North American locations (Fig. 1) in light or pheromone bait traps. At Lamberton, Minnesota, and Rosemount, Minnesota, season-long monitoring separated peaks of uni- and bivoltine ecotypes. Two pheromone bait traps, one *E*- and the other *Z*-, were placed in proximity at Cape Elizabeth, and Oxford Maine. An *E*-pheromone trap was used at the Newark, Delaware location. The last sample site was the Ithaca, New York colony of *Z*- pheromone individuals were originally collected May 1996 near Geneva, New York, or *E*-pheromone individuals were in continuous culture since April 1994, after initial collection from fields in Bouchville, New York. The laboratory colony was maintained at the New York State Agricultural Experiment Station, Geneva, New York. All other samples were collected in light or pheromone traps (see Fig. 1). DNA extraction was performed on individual thoraces as described by Marcon *et al*. (1999). DNA was suspended in 100 µl of TLE (10 mM Tris, 0.1 mM EDTA, pH 7,5), and DNA concentrations were adjusted to 50 ng/µl with TLE, and stored at −20° C.

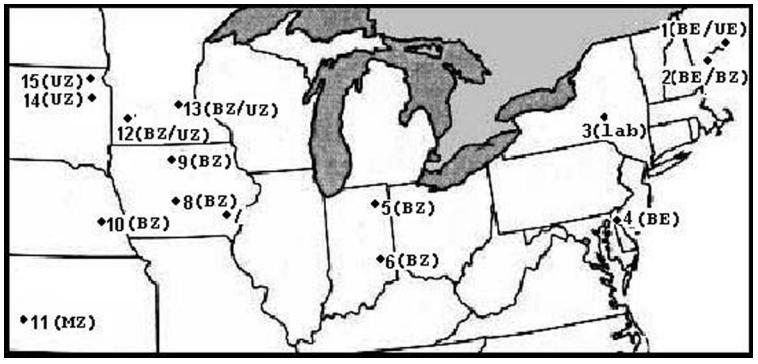

Figure 1. The location of 15 North American *Ostrinia nubilalis* collection sites. Ecotypes obtained from each collection site are indicated; univoltine Z pheromone race (UZ), bivoltine Z pheromone race (BZ), univoltine E pheromone race (UE), or bivoltine E pheromone race (BE). The New York laboratory colony data (site 3; lab) was omitted from statistical analysis due to haplotype fixation. Collection site ID: 1 Oxford Maine; 2 Cape Elizabeth, Main, 3 Ithaca, New York; 4 Newark, Delaware; 5 Columbia City, Indiana; 6 Franklin County, Indiana; 7 Crawfordsville, Iowa; 8. Hubbard, Iowa; 9 Kanawha, Iowa; 10 Mead, Nebraska; 11 Garden City, Kansas; 12 Lamberton, Minnesota; 13 Rosemount, Minnesota; 14 Brookings, South Dakota; 15 South Shore, South Dakota.

DNA sequencing

Mitochondrial *cox1*, *trn*L^{UUR}, and *cox2* gene sequences were compared among 14 individual adult corn borers. PCR of individual DNA samples used 50 µl reactions with 2.5 mM $MgCl_2$, 0.8 µM dNTPs, 10.0 pmol of each primer TY-J-1460 and TK-N-3785 (Simons *et al*. 1994), 400 ng of DNA, and 1.7 U of *Taq* polymerase (Promega, www.promega.com). A temperature cycle of 94° C for 3 min., followed by 40 cycles of 94° C for 40 sec, 53° C for 50 sec, and 72° C for 2 min was carried out on a PTC-100 thermocycler (MJ Research, http://www.mjr.com/). Purification of PCR reactions used Qiaquick PCR purification columns (Qiagen, www.qiagen.com) according to manufacturer directions, and 200 ng used in dye-terminator cycle sequencing quick start (DTCS-quick) primer extension reactions (Beckman-Coulter, www.beckman-coulter.com). Dye-terminator cycle sequencing (DTCS) reaction products were injected at 110kV for 15 sec onto a CEQ8000 capillary electrophoresis system (Beckman-Coulter, www.beckman-coulter.com) and separated at 110kV for 120min. Sequence reaction data were imported into Vector NTI Suite 7.0 (Informax, www.informax.com), and individual contigs were constructed. *Cox1* and *cox2* sequences were aligned with homologous regions the *O. nubilalis* (GenBank AF442957) and *O. furnicalis* (GenBank AF467260) mitochondrial genomes by using AlignX software (Informax; gap penalty of 5). Amino acid sequence was determined with Sequin 4.00 (www.ncbi.nlm.nih.gov).

