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Application of a dense genetic map for assessment of genomic responses to selection and inbreeding in *Heliothis virescens.*

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Abstract

Adaptation of pest species to laboratory conditions and selection for resistance to toxins in the laboratory are expected to cause inbreeding and genetic bottlenecks that reduce genetic variation. *Heliothis virescens*, a major cotton pest, has been colonized in the laboratory many times, and a few laboratory colonies have been selected for Bt resistance. We developed 350 bp Double-Digest Restriction-site Associated DNA-sequencing (ddRAD-seq) molecular markers to examine and compare changes in genetic variation associated with laboratory adaptation, artificial selection, and inbreeding in this non-model insect species. We found that allelic and nucleotide diversity declined dramatically in laboratory-reared *H. virescens* as compared with field-collected populations. The declines were primarily due to the loss of low frequency alleles present in field-collected *H. virescens*. A further, albeit modest decline in genetic diversity was observed in a Bt-selected population. The greatest decline was seen in *H. virescens* that were sib-mated for 10 generations, where more than 80% of loci were fixed for a single allele. To determine which regions of the genome were resistant to fixation in our sib-mated and Bt-selected lines, we generated a dense intraspecific linkage map containing 3 PCR-based, and 659 ddRAD-seq markers. Markers that retained polymorphism were observed in small clusters spread over multiple linkage groups. These markers are likely associated with genomic regions under balancing selection, thus preventing fixation of deleterious alleles.

Keywords: *Heliothis virescens*, genetic variation, colonization, inbreeding, ddRAD-seq, linkage map

Introduction

Laboratory-reared insect colonies are important resources for many types of entomological experiments. They are used to quantify physiological or behavioral differences between insect populations or species (Dekker *et al.*, 2006; Dobzhansky & Spassky, 1954; Fritz *et al.*, 2015; Groot *et al.*, 2005; Shaw *et al.*, 2000; Sokolowski, 1980; Tomaru *et al.*, 2000), identify the genetic architecture of insect traits (Gahan *et al.*, 2010, Mackay *et al.*, 2012, Oppenheim *et al.*, 2012), develop insect populations that express desirable traits (Collins, 1984; Goldman *et al.*, 1986; Gould *et al.*, 1995; Hoy, 1989; Pradeep *et al.*, 2005), and generate genetically modified species as a means of pest control (de Valdez *et al.*, 2011). A major concern for researchers maintaining insect colonies is the degree to which adaptation to the laboratory environment affects insect genotypic, and thereby phenotypic diversity (Boller, 1972; Huettel, 1976).

The phenotypic consequences of adaptation to the laboratory depend upon the trait of interest, and range from undetectable to severe (Baeshen *et al.*, 2014; Fox *et al.*, 2007; Gerloff *et al.*, 2003; Raulston, 1975; Roush, 1986). Observed phenotypic changes can be attributed to inadvertent selection for traits that are favorable in the laboratory environment (Roush, 1986), inbreeding depression (*i.e.* reduction in fitness caused by matings between related individuals; reviewed in Charlesworth & Willis, 2009; Mackauer, 1976), or the interaction of the two. Indeed, the selection that occurs during colony establishment creates conditions conducive to inbreeding (Roush, 1986). Families with higher fitness under laboratory conditions contribute disproportionately to the reproductive pool, thereby increasing the probability of matings between related individuals. Where selection is very strong, as in the production of an insecticide resistant colony, measures must often be taken to minimize the effects of inbreeding and thereby inbreeding depression (Gould *et al.*, 1995). Overall, the expectation is that the selection and inbreeding that takes place during insect colonization results in an overall loss of genetic

diversity (Munstermann, 1994), and concomitant genome-wide increase in homozygosity (reviewed in Etzel & Legner, 1999).

Previous studies that have examined genetic differences between field-collected, laboratory-adapted, and inbred populations of non-model insects have primarily focused on Dipteran species and were limited to small numbers of molecular markers (Mukhopadhyay *et al.*, 1997; Munstermann, 1994; Norris *et al.*, 2001). Such small numbers of markers allow for estimation of the genome-wide average change in genetic variability across populations, but cannot be used to examine fine-scale patterns of genomic change. Examination of these patterns allows for identification of where and how genetic variation, the raw material necessary for environmental adaptation, is maintained (Dobzhansky & Spassky, 1954). The relatively recent development of high-throughput sequencing combined with reduced-representation DNA library preparation techniques allows for the discovery of hundreds to thousands of new molecular markers, even in species for which genomic data are absent (Davey *et al.*, 2011). Here we used Double-Digest Restriction-Site Associated DNA Sequencing (ddRAD-seq; Peterson *et al.*, 2012), one type of reduced representation library, for *de novo* construction of molecular markers in the non-model species, *Heliothis virescens*.

The tobacco budworm, *H. virescens*, is an historically important pest of cotton throughout much of the Southeastern United States (Blanco, 2012). This non-model Lepidopteran species has been colonized a number of times for investigations of mating and host-selection behaviors (Sheck & Gould, 1995; Sheck & Gould, 1996; Sheck *et al.*, 2006), as well as detecting the underlying genetic basis for insecticide resistance (Gahan *et al.*, 2001; Gahan *et al.*, 2010; Taylor *et al.*, 1993). We used our newly developed ddRAD-seq markers to examine and compare the effects of colonization, selection, and sib-mating on *H. virescens* genome-wide measures of genetic diversity. To examine fine-scale patterns of change in genetic diversity, we also used our ddRAD-seq markers to generate a dense intraspecific

genetic map for *H. virescens*. This map consists of 659 high quality 350-bp markers which will serve as an important genomic resource to the entomological community.

Overall, our research aims to:

1. Quantify overall patterns of change in genomic diversity across field-collected, laboratory-reared (non-selected), Bt-selected, and sib-mated *H. virescens*.
2. Determine whether the observed degree of inbreeding in our sib-mated *H. virescens* calculated from our ddRAD-seq genotypic data matched theoretical expectations (Falconer & Mackay 1996).
3. Examine fine-scale patterns of genomic change by identifying which genomic regions were resistant to fixation in Bt-selected and sib-mated lines using ddRAD-enabled linkage mapping.

Results

We sequenced 204 *H. virescens* individuals from a total of 6 populations that were used in a population-level analysis of genomic change associated with laboratory colonization, artificial selection and inbreeding. These populations were comprised of 2 field-collected, 2 laboratory-reared, and 1 Bt-selected population, as well as specimens from a single inbred family following 10 generations of full-sibling mating (Table 1). Three of these populations, YDK, YHD2, and the inbred line were founded from a collection in Yadkin County, NC, in 1988 (Gould *et al.*, 1995), but were thereafter subjected to different rearing conditions, allowing us to make comparisons of population-genomic change within the same genetic background. In addition, 99 individuals (2 parents and 97 progeny) were sequenced for linkage analysis. This produced a total of 105,487,499 Illumina MiSeq reads (38,221,995 and 67,265,504 for linkage- and population-level analyses, respectively) that passed quality filters (data available upon request).

Genomic diversity among H. virescens *populations*

On average, 338,892 sequencing reads (*s.d.* = 113397) were produced per individual, and the variation in average read counts was spread uniformily across populations (Supplementary Figure 1). Ninety-four percent of individuals had read counts between 90,000 and 688,000, and were fed into the Stacks pipeline (Catchen *et al.*, 2011; 2013) for *de novo* locus construction (Supplementary Figure 1). Loci constructed by Stacks were sequenced to a 6× average depth of coverage per individual. In total, 4,281 polymorphic 350-bp ddRAD-seq markers (hereafter loci) were detected in at least one individual per population across all populations. Two well-documented challenges commonly encountered when working with moderate coverage reduced representation library data like ours are: 1) uneven distribution of missing data across sets of loci (Davey *et al.*, 2012; Xu *et al.*, 2014), and 2) under-sampling of heterozygotes (Li *et al.*, 2009; Nielsen *et al.*, 2011). Both reduce confidence in final genotypes called by genotyping-by-sequencing SNP calling algorithms, including the algorithm used in Stacks. To overcome these challenges, we examined several subsets of these 4,281 polymorphic loci for our downstream population genomic analyses. These subsets contained between 125-1231 loci, and were chosen based upon the overall proportion missing genotype calls in the subset. The smallest subset consisted of loci for which over 75% of individuals per population had genotypic data present and were therefore likely sequenced to greatest depth of coverage. Each larger subset allowed additional loci at the expense of coverage (*i.e.* more missing genotypic data were allowed; Table 2). By using multiple datasets, we were able to examine whether the presence of missing genotype calls influenced overall estimates of genomic diversity across populations.

