A feasibility trial of genomics-based diagnosis detecting insecticide resistance of the diamondback moth

A feasibility trial of genomics-based diagnosis detecting insecticide resistance of the diamondback moth[†]

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Abstract

BACKGROUND: Insecticide resistance management has been key for crop protection for over 70 years and is increasingly important because the development of new active ingredients has decreased in recent years. By monitoring the development of resistance in a timely manner, we can effectively prolong insecticide efficacy. Genomic-based diagnosis can reliably predict resistance development if information on resistant mutations against major pesticides is available. Here, we developed a feasibility trial of genomics-based diagnosis of insecticide resistance in diamondback moth (*Plutella xylostella*) populations in Nagano Prefecture, Japan. Amplicon sequencing analyses using a next-generation sequencer (Illumina MiSeq) for major insecticides, including diamides, pyrethroids, Bacillus thuringiensis (Bt) toxin (Cry1Ac), organophosphates, and spinosyns, were conducted.

RESULTS: Mutations related to the resistance of pyrethroids, organophosphates, and diamides (flubendiamide and chlorantraniliprole) prevailed, while those of a diamide (cyantraniliprole), Bt (Cry1Ac), and spinosyns were scanty, suggesting that they are still effective. The results of the genomics-based diagnosis were generally concordant with the results of bioassays. Resistance development tendencies were generally uniform across Nagano.

CONCLUSION: Insecticide-resistance management campaign can be conducted in Nagano Prefecture with a quick genomic-based diagnosis in early spring while bioassay is the only option for monitoring resistances whose mutations are unavailable. Our study is the first step in the future management of insecticide resistance in all significant pests.

Keywords: *insecticide resistance management, diamondback moth, next-generation sequencing, SNP, amplicon sequencing*

Introduction

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera; Plutellidae; hereafter referred to as DBM), is a wide-ranging and destructive pest of brassicaceous crops (Furlong et al. 2013; Talekar & Shelton 1993). It is the worst arthropod pest that develops pesticide resistance, second only to spider mites (pesticideresistance.org, accessed November 2021). DBM has a relatively short life cycle (seven-ten generations per year in Japan) and, coupled with strong vagility and fertility, can develop resistance to insecticides quickly. Resistance to all insecticides classified as pyrethroids and organophosphates developed until the early 1980s (Lee et al. 2007; Sonoda & Igaki 2010; Tsukahara et al. 2003). Resistance to Bt-toxin, which is an insecticidal protein produced by a specific class of bacterium (*Bacillus thuringiensis*), was detected soon after its use against DBM in Japan (Tanaka & Kimura 1991), and in Hawaii in the 1980s (Tabashnik et al. 1990). Spinosyn is a class of insecticides that originates as a natural product of the soil actinomycete, *Saccharopolyspora spinosa*. They were heavily used from the mid-1990s, and resistant populations were reported in the early 2000s in south-east Asian countries (Sayyed et al. 2004). Diamides are a relatively new class of insecticides that were first marketed in the mid-2000s. However, Troczka et al. (2017) reported resistance against diamides in Asian countries in the early 2010s. Cyantraniliprole, which is the newest diamide, was first marketed in 2014. It is currently the main insecticide used for DBM in Japan, but resistant populations were already reported in western Japan in 2017 (Jouraku et al. 2020). Fast and reliable estimation of resistant development in DBM has been desired in the practical crop fields in the world. Genomic-based diagnosis may provide such a solution if the information of the resistant mutations against major pesticides is available. DBM has been intensively studied to determine the mechanisms and causative genes of resistance against major insecticides. For example, target site mutations related to the resistance of diamides, pyrethroids, organophosphates, Bt-toxin (Cry1Ac), and spinosyn have been identified (Jouraku et al. 2020; Sonoda & Igaki 2010; Lee et al. 2007; Sonoda, Inukai, et al. 2017; Tsukahara et al. 2003). Recent advances in next-generation sequencing (NGS) have facilitated the identification of multiple mutations (including single nucleotide polymorphisms (SNPs) and small insertions/deletions (INDELs)), individually, with hundreds of samples simultaneously in a single sequencing run (Quail et al. 2012). Mutations (SNPs and small INDELs) that lead to the development of resistance can be used as simple genetic markers because many resistance traits of Lepidopteran insects are caused by target-site and single mutations (Beroza et al. 1974). There are also tractable characteristics of DBM for the regional diagnosis of resistance: large numbers of males can be captured by synthetic sex-pheromone traps. This method helps to capture more samples compared to the larval collection method (Reddy & Guerrero 2000). The moth is thought to be a strong disperser and, thus, the development of resistance in DBM is expected to be relatively uniform on a regional scale compared to the short dispersers, such as spider mites or thrips (Aliakbarpour & Salmah 2011; Dunley & Croft 1992). Therefore, a regional insect resistance management (IRM) strategy can be devised more quickly than that for short dispersers because it can be

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applied over a wide area.

We selected Nagano Prefecture for our test trial because of its geographical suitability (Figure 1). DBM, with 7-8 generations per year, causes substantial damage annually in Nagano Prefecture, located at the northern border of its overwintering habitat range. Although a small number of individuals can survive in winter, the majority emigrates from southern prefectures (Uesugi 2021). Prefectures in Japan are the practical governmental unit for decision-making in IRM, and Nagano Prefecture was keen to adopt this new technology. Considering a tractable character of DBM for NGS and a smooth technology transfer to crop fields, we conducted a genomics-based diagnosis for DBM using sex-pheromone trapping in Nagano Prefecture. Although there have been reports on one type of insecticide rather than various insecticides using simple genomic typing methods (Edwards et al. 2018; Guan et al. 2021; Wei, Shi, et al. 2013), we believe that our study is the first trial of genomics-based diagnosis for multiple insecticide resistances in DBM populations using NGS. The objectives of our study were as follows: 1) to report the results of genomics-based diagnosis of the multiple, major insecticide resistance using NGS, 2) to compare a conventional bioassay for all insecticides (except for organophosphates) with genomics-based diagnosis to confirm the validity of our method, and 3) to test the spatial structure in resistance development by measuring pairwise distances of genetic structure (F_{ST}) and permutation-based analysis of molecular variance (AMOVA) for mutations related to insecticide resistance among sites. Using these results, we evaluate the feasibility of the genomics-based diagnosis.

Materials and Methods

Study site

Nagano Prefecture is located at the centre of the Japanese islands and is the fourth largest prefecture in Japan (13,562 km^2, Figure 1). Nagano Prefecture is mainly covered by mountain ranges and is, therefore, suitable for highland agriculture.

Sampling

Male DBMs were collected for the genomic-based diagnosis using sex-pheromone traps with sticky sheets in four seasons: from April 21 to June 29 (16Sp) and from August 23 to December 6 (16Au) in 2016; from May 18 to June 20 (17Sp) and from August 26 to December 1 (17Au) in 2017. Although the sample dates range slightly over spring and autumn, we named them the spring (Sp) and autumn (Au) generations for convenience. The spring generations correspond to overwintering, and we assumed that their first generation had not experienced insecticide selection, whereas autumn generations were obtained after heavy selection. We collected at least 16 individuals per site; however, there were three exceptions when 13, 15, and 14 individuals were collected at sites NCK in 16Sp, NSA, and NNM in 16Au, respectively. The sample sizes and the locations are summarized in Figure 1 and Supporting Information1. The retrieved sticky sheets were kept in a refrigerator (under 8°C) for a maximum of eight months until all the samples were used for subsequent analyses. Immediately before the NGS analyses, individual samples were peeled from the adhesive sheets and kept in a 1.5 mL plastic microtube with 99.5% ethanol.

Library construction

Six libraries of multiplexed amplicon sequences for 2213 individuals collected in the four seasons, were constructed to identify genotypes for mutations related to insecticide resistance (Table 1). Each library included 384 individuals, except for the sixth run (293 individuals). The method of library construction is described in Supporing Information 1.

