

Influence of host plant, geography and pheromone strain on genomic differentiation in sympatric populations of *Ostrinia nubilalis*

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Abstract

Patterns of mating for the European corn borer (*Ostrinia nubilalis*) moth depend in part on variation in sex-pheromone blend. The ratio of (*E*)-11- and (*Z*)-11-tetradecenyl acetate (*E*11- and *Z*11-14:OAc) in the pheromone blend that females produce and males respond to differs between strains of *O. nubilalis*. Populations also vary in female oviposition preference for and larval performance on maize (*C*4) and nonmaize (*C*3) host plants. The relative contributions of sexual and ecological trait variation to the genetic structure of *O. nubilalis* remains unknown. Host-plant use (¹³C/¹⁴C ratios) and genetic differentiation were estimated among sympatric *E* and *Z* pheromone strain *O. nubilalis* males collected in sex-pheromone baited traps at 12 locations in Pennsylvania and New York between 2007 and 2010. Among genotypes at 65 single nucleotide polymorphism marker loci, variance at a position in the pheromone gland fatty acyl-reductase (*pgfar*) gene at the locus responsible for determining female pheromone ratio (*Pher*) explained 64% of the total genetic differentiation between males attracted to different pheromones (male response, *Resp*), providing evidence of sexual inter-selection at these unlinked loci. Principal coordinate, Bayesian clustering, and distance-based redundancy analysis (dbRDA) demonstrate that host plant history or geography does not significantly contribute to population variation or differentiation among males. In contrast, these analyses indicate that pheromone response and *pgfar*-defined strain contribute significantly to population genetic differentiation. This study suggests that behavioural divergence probably plays a larger role in driving genetic variation compared to host plant-defined ecological adaptation.

KEYWORDS

population genetics, reproductive isolation, sexual selection

1 | INTRODUCTION

Recent models of speciation predict that populations diverge primarily due to the accumulation of genetic changes coincident with

local ecological adaptation (Dieckmann & Doebeli, 1999; Nosil, 2012; Schluter, 2001; Schluter & Conte, 2009). For example, host plant shifts among herbivorous insects can increase genetic variance among sympatric subpopulations by disrupting random mating (Nason, Heard, & Williams, 2002; Pashley, 1986), or selecting for optimal responses to host plant defences (Berlocher & Feder, 2002;

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Drès & Mallet, 2002; Feder, Chilcote, & Bush, 1988). However, sexual selection can also contribute to population fragmentation and ultimately speciation by reducing the frequency of mating between individuals with variant sexual traits (Panhuis, Butlin, Zuk, & Tregenza, 2001), thus arguably functioning as a force independent of natural selection (Safran, Scordato, Symes, Rodríguez, & Mendelson, 2013). Sexual selection is capable of generating linkage disequilibrium (LD) between independently assorting loci due to assortative mating between mates with compatible attraction and response phenotypes (Kirkpatrick, 2017; Kirkpatrick & Ragnié, 2002). Divergent mating preferences for sexual traits are often one of the most important reproductive isolating barriers in sympatric populations (Dopman, Robbins, & Seaman, 2010; Grace & Shaw, 2011; Lackey & Boughman, 2017; Svensson, Eroukhanoff, & Friberg, 2006), although whether divergent preferences arise through sexual selection alone is still a matter of debate (Servedio & Boughman, 2017). Furthermore, sexual and natural selection might interact in various ways during speciation, and opportunities to study both in unison may provide unique insights into the mechanisms by which reproductive isolation is established and maintained (Butlin et al., 2012; Maan & Seehausen, 2011).

Populations of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), vary in ecological and sexual traits, and this species has emerged as a model for studying mechanisms of ecological diversification and the formation of incipient species (Coates, Dopman, Wanner, & Sappington, 2018; Dopman, 2011; Kozak et al., 2017; Lassance, 2016; Wang et al., 2017). *Ostrinia nubilalis* is a polyphagous lepidopteran native to Europe and western Asia. Multiple inadvertent introductions into North America on imported broomcorn from Europe occurred near Boston, Massachusetts, Amsterdam, New York, and St. Thomas, Ontario between 1909 and 1914 (Caffrey & Worthley, 1927; McLaine, 1922; Smith, 1920; Vinal, 1917). Populations subsequently expanded south and west to the Rocky Mountains (Chiang, 1972; Showers, 1993). Current *O. nubilalis* populations in the northeastern United States vary in the number of reproductive generations per year (voltinism), as well as sex pheromone communication systems (Glover, Knodel, Robbins, Eckenrode, & Roelofs, 1991; Roelofs, Du, Tang, Robbins, & Eckenrode, 1985; Sorenson, Kennedy, Van Duyn, Bradley, & Walgenbach, 1992). Specifically, E- and Z-strain females produce blends of 99:1 or 3:97 (E)-11- and (Z)-11-tetradecenyl acetate (11-14:OAc), respectively, (Klun & Brindley, 1970; Kochansky, Cardé, Liebherr, & Roelofs, 1975; Roelofs, Cardé, Bartell, & Tierney, 1972), to which corresponding males of the two strains preferentially respond (Glover, Tang, & Roelofs, 1987). Although pheromone blend specificity contributes to partial reproductive isolation (Dopman et al., 2010; Liebherr & Roelofs, 1975; Pélozuelo, Meusnier, Audiot, Bourguet, & Ponsard, 2007), hybrid females producing a 65:35 blend of E11- to Z11-14:OAc are found in nature (Durant, Fescemyer, Mason, & Udayagiri, 1995; Klun & Maini, 1979; Roelofs et al., 1985).

Genetic loci contributing to *O. nubilalis* sexual isolation are inherited independently (Löfstedt, Hansson, Roelofs, & Bengtsson, 1989), with female pheromone blend and male response respectively

controlled by autosomal *Pher* and sex-linked *Resp* loci (Dopman, Bogdanowicz, & Harrison, 2004; Glover, Campbell, Robbins, & Roelofs, 1990). *Pher* corresponds to the pheromone gland fatty acyl reductase gene (*pgfar*; Lassance et al., 2010), and fixed amino acid changes between *pgfar-e* and *pgfar-z* alleles cause differences in the biosynthesis of isomeric components of female pheromone blends (Lassance et al., 2013). Pheromone strains and hybrids can be distinguished by a single nucleotide polymorphism (SNP) fixed between *pgfar-e* and *pgfar-z* alleles. Estimates of hybridization and positive assortative mating based on this autosomal marker vary among natural populations (Coates, Johnson, et al., 2013). *Resp* is located within a region of the Z-chromosome that encodes a suite of genes putatively involved in neuronal development and function (Koutroumpa, Groot, Dekker, & Heckel, 2016). In addition to *Resp*, a major QTL for voltinism differences between strains is located on the Z-chromosome (Dopman et al., 2004). Several loci on the sex chromosome (*Tpi*, *OR1* and *OR3*) show strong differentiation between E- and Z-strain individuals while other sex chromosomal and autosomal loci do not (Dopman, 2011; Dopman, Pérez, Bogdanowicz, & Harrison, 2005; Lassance, Bogdanowicz, Wanner, Löfstedt, & Harrison, 2011). Thus, the contribution of divergence in sexual traits (sexual selection), to genome-wide substructure among sympatric *O. nubilalis* populations remains relatively unknown.