PCR-RFLP methods

Three PCR primer pairs, TY-J-1460 (Simons *et al*. 1994) with OnCox-D-R (5´-TCCA GGATTACCTAATTCAGCTC-3´), OnCox-H-F (5´-CACGAGCTTACTTTACCTCAGCA-3´) with OnCox-H-R (5´-CCAGCTAGCCCTAAGAAATGTTG-3´), and OnCox-SM-F (5´-GGCTA GC TGGTATACCTCGAC-3´) with OnCox-SM-R (5´-GGAGAGGCTCTATTTTGTAGACTA-3´) spanned polymorphic regions. All PCR amplifications used 1.5 mM $MgCl_2$, 0.5 uM dNTPs, 5 pmol of each primer, 0.225 U of *Taq* DNA polymerase (Promega), and 150 ng of DNA in a 12.5 µl reaction volume. Thermocycler conditions were 94° C for 2 min, followed by 40 cycles of 94° C for 30 sec, 52° C for 30 sec, and 72° C for 20 sec. Single 25 µl digests with *Dde*I, *Hae*III, *Msp*I, or *Sau3*AI including 5.0 µl of PCR product, 2.5 µl 10X buffer (Promega), 0.1 mg/µl bovine serum albumin, and 0.5 U of enzyme (Promega) were incubated at 37° C for 8 to 14 hr. Reactions were loaded on 1.0 mm by 16 cm 6% polyacrylamide (29:1 acrylamide: bisacrylamide) 0.5X Tris borate EDTA gels, and separated at 140 V for 4 h with a 25-bp step-ladder (Promega) for size comparison. Gels were stained with ethidium bromide, and digital images were taken on a PC-FOTO/

Eclipse documentation system (Fotodyne, www.fotodyne.com).

Data analysis

Pairwise effective number of migrants (*Nm*) were calculated from linearized F_{ST} estimates (Slatkin, 1995) and measured between Atlantic coast and Midwestern samples by using estimated *F*-statistic; $Nm = ((1/F_{ST}) - 1)$ (Hartl and Clark 1997). Genetic distance and analysis of molecular variance (AMOVA) were calculated as described by Weir and Cockerham (1984) and Excoffier *et al*. (1992) by using Arlequin v1.1 (Schneider *et al*. 1997). AMOVA tested regional population subdivision (Atlantic coast verses Midwestern samples) and sympatric voltinism ecotype differentiation at Lamberton and Rosemount collection sites. Haplotype relationships were investigated by phylogenetic analysis of PCR-RFLP and DNA sequence alignment data using parsimony that incorporated 1,000 bootstrap resampling steps performed using Seqboot, followed by Mix or DNAPars, and Consense programs in the PHYLIP package (Felsenstein 1989). A haplotype network was constructed from RFLP data using the program TCS version 1.12 (available at http://inbio.byu.edu/Faculty/kac/crandall_lab/programs.htm). Genetic differentiation by geographic distance model was tested by comparison of $\log_{10}$ distance (km) to $\log_{10}$ genetic distance estimates (Slatkin 1993) using NTSYS-pc v. 1.70 (Rohlf 1992), and MXCOMP of NTSYS-pc V. 1.70 to perform Mantel tests with 1000 iterations to test significance. Three levels of comparison were made using European corn borer genetic distance (D) estimates 1) among Atlantic coast and 2) among Midwestern sample sites, and 3) between Atlantic and Midwestern sample sites. ANOVA was performed using Proc Mixed of the SAS statistical software (v. 8).