Sequencing and genotyping

Each male diamondback moth, used for the amplicon sequencing, was placed in a 96-well PCR plate (0.2 mL). DNA was extracted from each sample by adding 200 μL of DNA extraction buffer (10 mM Tris-HCl pH 9.0, 1 mM EDTA, 0.1% Triton-X100) to each well, incubating in a thermal cycler for 15 min at 99.9°C, and centrifuging for 5 min at 750 × g. Each library was sequenced by Illumina MiSeq (six runs in total) using MiSeq Reagent Kit v3 - 600 cycles (used for three libraries in 2016), or v2 - 500 cycles (used for three libraries in 2017) (Illumina KK, Tokyo, Japan). Raw reads of each sequencing run were demultiplexed for each individual by an in-house Perl script. The script identifies individuals based on the combination of the internal barcode (5 bp) at the head of each read, and the pair of p5/p7 indices assigned to each read, and then trims the internal barcode. The demultiplexed reads were then filtered by Trimmomatic version 0.36 (Bolger et al. 2014) to trim the adapter and low-quality sequences. The cleaned reads were mapped to target scaffolds in the reference genome assembly of *P. xylostella*, available at Lepbase (accessed November 2020), using Bowtie2 version 2.2.6 (Langmead & Salzberg 2012). SNP calling of the mapped reads was performed by SAMtools 1.3.1 (Li, Handsaker, et al. 2009), and a genotype of each target mutation in Table S1, supporting information (susceptible-type homozygote, resistant-type homozygote, or heterozygote) was determined (genotype was not determined if the depth of the mapped reads was less than three). To remove individuals of non-DBM lepidopteran species (such as *Acrolepiopsis sapporensis* and *A. nagaimo*), which are morphologically similar to DBM and are known to be captured in synthetic sex-pheromone traps), the number of SNPs around the G4946E mutation site (target sequence: 5'- CGCGGCTCATCTGTTGGACGTG-GCTGTCGGGTTCAAGACGCTGAGGACTATCT -3' [53 bp] in a 190 bp region of a ryanodine receptor [RyR] gene amplified by the primer pair in Table S1), was checked and individuals were removed for further analysis if there were more than eight SNPs. We independently sequenced the individuals' cytochrome c oxidase I gene and confirmed that the sequences differed from that of DBM (data not shown). Consequently, 2116 of 2213 individuals were used in further analysis (87 individuals that were not DBMs, and 10 individuals caught in the summer [from July to early August]). The threshold of read depth and mapping quality (MQ) at each target site in genotyping was three and 20, respectively. For each target site, some individuals that failed to meet the threshold were excluded for further analysis. The information on resistant mutations of all individuals is available at OSF. The raw read data is available in the DDBJ Sequenced Read Archive under the accession numbers DRA011355 (16Sp), DRA011315 (16Au), DRA011316 (17Sp) and DRA011318 (17Au).

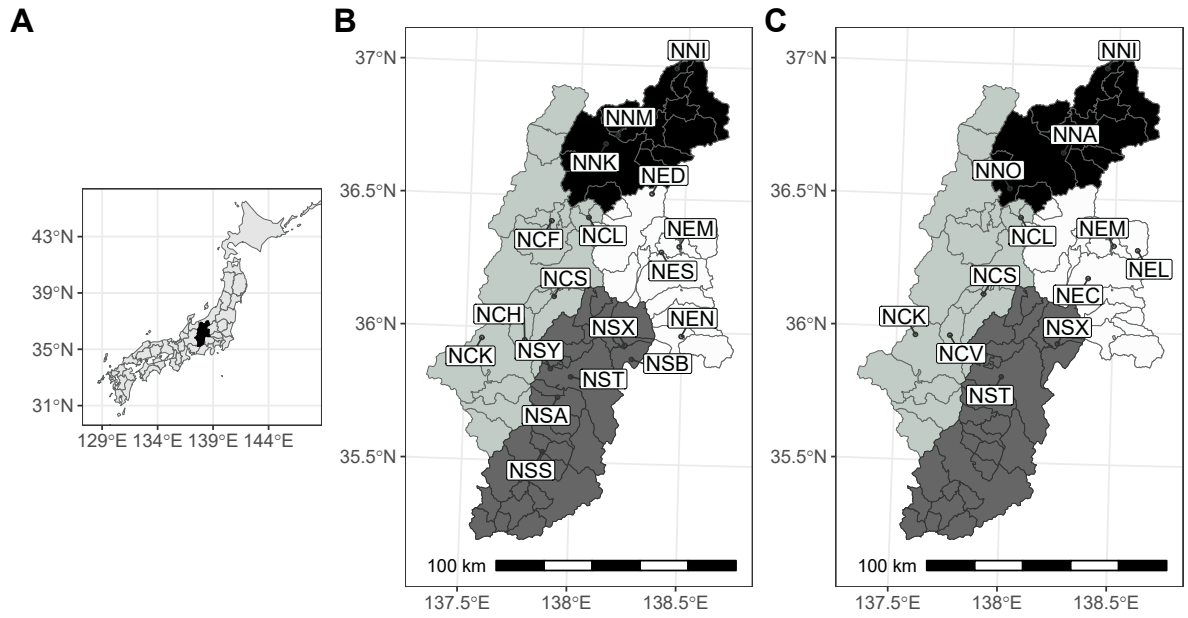

Figure 1. Locations of Nagano prefecture (the thick line in panel **A**), amplicon sequencing (panel **B**), and bioassay (panel **C**) study sites. Some sites were common between amplicon sequencing analyses and bioassays. The polygons are coloured by region: Hokushin (NN): black, Toushin (NE): white, Chushin (NC): light grey, Nanshin (NS): dark grey. The regions are coded as the first two letters of site codes.

Bioassay

Larvae for the bioassay were collected from the cabbage (or the other brassicaceous crop) fields just before harvesting, from June to September 2015-2017. The sampling locations and the insecticides tested are summarized in Figure 1 and Table S6, respectively. The main targets for the bioassay were larvae in the commercial crop fields. However, it was challenging to find enough specimen because of the low densities. Cabbages or the other brassicaceous crops were carefully investigated in the field, and plant tissues with larval damages were dissected to find larvae. Approximately 100 larvae were collected in the field, reared, and allowed to copulate randomly to secure sufficient genetic variability. Ten individuals were randomly selected from the F1-3 generation and were tested in each bioassay trial. The leaf-dip method was used to determine survival against practical doses of the insecticides (Hama et al. 1992). These standard recommended doses are generally used in the fields in Nagano Prefecture. The doses are generally highly toxic to kill susceptible individuals. A brassica (*Brassica rapa* var. *chinensis*) leaf disc, 7 cm diameter, was dipped into each diluted insecticide for 20 s and then dried. Ten individual fresh third-instar larvae were placed onto the leaf disc for 96 h (25°C, 60%-80% RH, 16L: 8D condition) and the survivors were counted. The tests were replicated three times. Ideally, the bioassays would have tested several dosages around the practical dose to develop a dose-response curve. However, the recommended dosage was set as the particular diagnostic threshold in our study because a dose-response analysis usually requires large sample sizes, making it unsuitable for testing wild individuals containing mixtures of homozygotes and heterozygotes (Roush & Miller 1986). No adjustment was made to remove the effect of background mortality since all individuals in the control survived the experiment. Some of the bioassay data for 2015 and 2017 have been published (Kitabayashi & Sato 2020). Twenty-one insecticides of the recommended dosage were tested, and nine of them are shown in the main text for comparison with the results of genomics-based diagnosis (see Table S6 for full results). Phoenix®with a 2000 dilution ratio (Nihon Nohyaku Co., Ltd., Tokyo, Japan), and Prebason®with a 2000 dilution ratio (FMC, Tokyo, Japan) contain two old classes of diamides, flubendiamide and chlorantraniliprole, were launched in Japan in 2007 and 2009, respectively (Kamimuro 2021). Verimark®with a 4000 dilution ratio (FMC, Tokyo, Japan), and Benevia®with a 4000 dilution ratio (FMC, Tokyo, Japan), contain a new diamide, cyantraniliprole, were launched in Japan in 2014 (Kamimuro 2021). For pyrethroids, Scout®with a 2000 dilution ratio (Nippon Soda Co., Ltd., Tokyo, Japan) was tested. Toarow®with a 1000 dilution ratio (Otsuka Chemical Co., Ltd., Tokyo, Japan) and XenTari®with a 2000 dilution ratio (Valent BioScience LLC, Libertyville, Illinois) contain Bt toxin. Spinoase®with a 5000 dilution ratio (Corteva Agriscience, Wilmington, DE) contains spinosad.

Pairwise F_{ST}

The spatial variation of the insecticide resistant mutants was elucidated by calculating the pairwise distances of genetic structure, i.e., Weir and Cockerham's pairwise F_{ST} (= θ) between all pairs of sites (Weir & Cockerham 1984) using the hierfstat package in R version 3.5.1 (Goudet & Jombart 2015; R Development Core Team 2020) because it is more suitable for data of unequal sample sizes than other methods. The significance of the positive relationships was tested firstly by simple linear regression between the pairwise F_{ST} and their corresponding geographical distances among sites. Then, the same relationship was tested by Mantel test with 999 permutations using the vegan package in R (Oksanen et al. 2019). We used the information on the seven mutations shown in Table 1, excluding 3aa DEL, since it was available

Table 1. Pesticide-resistance related markers in our study.

