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Source: Journal of Insect Science, 3(29) : 1-6

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.003.2901

Coates BS, Hellmich RL. 2003. Two sex-chromosome-linked microsatellite loci show geographic variance among North American *Ostrinia nubilalis.* 6pp. *Journal of Insect Science*, 3:29, Available online: insectscience.org/3.29

Journal of Insect Science

insectscience.org

Two sex-chromosome-linked microsatellite loci show geographic variance among North American *Ostrinia nubilalis*

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Received 28 April 2003, Accepted 30 July 2003, Published 9 September 2003

Abstract

PCR-based *O. nubilalis* population and pedigree analysis indicated female specificity of a $(GAAAAT)_n$ microsatellite, and male specificity of a CAYCARCGTCACTAA repeat unit marker. These loci were respectively named *Ostrinia nubilalis* W-chromosome 1 (ONW1) and *O. nubilalis* Z-chromosome 1 (ONZ1). Intact repeats of three, four, or five GAAAAT units are present among ONW1 alleles, and biallelic variation exists at the ONZ1 locus. Screening of 493 male at ONZ1 and 448 heterogametic females at ONZ1 and ONW1 loci from eleven North American sample sites was used to construct genotypic data. Analysis of molecular variance (AMOVA) and *F*-statistics indicated no female haplotype or male ONZ1 allele frequency differentiation between voltinism ecotypes. Four subpopulations from northern latitudes, Minnesota and South Dakota, showed the absence of a single female haplotype, a significant deviation of ONZ1 data from Hardy-Weinberg expectation, and low-level geographic divergence from other subpopulations. Low ONZ1 and ONW1 allele diversity could be attributed either to large repeat unit sizes, low repeat number, reduced effective population (*Ne*) size of sex chromosomes, or the result of recent *O. nubilalis* introduction and population expansion, but likely could not be due to inbreeding. These sequences have been deposited in GenBank AF442958, and AY102618 to AY102620.

Keywords: Ostrinia, Pyraloidea, microsatellite, sex-linked genetic markers

Abbreviation:
ONW1 *Ostrinia nubilalis* W-chromosome marker number 1
ONZ1 *Ostrinia nubilalis* Z-chromosome marker number 1

Introduction

Microsatellites are repetitive nucleotide elements that have a core repeat structure of 2 to 6 nucleotides (Chambers and MacAvoy, 2000). Microsatellite allele mutations predominantly occur via strand slippage during chromosome replication at a rate of 10^{-6} to 10^{-2} (Eisen, 1999), and is influenced by repeat unit length, number of repeat units in the array, microsatellite flanking sequence, and recombination (Chambers and MacAvoy, 2000).

Ostrinia nubilalis (Hübner), the European corn borer, is an invasive agricultural pest in North America introduced from Europe around 1917. The herbivorous *O. nubilalis* larvae are adapted to feeding on cultivated *Zea mays* (L.) and annually cause major economic losses (Mason *et al.* 1996). Phenotypic diversity in the North American population is present in two sex pheromone races, and three voltinism types (Showers 1993). Females of the two sex pheromone races are differentiated by their emission of either *E*- or *Z*-11-tetradecenyl acetate as the dominant pheromone component. Pheromone races show little allozyme marker differentiation (Harrison and Vawter 1977; Cardé *et al*. 1978; Glover *et al*. 1991). They interbreed in the laboratory and under natural conditions continual gene flow between the races apparently occurs (Roelofs *et al*. 1985; DuRant *et al*., 1995). In contrast, diapause ecotypes differ in the number of degree days required prior to adult emergence (Showers 1993), and the resultant asynchrony of adult mating cycles between voltinism types was suggested to minimize genetic exchange (Roelofs *et al*. 1985). Reduced gene flow between voltinism ecotypes was supported by RAPD-PCR marker data (Pornkulwat *et al*. 1998), but not by allozyme marker (Bourguet *et al*. 2000) or mitochondrial RFLP data (Marcon *et al*., 1999).