Results and Discussion

DNA sequence analysis

Mitochondrial *cox1*, tRNA-Leu^{UUR}, and *cox2* genes were sequenced from 14 *O. nubilalis* moths to survey genetic variation within the species. A 2,156 bp sequence alignment showed 26 point mutations of which 7 resulted in nonsynonymous amino acid changes (Table 1). Individual mutations were rare and resided in only one or two sequences. C*ox1* and *cox2* gene sequences were highly similar (> 99.5 and > 99.2%, respectively), and no mutations were observed in tRNA-Leu^{UUR}. Screening of 1,414 *O. nubilalis* moths from 15 collection sites (Fig. 1) revealed low polymorphism at *Dde*I, *Hae*III, *Msp*I, and *Sau*3AI sites, and a non-digested mitochondrial haplotype among 90% of individuals (1,267 out of 1,414; Table 2). Low *O. nubilalis* mitochondrial polymorphism agreed with reports by Marcon *et al*. (1999), and was congruent with allozyme markers (Harrison and Vawter 1977; Cianchi *et al*. 1980; Glover *et al*. 1991; Bourguet *et al*. 2000) and sex-linked microsatellite data (Coates and Hellmich 2003). Low mitochondrial variation may result from a genetic bottleneck at North American *O. nubilalis* introduction, selection, or mitochondrial DNA fixation by genetic drift. Similar results were observed from moth species, the gypsy moth, *Lymantria dispar* (Harrison *et al*. 1983), and tobacco budworm, *Heliothis virescens* (Roehrdanz *et al*. 1994), suggesting little intraspecific differentiation within the genera.

Phylogenetic methods and haplotype networks can construct haplotype relationships. Parsimony trees constructed from DNA sequence (Fig. 2) or PCR-RFLP haplotypes (not shown) were

Table 1. Twenty-six variable nucleotide positions observed between 14 North American *Ostrinia nubilalis* mitochondrial *cox1* and *cox2* sequences. Random collection of sequences used to survey level and location of polymorphism among samples, such that PCR-RFLP assays could be developed for higher sample throughput. Positions with respect to the *O. nubilalis* mitochondrial genome sequence (GenBank AF442957), and – indicates sequence identical to the consensus sequence. RFLPs: D = *Dde*I, H = *Hae*III, S = *Sau*3AI, and M = *Msp*I are indicated. See Table 2 for location detail of collection site ID.

	cox2																cox1										
	DNA sequence alignment position with respect to GenBank accession AF442957																										
Collection site ID / individual	1459	1529	2047	2062	2119	2161	2161	2169	2533	2531	2554	2829	2866	2928	2933	3050	3123	3429	3453	3462	3474	3477	3528	3544	3554	3555
Consensus	C	T	T	T	T	A	T	A	A	T	A	T	T	T	A	G	A	A	A	A	A	A	A	A	T	A
Complete Genome AF442957	A	C	C	A	-	T	G	-	T	-	G	G	-	G	-	C	-	-	-	-	-	-	-	-	G	-
Garden City, Kansas /♀17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	-	T	T	T	G	T	-	-	T
Hubbard, Iowa /♂25	-	-	-	-	-	-	-	-	-	C	G	-	-	-	-	-	-	C	-	C	-	-	-	G	-	-
Brookings, South Dakota /♀06	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	G	-	-
Lamberton, Minnesota (BZ) /♂24	-	-	-	-	G	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-
Mead, Nebraska /♂02	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kanawha, Iowa /♀10	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kanawha, Iowa /♀46	-	-	-	-	-	-	-	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lamberton, Minnesota (UZ) /♀26	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rosemount, Minnesota (UZ) /♀20	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxford, Maine (UE) /♀06	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-
Cape Elizabeth, Maine (BE) /♀47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-
Franklin Co., Indiana /♀04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	G	-	T
Crawfordsville, Iowa /♀46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-
Cape Elizabeth, Maine (UE) /♀46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
RFLP		D									H				M	S										

Table 2. The distribution of *Ostrinia nubilalis* mitochondrial RFLP frequencies and haplotypes in samples from 15 North American collection sites. Frequency of digestion (PCR-RFLP frequency), and number of *O. nubilalis* with a given haplotype at each collection site (mitochondrial haplotype number) are provided. Note: Maine (ME) and Minnesota (MN) samples were divided into ecotype (ET) for genetic distance estimation (see Table 4).