Gene code	Resistant gene	Target pesticide	Reference
G4946E	Ryanodine receptor (RyR)	Diamide (flubendiamide, chlorantraniliprole)	Sonoda, Inukai, et al. (2017)
I4790(M|K)	Ryanodine receptor (RyR)	Diamide (flubendiamide, chlorantraniliprole)	Jouraku et al. (2020) and Sonoda, Inukai, et al. (2017)
L1014F M918I	Sodium channel (SC)	Pyrethroid	Tsukahara et al. (2003)
Q525*	ABC transporters	Bt (Cry1Ac)	Supporting Infomation2
A298S	Acetylcholinesterase (AChE1)	Organophosphate	Lee et al. (2007) and Sonoda & Igaki (2010)
3aa DEL	A three amino acid deletion	Spinosyn (spinosad)	Wang, Wang, et al. (2016)

Note: E1338D is the resistant SNPs against diamides; T929I and A1101T against pyrethroids; and G324A against organophosphate. They were also tested in our genotyping analyses by MiSeq (results are not in the main text). We found that these were not major resistant mutations in Japan and/or coincide with the other major ones in our analysis. See the full list of markers in Table S1.

only in 2017.

AMOVA

To elucidate the relative contributions of regional and seasonal effects on the total genetic variation, permutation-based AMOVA (Analysis of MOlecular VAriance) was conducted using all the information on the seven resistance-related mutations shown in Table 1 (excluding that of 3aa DEL, since it was not tested in 2016). AMOVA is a standard technique used to assess the relative contributions of factors to the total genetic variation in a hierarchical population structure. Euclidean distances of the compositions in resistance-related markers among sites were modelled by two crossed factors, i.e., regions (four regions in Nagano Prefecture, see Figure 1) and seasons (2016 spring and autumn, 2017 spring and autumn). The four regions (Hokushin, Toushin, Chushin, and Nanshin) reflect geographical and cultivation differences in Nagano Prefecture. Statistical significance was assessed using 999 permutations (Larroque et al. 2019). We employed the adonis function in the vegan package for the permutation based AMOVA in R (R Development Core Team 2020).

Results

The failure to recover sequence data was generally small, less than 2.2%, except for SNPs against diamides (M918I, 9.45%), pyrethroids (L1014F, 11.8%), and Cry1Ac (Q525, 11.8%). The failure rate was not influenced by the duration of storage in the refrigerator but was dependent on the target SNPs. We confirmed that our protocol worked well for the genetic diagnosis.

The spatial and temporal patterns of the levels of homozygosity and heterozygosity with resistant mutations are shown in Figure 2. The frequencies of SNPs related to resistance against diamides (G4946E), pyrethroids (L1014F), and organophosphates (A298S) were high (approximately 50%) from the start of our study and were maintained across all seasons (Figure 2,Figure 3). Conversely, the frequencies of an SNP related to Bt toxin (Cry1Ac) resistance (Q525*) on the ABCC2 gene were generally low (0–0.174) across Nagano Prefecture, and in all four seasons. Since the Q525* mutation was not reported previously, the information should be provided, here. We first identified the Q525* mutation in the ABCC2 gene of the diamondback moth individuals that survived after treatment with 100 ppm of Cry1Ac toxin (GuardJet WP®, Kubota Corp., Osaka, Japan) by sequencing the cDNA of the ABCC2 gene (Supporting Infomation 2). Since the ABCC2 gene of the diamondback moth encodes a receptor for Bt Cry1Ac toxin, we assumed that the Q525* mutation introduced a destructive effect on the receptor function for the Cry1Ac toxin. However, the functional evidence should be confirmed in future analyses.

Similar to Bt toxin, the mutation related to spinosyn resistance, 3aa DEL, was not detected in 2017. The frequencies of I4790(K), which relates to a newest diamide class (cyantraniliprole), remained low, but gradually increased (0.05–0.158; Figure 2,Figure 3). Homozygosity in G4946E against diamides and in A298S against organophosphates increased in autumn both in 2016 and 2017 (Figure 2). We suspect that those are perhaps due to insecticidal selection during the cropping season. We also investigated the compound heterozygosity in mutations of the ryanodine receptor (Table S2). The majority (50-73%) of those with resistant mutations only had the marker G4946E without E1338D nor I4790(M|K). Individuals with all G4946E, E1338D and I4790(M) in the heterozygotes were the second largest combination (5-14%) but no individual had all the resistant mutations as the homozygotes. Individuals with I4790(K) of the new diamide were mainly heterozygotes and those heterozygotes with G4946E gradually increased. Heterozygotes with I4790(K) and (M) were found in 2017 spring samples. To determine the grade of selective sweeps in each resistant mutation, a variety of

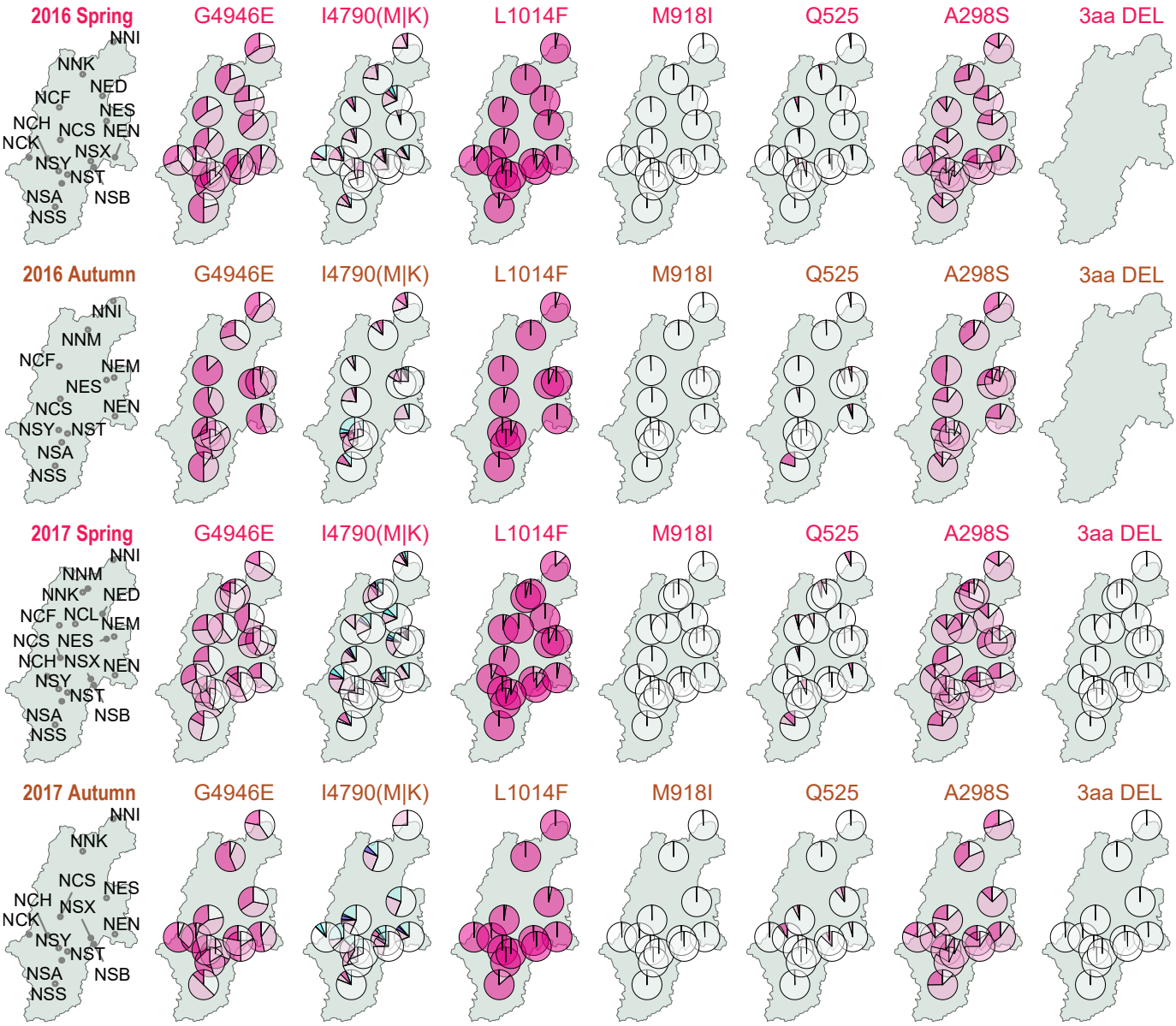

Figure 2. Spatial and temporal patterns of the genotype ratio of pesticide-resistance related markers. Red represents resistant homozygotes, pink represents heterozygotes, and light blue is susceptible homozygotes. For corresponding resistant genes, see Table 1. Please note, I4790(M) and (K) are on the same site but displayed separately to enhance the readability. Mutation M was treated as susceptible in I4790(K), K in I4790(M), and *vice versa*. Complete results of all markers can be found in Figure S1.