Host-plant interactions have long been recognized as factors that impact the diversity within insect populations (Ehrlich & Raven, 1964). Although *O. nubilalis* larvae can feed on over 200 monocot and dicot host plants (Hodgson, 1928; Lewis, 1975), evidence supports some degree of local host plant specialization on cultivated maize and other plant species (O'Rourke, Sappington, & Fleischer, 2010). Laboratory choice tests suggest that neonates may prefer alternate weedy hosts over maize (Tate, Tate, Hellmich, & Lewis, 2006), but survivorship is highest on maize (Calcagno, Thomas, & Bourguet, 2007; Fisher, Mason, Flexner, Hough-Goldstein, & McDonald, 2017). The relative proportion of carbon stable isotope ($\delta^{13}\text{C}$) integrated into developing insects is directly correlated with food source (Boutton, Archer, & Midwood, 1999; DeNiro & Epstein, 1978), and indicates history of C3- or C4 photosynthetic-pathways for host plants ingested by *O. nubilalis* as larvae (Malusa et al., 2005; Ponsard et al., 2004). Analysis of $\delta^{13}\text{C}$ demonstrated that both *O. nubilalis* strains fed mostly on C4 plants (predominantly maize), but a higher proportion of males from traps baited with the E-strain pheromone lure fed on nonmaize hosts as larvae in North America (O'Rourke et al., 2010). Nevertheless, host plant attraction for *O. nubilalis* oviposition can be influenced by plant phenology (Savinelli, Bacheler, & Bradley, 1988; Spangler & Calvin, 2000) and volatiles (Udayagiri & Mason, 1995), and may contribute to variation in host plant associations among *Ostrinia* species within Europe (Bethenod et al., 2005; Bontemps, Bourguet, Pélozuelo, Bethenod, & Ponsard, 2004; Malusa et al., 2008). In France, *Ostrinia* populations using the alternative pheromone blends of E11- and Z11-14:OAc were initially assumed to be E- and Z-strain *O. nubilalis*. Strong associations were demonstrated among moths using the Z-pheromone communication system for

maize (C4) and the E-system moths for C3 host plants (Bontemps et al., 2004; Bourguet et al., 2014) which were accompanied by significant levels of genetic differentiation (Bethenod et al., 2005; Malausa et al., 2007). However, subsequent studies concluded that *Ostrinia* using the E-pheromone system in France were *O. scapularis*, and a different species compared to sympatric Z-strain *O. nubilalis* (Calcagno, Bonhomme, Thomas, Singer, & Bourguet, 2010; Frolov, Bourguet, & Ponsard, 2007; Malausa et al., 2008). Thus, results of these European studies are not directly transferrable to E- and Z-strain *O. nubilalis* in North America which show significant, but less pronounced and spatially-dependent associations with C3 and C4 host plants, respectively (O'Rourke et al., 2010). The degree to which host plant preference is associated with any genetic differentiation within or between *O. nubilalis* pheromone strains in North America remains unknown.

Genetic drift may also contribute to differentiation among *O. nubilalis* populations. Based on anonymous microsatellite marker data, *O. nubilalis* from two locations in New York and Pennsylvania were genetically differentiated from sites in Ohio westward to Colorado (Kim, Coates, Bagley, Hellmich, & Sappington, 2011). Although the authors suggested landscape features within northeastern states, including the Appalachian Mountains and fragmented crop land might serve as a partial barrier to gene flow, the possible influence of pheromone strain differences among geographic regions (Glover et al., 1991; Liebherr & Roelofs, 1975) could not be ruled out. A separate study predicted a significant isolation-by-distance relationship when comparing only Z-strain populations along a north-south transect in the Midwest United States, although this pattern was driven by a small percentage of the sampled markers (15 of 194: Levy, Kozak, Wadsworth, Coates, & Dopman, 2015).

Of the handful of studies that address the relative roles of ecological adaptation, sexual selection, and genetic drift as drivers of genomic divergence, many find that sexual selection is potent only when coupled with ecological factors (van Doorn, Edelaar, & Weissing, 2009; Maan & Seehausen, 2011; Servedio & Boughman, 2017; Wagner, Harmon, & Seehausen, 2012), but in most systems limited details are available regarding ecologically and sexually relevant traits (Safran et al., 2013). By dissecting the short-term contributions of ecology, genetic drift, and sexual traits on patterns of genomic variation and gene flow among distinct subpopulations, subspecies, and incipient species, a broader understanding of their contributions to speciation across time scales may be gained. Using anonymous genomic SNP markers, this study estimates the influence of pheromone strain, host plant differences, and geographic distance on population genetic structure of *O. nubilalis* within the northeastern United States.

2 | MATERIALS AND METHODS

2.1 | Collection and phenotypic differentiation

Pheromone lures with a blend of 97:3 or 1:99 synthetic E11- and Z11-14:OAc (Trécé Incorporated, Adair, OK) were used to separately

bait one of a pair of Harstack wire cone traps at Pennsylvania and cloth Scentry traps at New York collection sites between 2007 and 2012 (12 sites total; Table 1; Figure 1; Table S1). Males were captured during the second flight of the bivoltine strain (i.e., adult offspring of the overwintering generation) and frozen at -20°C . Samples were shipped on dry ice to the USDA-ARS, Corn Insects & Crop Genetics Research Unit, Ames, Iowa, where thorax tissue and all four wings were dissected from each moth. Wings were placed in individual 0.2 ml wells of a 96 well PCR plate lined with aluminum foil and stored at -20°C until used for carbon isotope analysis. Total genomic DNA was purified from each thorax using the Qiagen DNeasy Blood and Tissue Extraction kit according to manufacturer instructions (Qiagen, Hilden, Germany). DNA concentration in resulting extracts was quantified on a NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, DE), diluted to 10 ng/ μl with deionized nuclease free water, and stored at -20°C .

Host plant history, C3 (nonmaize) or C4 (probable maize), was assigned to each male collected in pheromone traps based on carbon stable isotope analysis. For this, the four wings were dissected from each trap-collected male, and the ratio of ^{13}C to ^{14}C ($\delta^{13}\text{C}$) was estimated by continuous flow-isotopic ratio mass spectrometry (CF-IRMS) conducted at the University of California Berkley, Center for Stable Isotope Biogeochemistry (Berkley, CA). The results were converted to δ units relative to Pee Dee Belemnite carbonate ($\delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1,000$; R = the ratio of $^{13}\text{C}/^{12}\text{C}$ atomic percentage).

In addition, genetic assignment of pheromone strain was determined for each male based on SNP genotype of a marker at the *pgfar* locus. This assay detects alleles that are >98.7% correlated with E- or Z-pheromone strains (Coates, Johnson, et al., 2013). Specifically, the *TaqI* PCR-RFLP assay was used to assign homozygous *pgfar-e/pgfar-e* and *pgfar-z/pgfar-z* genotypes to respective strains, and *pgfar-e/pgfar-z* heterozygotes to a hybrid class. Additionally, capture of males in pheromone traps baited with the E- or Z-strain lure was used as a proxy for pheromone response phenotype, given that males of the two strains strongly differ in their flight towards E and Z pheromone blends (Roelofs et al., 1987).

