4-2018

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Abstract

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Disciplines
Ecology and Evolutionary Biology | Entomology | Genetics

Current Opinion in
Insect Science

Genomic mechanisms of sympatric ecological and sexual divergence in a model agricultural pest, the European corn borer

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The European corn borer, *Ostrinia nubilalis*, is a model species for elucidating mechanisms underlying adaptively differentiated subpopulations in the face of reciprocal gene flow, and is a major pest of cultivated maize in North America and Eurasia. Strains are characterized by different pheromone communication systems in combination with voltinism strains that are adapted to distinct local climate and photoperiod through adjustments in diapause traits. However, only partial barriers to inter-strain hybridization exist in areas of sympatry. Recent research shows that genes governing important strain-specific isolating traits are disproportionately located on the Z-chromosome. Furthermore, co-adapted combinations of some of these genes are non-recombining due to location within a large chromosomal inversion, and assist in maintaining strain integrity despite hybridization.

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Introduction

The accumulation of adaptive traits within a species is thought to most easily arise following geographic subdivision, from which distinct biological entities or species can emerge [1,2]. The genetic responses to natural selection that enable adaptation to local environmental conditions may also manifest as, or select for, barriers to mating or reduced hybrid fitness, resulting in populations that evolve along independent trajectories even when sympatric or upon secondary contact [3–5]. Nevertheless, when reproductive isolation is incomplete, limited gene flow between diverged strains and species can occur and contribute new raw material for further ecological adaptation. The emergence and maintenance of divergent adaptations, as well as the evolution of genetically subdivided populations, imposes important challenges for enacting effective pest control and resistance management programs targeting agricultural pest insect populations. The mechanism(s) that underpin these phenomena remain difficult to elucidate, but are being addressed using model systems.

The genus *Ostrinia* (Lepidoptera: Crambidae) is a globally distributed complex of greater than 20 closely related species and many additional ecologically diverse subspecies [6–8]. *Ostrinia* species are classified into three groups based on morphological features of the male genitalia [6] and supported by phylogenetic analyses of mitochondrial DNA [9]. Phylogenetic Group III contains several species with persistent intraspecific phenotypic variants [10], including two that feed on cultivated maize: the European corn borer, *Ostrinia nubilalis*, and the Asian corn borer, *Ostrinia furnacalis*. Across Group III, different blends of (E)11-tetradecenyl acetate and (Z)11-tetradecenyl acetate (E11-14:OAc and Z11-14:OAc) isomers constitute the predominant female-produced sex pheromone. Notable exceptions are the E12-14:OAc and Z12-14:OAc isomers produced by *O. furnacalis* [10] via a novel Δ14-desaturase, which evolved following a lineage-specific duplication of an ancestral Δ11-desaturase [11].

Despite differences in sexual communication systems some species boundaries are porous and reproductive isolation in nature is incomplete between *O. furnacalis* and *O. scapulalis* [12] and *O. furnacalis* and *O. nubilalis* [13••]. Laboratory experiments have also demonstrated the viability of hybrid offspring from crosses between *O. nubilalis* and *O. scapulalis*, *O. narynensis*, and *O. furnacalis* [12,14–16], and *O. furnacalis* and *O. scapulalis* [12]. The frequencies of neutral genetic markers fail to differentiate *Ostrinia* species [12,14], likely attributable to either incomplete lineage sorting of ancestral variation or to ongoing exchange of alleles between species. By contrast, markers positioned near or within strongly selected loci that underlie species-specific traits may be capable of defining lineages when genomic regions are in linkage disequilibrium with those loci [13••,17–19]. Of special interest are findings of gene flow between

sympatric populations of *O. nubilalis* and *O. furnacalis* in the Yili region of Xinjiang Province, China [13••]. Results of this study suggest introgression of alleles occurs from locally invasive *O. furnacalis* into resident *O. nubilalis* populations, and further imply that alleles responsible for ecological adaptations may be exchanged across species boundaries. A newly acquired adaptive allele presumably would increase in frequency in the local environment, as long as the allele remains phenotypically advantageous within the recipient genome architecture [13••]. Thus, introgression of locally-adapted alleles between incompletely isolated *Ostrinia* species could influence their evolutionary trajectories. The impact of interspecific gene flow on adaptation in agroecosystems or other ecological situations remains to be fully explored or understood.