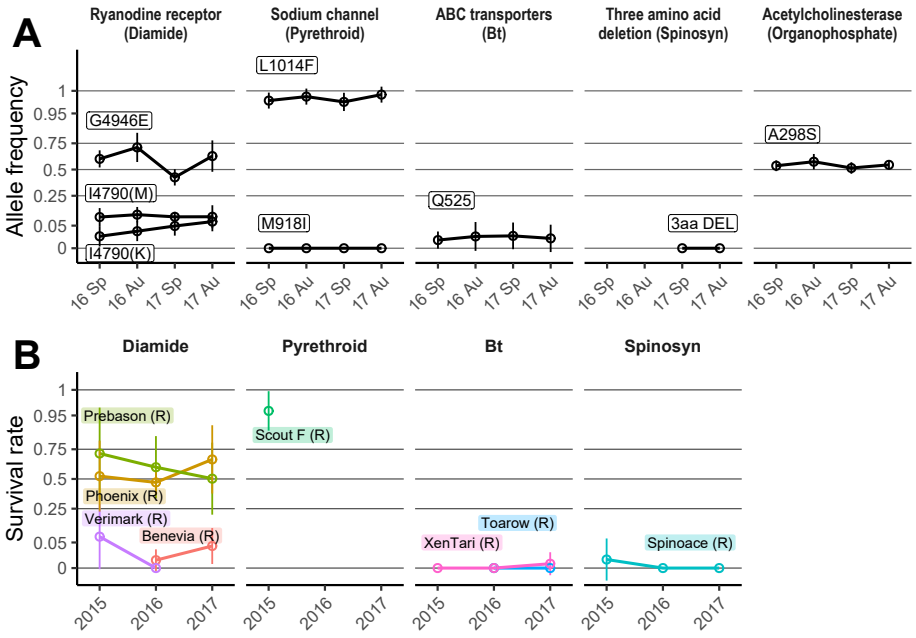

Figure 3. **A**) average trends of resistant mutations among sites in amplicon sequencing and **B**) those of resistant survivors in bioassay among sites. Note, scales of y-axes were arcsine and square root transformed so that the low frequencies were exaggerated. Error bars are the standard deviations among sites calculated after arcsine and square root transformation of the frequencies. Complete results of all markers and bioassay can be found in Figure S2, Figure S3 and Table S6.

haplotypes and their frequencies with resistant mutations, G4946E, A298S and/or G324A and L1014F were investigated (Table S3-5). We found that single haplotype (HT1) prevailed in all mutations (G4946E: 48-53%, A298S and/or G324A: 84-86%, L1014F: 70-94%). They were assumed to be a consequence of selective sweeps. Since G4946E had five major haplotypes (HT1-5) and the frequency of HT1 in G4946E was relatively low compared to A298S and/or G324A and L1014F, we suspect that independent evolutionary events occurred in the past and selective sweeps are still in progress.

The results of the bioassays (Figure 3) validate the results of our genomics-based diagnosis (Figure 3A; the full results of all insecticides can be found in Table S6 and Figure S3). For example, the old class of diamides, Phoenix and Prebason were less effective than the new ones, Verimark and Benevia. This corresponded with the genotyping results; the frequencies of G4946E were high, whereas those of I4790(K) remained low. The resistant mutant of pyrethroids (L1014F) prevailed in male moths and the pyrethroid, Scout, could not kill most of the larvae. However, we were unable to observe individuals containing the other pyrethroid mutant (M918I). The Bt toxins, Toarow and XenTari, and the spinosyn, Spinoase, exterminated almost all individuals in the bioassay, and related mutants (Q525* and 3aa DEL) were not detected in most male moths in the genomic-based diagnosis. Weir and Cockerham's pairwise F_{ST} (which is based on variation in seven markers; Table 1) was calculated in each season [see Figure 4]Weir.Cockerham1984. The records of 3aa DEL were excluded from the analysis because they were not available in 2016. Genetic diversity was low, especially in the spring (mostly <0.05) and there was no indication of spatial clustering, i.e., isolation-by-distance, according to the simple linear regression between the pairwise F_{ST} and their corresponding geographical distances among sites (Figure 4 confidence intervals). The Mantel's test also supported no spatial effects on genetic diversity (r = -0.111, p = 0.753 in spring 2016; r = -0.011, p = 0.408 in autumn 2016; r = 0.031; p = 0.392 in spring 2017; r = 0.137; p = 0.182 in autumn 2017). Variation in the seven markers was transformed into a dissimilarity matrix, i.e., Euclidean distances, and the effects of four seasons and sub-regions (four geographical regions in Nagano Prefecture: Hokushin, Toushin, Chushin, and Nanshin, Figure 1) were tested using a permutation-based AMOVA. The effect of seasons, sub-regions and interactions was statistically significant ($p < 0.001$, Table 2). However, the regional effect only contributed 0.8% to the total variance, even though the effect was significant. The effect of seasons was also significant, and the contribution was eight-fold larger than that of the regions (3.8%). No positive relationship was found between genetic and geographic distances (Figure 4). We inferred that the frequencies of resistant alleles were changing uniformly in the whole Nagano Prefecture.

Discussion

This study confirmed that genomic-based diagnosis could provide speedy estimates of resistance development in DBM. It was because we could have collected sufficient male moths in the early part of the cropping season by sex-pheromone traps. On the other hand, it was challenging to collect enough larvae for the bioassays to measure the development of resistance for the major pesticides used against DBM. DBM is a migratory species, and population densities are generally low in early spring, especially in northern Japan. In this study, enough larvae were obtained only at the end of the cropping season. Further time was spent in rearing the DMB for one to three generations. The bioassay used in this study presented methodological difficulties to detect low frequencies of resistant individuals. The genomic-based diagnosis

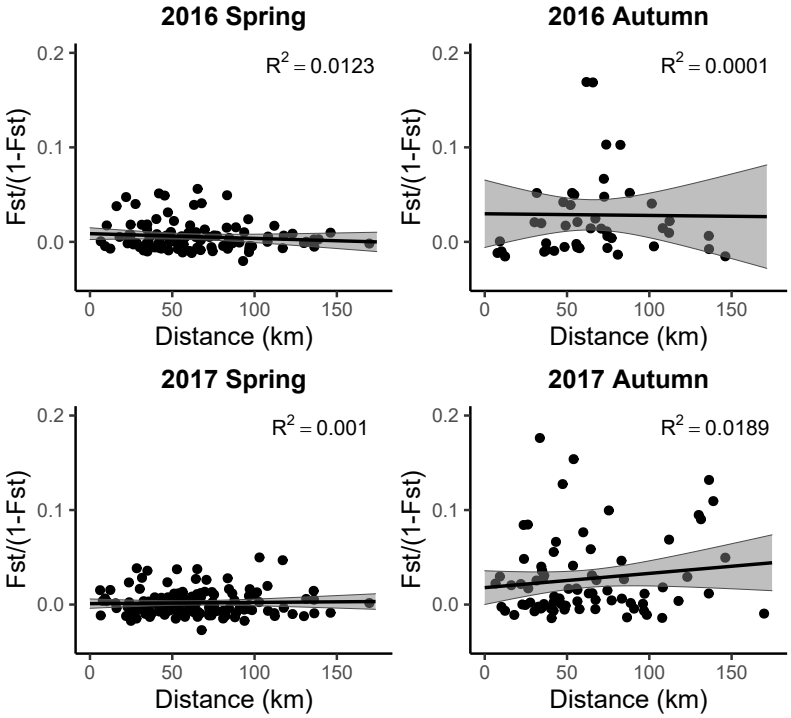

Figure 4. Isolation by distance represented as the gene differentiation among subpopulations measured by Weir and Cockerham's pairwise F_{ST}. According to Rousset Rousset1997,$F_{ST}/(1 - F_{ST})$ is plotted against distances between sites to increase the linearity. The resistance-related markers in Table 1 were used.

Table 2. Pesticide-resistance related markers in our study.

	df	SS	MS	*F*-stat	R^2	*p*
Season	3	305.0	101.7	28.2	0.038	$< 10^{-3}$
Region†	3	61.4	20.5	5.7	0.008	$< 10^{-3}$
Season × Region	9	168.5	18.7	5.2	0.021	$< 10^{-3}$
Residuals	2093	7542.8	3.6			
Total	2108	8077.7				

† Nagano prefecture is historically and geographically divided into four sub-regions (Hokushin, Toushin, Chushin, and Nanshin). The regions are coded as the first two letters of site codes in Figure 1.

provides twice the amount of chromosomal information from directly-captured male moths since they are diploid. In contrast, a bioassay requires 10 to 100-times more individuals to estimate low frequencies (e.g., less than 10%) of resistance development. In the bioassays, the larvae will have to digest the insecticide-treated food to show any response. Large numbers of larvae are required for such experiments. Recessive insecticide resistance is almost impossible to detect in bioassays because the number of resistant homozygotes is theoretically very low (Andow et al. 1998). NGS saves time and effort in obtaining high precision estimates of the early development of insecticide resistance. However, it should be noted that genomic-based diagnosis can be a promising tool only for specific markers with a known association with insecticides. Resistant individuals should first be identified in the field or laboratory and the mechanisms of resistance can be elucidated. Resistance-related mutations must be identified and utilized as genetic markers in monitoring programs. However, it is difficult to identify resistance-related markers when resistance is under polygenic control (Pittendrigh et al. 2013). Bioassays are the only option to detect such resistances in wild populations. In our study, twelve mutations associated with insecticide resistance were investigated. The resistance against old classes of diamides, pyrethroids, and organophosphates was high and maintained across all seasons (Figure 2, Figure 3). It is likely that the long-term and consistent use of these insecticides has maintained the resistant populations in Nagano Prefecture. A single haplotype prevailed in these mutations and could be a consequence of selective sweeps. Therefore, we have scientific confirmation for the cessation of these insecticides. Conversely, the frequencies of resistant mutations related to the Bt toxin (Cry1Ac) and to spinosyn were generally low in all four seasons (Figure 2, Figure 3). Populations resistant to Bt toxin (Cry1Ac) and spinosyn were found in several regions of the world (including Japan) until the early 2000s (**Sayyed.etal2004Tanaka.Kimura1991**;