We first examined all subsets of loci and determined the mean and maximum numbers of alleles per locus (Table 2). Across all sequenced populations (n = 192 total *H. virescens*), the mean numbers of unique alleles detected per locus ranged from 29 to 34 depending upon the number of loci included

in the analysis. As more loci were included, the average number of unique alleles detected per locus decreased. However, the maximum number of unique alleles detected increased from 86 in the smallest subset of loci to 94 in the 3 larger subsets. We also examined the proportion of loci that were fixed (*i.e.* only a single allele present) across populations. Across subsets, few loci were fixed for a single allele in laboratory-reared (5.6-10.9%), Bt-selected (5.3-7.3%) and field-collected (0-2.4%) populations (Table 2). Yet over 80% of loci were fixed in the inbred line following 10 generations of sib-mating (Table 2). Of the 125 loci with the fewest missing genotype calls, 86% were fixed in the inbred line. Expanding the number of loci to include those with more missing genotypes (n= 378, 573, 1231) reduced the percentage of fixed loci in the inbred line by up to 5% (Table 2).

We then determined the mean number of unique alleles present per locus for each subset of loci (Supplementary Figure 2). In general, we found no within population differences in the mean numbers of unique alleles detected among subsets of loci, and therefore we used a single, conservatively chosen subset of loci (n = 378) where at least 10 individuals were genotyped per population per locus for further analysis. The mean numbers of unique alleles per locus were 2.1 for the inbred line, 5.3 for the Bt-selected population, 5.4 and 4.4 for the non-selected, laboratory-reared populations (YDK and BENZ, respectively), and 18.4 and 17 for the field-collected populations (LA and TX, respectively). However, our sample sizes (*i.e.* numbers of individuals sequenced; see Table 1) differed for each population, and it was unclear whether differences between the aforementioned means were caused by sample size or population-level differences. To determine whether the mean number of unique alleles per locus differed according to population, where sample sizes were equal across populations, we randomly sampled 6, 12, 18, and 24 alleles per population per locus without replacement. We found that increasing the total number of alleles sampled led to an increase in the mean numbers of unique alleles per locus for all but the inbred line (Figure 1). Regardless of the number of alleles sampled,

152 field populations always exhibited the greatest allelic diversity, followed by selected and non-selected
153 colony populations. The lowest allelic diversity was observed in the inbred line.

154 When 18 alleles were randomly sampled per population per locus, we detected an average of
155 just over 1 unique allele per locus in the inbred line, indicating that most loci were fixed for a single
156 allele. For the inbred line, 52 loci did not reach fixation. Of these, forty-seven had 2 unique alleles,
157 four had 3 unique alleles, and one had 4 unique alleles out of the 18 that were randomly sampled. On
158 average, Bt-selected and non-selected colony strains each had *ca.* 3 unique alleles per locus, and field-
159 collected populations had *ca.* 9 unique alleles per locus (Figure 1). The majority of unique alleles
160 present in the field-collected populations (70.3% and 68.7% for LA and TX populations, respectively)
161 were observed only once (of 18 alleles; Figure 2). Low frequency alleles were less common in the Bt-
162 selected and non-selected laboratory populations (35.1% and 21.5%, respectively) and rare (0.7%) in
163 the sib-mated inbred line (Figure 2).

164 To further quantify and compare genetic diversity by population, we used the same random
165 sampling regime (n = 18 alleles without replacement), paired with the same conservative subset of loci
166 (n = 378) to calculate sample-size corrected S_K (Charlesworth & Charlesworth, 2010), and π (Nei 1978;
167 Nei & Li, 1979). These two measures are complementary: π is calculated as the proportion of
168 nucleotides that differ per two randomly chosen DNA sequences, averaged across all pairwise
169 comparisons per marker per population, and S_K is calculated as the number of unique single nucleotide
170 variants in a population at a single locus. When averaged across all markers (n = 378), the number of
171 variant sites (S_K) per 350 bp marker was 0.15 for the inbred line, and the maximum S_K was 2.95. Bt-
172 selected and non-selected laboratory populations had, on average, just over 1 nucleotide variant per 350
173 bp locus, with a maximum of *ca*. 6. Field-collected populations had the greatest number of variant sites
174 per 350 bp locus, where the genome-wide average was just over 5 nucleotide variants per locus, with a

maximum of *ca.* 15. Similar trends were observed for genome-wide and maximum nucleotide diversity (π) values. Relative to the laboratory-reared populations, genome-wide estimates of π were nearly an order of magnitude lower for the inbred line. The genome-wide π estimate for laboratory-reared populations ranged from 4.0×10^{-3} (Bt-selected population) to 6.7×10^{-3} (non-selected, YDK population), and 6.2×10^{-4} for the inbred line. Field-collected populations exhibited genome-wide π estimates of 9.3×10^{-3} and 9.2×10^{-3} for the LA and TX populations, respectively. All genome-wide and maximum π and S_K estimates, along with their corresponding 95% non-parametric bootstrapped confidence intervals (N = 5000) are reported in Table 3.

Inbreeding among laboratory-reared H. virescens *populations*

To determine whether the observed degree of homozygosity in our inbred line was consistent with that which would be expected following 10 generations of sib-mating, we compared the inbreeding coefficient *F,* as calculated according to pedigree- (Falconer & Mackay, 1996) and DNA marker-based information (Keller & Waller, 2002; Kim *et al.*, 2007). The expected inbreeding coefficient (F_t), following 10 generations of sib-mating was 0.89. This expected value fell within the bootstrapped 95% confidence intervals for marker-based inbreeding coefficients (F_{IT}) calculated from all subsets of markers. This indicated that there was no significant difference between the expected inbreeding coefficient and the observed inbreeding coefficient calculated using ddRAD-seq marker data. The genome-wide F_{IT} values (95% CIs) were 0.92 (0.88, 0.96), 0.89 (0.86, 0.92), 0.89 (0.87, 0.92), and 0.88 (0.86, 0.89), for the inbred line as calculated from 125, 378, 573, and 1231 ddRAD-seq loci, respectively.

198 *Linkage mapping*

199 Few genomic resources are available for *H. virescens*. Therefore, we determined the genomic
200 location of loci which were resistant to fixation by generating a dense ddRAD-seq linkage map. The
201 map was produced using a male informative cross (reviewed in Baxter *et al.*, 2009), and was comprised
202 of 659 informative polymorphic loci generated by ddRAD-seq, plus 3 partial gene sequences of
203 *ABCC2*, *HevCaLP*, and *DesatI*. Adding these partial gene sequences to our linkage map, all with
204 known locations in the *B. mori* genome allowed us to validate marker groupings for our linkage map.
205 All informative ddRAD-seq loci were grouped into 33 linkage groups, two more than the expected 30
206 *H. virescens* autosomes, and one segregating Z chromosome from the hybrid male parent used in our
207 cross. Linkage groups ranged in size from 7cM to 110cM (Figure 3), and yielded a total map length of
208 1919.5 cM. On average, there were 20 ddRAD-seq loci per linkage group, and the average spacing
209 was one locus per 3.5 cM. The smallest and largest linkage groups contained 3 and 53 loci,
210 respectively. The *HevCaLP*, *Desat1*, and *ABCC2* genes were grouped with linkage groups 15, 16, and
211 22, respectively. These linkage groups corresponded to *B. mori* chromosomes 6, 23, and 15 (Table 4),
212 where these candidate genes are known to reside (Gahan *et al.*, 2001; Gahan *et al.*, 2010; Mita *et al.*,
213 2004).