		PCR-RFLP frequencies				Mitochondrial haplotype numbers										
Subpopulation ID (collection site)	ET	D	H	S	M	w^+	D	H	S	M	DM	DS	MS	DH	DHM	Total (N)
1 Oxford, Maine[U]	UE	100	0	10.41	0	43	0	0	5	0	0	0	0	0	0	48
	UZ	100	0	18.75	0	13	0	0	3	0	0	0	0	0	0	16
2 Cape Elizabeth, Maine[U]	BE	100	0	6.25	0	90	0	0	6	0	0	0	0	0	0	96
	BZ	100	0	6.25	4.17	43	0	0	3	2	0	0	0	0	0	48
3 Ithaca New York[Y]	BE	100	0	0	0	48	0	0	0	0	0	0	0	0	0	48
(lab colony)	BZ	100	0	0	0	48	0	0	0	0	0	0	0	0	0	48
4 Newark, Delaware[R]	BE	97.92	2.08	2.08	2.08	46	0	0	1	0	0	0	0	0	1	48
5 ColombiaCity, Indiana[S]	BZ	97.96	3.06	2.04	2.04	90	1	2	2	2	0	0	0	1	0	98
6 Franklin Co, Indiana[S]	BZ	100	2.35	1.18	3.53	79	0	2	1	3	0	0	0	0	0	85
7 Crawfordville, Iowa	BZ	96.88	6.25	2.08	0	85	3	6	2	0	0	0	0	0	0	96
8 Hubbard, Iowa	BZ	100	7.29	2.08	2.08	86	0	7	1	1	0	0	1	0	0	96
9 Kanawa, Iowa	BZ	100	2.35	1.18	3.53	79	0	2	1	3	0	0	0	0	0	85
10 Mead, Nebraska[X]	BZ	98.96	8.33	3.13	1.04	82	1	8	3	1	0	1	0	0	0	96
11 GardenCity, Kansas[T]	MZ	96.47	4.71	4.71	5.88	81	2	4	4	4	1	0	0	0	0	96
12 Lamberton, Minnesota[V]	BZ	96.83	0	4.69	4.69	56	2	0	3	3	0	0	0	0	0	96
	UZ	96.43	10.71	0	0	40	2	6	0	0	0	0	0	0	0	48
13 Rosemount,Minnesota[V]	BZ	100	6.45	4.3	0	83	0	6	4	0	0	0	0	0	0	93
	UZ	87.5	18.75	9.38	9.38	32	0	6	2	2	0	0	1	0	0	43
14 Brookings, SouthDakota[Z]	UZ	94.44	1.85	3.7	3.7	46	3	1	2	2	0	0	0	0	0	54
15 South Shore,SouthDakota[Z]	UZ	100	3.74	0	5.61	97	0	4	0	6	0	1	0	0	0	108
Total		98.66	3.96	3.47	2.19	1267	14	54	45	27	1	2	2	1	1	1414

Ecotypes (ET) represented are univoltine Z pheromone race (UZ), bivoltine Z pheromone race (BZ), univoltine E pheromone race (UE), or bivoltine E pheromone race (BE). Haplotypes defined usingD = *Dde*I, H = *Hae*III, S = *Sau*3AI, M = *Msp*I, and w^+ (wildtype) uncleaved.

Collections provided by:
R. Charles Mason 2001
U. David Handley 2002
X. Ron Seymour 2001
S. John Obermeyer 2001
V. Bruce Potter 2001 & 2002
Y. Wendel Roelofs 2001
T. Lawrence Buschman 2001
W. William Hutchison 2002
Z. Micheal Catangui 2001

inconclusive with respect to haplotype relationships. The parsimony-based phylogenetic tree using DNA sequence data produced bootstrap branch support above a 50% threshold (n = 1,000 bootstrap resample steps) at only 4 of 10 nodes. Nested clade analysis (NCA; Templeton *et al*. 1992) was similarly unsuitable due to the generation of a circular haplotype-network (data not shown). Inability to resolve haplotype relationships using phylogenetic methods may reside in the absence of sufficient differentiation among *O. nubilalis cox*1 and *cox*2 haplotypes (Page and Holmes 1998).

Geographic population differentiation

Polymorphism was absent from Ithaca, New York laboratory colony samples, and these data were omitted resulting in 14 collection sites for analysis. An isolation-by-distance model was rejected as the basis of genetic differentiation between 14 North American *O. nubilalis* collection sites. Using the program NYSYS-pc v. 1.70 (Rohlf 1992), genetic distance versus geographic distance (km) matrices were plotted, and showed no correlation ($R^2 = 0.1132$, $P = 0.9759$). Therefore, geographic distance between sample sites may not be correlated with genetic differentiation. Alternatively, the geographic region of the collection site, Midwestern or Atlantic coast, was investigated as the basis of genetic variation. A direct comparison of *Hae*III haplotype frequency between Midwestern (5.1%; 54 of 1,055) and Atlantic coast samples (0.0%), indicated presence of a region specific haplotype (private allele; Table 2).