Zhao, Li, et al. 2002). These insecticides can now be recommended for the DBM again. The resistant mutation associated with the new diamide class, cyantraniliprole, was low but gradually increased with the season (Figure 2, Figure 3). This result is concerning because cyantraniliprole was first marketed in 2014 and was thought to have increased the practical longevity of the DBM. Therefore, the resistant mutation (I4790(K)) should be carefully and continuously monitored in preparation for future resistance development. Our genomic-based diagnosis concurred with the results of the bioassay. However, it is still unclear how the point mutation, as the cause of insecticide resistance, relates to the actual mortality of resistant individuals. The relationship between mutation and mortality is mainly dependent on the genotype and dominance, the mechanism of detoxification, and the properties of the pesticides (Scott 1995). It has been reported that many major resistant properties are point mutations and that they are recessive (Bourguet et al. 2000; Ffrench-Constant et al. 2004). In those cases, only homozygotes can survive after exposure to the insecticide applied at the practical dose. Consequently, the survival rates should be much lower than the resistance ratios estimated by genomic-based diagnosis, because the homozygotes are only the squared proportion of the allele frequency. In our study, the point mutation for resistance against older diamides was recessive (Steinbach et al. 2015; Wang, Khakame, et al. 2013). However, the allele frequencies with G4946E, and the survival rate with older diamides (Prebason and Phoenix) were similar, at 0.5–0.75 (Figure 2). We postulate that the larvae used for the bioassays were collected following insecticide application and the majority were resistant against the diamides. With a spatially extensive, genomic-based diagnosis, it is possible to determine the time and spatial extent for deploying IRM strategies for DBM. In our study, the pairwise-F_{ST} was generally small, and there was no evidence of isolation-by-distance. In addition, contributions of the regional (0. 8%) and seasonal (3.8%) effects were small, even though both were statistically significant. We assume that this is a typical trend of strong dispersers since the DBM population in Nagano Prefecture receives several immigrants annually from the southern prefectures (Uesugi 2021). No genetic differentiation related to insecticide resistance was found in the provinces of China (Wei, Shi, et al. 2013) although differences were observed on a continental scale (Li, Feng, et al. 2016). This study recommends that future IRM campaigns can be carried out in Nagano Prefecture as a whole, i.e., one or a few monitoring sites in spring will be sufficient for the year if enough samples are secured. Since NGS provides prompt estimates of resistance, a quick diagnosis will effectively contribute to the decision of which pesticides to use for those resistant mutations in the following cropping season. It is still necessary to conduct bioassays for unknown mutations, although it takes more time than the genomic-based diagnosis. Genomic-based diagnosis and bioassays should be used synergistically. Regional trials of genomics-based diagnosis for insecticide resistance had been conducted in practice against sanitary insect pests, which act as vectors for lethal human diseases e.g., *Anopheles* mosquitos (Donnelly et al. 2016). It is now being applied to agricultural pests. These trials have started from mutations against one specific insecticide (Edwards et al. 2018; Guan et al. 2021; Wei, Guan, et al. 2021). The high cost of amplicon sequencing analyses with NGS has precluded its use in agriculture. Currently, it costs approximately 1,700 USD to identify 10 SNPs from 384 individual DBM by Illumina MiSeq. This cost will be substantially reduced with the availability of a high-speed NGS, such as HiSeq X (accessed November 2021). Reducing sequencing costs would allow for research institutes to carry out genomic analysis to get prompt results. We could have tested twelve resistant mutations, since the pest is associated with a long history of research aiming to reveal the mechanisms of insecticide resistance (Furlong et al. 2013; Li, Feng, et al. 2016; Talekar & Shelton 1993). However, exploring resistant mutations is a laborious and endless task. There is a need to explore genetic markers of the other mutations associated with the same pesticides since variants with the same detoxification function may be present. For example, any mutations resulting in loss of nAChR a6 function may cause spinosyn resistance (Baxter et al. 2010). For Cry1Ac, two recent studies have confirmed that loss of function for ABCC2 and ABCC3 is associated with resistance (Liu et al. 2020; Zhao, Jiang, et al. 2021). The utility of genetic markers and a comparison of the results of bioassays with wild populations should be investigated. Continuous updates for new resistant makers published in scientific journals are also required. Identification of mutation markers related to unknown resistance mechanisms is expensive. Recent advances in genomic and proteomic methods enable rapid targeting of genes of resistance (Pittendrigh et al. 2013). The expressions of tens of thousands of genes can be simultaneously compared at a single time between resistant and susceptible individuals by microarray or RNA-seq. Quantitative real-time reverse transcription polymerase chain reaction, metabolic evaluation of insecticides by mass spectrometry, and evaluation of increased susceptibility by RNAi mediated knockdown can be used effectively to verify the roles of candidate genes. Although limited genetic markers are available to diagnose resistance by increased metabolic detoxification, more information on detoxification-related genes is available through genome-wide NGS data. It is hoped that markers related to the polygenic control of pesticide resistance will be identified. Although bioassays are still needed to associate resistance in the field with related mutations, prompt diagnoses over a wide region can be achieved by NGS. Our study represents the first step towards the labour-saving, rational management of insecticide resistance in all major pests.

Data Accessibility

The data that support the findings of this study are openly available in OSF at:
https://osf.io/z5hws/

Competing Interests

The authors declare no conflict of interest.

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References

Aliakbarpour H & Salmah MRC (2011). Seasonal abundance and spatial distribution of larval and adult thrips (Thysanoptera) on weed host plants in mango orchards in Penang, Malaysia. *Appl Entomol Zool* 46: 185–194. DOI: 10.1007/s13355-011-0030-5.

Andow DA, Alstad DN, Pang Y.-H, Bolin PC & Hutchinson WD (1998). Using an F_2 screen to search for resistance alleles to *Bacillus thuringiensis* toxin in European corn borer (Lepidoptera: Crambidae). *J Econ Entomol* 91: 579–584. DOI: 10.1093/jee/91.3.579.

Baxter SW et al. (2010). Mis-spliced transcripts of nicotinic acetylcholine receptor 6 are associated with field evolved spinosad resistance in *Plutella xylostella* (L.) *PLoS Genetics* 6: e1000802. DOI: 10.1371/journal.pgen.1000802.

Beroza M et al. (1974). Large field trial with microencapsulated sex-pheromone to prevent mating of gypsy moth. *J Econ Entomol* 67: 659–664. DOI: 10.1093/jee/67.5.659.

Bolger AM, Lohse M & Usadel B (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120. DOI: 10.1093/bioinformatics/btu170.

Bourguet D, Genissel A & Raymond M (2000). Insecticide resistance and dominance levels. *J Econ Entomol* 93: 1588–1595. DOI: 10.1603/0022-0493-93.6.1588.

Donnelly MJ, Isaacs AT & Weetman D (2016). Identification, validation and application of molecular diagnostics for insecticide resistance in malaria vectors. *Trends in parasitology* 32: 197–206. DOI: 10.1016/j.pt.2015.12.001.

Dunley JE & Croft BA (1992). Dispersal and gene flow of pesticide resistance traits in phytoseiid and tetranychid mites. *Experimental & Applied Acarology* 14: 313–325. DOI: 10.1007/BF01200570.

Edwards OR et al. (2018). A genomic approach to identify and monitor a novel pyrethroid resistance mutation in the redlegged earth mite, *Halotydeus destructor*. *Pesticide Biochemistry and Physiology* 144: 83–90. DOI: 10.1016/j.pestbp.2017.12.002.

Ffrench-Constant RH, Daborn PJ & Le Goff G (2004). The genetics and genomics of insecticide resistance. *Trends in Genetics* 20: 163–170. DOI: 10.1016/j.tig.2004.01.003.

Furlong MJ, Wright DJ & Dosdall LM (2013). Diamondback moth ecology and management: Problems, progress, and prospects. *Annu Rev Entomol* 58: 517–541. DOI: 10.1146/annurev-ento-120811-153605.

Goudet J & Jombart T (2015). hierfstat R package version 0.04-22. URL: https://cran.r-project.org/web/packages/hierfstat/index.html.

Guan F et al. (2021). Multiple origins of a single point mutation in the cotton bollworm tetraspanin gene confers dominant resistance to Bt cotton. *Pest Management Science* 77: 1169–1177. DOI: 10.1002/ps.6192.