To date, locus specific genetic markers used for *O. nubilalis* population analysis have inherent limitations that restrict the levels of variation detected, which might contribute to the conflicting evidence provided between *O. nubilalis* ecotypes. Intraspecific

diversity of allozyme markers is reduced by negative selection on nonsynonymous changes and environmental influence on post translational modification pathways (Hartel 1988). Mitochondrial DNA is also prone to fixation by genetic drift due to a reduced effective population size of the molecule compared to chromosomes (Avise *et al*., 1988). Given the limitations of previously used markers, unresolved questions regarding gene flow among North American ecotypes, and our desire to estimate both male- and female-based population differentiation (movement), we developed sex-specific *O. nubilalis* nuclear microsatellite markers. In the following allele variation of the North American population is characterized at two such markers, ONW1 (*Ostrinia nubilalis* W-chromosome 1) and ONZ1 (*Ostrinia nubilalis* Z-chromosome 1). Genotypic and haplotypic data were used to evaluate intraspecies differences, infer the presence of any population subdivision, and estimate the levels of genetic exchange (migration) among *O. nubilalis* voltinism ecotypes and geographically distinct North American subpopulations.

Materials and Methods

Satellite DNA Isolation

Genomic DNA from *O. nubilalis* was enriched for CA microsatellites by methods described by Lyall *et al*. (1993). Resultant PCR products were ligated into pGEM-T easy cloning vector (Promega, www.promega.com) according to manufacturer instructions. Electroporation competent *E. coli* SURE® cells (Stratagene, www.stratagene.com) were transformed on a MicroPulsar apparatus (BioRad, www.bio-rad.com), and clone selection and blue white screening was performed. Positive clones were propagated overnight at 37° C in 25 ml Terrific Broth that contained ampicillin. Plasmid DNA was isolated with a QIAprep spin miniprep kit (Qiagen, www.qiagen.com) according to manufacturer's directions. Template was sequenced at the DNA Sequencing and Synthesis Facility at Iowa State University, Ames, IA, USA.

Marker Screening and Analysis

Ostrinia nubilalis samples were collected from three light traps in Iowa, and samples obtained from collaborators (Table 1). DNA extractions were performed on moth thoracic tissue as described by Marcon *et al.* (1999). DNA pellets were diluted to 50ng/µl with TLE (10 mM Tris, 0.1 mM EDTA, pH 7.5) and stored at –20° C prior to use. Primer pairs ONW1-F (5'-TGGAAGTTGATCGGAATAAGAAGTC –3') with ONW1-R (5'-TGGAAGAGCGGTAACCTCCT –3'), and ONZ-M1F (5'-GGTGGGACCTCCATGCGC-3') with ONZ-M1R (5'-GCTGGGGCGTCTTCGAGGT-3') were designed from respective DNA sequence data using Primer3 (Rozen and Skaletsky 1998). Primers were synthesized at Integrated DNA Technologies (www.idtdna.com), and 5 pmol of each used along with 0.425 U *Taq* polymerase (Promega), 1.25 µl 10X thermal polymerase buffer (Promega), 2.5 mM $MgCl_2$, and 150 µM dNTPs to PCR amplify 150 ng of individual *O. nubilalis* DNA sample in a 12.5 µl reaction volume. Thermocycler reactions used 94° C for 3 min., followed by 40 cycles of 94° C for 20 sec., 53° C for 30 sec. (ONW1) or 50° C for 30 sec. (ONZ1), and 72° C for 15 sec. ONW1 locus amplification from eighteen other species used modified

Table 1. North American *O. nubilalis* male ONZ1 genotypic, and heterogametic female ONW1 and ONZ1 haplotype frequencies in twelve subpopulations. WA_3, WA_4, and WA_5 represent ONW1 allele with three, four, and five GAAAAT repeats, respectively. ZA_1 = ONZ1 allele with one CAYCARCGTCACTAA consensus repeat, and ZA_3 = ONZ1 allele with three CAYCARCGTCACTAA consensus repeats.