Ostrinia nubilalis is native to Europe, Northern Africa, and Western Asia, but is invasive in North America following multiple inadvertent introductions in the early 1900s and it now occupies the eastern two-thirds of the United States [20]. The species is a major pest of cultivated maize [12], and is a primary control target of transgenic maize varieties that express insecticidal proteins derived from *Bacillus thuringiensis* (Bt). While Bt-maize remains effective, there are concerns that the intense selection pressure imposed by the wide-spread adoption of Bt-maize could lead to the evolution of resistant *O. nubilalis* populations [21]. Insect resistance management (IRM) plans have been designed and implemented to slow the evolution of resistance, and their effectiveness depends critically on gene flow within and between populations [22,23].

Populations of *O. nubilalis* harbor phenotypic variation in reproductive and life-history traits, including sexual communication and voltinism controlled by the seasonal timing of induction and duration of overwintering diapause. Trait variation originated in Europe prior to their establishment in the northeastern United States and Canada, presumably through multiple introductions, although post-introduction gene flow in North America may have allowed for novel phenotypic combinations [20,24]. Populations using E pheromone ('E-strain') have remained restricted to the eastern United States where they are sympatric with Z pheromone populations ('Z-strain'). The distribution of the latter extends through the Corn Belt to the Rocky Mountains [20,24]. The reason E-strain phenotypes have not expanded its range westward into the Corn Belt is unknown, but ecological constraints could be involved. Both pheromone strains are polyphagous, but the E-strain may be more likely to develop on non-maize hosts [25]. Obligate univoltine populations are confined to the northern U.S. and southern Canada, while the facultative multivoltine populations are found throughout the species distribution except in the far north.

A number of scenarios can account for maintenance of adaptive divergence within a population, including both divergent selection and nonrandom mating [26,27]. Current evidence suggests that variation in *O. nubilalis* pheromone communication systems among partially isolated strains is the result of divergent sexual selection, and along with variation in voltinism, makes this species an excellent model for the study of incipient speciation and persistence of ecologically relevant phenotypic variation in sympatry [24,28•,29••]. Polymorphism in voltinism traits is an important adaptive mechanism by which *O. nubilalis* can optimize its phenology relative to local photoperiod and climate, including, perhaps, in response to long-term climatic fluctuations. While the number of case studies describing the behavioral and ecological adaptations involved in maintaining the integrity of divergent populations in sympatry are accumulating [27], a great challenge remains in uncovering the underlying genetic mechanisms. Here, we highlight recent work that dissects the genetic basis of strain-specific adaptive traits in *O. nubilalis*, which provides clues to their maintenance despite inter-strain gene flow. We review evidence for genetic variation between pheromone strains, as well as clinal variation in allele frequencies among voltinism ecotypes at loci implicated in the control of circadian rhythm. Understanding how adaptive phenotypes have emerged in *O. nubilalis* provides insights into the evolution of subpopulations in this and in other agricultural pest insects that can impact the efficacy of insect control and IRM strategies.

Sexual communication

E-strain and Z-strain females of *O. nubilalis* produce, and males preferentially respond to, pheromone blends of 99:1 or 3:97 *E*11-14:OAc and *Z*11-14:OAc, respectively [16]. Quantitative trait locus (QTL) mapping identified an autosomal locus, *Pher*, that determines *O. nubilalis* strain differences in isomeric ratios of female pheromone blends, and a sex (Z-chromosome)-linked locus, *Resp*, which governs male pheromone response [30]. Due to the ZW sex determination system in Lepidoptera, homogametic ZZ males and heterogametic ZW females show sex-based copy number variation at all Z chromosome loci. The pheromone gland-expressed fatty acyl reductase gene, *pgfar*, was later shown to correspond to *Pher* [18]. Non-synonymous mutations between *pgfar-e* and *pgfar-z* alleles cause functional differences in ligand specificity and production of pheromone blends with opposite major ratio components [31]. This research demonstrates an important genetic mechanism by which isomeric pheromone blend differences can arise, and a link between genotype and phenotype. Based on this work, a single nucleotide polymorphism (SNP) at the *pgfar* locus was detected that is >98% correlated with pheromone strain [32]. Specifically, two strain-specific alleles, *pgfar-e* and *pgfar-z*, were highly correlated with female pheromone phenotype determined by gas

chromatography among pure colony-derived strains and intercrossed lines. This work provided an important marker for *O. nubilalis* population and ecological research. For example, genotyping of light trap collected *O. nubilalis* at the *pgfar* SNP locus showed the complete fixation of the *pgfar-z* allele in the Midwestern United States, where only Z-strain populations are found. Genotypes were mixed in regions of pheromone strain sympatry in the Northeast.