Hama H, Suzuki K & Tanaka H (1992). Inheritance and stability of resistance to *Bacillus thuringiensis* formulations of the diamondback moth, *Plutella xylostella* (LINNAEUS) (Lepidoptera: Yponomeutidae). *Appl Entomol Zool* 27: 355–362. DOI: 10.1303/aez.27.355.

Jouraku A et al. (2020). Ryanodine receptor mutations (G4946E and I4790K) differentially responsible for diamide insecticide resistance in diamondback moth, *Plutella xylostella* L. *Insect Biochem Molec* 118: 103308. DOI: 10.1016/j.ibmb.2019.103308.

Kamimuro T (2021). Resistance to diamide insecticides and its inheritance in the oriental tea tortrix, *Homona magnanima* (lepidoptera: tortricidae), in Kagoshima prefecture, Japan. *Jpn J Appl Entomol Zool* 65: 87–97. DOI: 10.1303/jjaez.2021.87.

Kitabayashi S & Sato T (2020). Insecticide susceptibility to various insecticides of diamondback moth, (*Plutella xylostella*) in Nagano prefecture. *Annual Report of the Kanto-Tosan Plant Protection Society* 67: 65–68. DOI: 10.11337/ktpps.67.65.

Langmead B & Salzberg SL (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9: 357–359. DOI: 10.1038/nmeth.1923.

Larroque J et al. (2019). Temporal variation in spatial genetic structure during population outbreaks: Distinguishing among different potential drivers of spatial synchrony. *Evolutionary Applications* 12: 1931–1945. DOI: 10.1111/eva.12852.

Lee DW et al. (2007). Mutations of acetylcholinesterase1 contribute to prothiofos-resistance in *Plutella xylostella* (L.) *Biochemical and Biophysical Research Communications* 353: 591–597. DOI: 10.1016/j.bbrc.2006.12.088.

Li H, Handsaker B, et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25: 2078–2079. DOI: 10.1093/bioinformatics/btp352.

Li Z, Feng X, Liu S.-S, You M & Furlong MJ (2016). Biology, Ecology, and management of the diamondback moth in China. *Annu Rev Entomol* 61: 277–296. DOI: 10.1146/annurev-ento-010715-023622.

Liu Z et al. (2020). Resistance to *Bacillus thuringiensis* Cry1Ac toxin requires mutations in two *Plutella xylostella* ATP-binding cassette transporter paralogs. *PLoS Pathogens* 16: e1008697. DOI: 10.1371/journal.ppat.1008697.

Oksanen J et al. (2019). vegan R package version 2.5-6. URL: https://cran.r-project.org/web/packages/vegan/index.html.

Pittendrigh BR et al. (2013). "Understanding resistance and induced responses of insects to xenobiotics and insecticides in the age of "omics" and systems biology". *Insect Resistance Management*. Ed. by DW Onstad. 2nd ed. Amsterdam: Academic Press: 55–98. URL: https://books.google.com.ag/books?id=hjnisaYap4oC.

Quail MA et al. (2012). A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* 13: 341. DOI: 10.1186/1471-2164-13-341.

R Development Core Team (2020). *R: a language and environment for statistical computing*. URL: https://www.r-project.org/.

Reddy GVP & Guerrero A (2000). Pheromone-based integrated pest management to control the diamondback moth *Plutella xylostella* in cabbage fields. *Pest Management Science* 56: 882–888. DOI: 10.1002/1526-4998(200010)56:10<882::AID-PS226>3.0.CO;2-T.

Roush RT & Miller GL (1986). Considerations for design of insecticide resistance monitoring programs. *J Econ Entomol* 79: 293–298. DOI: 10.1093/jee/79.2.293.

Sayyed AH, Omar D & Wright DJ (2004). Genetics of spinosad resistance in a multi-resistant field-selected pop-

ulation of *Plutella xylostella*. *Pest Management Science* 60: 827–832. DOI: 10.1002/ps.869.

Scott JA (1995). The molecular genetics of resistance: Resistance as a response to stress. *FLA Entomol* 78: 399–414. DOI: 10.2307/3495526.

Sonoda S, Inukai K, Kitabayashi S, Kuwazaki S & Jouraku A (2017). Molecular evaluation of diamide resistance in diamondback moth (Lepidoptera: Yponomeutidae) populations using quantitative sequencing. *Appl Entomol Zool* 52: 353–357. DOI: 10.1007/s13355-017-0482-3.

Sonoda S & Igaki C (2010). Characterization of acephate resistance in the diamondback moth *Plutella xylostella*. *Pesticide Biochemistry and Physiology* 98: 121–127. DOI: 10.1016/j.pestbp.2010.05.010.

Steinbach D et al. (2015). Geographic spread, genetics and functional characteristics of ryanodine receptor based target-site resistance to diamide insecticides in diamondback moth, *Plutella xylostella. Insect Biochem Molec* 63: 14–22. DOI: 10.1016/j.ibmb.2015.05.001.

Tabashnik BE, Cushing NL, Finson N & Johnson MW (1990). Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *Journal of Enonomic Entomology* 83: 1671–1676. DOI: 10.1093/jee/83.5.1671.

Talekar NS & Shelton AM (1993). Biology, ecology, and management of the diamondback moth. *Annu Rev Entomol* 38: 275–301. DOI: 10.1146/annurev.en.38.010193.001423.

Tanaka H & Kimura Y (1991). Resistance to BT Formulation in diamondback moth, *Plutella xylostella* L., on watercress. *Jpn J Appl Entomol Zool* 35: 253–255. DOI: 10.1303/jjaez.35.253.

Troczka BJ, Williamson MS, Field LM & Davies TGE (2017). Rapid selection for resistance to diamide insecticides in *Plutella xylostella* via specific amino acid polymorphisms in the ryanodine receptor. *NeuroToxicology* 60: 224–233. DOI: 10.1016/j.neuro.2016.05.012.

Tsukahara Y, Sonoda S, Fujiwara Y, Nakasuji F & Tsumuki H (2003). Molecular analysis of the para-sodium channel gene in the pyrethroid-resistant diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Appl Entomol Zool* 38: 23–29. DOI: 10.1303/aez.2003.23.

Uesugi R (2021). Historical changes in the lethal effects of insecticides against the diamondback moth, *Plutella xylostella* (L.) *Pest Management Science* 77: 3116–3125. DOI: 10.1002/ps.6344.

Wang J, Wang X, et al. (2016). A three amino acid deletion in the transmembrane domain of the nicotinic acetylcholine receptor 6 subunit confers high-level resistance to spinosad in *Plutella xylostella. Insect Biochemistry and Molecular Biology* 71: 29–36. DOI: 10.1016/j.ibmb.2016.02.001.

Wang X, Khakame SK, Ye C, Yang Y & Wu Y (2013). Characterisation of field-evolved resistance to chlorantraniliprole in the diamondback moth, *Plutella xylostella*, from China. *Pest Management Science* 69: 661–665. DOI: 10.1016/j.ibmb.2016.02.001.

Wei SJ, Shi BC, et al. (2013). Genetic structure and demographic history reveal migration of the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) from the southern to northern regions of China. *PLoS One* 8: e59654. DOI: 10.1371/journal.pone.0059654.

Wei Y, Guan F, Wang R, Qu C & Luo C (2021). Amplicon sequencing detects mutationsassociated with pyrethroid resistance in *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Pest Management Science* 77: 2914–2923. DOI: 10.1002/ps.6327.

Weir BS & Cockerham CC (1984). Estimating F-Statistics for the analysis of population structure. *Evolution* 38: 1358–1370. DOI: 10.2307/2408641.

Zhao J.-, Li Y.-, et al. (2002). Monitoring and characterization of diamondback moth (Lepidoptera: Plutellidae) resistance to spinosad. *J Econ Entomol* 95: 430–436. DOI: 10.1603/0022-0493-95.2.430.

Zhao S, Jiang D, et al. (2021). Independent and synergistic effects of knocking out two ABC transporter genes on resistance to *Bacillus thuringiensis* toxins Cry1Ac and Cry1Fa in diamondback moth. *toxins* 13: 9. DOI: 10.3390/toxins13010009.

Supporting Information:

Feasibility trial of genomics-based diagnosis detecting insecticide resistance of diamondback moth

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The final published version is available at: https://doi.org/10.1002/ps.6776.

