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ABSTRACT The refuge plus high-dose strategy for resistance management assumes that the frequency of resistance alleles is low. We used an $F_2$ screen to estimate the frequency of resistance to transgenic corn that produces Bacillus thuringiensis Berliner Cry1Ab toxin (Bt corn) in an Iowa population of European corn borer, Ostrinia nubilalis (Hübner). We also proposed a modification to the statistical analysis of the $F_2$ screen that extends its application for nonuniform prior distributions and for repeated sampling of a single population. Based on a sample of 188 isofemale lines derived from females caught at light traps during the 2nd flight of 1997, we show with 95% confidence that the frequency of resistance to Bt corn was $<3.9 \times 10^{-3}$ in this Iowa population. These results provide weak evidence that the refuge plus high-dose strategy may be effective for managing resistance in O. nubilalis to Bt corn. Partial resistance to Cry1Ab toxin was found commonly. The 95% CI for the frequency of partial resistance were $[8.2 \times 10^{-4}, 9.4 \times 10^{-3}]$ for the Iowa population.

Variable costs of the method were $14.90 per isofemale line, which was a reduction of 25% compared with our initial estimate.

KEY WORDS Ostrinia nubilalis, $F_2$ screen, transgenic corn, resistance management

The refuge plus high-dose strategy is the recommended strategy used for insect resistance management of most varieties of transgenic insecticidal corn expressing crystal protein endotoxin genes from Bacillus thuringiensis Berliner (Bt) (NC-205 1998). Three key assumptions underlie this strategy (Alstad and Andow 1996, Andow and Hutchison 1998); the plant must express the toxin at high levels so that resistance is functionally recessive, resistant insects must mate randomly with susceptible individuals surviving in the refuge, and resistance alleles must be rare. Roush (1994, 1996) suggested that resistance allele frequency should be $<1 \times 10^{-3}$ for successful application of the refuge plus high-dose strategy. The challenge is to estimate the frequency of uncommon, recessive resistance alleles in natural populations. Previous approaches, including discriminating-dose laboratory assays, surveys of Bt field corn, and screening against test stocks (Gould et al. 1997), have practical limitations reviewed by Roush and Miller (1986) and Andow and Alstad (1998). Two additional techniques have recently joined this list. One involves the monitoring of Bt sweet-corn sentinel plots (Andow and Hutchison 1998); the 2nd is an $F_2$ screen, in which an inbreeding step allows expression of recessive alleles (Andow and Alstad 1998, 1999; Schneider 1999). We applied this screen to a sample of European corn borer, Ostrinia nubilalis (Hübner), collected near LeSueur, MN (Andow et al. 1998). Here we extend the analysis to provide more general Bayesian statistics for successive samples from the same population and apply these methods to a larger sample of O. nubilalis.

Materials and Methods

All methods follow those described in Andow et al. (1998) except as described below. Adult O. nubilalis were collected near Ames, IA, at light traps during the 2nd flight of 1997. All trapped moths were prepared for shipping the morning after capture. One group was shipped via overnight express mail, and from that shipment 92 females were set up in small oviposition cages (Leahy and Andow 1994) immediately upon receipt. Of these, 12 females died by the next day without laying any eggs. An additional 25 females did not lay any eggs. A 2nd group was picked up at the USDA-ARS Genetics Laboratory in Ames, IA, and placed in a cooler and brought back to Minnesota the same day. From this group, 308 females were set up in small oviposition cages. Of these, 35 females were dead by the next day, and 76 of the survivors did not lay any eggs (9 of these were still alive after 8 d). For both shipments, other females replaced the females that died during the 1st d. A total of 400 isofemale lines was set up and 31% did not produce any eggs. There was...
no difference in the proportion not producing eggs between the 2 shipping methods. We did not determine the mating status of the nonlaying females.

We were able to complete \( F_2 \) screening on 188 of the 275 cultured \( F_1 \) females that laid eggs (68%). Several lines were discarded because of putative infection by \textit{Nosema pyrausta} (Pallo), but most were lost because molds overgrew the diet. Consequently, each isofemale line represents 1 family line, and the egg hatch rate. Plants were carefully examined several times after inoculation, and they were dissected to search for live larvae. Detection probabilities were calculated using the methods described in Andow and Alstad (1998).