Ostrinia nubilalis ID	Subpopulations	Pt	♂ ONZ1 Genotypes N	ZA_1ZA_1	ZA_1ZA_3	ZA_3ZA_3	♀ ONZ1 & ONW1 haplotypes N	ZA_1WA_3	ZA_1WA_4	ZA_1WA_5	ZA_3WA_3	ZA_3WA_4	ZA_3WA_5
1	Brookings, SD	UZ	36	0.861	0.139	0.000	23	0.217	0.749	0.000	0.044	0.000	0.000
2	South Shore, SD	UZ	31	0.968	0.000	0.032	65	0.215	0.769	0.000	0.015	0.000	0.000
3	Lamberton, MN	UZ	24	0.875	0.042	0.083	20	0.050	0.950	0.000	0.000	0.000	0.000
4	Lamberton, MN	BZ	29	0.862	0.035	0.103	26	0.231	0.769	0.000	0.000	0.000	0.000
5	Kanawa, IA	BZ	48	0.979	0.021	0.000	45	0.089	0.733	0.178	0.000	0.000	0.000
6	Hubbard, IA	BZ	46	0.913	0.022	0.065	38	0.184	0.526	0.290	0.000	0.000	0.000
7	Crawfordsville, IA	BZ	40	0.947	0.026	0.026	47	0.128	0.872	0.000	0.000	0.000	0.000
8	Mead, NE	BZ	48	0.917	0.063	0.021	40	0.225	0.750	0.025	0.000	0.000	0.000
9	Garden City, KS	MZ	47	0.915	0.085	0.000	61	0.000	0.967	0.016	0.016	0.000	0.000
10	Columbia City, IN	BZ	48	1.000	0.000	0.000	46	0.087	0.714	0.196	0.000	0.000	0.000
11	Franklin Co., IN	BZ	48	1.000	0.000	0.000	37	0.027	0.757	0.216	0.000	0.000	0.000
12	Newark, DE	BE	48	0.854	0.083	0.063	0	NA	NA	NA	NA	NA	NA
	Totals		493	458	21	14	448	59	351	38	3	0	0
	O. furnicalis	NA	38	0.947	0.000	0.053	41	0.000	0.871	0.129	0.000	0.000	0.000
	O pentitalis	NA	1	0.000				0.000	1.000	0.000	0.000	0.000	0.000

Table 2. Male AMOVA and F_{ST}, and female AMOVA and θ_{ST} values from test of *O. nubilalis* geographic variation assuming four South Dakota and Minnesota (north latitude) subpopulations are subdivided from the remainder of the North American *O. nubilalis* population.

♂ ONZ1 Genotypes	df	Sum of Squares	Variance Component	% of variation
Among groups	1	0.55	0.0014 Va	3.78
Among subpops within groups	9	0.5	0.0002 Vb	0.63
Within subpops	879	31.65	0.0360 Vc	95.59
Total	889	32.7	0.0376	100

Fixation Indices		p-values
*F*sc (F_{IS})	0.007	0.037±0.005
*F*st	0.044	0.022±0.003
*F*ct (F_{IT})	0.038	0.004±0.002

♀ ONZ1 & ONW1 Haplotypes	df	Sum of Squares	Variance Component	% of variation
Among groups	1	4.14	0.0090 Va	4.02
Among subpops within groups	9	19.97	0.0516 Vb	23.09
Within subpops	437	71.23	0.1630 Vc	72.89
Total	447	36.34	0.2236	100

Fixation Indices		p-values
θ_{SC}(θ_{IS})	0.241	0.000±0.000
θ_{ST}	0.271	0.000±0.000
θ_{CT} (θ_{IT})	0.040	0.106±0.014

amplification conditions (Table 2). PCR products were separated at 150V for 10.5 hr. on a 0.1 x 20 cm 8% polyacrylamide (19:1 acrylamide:bisacrylamide) 1X TBE gel with a 25 base pair ladder (Promega) for size comparison. Bands were visualized after ethidium bromide staining, and image capture took place on a PC-FOTO/ Eclipse Electronic Documentation System (Fotodyne, www.fotodyne.com). Basis of variation was determined by cloning ≥ 2 loci of each observed fragment size for ONW1 and ONZ1. Plasmid DNA isolation and DNA sequencing was carried out as previously described.

ONW1 allele and ONZ1 genotypic frequency data was used to evaluate *O. nubilalis* population structure, and estimate male and female migration (M) between subpopulations. Population structure assumed genetic separation of univoltine *Z*-pheromone subpopulations (UZ; ID 1 to 4), from bivoltine and multivoltine *Z*-pheromone (Table 1; ID 5 to 11). Calculations of geographic-based population subdivision assumed three groups: group 1, subpopulations with ID 1 to 4; group 2, ID 5 to 9; group 3, ID 10 and 11. A second analysis assumed north-south population subdivision assumed two subpopulation groups; north: subpopulations with ID 1 through 4, and south ID 5 to 11 (Table 1). Statistical evaluations included analysis of molecular variance (AMOVA), and *F*-statistic or haploid allele data modified *F*-statistic (θ; Excoffier *et al*., 1992; Weir and Cockerham, 1984; Weir, 1996). The Hardy-Weinberg equilibrium of subpopulation ONZ1 was tested using 10,000 Markov chain steps, and significance p values set at 0.05. All calculations were performed with the Arlequin software package (Schneider *et al*., 1997).