214 In total, 99 of the 659 mapped ddRAD-seq loci could be aligned uniquely to a single locus in
215 the *B. mori* genome. Twenty-two linkage groups contained ddRAD-seq loci that could be aligned to a
216 single *B. mori* chromosome (Table 4), while 8 linkage groups did not contain any that could be aligned.
217 Linkage groups 19, 25, and 33 contained ddRAD-seq loci that aligned uniquely to more than one *B.*
218 *mori* chromosome. This was unlikely caused by spurious associations between ddRAD-seq loci;
219 increasing the LOD score to 8 failed to break up associations for those three linkage groups.
220

Identification of genomic regions resistant to fixation

To determine where heterozygosity was being maintained in the genome, we examined nucleotide diversity at the 659 mapped ddRAD-seq loci for Bt-selected (YHD2), non-selected (YDK), and inbred line. Of these 659 mapped loci, 302 (46%) were previously included in our population-level analyses, and 357 were newly discovered. Nucleotide diversity (π) was only calculated for a population if at least 3 individuals were genotyped at a locus. Therefore only 441 loci were examined for the inbred line, and 658 loci were examined for both Bt-selected and non-selected populations. In total, 60 (13%) of the mapped loci retained polymorphism in the inbred line, whereas 645 (98%) and 583 (86%) of mapped loci retained polymorphism in the Bt-selected and non-selected (YDK) populations, respectively.

DdRAD-seq loci that retained polymorphism in the inbred line were spread over 16 linkage groups (Figure 4), and often clustered together within a linkage group (Supplementary Figure 3). We used Tajima's D, a statistical test which identifies departures from the neutral model of molecular evolution, to determine whether any of these polymorphic loci were under balancing selection in the inbred line. Of these 60 polymorphic sites, 20 had statistically significant Tajima's D values ($q > 0.05$) indicative of balancing selection (Table 5). Linkage groups 3, 11, 14, and 15 each contained multiple polymorphic sites that appeared to be under balancing selection. Whereas 5 polymorphic loci on linkage group 11 were spread across the length of the linkage group, linkage groups 3, 14, and 15 contained multiple polymorphic loci clustered together within 5cM of one another.

We examined which, if any, of these polymorphic loci with strongly positive Tajima's D values were shared between the inbred line, the Bt-selected population, and their ancestral laboratory-reared *H. virescens* population (YDK). Following a bayesian adjustment for multiple comparisons (Efron 2001), no loci showed significantly positive Tajima's D values for the Bt-selected population, but 35

loci had positive Tajima's D values in the non-selected YDK population. While only 3 loci under balancing selection were shared between the inbred line and the non-selected (YDK) populations (n = 3), many of the same genomic regions were shared between populations. For example, both populations shared signatures of balancing selection on chromosome 15, for locus Hv20150 at 61 cM. A complete list of the shared genomic regions showing signatures of balancing selection can be found in Table 6.

Discussion

Here, we examined the degree to which colonization, artificial selection, and intense inbreeding influence genome-wide and fine-scale patterns of diversity. In the absence of a publicly available *H. virescens* reference genome, we used ddRAD-seq *de novo* locus construction to identify multiple subsets of polymorphic loci ranging in size from 125 (where at least 75% of individuals per population contained genotypic data), to 1231 (where at least 25% of individuals had genotypes present per population). Genome-wide measures of allelic diversity, F_{IT} values, and the degree of homozygosity were either unaffected (Supplementary Figure 2), or minimally affected (Table 2) when markers with high levels of missing genotypic data were present. Therefore, any biased genotype calls made by the Stacks SNP calling algorithm due to our moderate depth of sequencing coverage had little impact on our overall genome-wide estimates of diversity. Our results demonstrate that moderate coverage ddRAD-seq data can be used with confidence when conducting population genomic comparisons of genome-wide means.

We observed a precipitous decline in nucleotide and allelic diversity following long-term laboratory colonization, selection, and inbreeding for *H. virescens*. Despite the decline in genomic diversity for non-selected and Bt-selected laboratory-reared populations, fewer than 10% of loci were

fixed. While our Bt-selected population did not retain the level of genetic diversity that their ancestral (YDK) laboratory-reared population did, they consistently had higher measures of genomic diversity than did the non-selected (BENZ) population. Retention of higher levels of polymorphism in our Bt-selected line was likely due to the measures taken during its generation to ensure genomic diversity was maintained in the face of strong selection (Gould, 1995). Alternatively, strong genetic bottlenecks in the non-selected (BENZ) population prior to their use in our study could explain why our Bt-selected line was more genetically diverse than the non-selected line. Overall, differences among Bt-selected and non-selected laboratory-reared populations were modest; when 18 total alleles were sampled, laboratory-reared populations retained *ca*. 3 alleles per 350-bp locus relative to 9 alleles per 350-bp locus present in field-collected *H. virescens*. However, few low frequency alleles remained in the laboratory-reared populations relative to the field-collected populations (Figure 2), which has been observed elsewhere (Munstermann, 1994). For this reason, laboratory-reared populations are generally considered inbred (Roush, 1986). In the case of *H. virescens*, our results clearly show that a great deal of genomic diversity is retained, even following decades in colony. Few genome assembly algorithms accommodate polymorphism well (Kajitani *et al.*, 2014), and it is clear that the reductions in heterozygosity in our inbred line will be useful for production of a high quality *H. virescens* reference genome assembly.

Following 10 generations of inbreeding, > 80% of markers went to fixation in our sib-mated *H. virescens* population. Indeed, our inbreeding coefficient F_{IT}, as observed from our ddRAD-seq data, met theoretical expectations for all subsets of loci. Our *H. virescens* laboratory population was more amenable to inbreeding than other insect species (Munstermann, 1994; Rumball *et al.*, 1994; Turissini *et al.*, 2014; You *et al.*, 2013), despite their relatively high levels of genomic diversity (Figure 1) and genetic load (Supplementary Figure 4). Higher than expected allelic diversity has been observed in

several other insect species following experimental inbreeding attempts (Munstermann, 1994; Rumball *et al.*, 1994; Turissini *et al.*, 2014; You *et al.*, 2013). As one example, only 57% of the *An. gambiae* genome went to fixation, as observed according to SNP markers, following 10 generations of inbreeding (Turissini *et al.*, 2014). Observed differences between our *H. virescens* population and other insects could be species specific, but is more likely related to the proportion of the genome containing balanced lethal systems (Falconer & Mackay, 1996).

To determine where heterozygosity was being maintained in our inbred line, we developed a high density genetic linkage map for *H. virescens*. Our map contained 659 newly developed 350 bp ddRAD-seq markers that are long enough for future primer design and direct sequencing. This map represents a new tool for an historically important pest species that lacks genomic resources. No ddRAD-seq markers remained unlinked following mapping, which indirectly speaks to the quality of our linkage map. However, the number of groups in our linkage map was 2 more than expected (n = 31). This is likely due to the relatively small mapping population size used in this work (Silva *et al.*, 2007). Other mapping studies that analyzed segregating populations of a similar size have also reported genetic maps with excess numbers of linkage groups (Pootakham *et al.*, 2015; Singh *et al.*, 2009). Another possible explanation for the disparity between our observed and expected number of linkage groups is that polymorphic markers may not be evenly distributed over the chromosomes (Paterson, 1996). Perhaps the markers in several of our smaller linkage groups are found on opposite ends of *H. virescens* chromosomes, and thus are unlikely to show strong associations.

When we applied our linkage map to examine fine-scale patterns of genomic change following 10 generations of sib-mating, we found several linkage groups contained clusters of loci that did not go to fixation (Figure 4). Twenty out of the 60 ddRAD-seq loci not fixed for a single allele showed signatures of balancing selection (Table 5). These loci were likely genetically linked to balanced lethal

systems, or perhaps chromosomal inversions. Twelve loci spanning the entire length of linkage group 11 (syntenic to *B. mori* chromosome 9) did not go to fixation in the inbred line, and 5 appeared to be under balancing selection. Furthermore, several loci on this linkage group were under balancing selection in our non-selected (YDK) population, suggesting that a large chromosomal inversion may be present on *H. virescens* linkage group 11.