**Generalized Bayesian Statistics.** In our previous work (Andow and Alstad 1998), we assumed a uniform prior distribution for the expected allelic frequency. Because this assumption does not readily allow us to update our estimate of allelic frequency as additional data become available, we have modified these methods to use beta prior distributions (see Andow and Alstad 1999). A beta distribution, \( \text{Beta}(u,v) \), is a two-parameter, unimodal probability distribution that varies between 0 and 1. One parameter, \( u \), influences the left-hand tail and the other, \( v \), influences the right-hand tail of the distribution. A higher value of either parameter makes the respective tail smaller. The mean of the distribution is given by \( \mu = u/(u+v) \), and the concentration parameter, \( c = u+v \), determines how strongly the probability is concentrated around the mean. Mathematically, \( \text{Beta}(u,v) \) is

\[
\text{Beta}(u,v) = \frac{\Gamma(u+v)}{\Gamma(u)\Gamma(v)} P^{u-1}(1-P)^{v-1}, \quad 0 < P < 1.
\]

[1]

Similar to our previous work (Andow and Alstad 1998), we note that each isofemale line represents 1 independent Bernoulli trial, where a “success,” \( S \), is a true positive from the \( F_2 \) screen, which is phenotypically determined as a line that feeds and develops on \textit{Bt} corn. Consequently, \( S \) is distributed as a binomial \( (N,P) \), where \( N \) is the number of \( F_2 \) lines evaluated and \( P \) is the phenotypic probability of success (i.e., an isofemale line expressing resistance).

We assume the prior distribution of \( P \) is \( \text{Beta}(u,v) \). When \( u = v = 1 \), the prior distribution is unimodal, and is the appropriate assumption when no prior data are available. In this case, the Beta distribution is the same as a uniform distribution, simplifying to the case in Andow and Alstad (1998, 1999). When either \( u \neq 1 \) or \( v \neq 1 \), the prior distribution is unimodal. As discussed in the example below, \( u \) and \( v \) have a relation to the number of previously observed successes \( (S_0) \) and failures \( (N_0-S_0) \). Under these conditions, the posterior distribution of \( P \) is \( \text{Beta}(S+u, N-S+v) \) with mean \( \mathbb{E}[P] = (S+u)/(N+u+v) \) and variance \( \text{Var}[P] = \mathbb{E}[P](1-\mathbb{E}[P])/(N+u+v+1) \) (Brunk 1975).

Previously we have estimated the allele frequency for a rare allele, \( p_R \), using the relation \( \mathbb{E}(P) = 4p_R \) for monandrous females, where \( p_R \) is the gene frequency of the resistant allele, and \( 1-p_R \) is the frequency of the susceptible allele (Andow and Alstad 1998, 1999). A success, \( S \), occurs when either the mother or father carries at least 1 copy of the resistant allele. Under random mating, the probability that the mother does not carry even 1 copy of the resistance allele is \( (1-p_R)^2 \), which is the same for the father. Consequently, \((1-(1-p_R)^2)\) is the probability neither mother nor father have 1 or more copies of the resistance allele, and \( 1-(1-p_R)^2 \) is the probability that either 1 or both parents have at least 1 resistance allele and is

\[
P = 1-(1-p_R)^4.
\]

This can be solved for \( p_R \), as

\[
p_R = 1-(1-P)^{1/4}.
\]

[2]

When \( P \) is small this simplifies to \( p_R = P^{1/4} \), our previous result (Andow and Alstad 1998, 1999). Thus,

\[
\mathbb{E}[p_R] = \frac{(S+u)}{4(N+u+v)} \quad \text{P small}
\]

[3]

\[
\mathbb{E}[p_R] = 1 - \left[ 1 - \frac{(S+u)}{(N+u+v)} \right]^{1/4} \quad \text{P large},
\]

where \( \mathbb{E}[p_R] \) is expected resistance allele frequency. A similar substitution enables calculation of the variance.