Figure S1

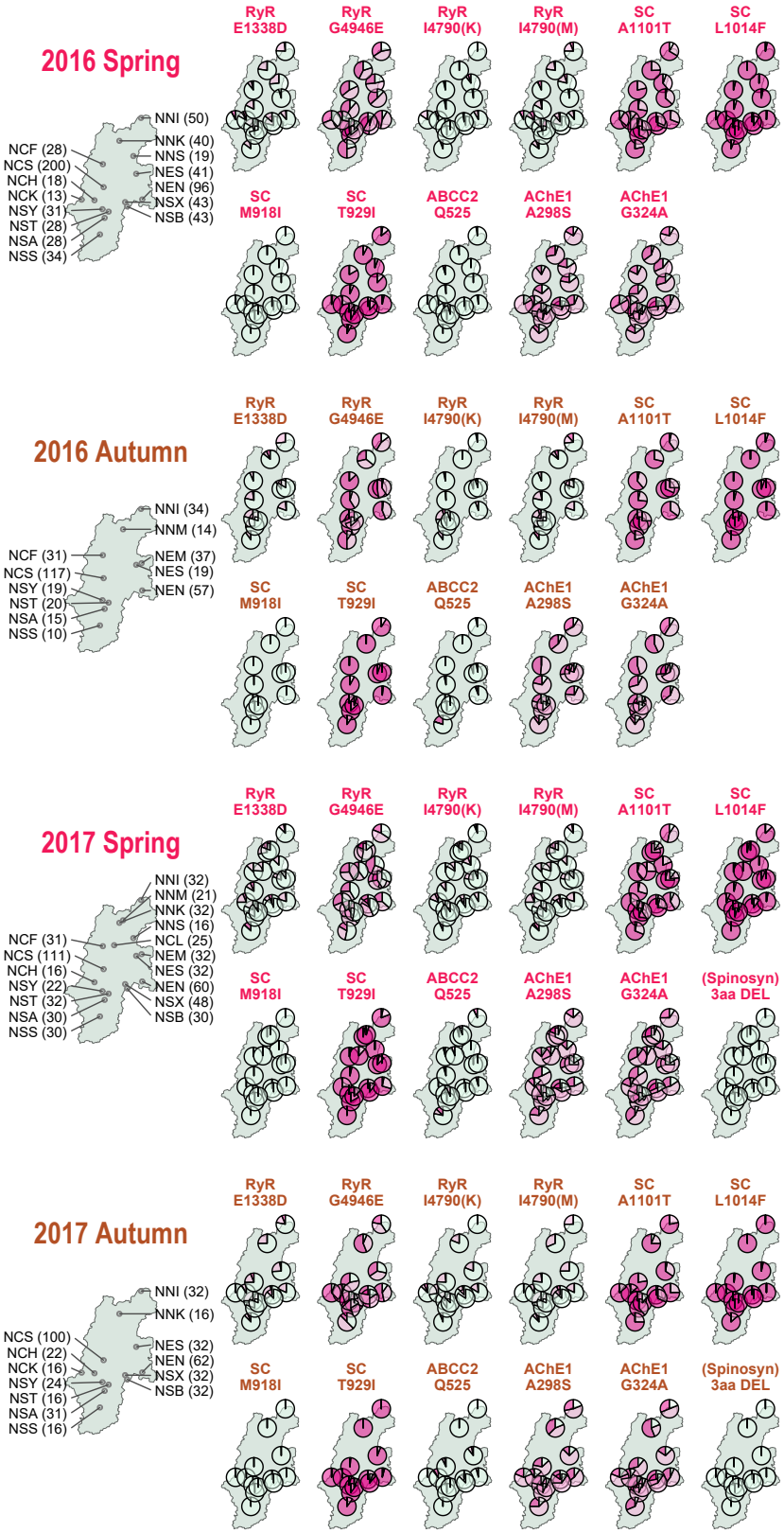

Figure S1. Spatial and temporal patterns of the genotype ratio of pesticide-resistance related markers. Red represents resistant homozygotes, pink represents heterozygotes, and light blue is susceptible homozygotes. Locations of the sampling sites and numbers of samples are depicted on the left map for each season. For corresponding resistant mutations, see Table S1.

Figure S2

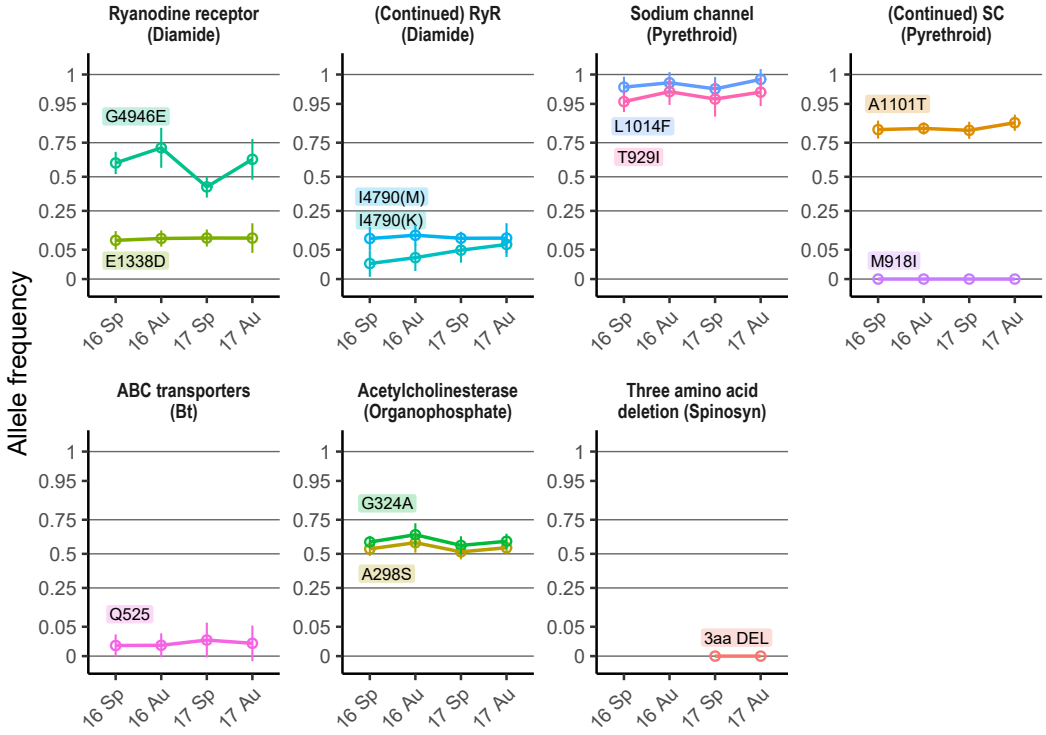

Figure S2. Mean percentage of resistant mutations among sites. Y-axes are arcsine square-root-transformed to exaggerate the low frequencies. Error bars represent the standard deviations among sites calculated after arcsine square-root-transformation of frequencies.