Several smaller genomic regions with signatures of balancing selection were also shared among our non-selected population (YDK) and inbred line (Table 6). These regions are likely associated with deleterious alleles that were sampled from the original field population in Yadkin County, NC from which our YDK population derived. It is possible that those deleterious alleles were never purged from YDK, but instead increased in frequency creating several balanced lethal systems. YDK is ancestral to the inbred line, so these balanced lethals were likely transmitted the inbred line as it was generated.

Conclusions

This work serves as one of the most thorough attempts to quantify the effects of genomic responses to selection and inbreeding in a non-model insect species. We demonstrated that laboratory-reared *H. virescens* have reduced allelic and nucleotide diversity relative to field-collected populations, and that inbreeding further diminishes genetic diversity. Although we identified several loci that did not go to fixation in *H. virescens* following 10 generations of inbreeding, our ddRAD-seq marker-based F_{IT} values met theoretical expectations. The genomic regions that retained polymorphism were likely due to deleterious alleles that were present in the founding individuals, and would unlikely go to fixation. This work demonstrates the difficulty involved in producing fully homozygous insect strains, which are currently critical to producing high-quality, complete reference genomes.

Methods

Field-collected H. virescens

Adult male moths were collected from Bossier Parish, Louisiana, and College Station, Texas using pheromone-baited live traps. Collections took place in LA from May through September, 2012, and in TX from May through October, 2012. Moths from each collection date were immediately placed in bottles of 95% ethanol for long-term storage. All bottles were held at -20 °C until DNA isolations took place.

H.virescens *colonies*

H.virescens were collected from Yadkin County, NC in 1988 (Gould *et al.,* 1995). This original population founded two of the colonies used in this study. YHD2 was selected for high levels of Bt resistance for 4 years on MVP- treated (0.864 mg/mL diet; Mycogen, San Diego, CA) corn-soy diet (Gould *et al.,* 1995), whereas a non-selected population (YDK) was reared on corn-soy diet alone. A third population (BENZ) originating from Stoneville, MS, was acquired from Benzon Research Incorporated (Carlisle, PA). BENZ *H. virescens* were acquired in their pupal stage, and newly eclosed adults were used for population-level comparisons. To produce an inbred population, single pair matings (SPMs) were set up between YDK siblings for 10 generations. An initial 37 SPMs were used to establish 29 lineages in filial generation one (8 single pair matings did not produce progeny). When SPMs failed to produce offspring, likely due to inbreeding depression, surviving lineages were expanded (Supplementary Figure 4). This was done to extend inbreeding for as many generations as possible, thus promoting as complete a reduction in heterozygosity as possible. Adult males from each laboratory-reared population were killed by freezing (-20 °C), and stored at -80 °C until DNA isolation.

359

360 *Mapping cross*

361 A non-selected female from the BENZ population was crossed to a Bt-selected (YHD2) male in
362 a single pair mating. One hybrid male offspring was then back-crossed to a Bt-selected (YHD2)
363 female, and their progeny were reared to adulthood on untreated corn-soy diet according to Joyner and
364 Gould (1985). Of the 120 progeny, 97 reached adulthood. Parents and their 97 adult progeny were
365 killed by freezing and stored until DNA isolation as described above.

366

367 *Genomic DNA library preparation*

368 All DNA was isolated from the adult thorax using a Qiagen Dneasy Blood and Tissue Kit
369 (Qiagen, Inc., Valencia, CA, U.S.A.). Genomic DNA samples were prepared for Illumina sequencing
370 according to the Poland *et al.*, (2012) protocol with minor modifications. Two-hundred ng of DNA per
371 individual were digested with EcoRI and MspI. For each individual, the overhang sites were ligated to
372 standard Truseq Universal adapters (Illumina, Inc. San Diego, CA). These adapters, which were
373 ligated to EcoRI overhang sites, contained one of 48 unique barcodes (Elshire *et al.*, 2011;
374 Supplementary Table 1). DNA fragments from each individual were assigned a unique barcode, and
375 individuals were combined into pools of no more than 48 individuals. A Pippin Prep (Sage Science,
376 Inc., Beverly, MA) was used to select adapter-ligated DNA fragments ranging from 450-650 bp from
377 each pool. Size-selected DNA fragments were amplified in a Peltier PTC200 thermalcycler (here and
378 throughout) using Illumina primers (Supplementary Table 2) under the following conditions: 72 °C for
379 5min, 18 cycles of 98 °C for 30 sec, 65 °C for 20 sec, 72 °C for 30 sec, followed by 72 °C for 5 min.
380 For each pool, 1 of 4 Illumina indices was added via PCR to the MspI adapter. Therefore, sequences
381 from each individual could be identified by the unique combination of barcode and index. A complete

list of barcodes and indices used in this study can be found in the Supplementary Tables 1 and 2, respectively. Amplified libraries were pooled, cleaned with a Qiaquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, U.S.A.), and diluted to 4nM prior to sequencing. Prepared genomic DNA libraries constructed from 303 *H. virescens* individuals were spread across 9 full and partial Illumina MiSeq runs.

De novo *marker formation*

Overlapping paired-end reads were merged with FLASH (Magoc & Salzberg, 2011), and Stacks v. 1.09 (Catchen *et al.*, 2011; 2013) was used for demultiplexing and *de novo* formation of loci. Merged paired-end reads were filtered for quality using the process_radtags script. Further quality filtering entailed removal of reads when: 1) they did not have an intact EcoRI cut site, 2) had a quality score < 30, or 3) were smaller than 350 bp. We did not allow process_radtags to rescue reads where barcode sequences contained an error. All remaining merged reads were truncated at a length of 350 bp, and fed into the Stacks pipeline.

Stacks parameter settings

Reads from all individuals were run through ustacks with the following parameter settings: -m 3, -M 14 (allowing for 5% nucleotide mismatch rate between alleles per individual), -max_locus_stacks 2, --alpha 0.05. A consensus catalog of loci was first formed using the parents of the mapping cross with cstacks, where the -n 14 parameter allowed for a 5% between individual nucleotide mismatch rate. For the mapping family, genotype calls were made using sstacks prior to field- and colony-strain alleles being added to the catalog. Progeny genotypes were automatically corrected using the Stacks genotypes script. Twenty-four individuals of each colony and field-collected strain collected in 2012

were later added to the catalog, and all field-collected and laboratory-reared populations were also genotyped using sstacks.

Data analyses

All population genomic and linkage analysis were conducted in R version 3.1.2 (R core team, 2014).

Genomic diversity among H. virescens *populations*

In total, we sequenced the 13 surviving males from an inbred line subjected to 10 generations of sib-mating, 42-46 males per colony strain, and 30 males per field-collected population (Table 1). Prior to running sequence data through the Stacks pipeline, we checked individual read counts across populations to ensure uniformity (Supplementary Figure 1). Twelve of the 204 individuals sequenced had too few (< 90,000) or too many reads (> 688,000) and were removed from the dataset prior to analysis, following Bi *et al.* (2013). From our Stacks output, we constructed 4 different sets of consensus loci present across populations. These subsets, containing a core overlapping set of 125 loci, and increasing in size from 125 to 1231, consisted of marker sets with varying percentages of missing genotype calls (range = 11.2-29.5%) (Table 2). We used these 4 different subsets to examine and compare changes in genomic diversity across populations.

We estimated the mean number of unique alleles present per locus, and corresponding 95% non-parametric bootstrapped confidence intervals (N = 5000) across populations using a custom-written R script. Within a population, each locus was randomly sampled without replacement to a depth of 6, 12, 18, and 24 haplotypes, and then unique alleles per locus were counted. Due to their small sample size resulting from intensive inbreeding, we only sampled 6, 12, and 18 haplotypes per locus for the inbred

line. We focused our analysis on the subset of loci containing 378 consensus loci because genotype calls were present for at least 10 individuals per population. Increasing the number of loci analyzed did not significantly change the mean number of unique alleles per population (Supplementary Figure 2). We also calculated two measures of nucleotide diversity per 350 bp locus using the R package, pegas (v. 0.6; Paradis, 2010): π (Nei, 1987) and S_K corrected for sample size (Charlesworth & Charlesworth, 2010; Watterson, 1975). We then generated population-level genome-wide means and 95% non-parametric bootstrapped (N = 5000) confidence intervals for each metric (Table 3).