The 95% credibility interval can be calculated using \( F \)-tables as described in Andow and Alstad (1999). This method is particularly useful if adequate computational power is unavailable. A \( \text{Beta}(S+u, N-S+v) \) distribution can be transformed to the \( F \) distribution. Letting \( a = S+u \) and \( b = N-S+v \), the quantity \( z = (P/a) / [(1-P)/b] \) has an \( F \) distribution with 2\( a \) and 2\( b \) degrees of freedom. Tabled values of the 0.025 tail of the \( F \) distribution can be used to calculate 95% credibility intervals for all cases except \( u = 1 \) and \( S = 0 \). For this special case, the 0.05 upper tail of the \( F \) distribution should be used to determine the upper 95% credibility interval (Schneider 1999). An example using this method is provided in Andow and Alstad (1999). Here we calculate credibility intervals directly by integrating the Beta distribution to the appropriate limits

\[
P_{\text{low}} = 0 \int_{0}^{p_{\text{low}}} \text{Beta}(S+u, N-S+v) dP \leq 0.95 \quad \text{for} \quad S = 0
\]

[4]
would be Beta(1, 101).

If we were to sample an additional 200 lines and we found 1 line with resistance, we would have $S_0 = 0$ and $N_0 = 100$. We could use equation 3 to calculate the expected frequency of resistance using a Beta prior with $u = v = 1$. The posterior distribution would be $\text{Beta}(S_0+1, N_0 - S_0+1) = \text{Beta}(1, 101)$.

We performed a extraordinary screening of the 100 isofemale lines and found 1 line with resistance, and $N = 200$. We should use the previous data from the 100 lines to describe the prior distribution of $P$ for the next data. This prior distribution will be $\text{Beta}(u, v)$ with $u = S_0+1$ and $v = N_0 - S_0+1$. This shows the relation between the current prior distribution and the previously observed successes ($S_0$) and failures ($N_0 - S_0$).

The posterior distribution of $P$ after adding in the additional 200 lines will be $\text{Beta}(S+u, N - S+v) = \text{Beta}(S+S_0+1, N+N_0) = \text{Beta}(S+S_0+1) = \text{Beta}(2, 300)$.

Using these formulas it is possible to update allele frequency estimates for any number of prior observations.

Further Testing of Partially Resistant Lines. We selected the isofemale lines that caused the greatest feeding injury, had mature 1st instars, or lived and dispersed larvae. None of these non-Bt plants suffered feeding, suggesting that the larvae did not wander from plant to plant. As noted in Andow et al. (1998), feeding injury to the plants in the greenhouse was localized to a 5–10 cm band across 1–2 leaves. Four lines were inoculated on non-Bt corn to evaluate their vigor. All 4 lines readily consumed the non-Bt corn, causing extensive shot-holing injury.

Each line that produced sufficient eggs was also screened on meridic diets in at least 2 independent dishes with 3 $\mu$g Cry1Ab toxin per milligram incorporated into the diet (Andow et al. 1998).

Cost of the $F_2$ Screen. We estimated crude variable costs of the $F_2$ screen, including labor, insect diet, and field supplies, excluding the retesting costs and the costs for collecting the moths. The time required to collect the moths was minimal. We were able to minimize labor costs because people had been hired for other purposes and labor was estimated as actual hours worked rather than a percentage of annual salary. We calculated labor at $10.00/h (Andow et al. 1998, Bolin et al. 1998). Fixed and capital costs, such as overhead, insect cages and dishes, and growth chambers were not included in this cost estimate.

Results and Discussion

Resistance Allele Frequency. One hundred and eighty-eight isofemale lines were screened on Bt corn plants. None of the family lines fed extensively on the Bt corn, and no larva survived to the 2nd instar. Nearly all of the feeding injury on the Bt corn plants was characteristic of neonate feeding. Only 9 lines caused feeding injury with larger 2-mm holes or slightly elongated lesions of 2.3 mm. The inoculated Bt plants were dissected to count surviving larvae on 15, 19, 22, and 27 September 1997. Nearly all of the lines (85 lines) had live larvae on the Bt plants at the time of dissection. Most of these lines were probably younger larvae. Seven of the lines had at least 1 larva surviving that had grown to a mature 1st instar, 3 lines had $\geq 20$ larvae surviving, and 11 lines had larva that survived $\geq 7$ d on the Bt corn. Line 399 had several elongated feeding lesions and line 67 had a larva surviving $\geq 7$ d. No line had larva that matured to 2nd instar.