2.2 | Population genetic differentiation

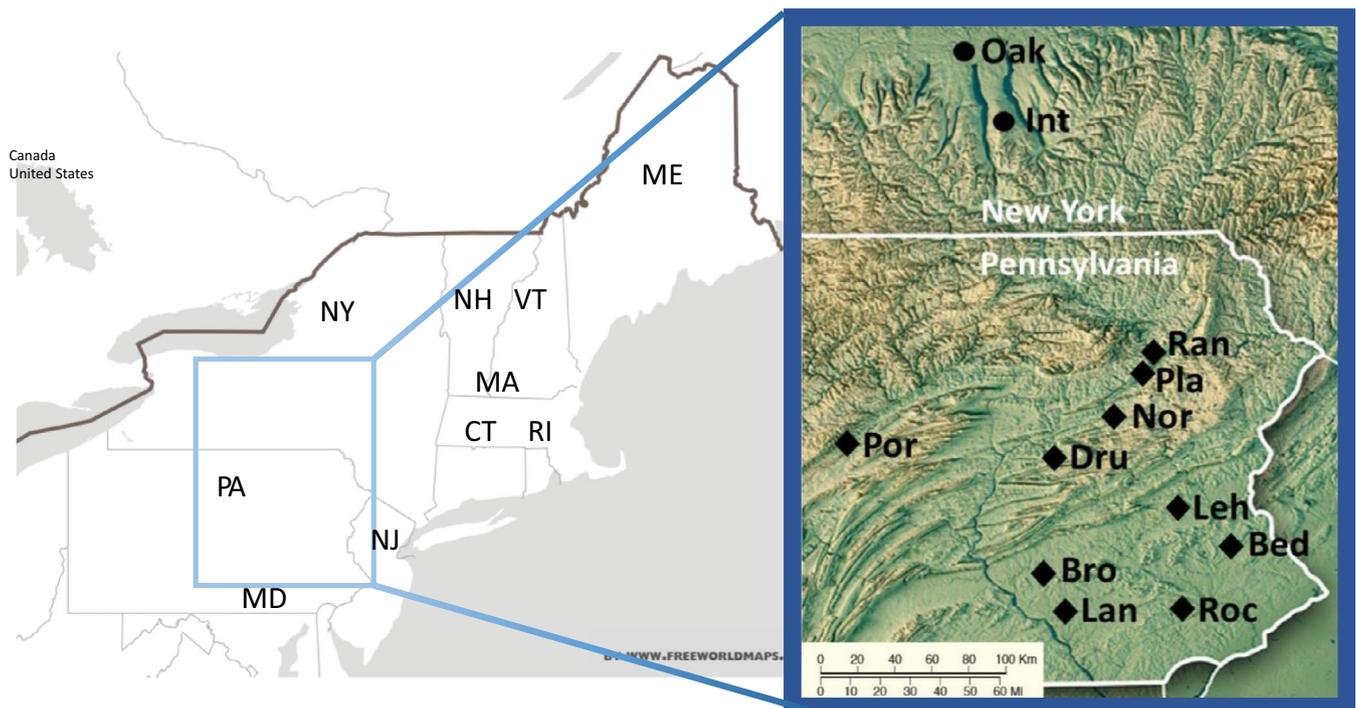
A set of 108 anonymous biallelic SNP markers was used to genotype each male by single base extension assay products analyzed on a Sequenom MassARRAY located at the Iowa State University, Center for Plant Genomics, Ames, IA. In brief, loci containing a priori defined SNPs were amplified in four PCR multiplexes (W1, W2, W3 and W4; Coates et al., 2011), and subsequently used as template for SBE assays that incorporated allele-specific mass-modified dideoxynucleotides using the iPLEX-Gold Mastermix (Sequenom; Tang et al., 1999). Genotypes were determined from raw Sequenom MassARRAY output as described by Coates, Sumerford, Siegfried, Hellmich, and Abel (2013).

Processed Sequenom SNP and *pgfar* PCR-RFLP genotype data were assessed for Hardy-Weinberg equilibrium (HWE), with

TABLE 1 Sample locations within and across years for 12 sites in the northeast United States where *Ostrinia nubilalis* E- and Z-pheromone strains are sympatric

No.	ID	Location	Year	Trap counts		Pheromone strain (host plant C3/C4)		
				E-lure	Z-lure	<i>pgfar-e/-e</i>	<i>pgfar-z/-z</i>	<i>pgfar-e/-z</i>
1	Bed09	◆Bedminster, PA	2009	21	48	4 (0; 4)	47 (11;36)	18 (5;13)
2	Bro10	●Brooklawn, PA	2010	48	48	26 (0; 26)	43 (0;43)	27 (3;24)
3	Dru09	◆Drums, PA	2009	48	48	10 (1; 9)	65 (20;45)	21 (3;18)
4	Int07	●Interlaken, NY	2007	46	48	42 (15; 27)	46 (6;40)	6 (2; 4)
5	Lan08	◆Landisville, PA	2008	32	45	18 (0; 18)	46 (6;40)	13 (0;13)
	Lan09	◆Landisville, PA	2009	48	45	15 (1; 14)	49 (21;25)	29 (6;23)
	Lan10	◆Landisville, PA	2010	48	48	29 (3; 26)	46 (16;33)	21 (4;17)
6	Leh10	◆Lehigh, PA	2010	20	48	9 (0; 9)	46 (19;27)	13 (2;11)
7	Nor10	◆Northampton, PA	2010	3	48	0 (0; 0)	44 (8;36)	7 (2; 5)
8	Oak07	●Oaks Corners, NY	2007	48	48	22 (0;22)	49 (1;49)	25 (1;24)
9	Pla09	◆Plains, PA	2009	48	18	20 (5;15)	16 (6;10)	30 (4;26)
	Pla10	◆Plains, PA	2010	28	11	19 (1;18)	8 (3; 5)	12 (0;12)
10	Por09	◆Port Matilda, PA	2009	48	18	16 (1;15)	19 (4;15)	31 (0;31)
11	Ran09	◆Ransom, PA	2009	47	48	0 (0; 0)	48 (13;35)	47 (4;43)
	Ran10	◆Ransom, PA	2010	44	22	36 (1; 35)	20 (3;16)	10 (0;10)
12	Roc08	◆Rockspring, PA	2008	32	32	24 (0; 24)	33 (0;33)	7 (0; 7)
	Roc09	◆Rockspring, PA	2009	47	46	30 (28; 2)	42 (1;41)	21 (8;13)
	Roc10	◆Rockspring, PA	2010	48	48	35 (1;34)	44 (2;42)	17 (2;15)
				704	717	355 (57;298)	711 (140;571)	355 (46;309)

Note: Males were captured in pheromone traps at each location baited with either an E-lure (99:1 E::Z11-14:OAc) or Z-lure (3:97 E::Z11-14:OAc). Pheromone strain of the same males was determined independently based on *pgfar* genotype (homozygous *pgfar-e/-e* and *pgfar-z/-z*; heterozygous *pgfar-e/-z*). The count of males from which larval host plant as determined from wing stable carbon isotope content ratio of $^{13}\text{C}/^{14}\text{C}$ (i.e., $\delta^{13}\text{C}$) are indicated within brackets (C3 $\delta^{13}\text{C}$ range of -22 to -32 ; C4 $\delta^{13}\text{C}$ range of -8 to -17). ◆, Harstack wire cone trap; ●, cloth Scentry trap.

**FIGURE 1** Topographic map of geographic locations for 12 collection sites (location abbreviations in Table 1 and coordinates in Table S1). ◆, Harstack wire cone trap; ●, cloth Scentry trap

departures of observed heterozygosity (H_O) from expected heterozygosity (H_E) across collection sites tested for significance ($\alpha = 0.05$) using Markov chain exact tests implemented in the ARLEQUIN software package (version 3.5.2; Excoffier, Laval, & Schneider, 2005). HWE tests were performed for E-, Z- and hybrid *pgfar* PCR-RFLP-defined partitions within location. Loci were subsequently excluded from further analyses if monomorphic, genotypic data were missing for $\geq 25\%$ of individuals, or genotypic proportions showed a significant departure from HWE expectation in $\geq 20\%$ of populations. Exceptions were made in instances where no HWE departures were predicted across all E- or Z-strain populations. For loci that passed the preceding filters, Fisher's exact tests were conducted to predict linkage disequilibrium (LD) between all pairs of unphased loci, and significance determined by 10,000 Markov Chain permutation steps using ARLEQUIN 3.2.2 (Excoffier et al., 2005). Any pair of SNP loci in LD were compared to known map positions on a consensus *O. nubilalis* genetic linkage map (Levy et al., 2015). Linkage groups (LGs) were assigned to physical location on *Bombyx mori* chromosomes by querying the *B. mori* genome assembly database with *O. nubilalis* contig sequences from which SNP markers were developed (Coates et al., 2011) using the BLASTX algorithm at KAIKOBASE (Shimomura et al., 2009). Additionally, the *B. mori* fatty acyl-reductase involved in bombykol production (Moto et al., 2003; GenBank accession BAC79425) is the nearest ortholog to *O. nubilalis pgfar* (Lassance et al., 2010), and was used to predict the relative orthologous position in *O. nubilalis*. All BLAST database results were filtered for E -values $\leq 10^{-50}$.