Population differentiation imparted by the absence of *Hae*III haplotypes from Atlantic coast samples was investigated using modified fixation indices (θ-statistics; Weir and Cockerham, 1984; Excoffier *et al*. 1992), analysis of molecular variance (AMOVA; Excoffier *et al*. 1992), and genetic distance estimates

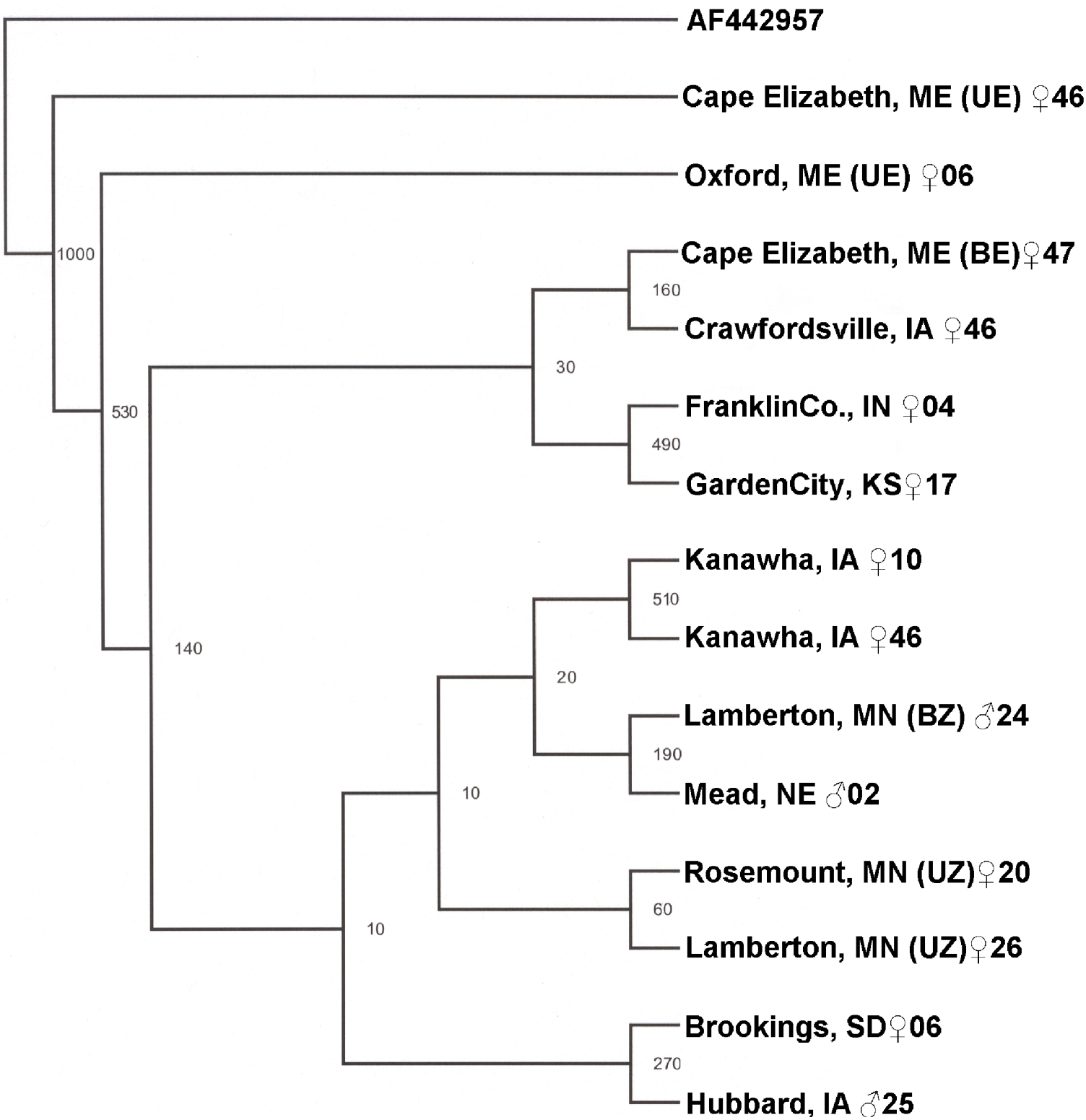

Figure 2. Parsimony tree constructed from a 2,156 bp alignment of 14 mitochondrial *cox1*, tRNA-Leu^{UUR}, and *cox2* DNA sequence (Table 1) randomly selected from the 1,414 *Ostrinia nubilalis* samples evaluated.