Results

Two polymorphic tandem repetitive loci, *O. nubilalis* Z-chromosome 1 (ONZ1) and W-chromosome 1 (ONW1), were sequenced from separate clones in a microsatellite enriched partial genomic library. The ONW1-containing clone, pGEM-OnCA09, had two repetitive regions, ten tandem GT/CA repeat units and four consecutive GAAAAT repeats (Fig. 1a; GenBank AF442958), and population analysis revealed three alleles of 141 (3 repeats; allele WA_3), 147 (4 repeats; allele WA_4), and 153 base pairs (5 repeats; allele WA_5). Two samples from each of three observed ONW1 allelic types (electromorphs) were DNA sequenced, and full GAAAAT repeat units were identified as the basis of size variation without flanking region mutation (data not shown). Successful PCR results were obtained from the ONW1 hexanucleotide repeat only from female DNA samples (data not shown). The ONZ1-containing clone pGEM-OnCA01 had a fifteen nucleotide-long imperfect repeat element, CAYCARCGTCACTAA, (underlined nucleotides optional; Fig 1b; GenBank AY102618 to AY102620). ONZ1 was PCR amplified from male derived DNA and all attempts to amplify female DNA failed (data not shown). Six ONZ1 alleles were sequenced, three from each of two size classes. Two ONZ1 allele size range variants 95 to 97 (ZA_1) and 106 to 107 base pairs (ZA_2) were characterized with one insertion/deletion and two substitutions within both the repeat and the flanking region (Fig 1b). Since single base changes were not resolved by polyacrylamide gel electrophoresis methods, two alleles (ZA_1 and ZA_2) were scored and used for genotype data. Three pedigrees with ≥ 46 F2 progeny confirmed the male specificity of ONZ1 and female specificity of ONW1. More specifically, ONW1 and ONZ1 were hemizygous in females (single locus with no corresponding homologous pair), and males were diploid at ONZ1 and lacked the ONW1 locus.

ONZ1 and ONW1 were PCR amplified from 448 female, and ONZ1 from 493 male, DNA samples from 11 North American subpopulations of *O. nubilalis*. Male ONZ1 genotypic and ONZ1 and female ONW1 haplotype frequencies were calculated (Table 1). Male genotypic and female haplotype data were separately evaluated by AMOVA and fixation indices, and independently showed stronger evidence for geographic- compared to voltinism-based population structuring (Table 2).

Discussion

Low allele size diversity was present at *O. nubilalis* W chromosome (ONW1) and Z-chromosome (ONZ1) microsatellite

A:

```
nt
001 G AACAGGTAA GTTTGCGTGG TATACGAAGA CGAGTAAGAT TTGGAAGTTG ATCGGAATAA
                                          ONW1-F >>>>>>>>> >>>>>>>>>>
061  GAAGTCCATG AAAATGAAAA TGAAAATGAA AATGTTTTAT TTGATAAATA AAGTGGATTA
     >>>>>>
121  GCAAGATATT TTGGTAACCA TCCTTTTAAG TATAAGAATA CCTGTAGTAG GAGGTTACCG
                                          << <<<<<<<<<<
181  CTCTTCCAAA AGTATTAACA TACATTCTTA GTTAGTGTGT TTGTGGACTT TGTGGTGTAT
     <<<<<<<< ONW1-R
241  TGAGTGAATT TGTGTGTGTG TGTGTGTGTG AA
```

B:

```
nt
001  GGTGGGACCT CCATGCGCA* CAGCGTCACT CA*CAGCGTC ACTAACACAG CGTCAAACC OnCA01
001  ---------- ---------* ---------- --A--A---- ---------- ------A*- Allele ZA2-1
001  ---------- ---------* ---------- --A--G---- ---------- ------AC- Allele ZA2-2
001  ---------- ---------T ********** ********** *--------- ------AC- Allele ZA1-1
001  ---------- ---------T ********** ********** *--------- ------CC- Allele ZA1-2
001  ---------- ---------* ********** ********** *--------- ------A*- Allele ZA1-3
     >>>>>>>>>> >>>>>>>>
      ONZ-M1F     CA RCGCAY CARCGTCACT CAYCARCGTC ACTAACA CARCGTCA = consensus repeats

061  TTTTTCCCAT GCCACTCACC ATGTTCCAAC AAGTGTCGGT ACCTCGAAGA CGCCCCCAT OnCA01
061  ---------- -------A-- ---------- ---------- ---------- --------- ZA2-1
061  ---------- -------A-- ---------- ---------- ---------- --------- ZA2-2
061  ---------- -------A-- ---------- ---------- ---------- --------- ZA1-1
061  ---------- -------A-- ---------- ---------- ---------- --------- ZA1-2
061  ---------- -------T-- ---------- ---------- ---------- --------- ZA1-3
                                                <<<<<<<<< <<<<<<<<< ONZ-M1R
```