Locus-by-locus F_{ST} , F_{IS} , and F_{IT} estimates were made based on homozygous *pgfar* genotypes of males partitioned by having been collected in traps baited with E11- or Z11-14:OAc pheromone lure (male pheromone response) or by *pgfar* genotype. Global F -statistics and analysis of molecular variance (AMOVA) were estimated for genotypes partitioned by pheromone response and *pgfar* genotype, as well as by host plant history (C3 or C4), across the 12 collection sites. Pairwise F_{ST} estimates were made between collection sites, and within collection site between pheromone response, *pgfar* genotype, and host plant history. Significance threshold (false discovery rate) for each statistic was adjusted for multiple testing using the BH procedure (Benjamini & Hochberg, 1995). All estimates of subpopulation differentiation were assessed using ARLEQUIN version 3.5.2 (Excoffier et al., 2005). A filtered SNP data set of 64 loci (omitting *pgfar*) was used in all instances, except for comparisons of pheromone response in which the corresponding SNP data set of 65 loci (including *pgfar*) was also assessed.

Principal coordinate analysis (PCoA) was conducted using the covariance matrix of pairwise F_{ST} s using GENALEX version 6.503 (Peakall & Smouse, 2006). Pairwise F_{ST} estimates were made for 52 groups ($n = 1,421$ genotypes) using the 65 SNP loci that included the *pgfar* locus, and again with 64 loci after excluding the *pgfar* locus. The 52 groups represent the three *pgfar* genotypes in the 12 collection sites for each year; note that there were no homozygous *pgfar-e/pgfar-e* individuals in two of the collections (Table 1), so those combinations were not represented in the analyses. Pairwise F_{ST} estimates were also made for the same 52 *pgfar* delimited groups with collection

sites further partitioned by the two host plant history categories of C3 and C4 ($n = 1,411$ individuals), creating 91 total groups; note that there were no individuals with C3 host plant history in 13 of the *pgfar* plus collection site groups, so those combinations were not represented in the analyses. Factor scores along the two principal coordinates that accounted for the most variation were used to construct a scatter diagram to visualize genetic relationships among populations.

2.3 | Bayesian clustering

Population structure was inferred using a model-based clustering method implemented by STRUCTURE 2.3.4 (Falush, Stephens, & Pritchard, 2007; Hubisz, Falush, Stephens, & Pritchard, 2009; Pritchard, Stephens, & Donnelly, 2000). STRUCTURE provides likelihood estimates of the number of distinct populations (K) from unlinked genetic marker data using a Markov Chain Monte Carlo (MCMC) method. Estimates were obtained by iteratively running STRUCTURE 2.3.4 with K values from 1 to 10. Each run consisted of 10 replications with 1×10^7 iterations preceded by a burnin of 10^5 iterations. Runs used an admixture model of individual ancestry and a uniform prior for ALPHA. The LOCPRIOR option (Hubisz et al., 2009) was used on genotypes partitioned into 12 collection sites (1,421 genotypes, 12 partitions by location), pheromone response combined across the 12 sample sites (1,421 genotypes, two partitions by collection in traps baited with E11- or Z11-14:OAc pheromone lure), and C3 and C4 host plant history ($\delta^{13}C$ estimates for C4 host = -8 to -17 and C3 host = -22 to -32). All runs were conducted using the set of 64 SNP genotypes (*pgfar* omitted), then repeated using the set of 65 SNP genotypes (*pgfar* included). For all STRUCTURE results, the median value of the estimated log probability for each clustering analysis, conditional on K , $\ln \Pr(X|K)$, was used to compute the posterior probability of K , $\Pr(K|X)$, assuming a uniform prior distribution for K . Optimized values for K were derived from the resulting population coancestries (Q -matrix) using the ΔK statistic (Evanno, Regnaut, & Goudet, 2005). Individual Q -matrix data were visualized using DISTRUCT (Rosenberg, 2004).

2.4 | Ecological and behavioural relationships

Possible isolation-by-distance (IBD; Slatkin, 1993) was assessed by regressing pairwise genetic distance (based on estimates of F_{ST} among 12 sample locations) on geographic (km) distance. IBD analyses were performed using GENALEX (Peakall & Smouse, 2006) with the associated probabilities determined using Mantel's Test, and significance determined based on 999 permutations. The significance of any differences in the distribution of $\delta^{13}C$ estimates between *pgfar*-defined E-, Z- and hybrid (three partitions) or pheromone response (two partitions) grouped by sample site were made via single-factor ANOVA using SAS 9.2 (SAS Institute, Cary, NC, USA). Additionally, a distance-based redundancy analysis (dbRDA; Legendre & Anderson, 1999; Oksanen et al., 2011) was used to test the relative roles of host plant use (C3:C4), sexual traits (*pgfar* or pheromone response), and

geographic distance on population genetic structure. Redundancy analysis allows for multiple predictor variables to be analyzed simultaneously and each parsed into distinct factors unlike the partial Mantel Test (Legendre & Anderson, 1999; Legendre & Fortin, 2010). At each sampling site, males were classified as E- or Z-strain in two different ways: (a) by pheromone response (collected in traps baited with E11- or Z11-14:OAc pheromone lure), and (b) by male *pgfar* genotype (*pgfar-e/-e* as E-strain individuals and *pgfar-z/-z* as Z-strain individuals; all *pgfar-e/-z* were excluded). Since very few E-strain individuals were collected at Northampton, PA (Nor), and due to proximity (~5 km) of this site to Lehigh, PA (Leh), Nor was removed from analysis, resulting in 22 total populations (11 E- and 11 Z-strain). For each population, the mean $\delta^{13}\text{C}$ value was calculated, and the matrix of geographic distances (km) was transformed into component eigenvectors for each location using the PCNM function in the VEGAN R package (<http://cc.oulu.fi/~jarioksa/softhelp/vegan/>). Subsequent dbRDA was performed using the CAPSCALE function in the VEGAN R package (<http://cc.oulu.fi/~jarioksa/softhelp/vegan/html/capscale.html>). The constrained matrix was composed of multilocus F_{ST} estimates (from the data set of 64 SNPs [*pgfar* excluded]), and the predictor variables were set to pheromone (a binary trait, 0 = same pheromone, 1 = different pheromone), mean $\delta^{13}\text{C}$, and the first three geographic distance eigenvectors. Significance of each predictor (while accounting for other factors) was determined by permutation ($N = 1,000$). The dbRDA was replicated using latitude and longitude as factors instead of the eigenvectors to verify that the results were not influenced by the geographic distance transformation. Partial Mantel Tests were also performed in VEGAN using distance matrices and conditioning pairs of factors (geographic distance, pheromone difference, and mean $\delta^{13}\text{C}$ difference) sequentially on each other. The p -values were corrected for multiple testing using the Bonferroni method. In addition, an individual-based analysis was performed in which genetic distance was calculated as the number of allelic differences between two individuals (allelic dissimilarity) in the PPOPR package (Kamvar et al., 2014). The predictor variables in the dbRDA were pheromone (*pgfar* or pheromone response), $\delta^{13}\text{C}$ value, and the first four distance eigenvectors (due to the larger number of eigenvectors in the individual data set).