The strain-specific communication system logically should provide a mechanism for reproductive isolation by assortative mating [24]. Based on genotypes at the *pgfar* SNP locus in moths collected at 11 sympatric sites, reproductive isolation between pheromone strains was indeed stringent in some sites, but up to 42% of individuals from other sites were hybrids [32]. Thus, assortative mating seems to occur in some locations but not in others, suggesting variation in gene exchange between populations. This perspective is consistent with a recent study showing that genetic differentiation between strains may depend on a combination of pre-reproductive barriers, including both pheromone system and voltinism (number of reproductive generations per year; see section on 'Voltinism') [29••].

Annotations of genes in the region near the Z-chromosome-linked QTL for male pheromone response, *Resp* [24], suggest their involvement in neurogenesis [33••]. Pheromone strain-specific polymorphisms in these genes may determine differing male neural organization responsible for discriminating pheromone blend ratios. Earlier research implicated transmembrane odorant receptor (OR) proteins, encoded within a cluster of tandemly duplicated paralogs on the Z-chromosome [34], as likely candidates determining specificity of male pheromone responses. ORs showed higher expression in male compared to female antennae [35–37], and the *in vitro* OR activation pattern matched *in vivo* activation of pheromone-sensitive olfactory neurons in a species-specific and strain-specific manner [35,37]. Recent work has examined pheromone binding protein specificities [38] and male behavioral responses after gene editing-based knockout of the OR co-receptor [39], but the functional role of ORs in variable male sexual response remains elusive. However, the findings by Koutroumpa *et al.* [33••] implicate genes controlling nervous system structure as major factors influencing pheromone discrimination by *O. nubilalis* males. Olfactory neurons responding to the major and minor pheromone components are physically connected to specific macroglomeruli of different sizes in the antennal lobe of the brain, and these neural connections are identical but physiologically reversed between E-strain and Z-strain *O. nubilalis* [40,41]. Furthermore, among F₁ hybrids, the macroglomeruli are of approximately equal size, and inheritance of

glomerulus size among backcross progeny is co-dominant and sex-linked [42].

Voltinism

Ostrinia nubilalis overwinter as mature larvae in diapause, a state of developmental arrest associated with metabolic changes and increased cold-hardiness [43–47]. Diapause improves survival during periods of reduced temperature and resources. Diapause initiation and termination thresholds among individuals in response to local photoperiod and temperature conditions contribute to the predominant voltinism expressed at the population level. Compared to obligate univoltine populations or ecotypes, the initiation of facultative diapause in multivoltine ecotypes corresponds to a greater number of accumulated degree days, while termination occurs in fewer degree days [48]. Hence, populations with a single mating generation per year are restricted to northern latitudes [20]. In regions where uni-voltine and multivoltine *O. nubilalis* populations are sympatric, differences in time of diapause termination and initiation limits temporal overlap in mating periods between ecotypes, which promotes allochronic reproductive isolation [17,24]. Genetic mapping identified a single major QTL on the Z-chromosome determining post-diapause development (*Pdd*) duration, which is longer in univoltine than in multivoltine *O. nubilalis*. *Pdd* is located near, but distinct from *Resp* [17]. Additionally, inheritance of early diapause initiation in univoltine *O. nubilalis* is sex-linked [49], as also seems to be the case for *O. furnacalis* [50,51], but causal genetic loci or their relative positions on the Z-chromosome are yet to be determined.

In multivoltine *O. nubilalis* populations, both genetic and environmental factors influence the seasonal timing of initiation and termination of facultative diapause, which in concert determine voltinism at a given latitude. These adaptive traits are crucial for maximizing reproductive potential by facilitating additional mating generations when environmental conditions are favorable, balanced against the risk of decreased overwinter survival if development to the diapause stage (mature larvae) cannot be achieved in the last generation before the onset of winter. Photoperiod interacts with temperature in dictating seasonal timing of diapause induction in *O. nubilalis* [48]. The southward range expansion from the northeastern to the southeastern United States seems to have been slowed by the need for populations to progressively adapt to the dynamic interactions of temperature and photoperiod at decreasing latitudes [20]. Furthermore, adaptive response to these environmental interactions must occur at several loci that contribute to seasonal timing of diapause. Variation in voltinism among *O. nubilalis* populations is correlated with clock genes involved in circadian rhythm [52••]. Specifically, genotypes of *O. nubilalis* collected across a North-South cline from Minnesota to Missouri in the United States show a cyclical spatial