Estimating the inbreeding coefficient

To estimate our marker-based inbreeding coefficient, we examined multiple sets of loci (Table 2) and found that trends across all datasets were similar (data not shown). However, we reported F_{IT} values from a set of 378 loci because the dataset contains few missing genotypes per population, while still making inferences from several hundred loci. We calculated F_{IT} for the inbred line relative to the non-selected (YDK) population after Keller and Waller (2002), where $(1-F_{IS})(1-F_{ST}) = 1-F_{IT}$. F_{IS} was the level of inbreeding within the inbred line, calculated as $F_{IS} = 1-(H_O/H_E)$, where H_O and H_E were calculated for each locus using the R package adegenet (v. 1.4-2; Jombart, 2008). F_{ST} was the accumulated effect of inbreeding over time, calculated as $1-(H_E(\text{inbred line})/H_E(\text{YDK}))$.

Amplification and genotyping of PCR-based markers for the mapping family

Progeny from the mapping family were genotyped at three additional loci, and these loci were mapped alongside our ddRAD-seq markers to validate our linkage groupings. We targeted the previously described *H. virescens* genes *DesatI*, *ABCC2*, and *HevCaLP* via PCR followed by gel electrophoresis, or direct sequencing. Amplification and genotyping protocols were as follows.

451 A 468 bp fragment from *DesatI* was amplified in a 30 µl reaction with forward and reverse
452 primers [5'-TGAGGGACCATCGTCTCCAT-3'] and [5'-CACTGCTACATTTTGGGCAG-3'],
453 respectively (Ward, 2009). Each reaction contained 6 µL of 5× GoTaq buffer (ProMega), 29 µM per
454 dNTP, 92 ng per primer, 0.75 U GoTaq polymerase, and *ca.* 1 µg genomic DNA. Sample DNA was
455 amplified alongside a negative control (here and throughout), where pcr-grade H_2O was substituted for
456 genomic DNA. Reactions were incubated at 95ºC for 1 min followed by 35 cycles of 95ºC for 1min,
457 52ºC for 1min, and 72ºC for 2 min. PCR products were purified using a standard ethanol precipitation,
458 and directly sequenced on an ABI3730xl (Applied Biosystems, San Francisco, CA). A single
459 nucleotide polymorphism (cytosine to tyrosine substitution) at bp 36 was found in the YHD2 parent of
460 the mapping cross. Offspring were genotyped at this locus using PolyPhred (Nickerson *et al.*, 1997),
461 and genotype calls were visually confirmed using consed (Gordon *et al.*, 1998).
462 An intronic region of the *ABCC2* gene previously described by Gahan *et al.* (2010) was
463 amplified using primers Hs-ABC2dU02-F1 [5' – TGGTTACAAGAAATAGAAAATGCAAC-3'] and
464 Hs-ABC2eU03-R2 [5' – CTTTCAAACTGAACCGCATCAC – '3]. Each 30 µl reaction volume
465 consisted of 6 µL of 5× GoTaq buffer, 29 µM per dNTP, 73 ng per primer, 0.75 U GoTaq polymerase,
466 and 1 µg genomic DNA. Reactions were held at 95ºC for 2 min followed by 30 cycles of 95ºC for 30
467 sec, 58ºC for 30 sec, and 72ºC for 40 sec, and the resulting products were cleaned via ethanol
468 precipitation. Following sequencing on an ABI3730xl, chromatogram files were visualized using
469 FinchTV (version 1.3.1, PerkinElmer, Inc., Seattle, WA). As described by Gahan *et al.* (2010), the
470 YHD2 parent was homozygous for a 22 bp deletion, whereas the F_1 parent was heterozygous for this
471 deletion. Therefore, we examined the segregation of this deletion in the mapping family offspring,
472 which was detectable by the presence of a TAT sequence near amplicon bp 40.
473 Finally, the *HevCaLP* locus described by Gahan *et al.* (2007) was amplified in a multiplexed

reaction using three primers: the universal reverse primer [5'-ATACGAGCTGACGACACGCTGGGAGA-3'], one forward primer that targets a retrotransposon insertion conferring resistance to *Bacillus thuringiensis* [5' – CGCAACGCGCGATCTACTCTTGTCACC – 3'], and another forward primer that targets wild-type sequence [5' – AAGTGTCCCAGTCGATGCTGAA – 3']. An initial 20-µl reaction contained 4 µl 5× GoTaq buffer, 29 µM per dNTP, 56 ng per primer, 0.5 U GoTaq polymerase, and 1 µg genomic DNA. Reactions were incubated at 95ºC for 2 min, followed by 30 cycles of 95ºC for 30 sec, 58ºC for 20 sec, and 72ºC for 40 sec. A reconditioning reaction, aimed at reducing heteroduplex formation, was set up as above, but incubated for 3 rather than 30 cycles. These reactions were capable of producing two amplicons, which differed in length by 76 bp. The YHD2 parent was homozygous for the long amplicon (*ca.* 800 bp) containing the insertion that confers resistance to *Bacillus thuringiensis*, whereas the F_1 parent was heterozygous for a long and short amplicon. PCR products from mapping family offspring were run on a 2% agarose gel alongside Hyperladder I (Bioline, Taunton, MA) for visualization and genotype scoring.

Linkage mapping

Double-digest RAD-seq markers present in fewer than 75% of the mapping family offspring were filtered out, and the remainder were checked using a chi-square test for mendelian segregation ($\alpha = 0.01$). PCR-based markers, as well as those ddRAD-seq markers that segregated in a mendelian fashion were assigned to linkage groups (LOD = 5, maximum recombination fraction = 0.3) using the onemap package (Margarido *et al.*, 2007) in R. We validated groupings by aligning all markers to the *Bombyx mori* genome using Blastn in Kaikobase version 3.2.2 (http://sgp.dna.affrc.go.jp/KAIKObase/). Furthermore, we confirmed that the locations of the *DesatI*, *ABCC2*, and *HevCaLP* pcr-based markers,

as well as ddRAD-seq markers found in their respective linkage groups aligned to the same *B. mori* chromosomes (Table 4). Markers on each linkage group were ordered using the recombination counting and ordering algorithm (RECORD; Van Os *et al.*, 2005). RECORD was chosen based upon previous studies demonstrating the reliability of it's performance (Collard *et al.*, 2009, Mollinari *et al.*, 2009).

Assessment of fine-scale differences in nucleotide diversity across laboratory-reared populations

Markers that retained polymorphism in the inbred line were examined for signatures of balancing selection in the following populations: non-selected (YDK), Bt-selected (YHD2), and the inbred line. We used a Tajima's D test as calculated by the R package, pegas (v. 0.6; Paradis, 2010). A bayesian false discovery rate methodology (Efron *et al*., 2001) implemented with the R package fdrtool (v.1.2.15; Strimmer 2008) was applied to Tajima's D p-values to account for multiple hypothesis tests.