3 | RESULTS

3.1 | Collection and phenotypic differentiation

A total of 1,421 adult male *O. nubilalis* were collected at 12 trap locations in New York and Pennsylvania from 2007 to 2010 (Figure 1), with 704 and 717 collected from E- and Z-pheromone baited traps, respectively (Table 1). Results of the *pgfar*-specific PCR-RFLP genotyping assay yielded three distinct genotypes which were assigned to E-strain (*pgfar-e/pgfar-e*; $n = 355$), Z-strain (*pgfar-z/pgfar-z*; $n = 711$), or a heterozygote class (*pgfar-e/pgfar-z*; $n = 355$; Table 1; <https://doi.org/10.5061/dryad.kj34mq9>). Assessment of temporal (year-to-year) change in heterozygosity at *pgfar* across locations in 2009 vs. 2010 indicated a significant difference ($p = .043$; Figure

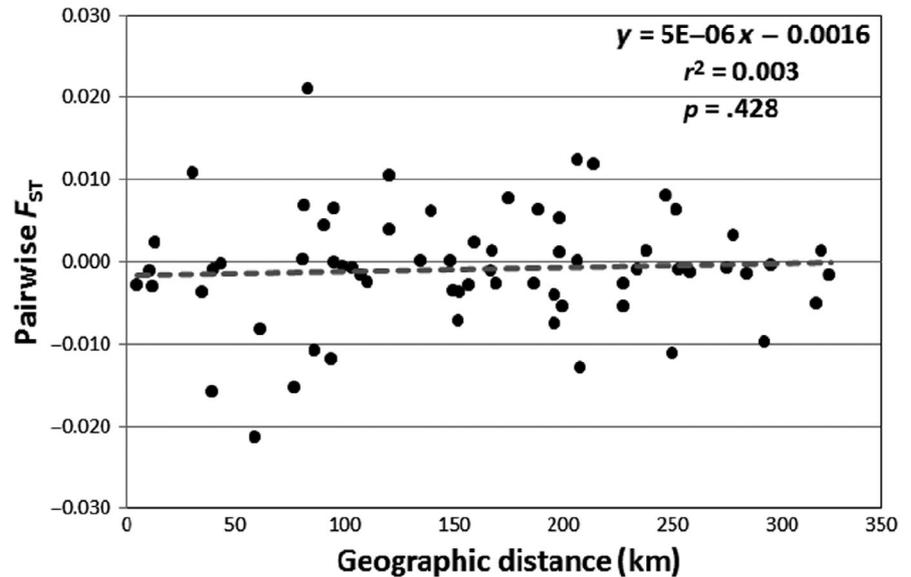
S1a). The $\delta^{13}\text{C}$ values calculated for 1,415 males from which isotope readings were collected showed a bimodal distribution, with mode peaks at -10 and -28 (Figure S1b). The estimates of $\delta^{13}\text{C}$ between -8 to -17 and -22 to -32 were respectively assigned to the C4 ($n = 1,160$; 82.0% of total) and C3 ($n = 255$; 18.0%) groups. The host plant history of five individuals with $\delta^{13}\text{C}$ estimates ranging between -18 and -21 were considered ambiguous and were excluded from further analyses involving host plant. For this data set, the proportion of males derived from C3 host plants did not differ significantly between 2009 and 2010 ($p = .109$; Figure S1c). These male pheromone response, *pgfar*-defined pheromone strain, and host plant history partitions of the data were used in subsequent analyses.

3.2 | Population genetic differentiation

Of the 108 SNP markers, 65 (including *pgfar*) passed stringency criteria and were retained within the final data set (Dryad database accession <https://doi.org/10.5061/dryad.kj34mq9>). Of those markers that were excluded, 31 were not in HWE. Subsequent locus-by-locus F_{ST} estimates indicated five SNP loci, *pgfar*, contig00078.322, contig05852.999, contig06634.250, and contig06864.843, differed significantly between groups of males defined by response to E- or Z-strain pheromone lure (Table S2a). Similarly, markers contig00078.322, contig01257.497, contig01453.160, contig05858.945, contig06634.250, and contig06864.843 showed locus-by-locus differentiation between pheromone strains defined by *pgfar* (Table S2b; significance of *pgfar* ignored). The *pgfar* locus accounted for 64% of the total genetic variation between samples trapped with different pheromone lures, and the SNP marker locus contig01257.497 accounted for $\geq 10\%$ of the total genetic variance in both locus-by-locus analyses. Tests of independence among the 65 filtered loci detected LD only between *pgfar* and contig01257.497 ($p < .0001$; results of other locus pairs not shown). Secondary confirmation of linkage came from the mapping of ortholog positions. Specifically, the predicted genome position of the nearest *B. mori* ortholog of *O. nubilalis pgfar*, BAC78425 (Lassance et al., 2010), is synonymous with the *B. mori* gene model BGIGMGA010458 (E -values $\leq 8 \times 10^{-39}$; 84.5%–100% identity across exons) located on *B. mori* chromosome 12 (positions 2,426,074–2,437,943). The nearest ortholog to *O. nubilalis* SNP marker locus contig01257.497 is a ubiquitin conjugating enzyme E2 (E -values $\leq 10^{-56}$; 80.0%–100% identity across exons) on *B. mori* chromosome 12 positions 5,150,695–5,153,581 or ~2.7 Mbp away from the *pgfar* ortholog. The SNP marker contig01257.497 was previously positioned on LG11 of an *O. nubilalis* genetic map, and also assigned to an orthologous position on *B. mori* chromosome 12 (Levy et al., 2015). Contig06721.1113 was the only SNP marker on the Z-chromosome and F_{ST} estimates did not differ significantly between strains defined either by pheromone response (Table S2a) or *pgfar* (Table S2B).

Based on the entire data set of genotypes from 65 SNP loci (inclusive of *pgfar*), F_{ST} estimates between the 12 collection sites were significant for 13 of the 66 (19.7%) pairwise comparisons (Table S3A). This same set of genotypes was partitioned across sample sites

FIGURE 2 Influence of geographic distance on genetic differentiation between *Ostrinia nubilalis* subpopulations (collection sites; Table 1). Tests for an isolation-by-distance model between pairwise genetic distance (F_{ST}) and geographic distance. Associated R^2 (goodness-of-fit) and p -values estimated from 1,000 iterations performed using Mantel Tests



based on response to either E- or Z-strain pheromone lures ($n = 24$ groups). Resulting F_{ST} estimates ranged from -0.09583 to 0.05852 and 144 of 276 comparisons (52.2%) were significant, of which 125 (86.8%) were significant between groups responded to different lures (Table S3b). Eleven of the 12 (92%) pairwise F_{ST} comparisons were significant between sympatric (within location) groups collected in E- or Z-pheromone-baited traps. The global estimate of differentiation between E- and Z-strain males across locations was also significant ($F_{ST} = 0.0322$, $p < .0001$), and AMOVA implicated pheromone response as accounting for 3.22% of the total variation between subpopulations (Table S4a). Conversely, omission of the *pgfar* locus from comparisons of pheromone response resulted in a lower number of significant pairwise F_{ST} estimates (15.2%; Table S3c), and a reduced global estimate of subpopulation differentiation ($F_{ST} = 0.0047$, $p < .0001$; Table S4b). Interestingly, significant genotypic differences between sympatric E- and Z-strains were only detected at northern collection sites (Oak, Int, Ran, Plan and Roc; Figure 1) where pairwise F_{ST} estimates were ≤ 0.0087 ; Table S3c). Five of 12 (42%) pairwise F_{ST} comparisons were significant between sympatric (within location) E- and Z-pheromone response groups, and were located at a significantly higher latitude (mean 41.79°N) than those that were nonsignificant (mean latitude 40.54°N ; $T_{4.78} = 2.81$, $p = .039$).

Pairwise F_{ST} estimates from 64 SNP loci (excluding *pgfar*) between E- and Z-strains identified by homozygous genotypes at the *pgfar* marker locus across 21 trap locations where the strains co-occurred (Table 1) were significant in 210 of 276 comparisons (76.1%; Table S3d). Among these, F_{ST} estimates between E- and Z-strains within and across sites were significant in 113 of 132 instances (85.6%; 10 of 11 sympatric E-Z comparisons). Global differentiation across locations between E- and Z-strain males based on *pgfar* genotype was low but significant ($F_{ST} = 0.0085$, $p < .0001$), and AMOVA implicated *pgfar*-defined pheromone strain as accounting for 6.78% of the total variation (Table S4c).