Figure 1
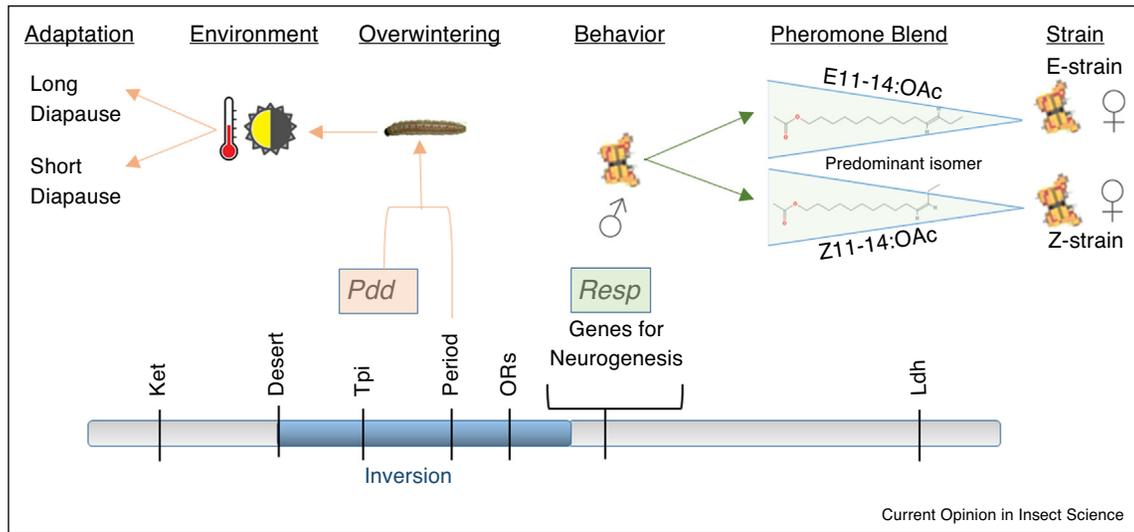
Linear order of genes on the *Ostrinia nubilalis* Z-chromosome showing the location of an approximately ∼7 cM inversion (blue). Within the inversion, a quantitative trait locus (QTL) for post-diapause development time, *Pdd*, maps near the triosphosphate isomerase (Tpi) marker [21] and the *Period* gene, a regulator of circadian rhythms [46]. *Pdd* and *period* are putatively involved in larval response to the interaction between temperature and photoperiod, which leads to adaptive responses of diapause duration among voltinism ecotypes. A second QTL within the co-linear region of the Z-chromosome is involved in male pheromone response, *Resp*, determining behavioral attraction to either E11-tetradecenyl acetate or Z11-tetradecenyl acetate (E11-14:OAc or Z11-14:OAc) as the major isomeric component of female pheromone blends [24]. *Resp* spans a Z-chromosome region encoding genes putatively involved in neurogenesis, and is independent of a locus encoding a tandem repeat of odorant receptor (OR) paralogs [26].

pattern in allelic frequency variation in the circadian clock genes, *cryptochrome 1* and *period*. These spatial allelic frequencies repeat across latitude in a 'sawtooth' pattern as population demography transitions between one, two, and three reproductive generations per year [52••]. This research implicates the circadian clock in an adaptive response to clinal changes in climatic conditions such as photoperiod and temperature (spatial selection), and suggest that *cryptochrome 1* and *period* alleles play a role in photoperiodic diapause responses that are timed to maximize generation number. Supporting this hypothesis, *period* is physically linked to *Pdd* [52••,53•], the locus underlying variation in timing of diapause termination.

A polymorphic rearrangement that is likely an inversion spans an approximate 7 cM (3.9 Mbp) region on the Z-chromosome that overlaps with *Pdd*, *period*, and tandemly-repeated OR loci, but genes underlying male pheromone blend response (*Resp*) are in a collinear chromosomal segment [29••,53•] (Figure 1). Thus, this rearrangement has captured genes involved in voltinism differences and male detection of female pheromones. In general, a chromosomal inversion impacts the evolution of genes within it by increasing linkage disequilibrium (decreasing recombination rates), such that the genome region functionally evolves as a single haplotype. Thus, mutations can accumulate independently by genetic drift and selection within inverted and standard

chromosomes [53•]. Although ORs and *Resp* play different roles in male behavior, the effects of any genetic interactions at these loci remain unknown (e.g. epistasis). Furthermore, the influence of rearranged (inverted) and standard sex chromosome configurations on the evolution of male pheromone detection and behavioral responses remain to be elucidated. Variation in voltinism does occur among populations with rearranged as well as collinear Z chromosomes, the latter of which can allow for disentangling interactions between *Pdd* and *period* on voltinism. The inversion is segregating in both pheromone strains and thus is not fixed at the population level, but it does reach high frequency in sympatric populations that differ in both pheromone system and voltinism. This suggests the inversion has a significant, yet unresolved, impact on adaptation in *O. nubilalis*. One possibility is that individuals from these populations are at risk of producing less-fit hybrid offspring through breakup of parental co-adapted gene complexes, and that the chromosomal rearrangement protects against segregation into suboptimal or maladaptive allelic combinations [29••].