(Nei 1972; Schneider *et al*. 1997). A comparison of Midwestern and Atlantic coast *Dde*I, *Hae*III, *Msp*I, and *Sau*3AI RFLP haplotype frequencies generated a modified fixation index, θ_{ST}, of 0.024 that indicated moderate inbreeding and significantly higher relatedness of haplotypes within each region ($P < 0.0001$). Low but significant mitochondrial haplotype differentiation between *O*. *nubilalis* corroborates prior studies (Harrison and Vawter 1977; Cianchi *et al*. 1980; Glover *et al*. 1991; Coates and Hellmich 2003). Analysis

Table 3. AMOVA and modified *F*-statistics (Theta (θ)-statistics; Weir and Cockerham, 1984). **A)** Subdivision of North American *Ostrinia nubilalis* haplotypes into Atlantic coast (sites 1, 2, and 5) and Midwestern collection sites (sites 5–15, Table 2; the Ithaca, New York laboratory colony haplotype data were omitted). **B)** Subdivision of sympatric univoltine (UZ) and bivoltine (BZ) ecotypes from Lamberton and Rosemount, Minnesota collection sites (sites 12 and 13; Table 2).

A) Atlantic and midwestern subdivision

Comparison	df	Sum of Squares	Variance component	Percent of variance
Among regions	1	0.946	0.00188 Va	1.64
Among subpops within regions	14	2.757	0.00082 Vb	0.71
Within subpops	1300	146.141	0.11242 Vc	97.65
	1315	149.844	0.11512	100.00

Fixation Indices		P-value and SE
$Theta_{SC}$ (F_{IS})	0.00725	0.00098 ± 0.00098
$Theta_{ST}$ (F_{ST})	0.02350	0.00000 ± 0.00000
$Theta_{CT}$ (F_{IT})	0.01637	0.00098 ± 0.00098

B) Minnesota sympatric voltinism

Comparison	df	Sum of Squares	Variance component	Percent of variance
Among regions	1	0.529	0.00227 Va	1.53
Among subpops within regions	2	0.544	0.00212 Vb	1.43
Within subpops	244	35.008	0.14348 Vc	97.03
	247	36.081	0.14786	100.00

Fixation Indices		P-value and SE
$Theta_{SC}$ (F_{IS})	0.01455	0.30108 ± 0.01070
$Theta_{ST}$ (F_{ST})	0.02350	0.00782 ± 0.00313
$Theta_{CT}$ (F_{IT})	0.01637	0.33138 ± 0.00000

of molecular variance (AMOVA) comparing Midwestern and Atlantic coast haplotype frequencies indicated 1.64% of total variation within North America might be due to regional differences (Table 3; Panel A). The presence of a single non-digesting haplotype among 90% of the individuals (w^+; Table 2) may contribute to low variation between geographic regions. The absence of the *Hae*III haplotype from the Atlantic coast samples suggest that it may be the basis of the low-level geographic (regional) differentiation, and its relative contribution was estimated by excluding it from fixation index estimation and comparison to original values. After removal of *Hae*III PCR-RFLP haplotypes, the fixation index (θ_{ST}) decreased to 0.017 ($P < 0.010$; data not shown), suggesting that *Hae*III haplotypes contributed 29% of the measurable increase in relatedness of haplotypes within each geographic region ((0.024-0.017)/0.024 @ 0.29). These analyses and raw haplotype frequency data suggest the *Hae*III haplotype might have regional geographic specificity or reflect North American *O. nubilalis* population subdivision.