A total of 55 of 276 (19.9%) pairwise F_{ST} estimates were significant between partitions of the SNP genotype data defined by both collection site and host plant history (C3 or C4) (Table S3e). Twenty-six of these 55 (47.3%) significant comparisons were between genotypes assigned to different host plant history groups, including within three sites (Interlaken [Int], Ransom [Ran], and Rockspring [Roc]). Overall global estimates of F_{ST} between C3 and C4 host plant history groups across all collection sites was low and nonsignificant ($F_{ST} = 0.0004$; $p = .1779$; Table S4d).

There was no significant correlation of pairwise F_{ST} estimates with geographic distance as tested by the IBD model ($p = .4280$; Figure 2). The results of principal coordinate analyses (PCoA) based on genotype data from all 65 SNP loci (including *pgfar*) indicated that factor scores along Coordinate 1, accounted for 25.1% of the total variation, and grouped E-, Z- and heterozygote individuals into unique clusters along the Coordinate 1 axis (Figure 3a). This contrasted with results of PCoA based on SNP data from the 64 loci excluding the *pgfar* locus, where factor scores for E-, Z- and heterozygotes groups were not distinct (Figure 3b). PCoA revealed no discernable pattern in clustering of groups that accounted for *pgfar* genotype (pheromone strain) and location when assigned to C3 or C4 host plant history groups (Figure 3c).

3.3 | Bayesian clustering

An iterative Bayesian simulation approach estimated the optimal number of clusters (K), or subpopulations, represented by multilocus *O. nubilalis* SNP genotypes. STRUCTURE 2.3.4 predicted an optimal number of $K = 3$ both when *pgfar* was either included (65 loci; $\Delta K = 47.7$) and excluded (64 loci; $\Delta K = 36.3$) from the genotypic data set partitioned by pheromone response as the prior. Coancestries (clusters) were relatively homogenous between groups based on E- and Z-strain response using the data set with 64 loci (Figure S2a). In contrast, inclusion of *pgfar* (65 loci) resulted in a mean proportion of Cluster 1 among E-pheromone response males of 0.7786

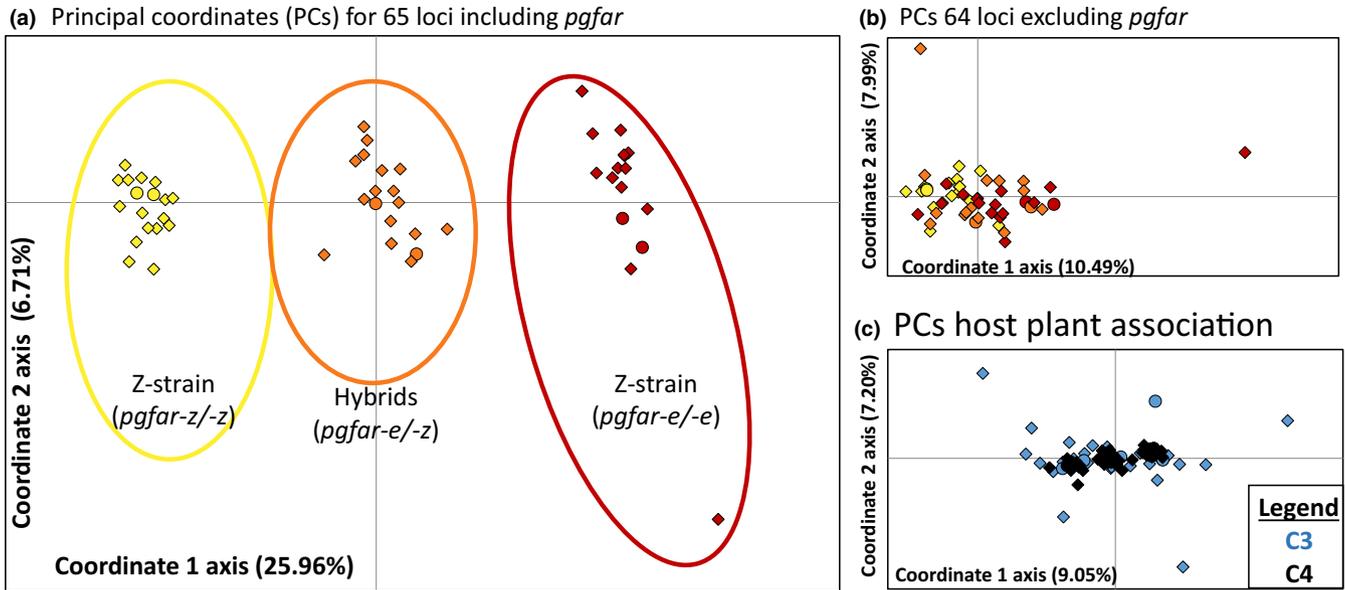


FIGURE 3 Scatter plot of factor scores along the major principal coordinates (PCs) accounting for the greatest variation among *Ostrinia nubilalis* population-derived single nucleotide polymorphism (SNP) genotype data partitioned into (a) pheromone strain using 65 SNP markers including the *pgfar* locus, (b) pheromone strain using 64 SNP markers excluding the *pgfar* locus, and (c) host plant history using 64 SNP markers excluding the *pgfar* locus. ◆, Harstack wire cone trap; ●, cloth Scentry trap

compared to 0.0451 among Z-pheromone response males (Figure S2a). Assessment of these differences in assignment of coancestries revealed that the proportion of homozygous *pgfar-z/z* males that responded to the Z-strain lure (mean = 0.8972 ± 0.0512 ; Table S5) was significantly greater than the proportion of homozygous *pgfar-e/e* genotypes that responded to the E-strain lure (0.4305 ± 0.2249 ; one-way ANOVA $F = 49.12$, $p < .0001$, $df = 1, 22$).

Partitioning data using the 12 collection sites as a priori information under the LOCPRIOR option yielded similar results. Specifically, for 65 loci (including the *pgfar* locus), STRUCTURE 2.3.4 estimated $K = 3$ ($\Delta K = 28.5$) with clusters 1 and 2 corresponding to E- (E-pheromone response-*pgfar-e/e*) and Z-strain (Z-pheromone response-*pgfar-z/z*), respectively (Figure S3). Use of 64 loci (*pgfar* omitted) resulted in $K = 2$ ($\Delta K = 29.4$), and no structure among pheromone response groups. Approximately equal mean proportions individual coancestries (population Q-score) of 0.850 ± 0.240 and 0.830 ± 0.241 were assigned to Cluster 1 for *pgfar-e/e* and *pgfar-z/z* genotypes, respectively (remaining data not shown).

Use of host plant history (C3 or C4 designation) based on $\delta^{13}\text{C}$ estimates as a priori information for Bayesian clustering of *O. nubilalis* genotypes across 64 SNP loci (excluding *pgfar*) resulted in an optimum K of 3 ($\Delta K = 42.0$). There was no difference in the mean proportion of coancestries assigned to Cluster 1 between C3 and C4 plants for comparisons either with E- (C3: 0.892 ± 0.148 ; C4: 0.873 ± 0.147) or Z-strain (C3: 0.836 ± 0.218 ; C4: 0.837 ± 0.226) defined by *pgfar* genotype (Figure S2b). Bayesian clustering of genotypes from 65 SNP loci (including *pgfar*) resulted in an optimum $K = 3$ ($\Delta K = 569.6$) with shared coancestries differentiated into clusters corresponding to *pgfar-e/e*, *pgfar-e/z* and *pgfar-z/z* despite $\delta^{13}\text{C}$ estimates (C3 or C4 host plant history) having been set as the prior (Figure S2b).