Conclusions
Major insights have been gained recently regarding the genetic mechanisms underlying variation in the pheromone system and diapause in *O. nubilalis*. Genes, as well as changes in the architecture, on the Z-chromosome play a major role in facilitating the maintenance of differing

pheromone preference and diapause between pheromone strains and voltinism ecotypes. However, many uncertainties remain. For instance, although *period* likely influences multivoltine *O. nubilalis* response to photoperiod and is tightly linked to *Pdd*, the underlying genetic basis of diapause variation is yet to be resolved. Similarly, genes involved in neurogenesis are tightly linked to male pheromone response preferences, but the precise genetic and biochemical mechanisms, as well as the potential structural and functional interactions with any of the ORs, remain unknown. At the population level, the segregating Z-chromosome inversion may influence the tempo of evolution of pheromone preference and diapause, potentially by influencing the range of allelic combinations that determine these traits.

Any inhibition of random mating or gene flow across subpopulations or incipient species boundaries might impact the efficacy of IRM strategies designed to delay or circumvent the onset of resistance to pesticides [54–56]. Specifically, slight changes in gene flow parameters drastically alter model predictions of transgenic crop durability [23] and eventually real-world efficiency of agricultural production [57]. The genomic architecture of traits underlying ecological adaptations in *O. nubilalis* is relatively simple, involving a bias for linkage with genes located on the Z-chromosome. Recombination of causal Z-chromosome-linked genes is reduced by hemizygosity in females, as well as by being located within an inversion, suggesting their tight co-ancestry. Pheromone system and voltinism traits serve as pre-reproductive isolating barriers between populations. Therefore, the underlying genes on the Z-chromosome and within the inversion may restrict gene flow across much of the sex chromosome. Nevertheless, our understanding of the consequence of this rearrangement on gene flow is complicated by observations that standard and rearranged chromosomes remain segregating rather than being differentially fixed between E-strain and Z-strain populations.

Effective integrated pest management (IPM) practices depend, in part, on the relative local temporal and spatial abundance, and ecological differences of *O. nubilalis* strains. For example, fidelity to maize as a host and adult dispersal distances may differ between voltinism and pheromone strains [25,58–60] recently suggested that E-strain adults emerge in spring when maize is phonologically less-attractive for oviposition, making infestation of non-maize crops more likely. Thus, understanding strain composition of a local population becomes most crucial in regions of sympatry and localities with more diverse cropping systems, in part because of possible high proportions of hybrids [25]. Identifying which genetic configurations are less prone to recombination after inter-strain hybridization is important to predicting predominant local insect phenology and population dynamics, which could be addressed through the association of

alleles between relevant loci linked to diapause and sexual communication. Also, the correspondence between strain-specific alleles at unlinked *Resp* and *Pher* (*pgfar*) loci may influence the reproductive dynamics within sympatric populations, and thus the probability of inter-strain gene flow (or introgression) that could impact host plant utilization. Additionally, voltinism of a local population impacts both the timing and intensity of pest pressure. This occurs via increasing population size following successive multivoltine mating generations or a succession of feeding damage inflicted by uni-voltine and multivoltine ecotypes when in sympatry, and is important in modeling population dynamics. Voltinism of a local population will also affect synchrony of pest pressure relative to crop phenology, as well as the proportion of a population successfully entering diapause and emerging the next spring. Understanding how populations have adapted in their diapause response in the past to differences in climate during range expansion across latitudes [52••] will help predict rates and forms of adaptations in response seasonal and long-term climatic changes at a given latitude.

Despite the many mysteries that remain regarding *O. nubilalis* pheromone and voltinism strains and their ecological interactions, the advances in identification of the genes involved and their arrangement in the genome promises to increase understanding of population-level processes that impact their management in agroecosystems. Indeed, much exciting research into these topics is on the horizon, which will not only expand our understanding of these complex genomic and ecological interactions in *O. nubilalis*, but shed light on the dynamics of speciation itself.

Acknowledgements

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