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Figures

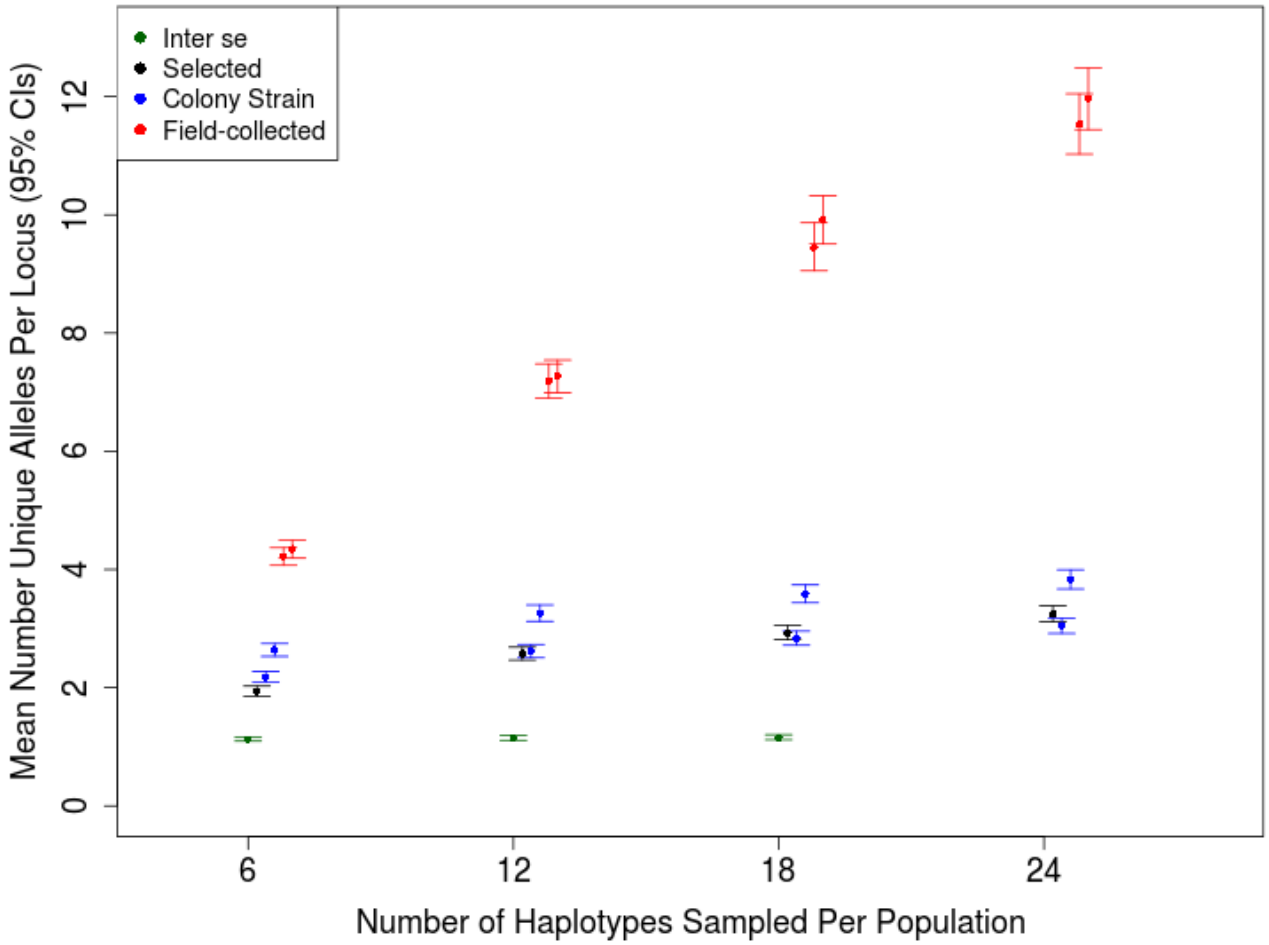


Figure 1 – Mean numbers of unique alleles detected among 378 loci depend upon the number of haplotypes sampled per population. For all but the inbred line (in green), as sample size increases, so does the number of unique alleles detected per locus. Due to low survivorship in the inbred line, no mean was computed for a haplotype sample size per locus of 24.