Genetic distance estimates from 14 *O. nubilalis* collection sites were generated in a pairwise manor (Ithaca, New York laboratory colony haplotypes omitted), and comparisons supported evidence of *O. nubilalis* geographic differentiation previously inferred from fixation indices and AMOVA (Table 3A). Genetic distances were estimated separately for Lamberton and Rosemount, Minnesota voltinism ecotypes, and Cape Elizabeth and Oxford, Main pheromone races (i.e. treated as separate subpopulations and analyzed as separate entities; Table 2; discussed in next section). Genetic distance estimates suggested differentiation between 16 of 153 comparisons using a Bonferroni corrected significance threshold ($P < 0.0003$; Table 4). No significant difference in genetic distance was predicted between any two collection sites or ecotypes on the Atlantic coast, suggesting greater genetic similarity within Atlantic coast regions compared to the Midwest. Geographic similarity was further investigated by one-way ANOVA comparing genetic distance (D) between Atlantic coast and Midwestern samples, and resulted in significant differentiation ($F = 14.53$; df = 2, 150; $P < 0.0001$).

Exclusion of the *Hae*III haplotype from Atlantic coast regions suggest 1) low frequency of *Hae*III haplotype migration, or 2) separate and distinct regional *O. nubilalis* introductions. Assuming Hardy-Weinberg equilibrium, a historic effective female migration rate ($N_fm = ((1/F_{ST}) - 1)$; Hartl and Clark 1997) between Atlantic coast and Midwestern regions was estimated at 40 to 41 female migrants per generation (using $\theta_{ST} = 0.024$; Table 3). Migration estimates are crude due to unrealistic island model assumptions (Hartl and Clark 1997), but suggest a historically moderate level of haplotype exchange. Migration rate estimates may be lower due to inability to differentiate many *O. nubilalis* mitochondrial haplotypes. The second hypothesis assumes that Atlantic and Midwestern differences result from separate North American *O. nubilalis* introductions. Records indicate *O. nubilalis* infestations in eastern North America near Boston (1913) and eastern New York State (1919), and a single Midwestern introduction near Lake Erie (Vinal 1917; Showers 1993). If Boston and New York introductions populated Atlantic coast regions and the single Lake Erie introduction populated the Midwest, present day genetic differentiation might be indicative of European founders. Alternatively, *Hae*III haplotype extinction in the Atlantic coast region could have resulted after introduction due to random genetic drift within a small founder population or selection within new habitats. Sampling error in the current study is unlikely to be due to collection sizes at multiple regional locations (Fig. 1; Table 2). Additional sampling is required for follow up confirmatory studies.

Sympatric ecotype differentiation

Coexisting (sympatric) pheromone races were present in the Oxford (site 1) and Cape Elizabeth, Main (site 2) pheromone traps. Comparison of mitochondrial haplotypes between pheromone races showed no significant inbreeding effects within ecotype at Oxford and Cape Elizabeth, Main locations ($\theta_{ST} = 0.021$; $P = 0.081$), suggesting gene flow between pheromone ecotypes and corroboration of pheromone hybrid females observed in the field (Roelofs *et al*. 1985; DuRant *et al*. 1995). Sympatric voltinism ecotypes also were collected from the same light trap at Lamberton (site 12) and Rosemount (site 13) Minnesota. Comparison of haplotype frequency among sympatric voltinism ecotypes may represent a measure of ecotype variation in the absence of confounding geographic effects. In contrast to pheromone ecotypes,

Table 4. Significantly different pairwise Nei genetic distance (D) comparisons from an 18 x 18 matrix of 14 *Ostrinia nubilalis* sample sites (Ithaca, New York laboratory colony data omitted). Empirical *P* values were determined after 10,000 Markov chain steps (permutations), and significance threshold was set using the Bonferroni adjustment for multiple tests (0.05/153 = 0.0003267). Note: Haplotypes from Lamberton and Rosemount, Minnesota (MN), and Cape Elizabeth and Oxford, Main (ME) collection sites subdivided into ecotype (Table 2). See Table 2 for collection site ID and ecotype (ET) definition.

ID	Collection site (ET)		ID	Collection site (ET)	D	*P*-value
1	Oxford, ME (UE)	vs.	6	Franklin Co., IN (BZ)	0.047	< 0.0001
			10	Mead, NE (BZ)	0.0869	< 0.0001
			12	Lamberton, MN (UZ)	0.0929	< 0.0001
			13	Rosemount, MN (UZ)	0.0785	< 0.0001
			15	South Shore, SD (UZ)	0.0326	< 0.0001
	Oxford, ME (UZ)		5	Columbia City, IN (BZ)	0.1005	< 0.0001
			12	Lamberton, MN (UZ)	0.0654	< 0.0001
2	Cape Elizabeth, ME (BE)	vs.	7	Crawfordsville, IA (BZ)	0.0278	< 0.0001
			8	Hubbard, IA (BZ)	0.0328	< 0.0001
			9	Kanawha, IA (BZ)	0.0225	< 0.0001
12	Lamberton, MN (UZ)	vs.	6	Franklin Co., IN (BZ)	0.0487	< 0.0001
			9	Kanawha, IA (BZ)	0.0487	< 0.0001
			2	Cape Elizabeth (BE)	0.0851	< 0.0001
			12	Lamberton, MN (BZ)	0.0534	< 0.0001
13	Rosemount, MN (UZ)	vs.	5	Columbia City, IN (BZ)	0.0424	< 0.0001
			9	Kanawha, IA (BZ)	0.0484	< 0.0001