Figure 1. A) 272 bp insert DNA sequence from clone pGEM-OnCA09 showing three tandem GAAAAT repeats (underlined) and primer binding sites (underscored by arrows indicating direction) used to PCR amplify the locus *Ostrinia nubilalis* W-chromosome marker number 1 (ONW1; GenBank accession: AF442958). **B)** Multiple DNA sequence alignment of the PCR amplified *O. nubilalis* Z-chromosome linked marker number 1 (ONZ1) alleles. Consensus sequence of imperfect alleles, CAYCARCGTCACTAA (underlined nucleotides optional), and primer binding sites (arrows indicating direction) are underscored, and – representing an invariable nucleotide identical to clone OnCA01, and * is a deletion.

loci. The ONW1 allele with four repeat units [$(GAAAAT)_4$; WA_4), was present among 76% of North American *O. nubilalis* females, and described from 87.1 % of 41 female *O. furnicalis* (Gunée) and a single *O. pentitalis* (Grote) sample, indicating that the allele likely is ancestral to the genus. The molecular basis of low ONW1 allele size variation might lie in the repetition of a hexanucleotide repeat that is less prone to strand slippage during recombination, the short array of 3 to 5 GAAAAT repeats, and the potential location of ONW1 in the nonrecombining region of the W chromosome that eliminates unequal crossover as a mechanism generating allele diversity. ONW1 WA_3 alleles outnumbered WA_5 alleles, 19.3% to 4.7% (Table 1), indicating a preponderance of microsatellite repeat unit loss, as opposed to gain that occurs typically at microsatellite loci (Rubinsztein *et al.*, 1995, Weber and Wong, 1993). Rose and Falush (1998) determined that a minimum of eight repeats was required for evolution of hypervariable microsatellites, and array lengths such as ONW1 that are below this threshold suggest insensitivity toward repeat number expansion bias. The ONZ1 locus is biallelic, with the dominant ZA_1 allele frequency at 95% (937 of 986) in males and 99% (445 of 448) in females. Near fixation of ZA_1 in female *O. nubilalis* and not in males might result from differences in effective population sizes (N_e) of the W chromosome compared to Z chromosomes. Assuming a 1:1 sex-ratio, lepidopteran W chromosomes have a three- and fourfold reduction in effective population size compared to Z and autosomal chromosomes respectively (Charlesworth *et al.*, 1987; Hartel, 1988), whereby associated loci are be more prone to fixation and interpopulation divergence (Charlesworth *et al.*, 1987). Despite being less prone to molecular fixation, ONZ1 locus allele diversity was lower than ONW1 that might result from its longer imperfect repeat, or be a result of population effects (see below).

No genetic divergence was detected between female ONW1 and ONZ haplotypes based on voltinism ecotype, as indicated by –6.44% (0.0%) of total population variation among groups. Similarly at the ONZ1 locus, grouping samples by voltinism showed an F_{ST} = 0.028, and 0.83% of total population variance among ecotypes (remaining data not shown). Results indicated no evidence of *O. nubilalis* voltinism-based ecotype structure and are in agreement with mitochondrial PCR-RFLP (Marcon *et al*. 1999) and allozyme data (Bourget *et al*. 2000), but in conflict with voltinism ecotype variation detected by RAPD-PCR (Pornkulwat *et al*. 1998). Additionally, a single *E*-pheromone subpopulation from Newark, Delaware did not show significant ONZ1 allele or genotype variance with *Z*-pheromone race subpopulations. A triose phosphate isomerase (TPI) marker was previously mapped to the Z chromosome and linked to inter-pheromone race behavioral or response differences in males (Glover *et al*. 1991), suggesting a Z chromosome location of ONW1 distant from TPI and male pheromone response

genes. Lack of both voltinism ecotype-based population differentiation or a correlation of ONZ1 with pheromone race might be an artifact of recent *O. nubilalis* range expansion across North America (Showers 1993), suggesting slowed ONW1 and ONZ1 lineage sorting (Avise et al. 1984) and allele extinction by genetic drift (Takahata 1983) between ecotypes.