3.4 | Ecological and behavioural relationships

The overall proportions of individuals with $\delta^{13}\text{C}$ values in the range of -24 to -32 (C3 host plant history group) among *pgfar-e/pgfar-e* (E-strain), *pgfar-z/pgfar-z* (Z-strain), and *pgfar-e/pgfar-z* (E-/Z-strain hybrids) were 0.161, 0.203, and 0.151, respectively. Single factor ANOVA showed no significant difference in the proportion of homozygous male *pgfar* genotypes assigned to the $\delta^{13}\text{C}$ bin (-8 to -32) across populations ($F = 3.42 \times 10^{-5}$, $p = 1.0000$, $df = 2, 22$), between groups defined as *pgfar* homozygotes across locations ($F = 1.75 \times 10^{-5}$, $p = .9967$, $df = 1, 23$), or strain defined by pheromone response across locations ($F = 2.59 \times 10^{-5}$, $p = .9981$, $df = 2, 22$).

Analyses of dissimilarity matrices using dbRDA showed that F_{ST} estimates (genetic differentiation) among populations differed significantly only by pheromone strain. E- and Z-strain pairwise comparisons had significantly higher F_{ST} estimates when classified by pheromone response or by *pgfar* genotype (Figure 4a,b; Table 2), but not by host plant (Figure 4c). These dbRDA analyses also indicated F_{ST} estimates were not significant for host plant history (C3:C4; $p \geq .268$) or geographic distance $p \geq .369$). Similarly, latitude and longitude (used as distance factors) or using partial Mantel tests indicated that pheromone strain (response and *pgfar* genotype) significantly contributed to genetic variance, but host plant history did not (Tables S6a,b and S7). Lastly, males responding to different pheromone lures, from different locations, or with different C3:C4 ratios showed significant levels of mean allelic differentiation; however, the effect of host plant history (C3:C4; $\eta^2 = 0.0009$) was weaker than those of distance ($\eta^2 = 0.0017$) or pheromone response ($\eta^2 = 0.0034$; Table S8).

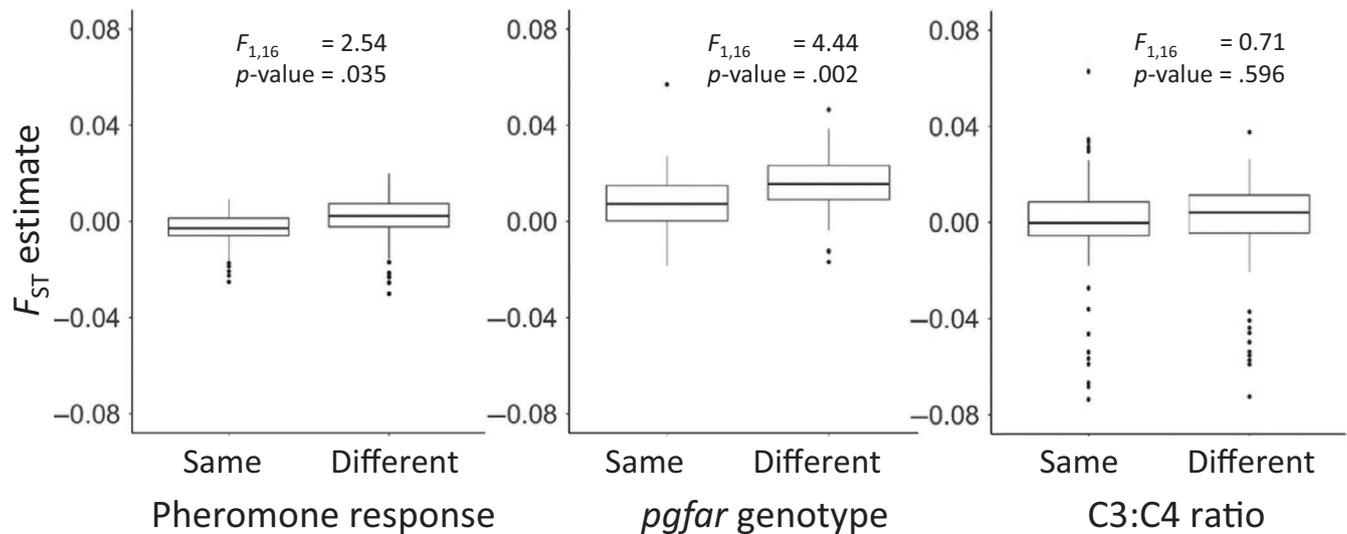


FIGURE 4 Influence of ecological factors on the genetic differentiation between *Ostrinia nubilalis* subpopulations (collection sites; Table 1) based on dbRDA analyses (64 loci, excluding *pgfar* genotypes). Range of F_{ST} estimates between E- and Z-strain when partitioned by pheromone lure (trap), homozygous genotypes from the *pgfar* genetic marker, or C3:C4 ratio (host plant). Significance as determined by dbRDA (Table 2) are shown for each comparison

4 | DISCUSSION

In many diverging populations of Lepidoptera, ecological factors such as host plant use co-vary with differences in sexual signals (Groot et al., 2008; Joyce et al., 2016), but in few cases has the relative contribution of these factors to genetic structure been tested (Gouin et al., 2017; Prowell, McMichael, & Silvain, 2004). *Ostrinia nubilalis* is an emerging model for speciation dynamics due to sympatric ecotypes or strains that vary in voltinism and sex-pheromone communication (Coates et al., 2018; Dopman et al., 2010; Kozak et al., 2017; Lassance, 2016; Olsson et al., 2010). Ecological differences in host plant preference occur between closely related *O. nubilalis* strains in North America (O'Rourke et al., 2010) and among *Ostrinia* species in Europe (Bethenod et al., 2005; Bourguet, Bethenod, Trouve, & Viard, 2000; Bourguet et al., 2014; Frolov et al., 2012; Pélozuelo, Malosse, Genestier, Guenego, & Frérot, 2004; Pélozuelo et al., 2007; Thomas, Bethenod, Pelozuelo, Frérot, & Bourguet, 2003). Although pheromone system and host plant use co-vary in *Ostrinia* (Alexandre et al., 2013), elucidating the relative contribution of various selective forces to differentiation in the presence of high rates of gene flow remains a challenge.

Our current study suggests that gene flow among northeastern populations of *O. nubilalis* remains relatively high at neutral genetic loci, as inferred by mostly nonsignificant pairwise F_{ST} estimates between collection sites (Table S3a) and by IBD modelling (Figure 2). Similarly, our dbRDA analyses suggest that geographic distance did not contribute significantly to variation among collection sites (Table 2, Tables S6 and S7). This conclusion is supported by earlier population genetics studies where low pairwise F_{ST} estimates and shallow IBD slopes suggested high gene flow over long distances (Bourguet, Bethenod, Pasteur, & Viard, 2000; Frolov et al., 2012; Kim, Bagley, Coates, Hellmich, & Sappington, 2009; Kim et al., 2011).

Additionally, in the current study, we detected no significant impact of host plant history on *O. nubilalis* population genetic structure (Table S4d) or in PCoA (Figure 3c). Likewise, dbRDA-based eigenvectors for variance in C3:C4 ratios among collection sites (Table 2, Table S6) and partial Mantel Tests (Table S7) were not significant. Finally, the proportion of individual coancestries assigned to subpopulations in Bayesian clustering analysis were strongly influenced by variation at the *pgfar* locus, not host plant despite the latter being used as the prior (Figure S2b). Together, these results indicate that host plant history is not a strong ecological factor impacting *O. nubilalis* population structure in North America. The scenario in Europe appears to be markedly different between the sympatric sibling species, *O. nubilalis* and *O. scapularis*. Specifically, *Ostrinia* from France that use the E-pheromone communication system are *O. scapularis* which predominantly feed and develop on mugwort (Frolov et al., 2007; Malausa et al., 2007), whereas *O. nubilalis* are Z-strain and mostly feed on maize. Thus, conclusions from the current study regarding the basis of ecological diversification between E- and Z-strain *O. nubilalis* may not be transferrable to the sibling species in Europe.