differences were detected when haplotypes from Minnesota collection sites were separated into voltinism ecotypes and analyzed using modified fixation indices (Weir and Cockerham, 1984; Excoffier *et al*. 1992), and analysis of molecular variance (AMOVA; Excoffier *et al*. 1992). The level of relatedness was significantly higher for haplotypes of the same voltinism ecotype at the Lamberton and Rosemount, Minnesota collection sites ($\theta_{ST} = 0.030$; $P = 0.008$; Table 3). This suggested inbr,eeding within and reduced gene flow between, voltinism ectypes. Furthermore, only 1.53% of total genetic variance between haplotypes was due to voltinism, again suggesting presence of low but significant levels of differentiation.

Sympatric voltinism ecotype differentiation was also detected via a pairwise comparison of genetic distance estimates (Table 4). Specifically, the sympatric uni- and bivoltine ecotypes from Lamberton, Minnesota showed a significantly different pairwise genetic distance (D = 0.0534, $P < 0.0001$; Table 4), and may represent a measure of ecotype variation without confounding geographic effects. In contrast, no significant genetic distance was observed between uni- and bivoltine haplotype frequencies from Rosemount, Minnesota (D = 0.0198; $P = 0.054$; data not shown). Genetic differentiation between sympatric voltinism ecotypes at Lamberton, Minnesota suggests a possible mating barrier that may be attributable to mating period asynchrony (Eckenrode *et al*. 1983; Roelofs *et al*. 1985). Recent northern bivoltine movement into Minnesota might be responsible for voltinism differences. Univoltine ecotypes migrated to Minnesota in the early 1940s (Chiang 1961) and reached North Dakota in 1950 (Chiang 1972), and a second *O. nubilalis* migration entered southern Minnesota in 1952 which was suggested to be a northern expansion of bivoltine moths (Chiang *et al*. 1965; McLeod 1978; Palmer *et al*. 1985). Bivoltine migration into univoltine areas, and minimal gene flow due to non-overlapping mating periods, may have maintained ecotype differences. Alternatively, genetic drift in smaller northern populations may cause yearly fluctuation in haplotype frequencies that caused the Rosemount, Minnesota samples to be above threshold when sampled. However, the Bonferroni-corrected significance thresholds may be unrealistically high.

Significant differences in genetic distance estimates, θ-statistics, and AMOVA suggest little interspecific gene flow. Genetic diversity observed between sympatric *O. nubilalis* voltinism types may reflect the effects of reproductive isolation or ecological adaptation (Showers 1979; Showers 1993). Ecotype differentiation not associated with change in plant host range appears rare except in the pea aphid, gallflies, soapberry bug, and brown plant hopper (see Berlocher and Feder 2002 for references). The extent of *O. nubilalis* ecotype (reproductive) isolation in regions of sympatry remains to be determined, but development of more polymorphic genetic markers and more detailed sampling might help address this question.

Conclusions

Low but significant geographic subdivision of the North American *O. nubilalis* population may exist (Table 2 and 4). Exclusion of an *O. nubilalis Hae*III mitochondrial haplotype from Atlantic coast populations might indicate a genetic bottleneck among

European founders, rarity of *Hae*III among immigrants from Midwestern region of the United States, or a migrant barrier imparted by the Appalachians. Data also showed genetic differentiation between sympatric *O. nubilalis* voltinism ecotypes, which may be maintained by asynchrony of mating periods and a reduced level of intermating (McLeod *et al*. 1979; Eckenrode *et al*. 1983), or may be anartifact of recent northern expansion of bivoltine ecotypes. Additional genetic markers and more detailed sampling are required to further investigate these observations and currently are under development.

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