Low but significant differentiation was previously shown between *O. nubilalis* ecotypes (Harrison and Vawter 1977; Cardé *et al*. 1978), but not between geographically distant samples (Bourget *et al*. 2000). Both sex-linked genetic markers, ONW1 and ONZ1, indicated greater contribution of geography to total genetic variance compared to voltinism. AMOVA analysis indicated that 3.78% of total population variance was between two groups; a "northern" group consisting of Minnesota and South Dakota subpopulations (ID 1 to 4), and all other subpopulations classified as "southern" (ID 5 to 11; Table 1). Combined ONZ1 and ONW1 female haplotype data indicated that 4.02% of total population variance was accounted for among "northern" and "southern" groups. Low-level geographic divergence of four subpopulations from northern latitudes might reside in their omission of the ZA_1WA_5 female haplotype (Table 1), and significant deviation of ONZ1 data from Hardy-Weinberg expectation at three of the four sites. In the "northern" geographic region, chi-square (χ^2) tests detected significant departure of ONZ1 genotypes from Hardy-Weinberg equilibrium within Lamberton, Minnesota bivoltine *Z*-pheromone ($p < 0.001$), Lamberton, Minnesota univoltine *Z*-pheromone ($p = 0.007$), and South Shore, South Dakota univoltine *Z*-pheromone subpopulations ($p = 0.016$). Deviation for HWE was not exhibited from any other subpopulation. Except for the Brookings, South Dakota sample, the basis for Hardy-Weinberg deviations among northern subpopulations likely reside in their decreased the average heterozygosity ($H_S = 2 \div 81 \cong 0.025$) compared to the average among the remaining subpopulations ($H_T = 19 \div 317 \cong 0.060$). Reduced heterozygosity might be attributed to the presence of null alleles, or a stronger effect of the 1/4 reduction in Z chromosome effective population size in smaller fringe populations. Alternatively, recent or recurrent population size effects might disproportionately disrupt *O. nubilalis* population Hardy-Weinberg equilibrium in northern regions. Increased severity of cyclical genetic bottlenecks due to larval over-wintering mortality in northern regions (Hudson and LeRoux 1986) might disturb mutation-drift equilibrium since N_e likely does not remain stable for $2N_e$ to $4N_e$ generations (Nei and Li 1976). Additionally, entry of univoltine *O. nubilalis* moths into Minnesota in the early 1940s (Chiang, 1961), South Dakota in 1948, and North Dakota in 1950 (Chiang 1972), followed by the northern migration of bivoltine subpopulations starting in the early 1950s (Chiang 1965), suggest recent range expansion might have caused a deviation from migration-drift equilibrium (Takahata 1983).

The level of migration was inferred from *F*-statistics derived from male genotype and female haplotype data (Table 2). Female haplotype analysis indicated substantial effects of inbreeding and genetic drift ($\theta_{ST} = 0.227$; $\theta_{IS} = 0.274$), whereas male ONZ1 genotypic analysis suggested little evidence of either ($F_{ST} = 0.028$; $F_{IS} = 0.020$; Table 2). Inbreeding prevalence among female *O. nubilalis* might be used to infer lower migration rates as compared to males. When considered in conjunction with increased levels of genetic drift among females ($\theta_{ST} = 0.227$) in relation to males ($F_{IS} = 0.020$), the reduced effective population size of the W chromosome and lack of homologous recombination during female gametogenesis might result in a strong influence of nucleotide fixation in female lineages. Thus differences between loci used in this study might reflect a background of chromosome position effects, and not truly be representative of population-based influences. Corroborating biological estimations of male and female flight distances are required to verify these results.

Low ONZ1 and ONW1 allele diversity was attributed to large repeat unit sizes, low repeat number, reduced effective population (N_e) size of sex chromosomes, the result of a genetic bottleneck that occurred when *O. nubilalis* was introduced, or genetic drift caused by continued population expansion. Analysis of male ONZ1 genotypes and female ONW1 and ONZ1 haplotypes showed no evidence for voltinism-based population structure. The similarity of west-central Minnesota and eastern South Dakota samples, compared to all other subpopulations, for both ONW1 and ONZ1 provided evidence of geographic population structure. Recent *O. nubilalis* population expansion into northern regions of the central United States and recurrent seasonal bottlenecks likely explain the maintenance of HW disequilibrium, and form the basis of geographic population subdivision instead of inbreeding.

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