Our study suggests that pheromone strain has a greater effect on *O. nubilalis* genetic differentiation than either geography or host plant use. Specifically, pheromone strain of captured males (*pgfar* homozygous genotype) or pheromone response phenotype (*Resp*) each represent significant factors accounting for *O. nubilalis* population genetic variance. This conclusion is most clearly drawn from dbRDA, wherein pheromone response phenotype and *pgfar* genotype are the most significant eigenvectors determining degree of genetic variance when compared to host plant history- and geography-based eigenvectors (Table 2; Figure 4; Tables S6, S8). Even though genetic loci determining female pheromone signal and corresponding male response are not physically linked in all species

TABLE 2 Distance-based redundancy analysis (dbRDA) of eigenvectors (EV) for the relative contributions to *Ostrinia nubilalis* population genetic structure of pheromone strain (assigned based on [a] male response [attraction] to either E-strain lure [99:1 E::Z11-14:OAc] or Z-strain lure [3:97 E::Z11-14:OAc], or [b] *pgfar* genotype), host plant history (C3:C4), and geographic distance (km) (*pgfar* genotype excluded)

Component eigenvector	Sum of Sq.	$F_{1,16}$	p	η^2
(a) Strain assigned by pheromone response (males in lure-baited traps)				
Pheromone strain (response)	0.00021459	2.5390	.03497	0.115
Mean C3:C4	0.00010939	1.2943	.26873	0.059
Geographic distance EV1	0.00005160	0.6105	.73826	0.028
Geographic distance EV2	0.00004956	0.5864	.71528	0.027
Geographic distance EV3	0.00008757	1.0361	.36963	0.047
Residual	0.00135226			
(b) Population by <i>pgfar</i> genotype				
Pheromone strain (<i>pgfar</i>)	0.0010539	4.4362	.001998	0.189
Mean C3:C4	0.0001699	0.7151	.596404	0.030
Geographic distance EV1	0.0001569	0.6603	.697303	0.028
Geographic distance EV2	0.0002069	0.8711	.489510	0.037
Geographic distance EV3	0.0001993	0.8389	.495504	0.035
Residual	0.0038010			

Note: The sum of squares (Sum of Sq.), F -statistic based on 1 and 16 degrees of freedom ($F_{1,16}$), proportion of the total variance attributed to each component (η^2), and associated p -values are shown.

of Lepidoptera studied (Groot, Dekker, & Heckel, 2016), including *O. nubilalis* (Dopman et al., 2004; Löfstedt et al., 1989; Roelofs et al., 1987), these loci remain in LD (Kirkpatrick, 2017; Kirkpatrick & Ravné, 2002). Several models have been proposed to explain the co-evolution of genetic loci contributing to mate attraction and selection (Groot et al., 2016), but lack of information on the genomic architecture of these traits hinders current research (Coates et al., 2018). Although we used capture of male *O. nubilalis* in traps baited with strain-specific pheromone lures as a proxy for pheromone attraction phenotype (*Resp*), a direct measure of allelic variation at the *Resp* locus is lacking. The Z-chromosome-linked *Resp* locus (Dopman et al., 2004) is located within a suite of genes putatively involved in neurogenesis (Koutroumpa et al., 2016). The specific gene or mutation(s) involved, or genetic markers, most strongly correlated with male response are yet to be determined. Thus, variation at the causal locus, and therefore potential impacts of selection could not be tested directly and remain the subject of future work.

Our results suggest that sexual selection is acting concurrently on unlinked loci contributing to female pheromone signaling and corresponding male response phenotypes in *O. nubilalis*. These characters appear to have a more significant impact on inter-strain genetic divergence and strain maintenance than the ecological factor of host plant use. These results are consistent with the observed high rates of assortative mating caused by male response to female pheromone and selection against hybrid

pheromone blends (Dopman et al., 2010; Linn, Musto, & Roelofs, 2007). However, observed levels of hybridization (as evidenced by heterozygosity at *pgfar*; Figure S1a) suggests assortative mating is incomplete and gene flow is occurring between E- and Z-strains. Hybridization probably explains why genome-wide structuring was not detected among strains when the *pgfar* locus genotype was removed from analyses. Future studies using a greater number of anonymous markers could clarify whether population differentiation in *O. nubilalis* is limited to *Pher* and *Resp*, and genome regions physically linked to these loci. It is also possible that other islands of differentiation in the genome are associated with loci promoting assortative mating (Lassance & Löfstedt, 2009; Royer & McNeil, 1992) including differences in circadian timing (Levy, Kozak, & Dopman, 2018; Liebherr & Roelofs, 1975).

Although we found no evidence that host-plant use contributes to genetic structure, sexual selection alone is not necessarily the singular evolutionary force maintaining *O. nubilalis* pheromone strains. Ecological variation in seasonal timing (voltinism) in conjunction with pheromone strain may also contribute to high genetic differentiation on the *O. nubilalis* Z-chromosome compared to on the autosomes (Kozak et al., 2017; Levy et al., 2015). This phenomenon may be due in part to a rearrangement (probably an inversion; Wadsworth et al., 2015) of a segregating region of the Z-chromosome which differs in frequency most among individuals that differ in pheromone strain and voltinism ecotype (Kozak et al., 2017). Voltinism is strongly correlated with latitude in Z-strain *O. nubilalis* (Showers, 1981). Based on SNP markers (excluding the *pgfar* locus), we detected significant F_{S-T} between E- and Z-pheromone response groups collected in the same location mostly at northern sites (Int, Oak, Pla, and Ran) where the strains are also known to differ in voltinism (bivoltine E- and univoltine Z-strain; Calvin & Song, 1994; Glover et al., 1991). These results suggest that interactions between certain ecological and sexual traits may reinforce one another to limit gene flow in *O. nubilalis*, and the subsequent development of reproductive isolation may be a multivariate process dependent on both natural and sexual selection (Butlin et al., 2012; Maan & Seehausen, 2011).

In this study, we found that female pheromone blend and male response contributes to a greater proportion of the genetic differentiation among populations of *O. nubilalis* in the north-eastern United States compared to geographic distance or host plant. These conclusions were drawn from congruent results from Bayesian clustering, PCoA, IBD modelling, and dbRDA. Data from this and earlier studies suggest that sexual selection may function as a component maintaining the observed level of genetic differentiation between sympatric pheromone strains in North America, but whether it is sufficient by itself to maintain the pheromone strains over evolutionary time remains unclear. The relative contributions of male pheromone response and voltinism on genomic variance between *O. nubilalis* pheromone strains may be more precisely quantifiable upon the future development of genetic markers that detect or are tightly linked to causal mutations. Regardless, these data are important for understanding the

relative contributions of ecological factors (host plant use), distance (geographic isolation), and sexual selection (maternal female pheromone blend and male response) in forming barriers to gene flow between *O. nubilalis* subpopulations, which may be propelling them along different evolutionary trajectories.

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AUTHOR CONTRIBUTIONS

B.S.C. designed experiments, analyzed data, and wrote the manuscript with contributions from other authors; G.M.K., K.S.K., and Y.W. each analyzed portions of the data; J.S. conducted laboratory experiments and collected data; S.J.F. designed strategy for location of traps and collection of samples; E.B.D., and T.W.S. provided advice and contributed to writing portions of the manuscript.

DATA AVAILABILITY STATEMENT

High throughput single nucleotide polymorphism genotype data for all samples are available at <https://doi.org/10.5061/dryad.kj34mq9>.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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