These results suggest that there were no major resistance alleles among the isofemale lines we screened. With $S = 0$ and $N = 188$, the expected resistance allele frequency in the Ames, IA, population was $1.3 \times 10^{-3}$, with a 95% CI of $[0.39 \times 10^{-3}]$. Thus, we can conclude with 95% confidence that the frequency of major resistance alleles in the area near Ames, IA, was $< 3.9 \times 10^{-3}$. This frequency estimate approaches that needed to support one of the assumptions of the refuge plus high-dose strategy for resistance management in Bt corn.

The probability that we could detect a resistance allele in each of these family lines is shown in Fig. 1.
Approximately 90% of the lines had a detection probability of >0.95, and <2% had a detection probability of <0.80. The experiment-wise detection probability was 0.98. This means that if there actually were a resistance allele in any of the 188 lines we tested, we would have detected it 98% of the time. These results compare favorably with our previous work (Andow et al. 1998), where only 53% of the lines had a detection probability of >0.90 and the experiment-wise detection probability was 0.88.

Retesting and Partial Resistance. Of the 21 retested lines, several caused more than neonate-type feeding injury to Bt corn in the greenhouse, but none survived to 2nd instar. Six lines caused minor injury to the sheath or stalk areas, and of these 6, line 399 caused 9 leaf-feeding holes >2 mm, the heaviest leaf injury observed. Line 67 also caused some injury to the stalk. Another line caused two 3- to 4-mm feeding holes in the leaves. Compared with feeding by neonates on non-Bt corn plants, this level of injury is minor. Line 399 was retained for additional future testing in the laboratory.

Seventeen of the lines produced sufficient eggs to screen on the Bt diet, and 2 lines showed significant growth and development, with several 2nd instars present after 10 d. Both lines were tested in 3 or more diet dishes with at least 500 larvae per dish, and survival to 2nd instar occurred in all 3 dishes. These lines corresponded to our original lines 67 (renamed B97-2) and 399 (renamed B97-1). Both of these lines were retained as potential partially resistant lines.

Partial resistance may be quite common in this population. Assuming that the lines that were not retested did not have partial resistance, the results imply that with S = 2 and N = 188, the expected frequency of partial resistance alleles was 3.9 \times 10^{-3} with 95% CI of [8.2 \times 10^{-4}, 9.4 \times 10^{-3}] in the Ames, IA, population.

Other researchers have inferred that partial resistance to Bt toxins is common in populations of O. nubilalis (Huang et al. 1997, Keil et al. 1997, Andow and Hutchison 1996, Bolin 1998). Previous work demonstrated that mass selection on laboratory colonies of O. nubilalis resulted in populations with elevated levels of resistance. Huang et al. (1997) report resistant ratios of 30–60, and Bolin (1998) reports ratios of 10–20. In both cases, the starting gene pool was small. Indeed for Bolin (1998) only 32 mated pairs (~128 haplotypes) contributed to the founding laboratory population. Our previous work on a Minnesota population showed partial resistance between 1 \times 10^{-3} and 1.5 \times 10^{-2} (Andow et al. 1998, Andow and Alstad 1999). The results reported here provide additional quantitative support for the inference that partial resistance alleles to Bt toxins are prevalent in natural populations of O. nubilalis. This is important, because partial resistance alleles may interact with a major resistance allele, accelerating the evolution of resistance (Alstad and Andow 1996).

Cost of the F2 Screen. The estimated variable cost per isofemale line for the F2 screen in the field was $14.90. This included 20 h to set up the 400 F2 lines, 32 h to collect eggs from these lines, 45 h to raise the F3, 45 h to collect eggs, and 42 h to inoculate and evaluate the plants in the field. Materials for rearing the lines were about $800. This is about a 25% reduction in variable costs compared with that reported in Andow et al. (1998), and considerably less expensive than larval assays for O. nubilalis (Bolin et al. 1998). If we had evaluated the plants solely based on feeding injury instead of dissections, this cost would have been reduced to $13.90 per line. It is unlikely that the cost will be much less even with additional improvements in record keeping and handling procedures. In summary, the F2 screen is economically feasible and could be used for resistance monitoring.

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