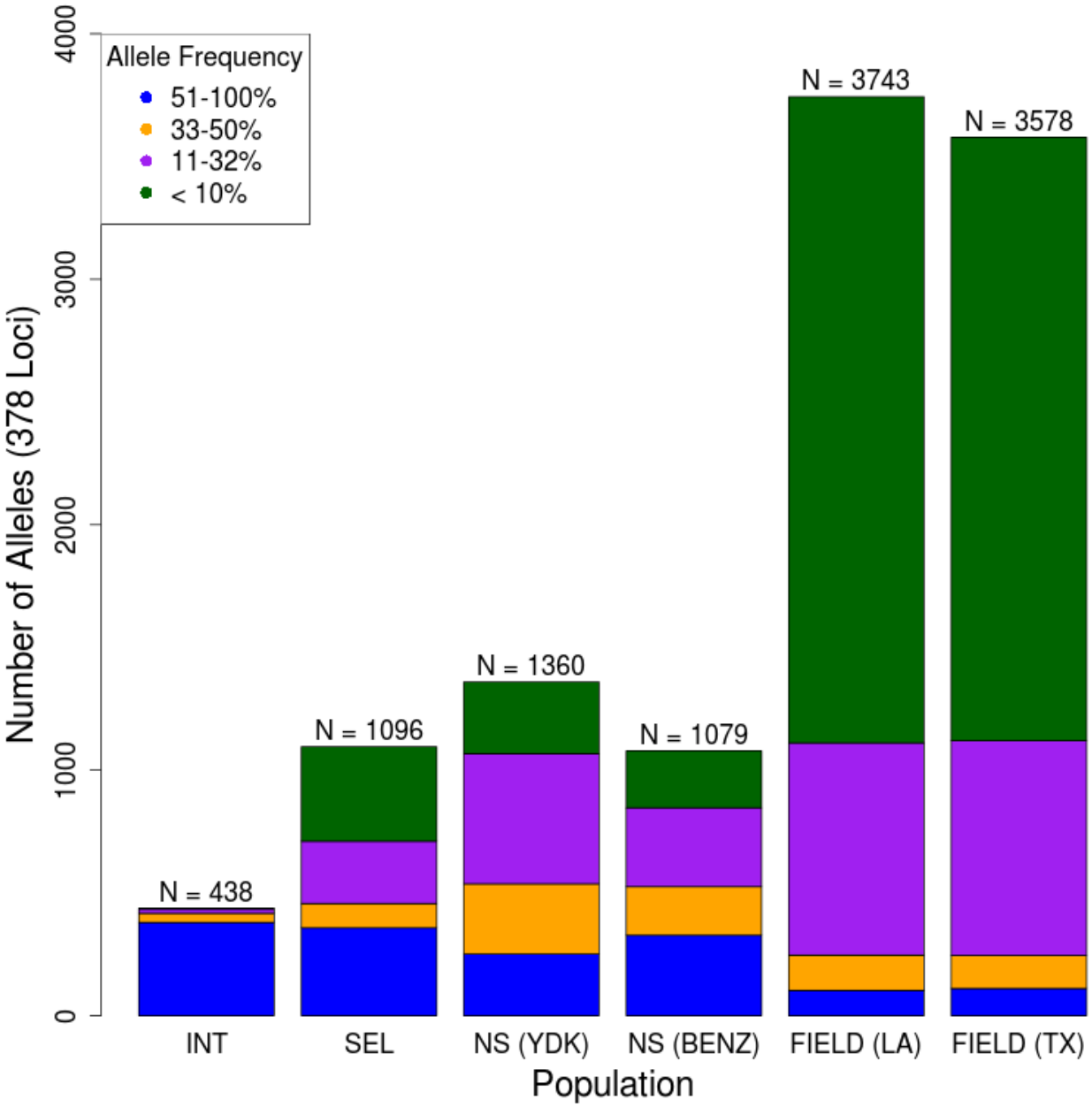
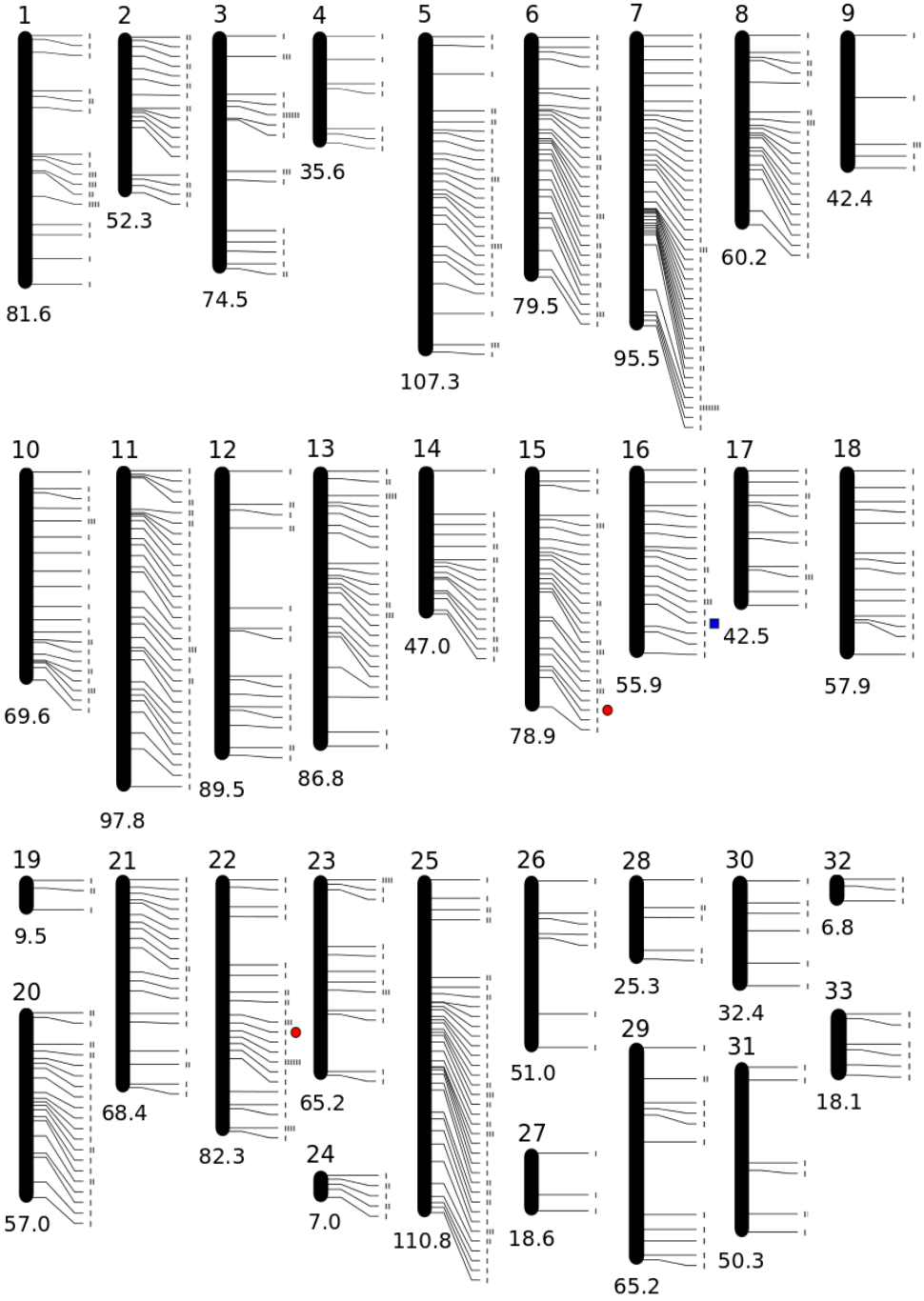
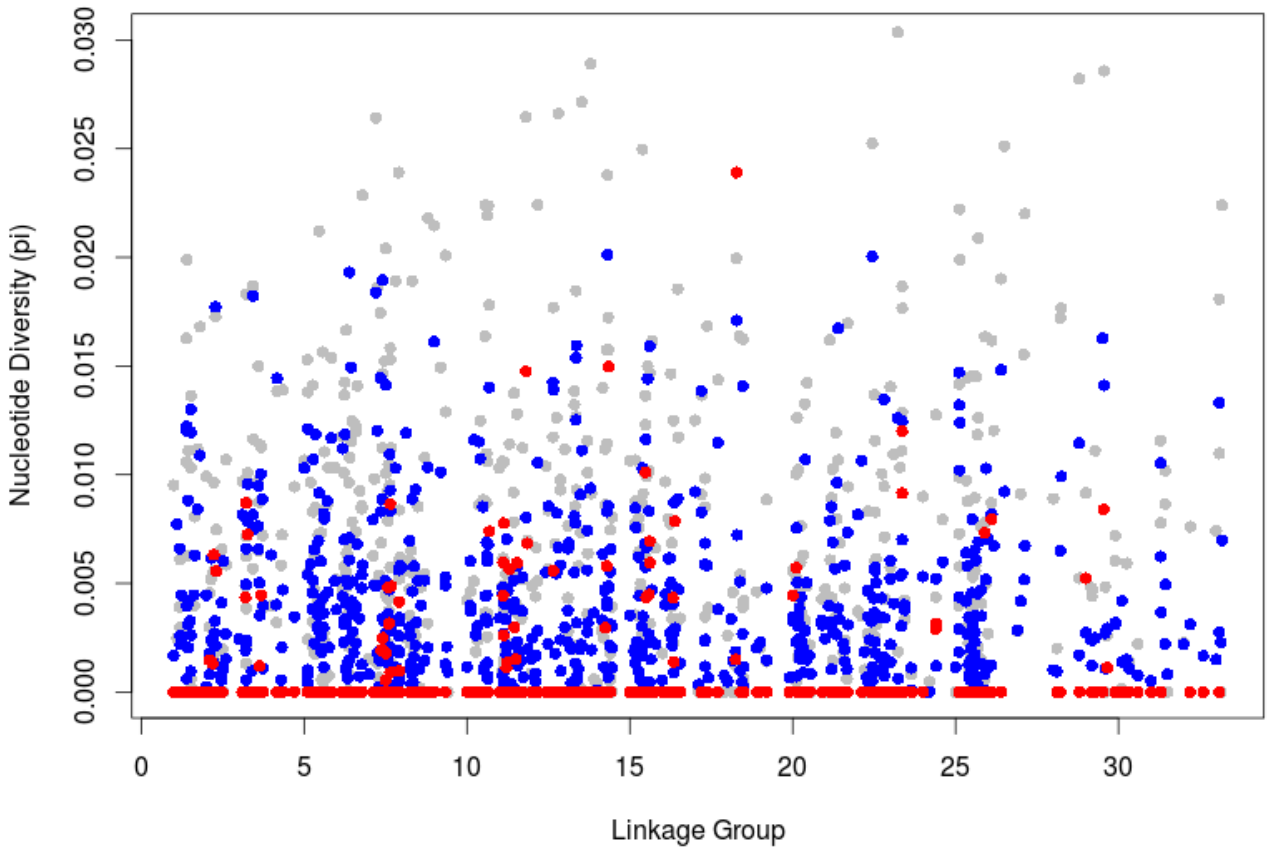


Figure 2 – Total numbers of unique alleles detected in sib-mated (INT), Bt-selected (SEL), non-selected (NS), and field-collected (FIELD) populations based upon random sampling of 18 haplotypes per population per locus. Alleles were binned and color-coded according to the frequencies at which they were present out of 18 total haplotypes. Numbers above each bar represent the total number of unique alleles found per population out of 378 loci.



715 **Figure 3** – *Heliothis virescens* linkage map with a total length of 1919.5 cM. Centimorgan lengths are
716 below each linkage group. Each tick mark represents an individual marker that mapped to a particular
717 position in the linkage group. Red circles next to linkage groups 15 and 22 represent the positions of
718 the *HevCaLP*, and the *ABCC2*, respectively. The blue square represents the position of the delta-11-
719 desaturase on linkage group 16.
720



721 **Figure 4 –** Nucleotide diversity per mapped marker site. Grey and blue circles represent nucleotide
722 diversity in non-selected (YDK), and Bt-selected (YHD2) populations, respectively. Red circles
723 represent nucleotide diversity in the inbred line following 10 generations of sib-mating.

Tables

Table 1 - Population history, sample sizes (N) and ddRAD-seq read summary data. Filtered N refers to the population sample size following removal of individuals with low read counts.

Population	Population History	Total N	Filtered N	Mean Number Reads per Individual
Inbred Line	10 generations of sib-mating	13	13	334689
YHD2	Bt-selected	43	41	346264
BENZ	Unselected	42	40	318636
YDK	Unselected	46	44	335728
LA2012	Field-collected	30	27	398336
TX2012	Field-collected	30	28	306164

Table 2 – Multiple sets of consensus loci used to calculate population genetics parameters. Consensus sets of loci containing 125, 378, and 583 loci are subsets of the largest set containing 1231 loci. The mean and maximum numbers of alleles per marker reported represent summary statistics for the entire multi-population dataset. The abbreviations Bt-sel and NS stand for Bt-selected and non-selected, respectively.

Number of Loci	Missing Genotypes (%)	Mean Number of Alleles per Marker	Max Number of Alleles per Marker	Fixed Loci (% of Total Examined)					
				Inbred line	Bt-Sel (YHD2)	NS (YDK)	NS (BENZ)	Field (LA)	Field (TX)
125	11.2	34	86	86.4	7.2	5.6	8.0	2.4	0
378	18.6	32	94	85.7	5.3	5.6	7.9	1.1	0.3
583	19.3	32	94	84.2	6.9	6.2	9.3	0.9	0.2
1231	29.5	29	94	81.3	7.3	7.5	10.9	1.4	0.9

Table 3 – Genome-wide nucleotide diversity values per 350 bp locus across populations. Values were generated by randomly sampling (without replacement) 18 haplotypes per population, then calculating π and S_K at each locus. Genome-wide values represent population-level π and S_K averaged across all loci. The abbreviations Bt-sel and NS stand for Bt-selected and non-selected, respectively.

Population	Genome-wide π (2.5, 97.5% CIs)	Max π	Genome-wide S_K (2.5, 97.5% CIs)	Max S_K
Inbred line	0.0006 (0.0004, 0.0008)	0.013	0.15 (0.10, 0.19)	2.95
Bt-Sel (YHD2)	0.0040 (0.0035, 0.0043)	0.020	1.67 (1.54, 1.81)	6.53
NS (YDK)	0.0067 (0.0061, 0.0073)	0.028	1.66 (1.53, 1.80)	6.20
NS (BENZ)	0.0051 (0.0046, 0.0056)	0.026	1.35 (1.24, 1.47)	5.46
Field (LA)	0.0093 (0.0086, 0.0100)	0.034	5.23 (4.92, 5.55)	15.40
Field (TX)	0.0092 (0.0085, 0.0099)	0.033	5.02 (5.15, 5.33)	15.63

Table 4 – Linkage group (LG) correspondence with *B. mori* chromosome (Chr). Linkage groups with an asterisk contained one or more markers that aligned uniquely to an unmapped *B. mori* sequence. Where linkage groups contained markers that aligned to more than one *B. mori* chromosome, italicized marker names correspond to the italicized *B. mori* chromosome.

LG	*B.mori* Chr	Number Markers Aligned to *B.mori* Chr	Total markers in LG	LG Length (cM)	Average Marker Spacing (cM)	Names of Markers Aligned to *B.mori* Chr
1	25	3	25	81.6	3.3	19754, 29329, 20282
2	8	6	23	52.3	2.3	22394, 27499, 21475, 17667, 17852, 25500
3*	17	3	24	74.5	3.1	376, 21931, 25112
4	-		7	35.6	5.1	
5*	4	5	36	107.3	3.0	2556, 29595, 22095, 8695,15160
6	5	6	40	79.5	2.0	18575, 4268, 21041, 23679, 66, 5723
7	22	9	49	95.5	1.9	13710, 2328, 1443, 3654, 13880, 22897, 3123, 2430, 19858
8*	11	9	25	60.2	1.7	22584, 939, 3060, 18820, 23156, 17932, 202, 22290, 21173
9	23	2	7	42.4	6.1	4275, 1286
10	3	5	26	69.6	2.7	5015, 5329, 4265, 4545, 23262
11	9	8	37	97.8	2.6	19938, 6113, 16969, 19392, 1572, 89, 22588,19343
12	-		18	89.5	5.0	
13	13	6	31	86.8	2.8	2074, 15962, 26388, 1193, 16684,18825
14	19	1	21	47.0	2.2	14242
15	6	7	35	78.9	2.3	25943, 4579, 25083, 1752, 3385, 2784, 18178
16	23	4	20	55.9	2.8	2485, 16350, 806, 20599
17	7	2	14	42.5	3.0	29343, 343
18	26	1	14	57.9	4.1	1113
19	11, *22*	2	4	9.5	2.4	3536, *16185*
20*	10	6	25	57.0	2.3	17820, 2978, 18970, 12277, 16316, 12984

Table 4 continued.

LG	*B.mori* Chr	Number Markers Aligned to *B.mori* Chr	Total markers in LG	LG Length (cM)	Average Marker Spacing (cM)	Names of Markers Aligned to *B.mori* Chr
21	21	3	21	68.4	3.3	26113, 20542, 7624
22	15	2	33	82.3	2.5	21411, 5773
23	14	1	17	65.2	3.8	4156
24			7	7.0	1.0	
25	12,*1*	4	53	110.8	2.0	20280, 9211,12382, *23604*
26			7	51.0	7.3	
27	2	1	3	18.6	6.2	5781
28			6	25.3	4.2	
29	16	1	12	65.2	5.4	23481
30			6	32.4	5.4	
31			7	50.3	7.2	
32			3	6.8	2.3	
33	14, *28*	2	6	18.1	3.0	6601, *20184*

774 **Table 5 –** Markers that retained polymorphism following 10 generations of inbreeding, and showed statistically significant ($\alpha = 0.05$)
775 signatures of balancing selection according to Tajima's D. N represents the number of individuals (of 13 total) that were genotyped per
776 locus. The q-value is derived from a bayesian false discovery rate methodology (Strimmer, 2008) that accounts for multiple comparisons.

Marker	LG	Position (cM)	N	π	Tajima's D	Unadjusted p-value	q-value
856	3	20	11	4.3×10^{-3}	2.2	0.028	0.043
19130	3	22	13	8.7×10^{-3}	2.7	0.006	0.038
2599	3	26	11	7.2×10^{-3}	2.5	0.011	0.042
6113	11	11	9	6.0×10^{-3}	2.4	0.017	0.043
19392	11	12	12	7.8×10^{-3}	2.1	0.006	0.050
20645	11	45	12	3.0×10^{-3}	2.1	0.037	0.050
13922	11	54	10	6.0×10^{-3}	2.5	0.014	0.042
21320	11	86	10	6.8×10^{-3}	2.1	0.032	0.049
68	14	29	11	5.8×10^{-3}	2.4	0.017	0.043
13550	14	34	11	1.5×10^{-2}	3.1	0.002	0.031
18240	15	48	10	1.0×10^{-2}	2.6	0.009	0.041
17301	15	50	11	4.3×10^{-3}	2.2	0.030	0.048
18676	15	59	12	4.5×10^{-3}	2.4	0.017	0.043
20150	15	61	11	6.9×10^{-3}	2.3	0.023	0.045
18178	15	61	11	5.9×10^{-3}	2.5	0.012	0.042
18240	16	32	10	4.3×10^{-3}	2.1	0.034	0.041
13777	18	27	10	2.4×10^{-2}	3.0	0.002	0.031
20662	20	0	11	4.5×10^{-3}	2.3	0.021	0.045
29612	23	36	8	1.2×10^{-2}	2.7	0.007	0.040
4851	26	1	12	8.0×10^{-3}	2.2	0.027	0.047

778 **Table 6 –** Genomic regions that share signatures of balancing selection for the inbred and non-selected
779 (YDK) populations of *H. virescens*. Numbers in parenthesis following cM ranges indicate the numbers
780 of markers with significant Tajima's D values at the corrected significance threshold of $\alpha = 0.05$ within
781 the cM ranges.
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	Range of Positions in cM	
Linkage Group	**Inbred Line**	**Non-selected**
3	20-26 (3)	22-26 (2)
11	11-86 (5)	14-86 (4)
14	29-34 (2)	30-31 (2)
15	48-61 (5)	61 (1)
23	36 (1)	22 (1)
26	1 (1)	1 (1)