Figure S3

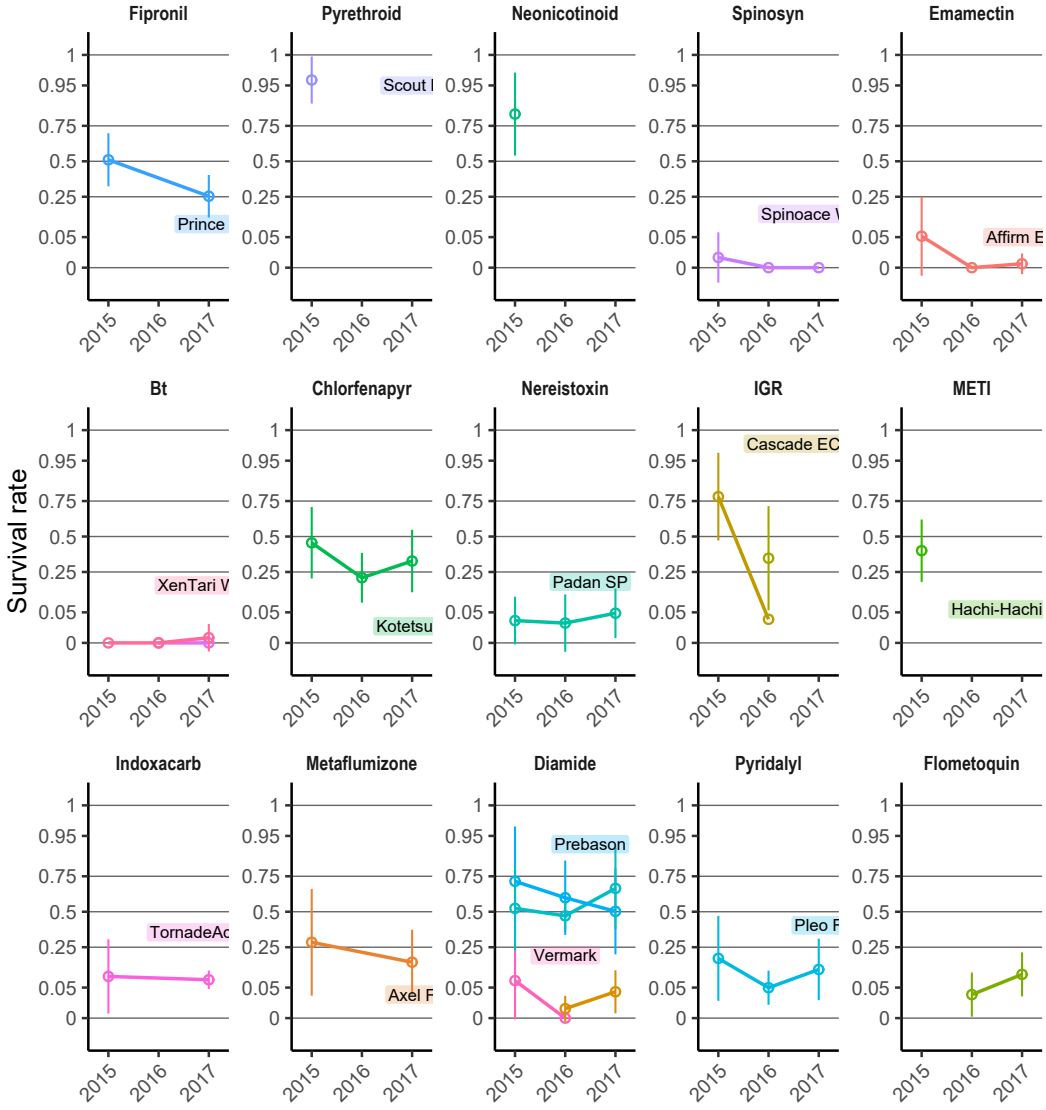

Figure S3. Mean survival rate among sites after insecticide treatments (data are the same as Table S6). Y-axes were arcsine square root-transformed to exaggerate the low frequencies. Error bars represent the standard deviations among sites calculated after arcsine square root-transformation of the frequencies to secure homoscedasticity in proportional data.

Table S1

Markers related to insecticide resistance (single nucleotide markers [SNPs] and small insertions/deletions [INDELs]) in our study. Markers in bold are used in the main analyses.

Gene code	Resistant gene	Target insecticide	Reference
G4946E **I4790M** E1338D	Ryanodine receptor (RyR)	Diamide (flubendiamide, chlorantraniliprole)	Sonoda, Inukai, et al. (2017)
I4790K	Ryanodine receptor (RyR)	Diamide (flubendiamide, chlorantraniliprole, cyantraniliprole)	Jouraku et al. (2020)
L1014F T929I **M918I** A1101T	Sodium channel (SC)	Pyrethroid	Tsukahara et al. (2003)
Q525*	ABC transporters (ABCC2)	Bt (Cry1Ac)	Supporting Information2
3aa DEL	A three amino acid deletion	Spinosyn (spinosad)	Lee et al. (2007) and Sonoda & Igaki (2010)
A298S G324A	Acetylcholinesterase (AChE1)	Organophosphate	Wang, Wang, et al. (2016)

E1338D are resistant SNPs against diamide
T929I and A1101T against pyrethroid
G324A against organophosphate
These were also tested through genotyping analyses by MiSeq (results not shown in the main text). These were not major resistant mutations in Japan and/or coincide with other major resistant mutations identified in our analysis.

Table S2

Mutation combinations of ryanodine receptor and their frequency.

No	Mutation combination			Frequency of mutation combination			
	E1338D	I4790(M|K)	G4946E	2016Spring	2016Autumn	2017Spring	2017Autumn
1	E	I	E	0.407	0.54	0.242	0.411
2	E	I	G/E	0.314	0.188	0.257	0.143
3	E/D	I/M	G/E	0.082	0.14	0.045	0.117
4	E	I	G	0.079	0.03	0.25	0.091
5	E/D	I/M	G	0.025	0.005	0.059	0.033
6	E	I/K	G/E	0.025	0.04	0.043	0.119
7	E/D	M	G	0.015	0.019	0.009	0.005
8	E/D	M	G/E	0.013	0.011	0	0
9	D	M	G	0.008	0.005	0.014	0.009
10	E/D	I	G/E	0.006	0	0	0
11	E	I/K	G	0.006	0	0.028	0.009
12	D	I/M	G/E	0.003	0.008	0.009	0.007
13	D	M	G/E	0.003	0	0	0
14	E/D	I	E	0.003	0	0	0.007
15	E/D	I	G	0.003	0	0	0
16	E	K	G/E	0.003	0.008	0	0
17	E/D	I/K	G/E	0.001	0	0	0
18	E	K	G	0.001	0	0.01	0.012
19	E	I/M	E	0.001	0.003	0	0
20	E	I/M	G	0.001	0.003	0.005	0
21	D	I/M	G	0	0	0.016	0.012
22	D	M/K	G	0	0	0.002	0
23	E/D	I/K	G	0	0	0.002	0
24	E/D	M/K	G	0	0	0.009	0.019
25	E	I/M	G/E	0	0	0.002	0.007

Table S3

Frequency of haplotypes with G4946E mutation of ryanodine receptor.

Haplotype ID	G4946E site haplotype GAG(E), GAA(E)	Count of haplotypes				Frequency of haplotypes				Sequence of haplotype †
		2016S	2016A	2017S	2017A	2016S	2016A	2017S	2017A	
HT1	GAG	423	280	209	241	0.476	0.505	0.525	0.479	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTGTACTCTCTGTGGTAC TTCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCCGCTCATCTGT TGGACGTGGCTGTTGAGTTCAAGACCCTGAGGACTATCTTGC
HT2	GAG	195	123	73	106	0.220	0.222	0.183	0.211	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTGTACTCTCTGTGGTAC TTCTCGTTCTCTGTGATGGGCAATTTCAACCACTTCTTCTTCGCCGCACATCTGT TGGACGTGGCTGTTGAGTTCAAGACTCTGAGGACTATCTTGC
HT3	GAG	123	57	71	80	0.139	0.103	0.178	0.159	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTCTACTCTCTATGGTAC TTCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCGGCTCATCTGT TGGACGTGGCTGTTGAGTTCAAGACTCTGAGGACTATCTTGC
HT4	GAG	106	68	23	51	0.119	0.123	0.058	0.101	GGAAGGCGGGCGTCACGATCACAGACAACTCTTTCCTCTACTCTCTATGGTACT TCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCGGCTCATCTGTT GGACGTGGCTGTCGAGTTCAAGACTCTGAGGACTATCTTGC
HT5	GAA	39	19	19	19	0.044	0.034	0.048	0.038	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTCTACTCTCTATGGTAC TTCTCGTTCTCTGTGATGGGTAACTTCAACCACTTCTTCTTCGCCGCTCATCTGT TGGACGTGGCTGTTGAATTCAAGACGCTGAGGACTATCTTGC
HT6	GAG	1	5	0	3	0.001	0.009	0.000	0.006	GGAAGGCGGGCGTCACGATCACAGACAACTCATTCCTCTACTCTCTATGGTAC TTCTCGTTCTCTGTGATGGGTAACTTCAACCACTTCTTCTTCGCCGCTCATCTGT TGGACGTAGCTGTTGAGTTCAAGACCCTGAGGACTATTCTGC
HT7	GAA	0	0	0	1	0.000	0.000	0.000	0.002	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTCTACTCTCTATGGTAC TTCTCGTTCTCTGTGATGGGTAACTTCAACCACTTCTTCTTCGCCGCTCATCTGT TGGACGTGGCTGTTGAATTCAAGACCCTGAGGACTATCTTGC
HT8	GAG	0	0	0	1	0.000	0.000	0.000	0.002	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTGTACTCTCTGTGGTAC TTCTCGTTCTCTGTGATGGGCAATTTCAACCACTTCTTCTTCGCCGCACATCTGT TGGACGTGGCTGTTGAGTTCAAGACCCTGAGGACTATCTTGC
HT9	GAG	0	1	0	0	0.000	0.002	0.000	0.000	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTGTACTCTCTGTGGTAC TTCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCCGCTCATCTGT TGGACGTGGCTGTTGAGTTCAAGACCTTGAGGACTATCTTGC
HT10	GAG	0	0	1	0	0.000	0.000	0.003	0.000	GGAAGGCGGGAGTCACGATCACAGACAACTCTTTCCTCTACTCTCTATGGTACT TCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCCGCTCATCTGTT GGACGTGGCTGTTGAGTTCAAGACGCTGAGGACTATTCTGC
HT11	GAG	0	0	1	0	0.000	0.000	0.003	0.000	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTGTACTCTCTGTGGTAC TTCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCGGCTCATCTGT TGGACGTGGCTGTTGAGTTCAAGACTCTGAGGACTATCTTGC
HT12	GAG	0	1	0	0	0.000	0.002	0.000	0.000	GGAAGGCGGGCGTCACGATCACAGACAACTCGTTCCTCTACTCTCTATGGTAC TTCTCATTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCCGCTCATCTGT TGGACGTGGCTGTTGAGTTCAAGACTCTGAGGACTATTCTGC
HT13	GAG	0	0	1	0	0.000	0.000	0.003	0.000	GGAAGGCGGGCGTCACGATCACAGACAACTCATTCCTCTACTCTCTATGGTAC TTCTCTTTCTCTGTGATGGGTAACTTCAACCACTTCTTCTTCGCTGCACATCTGT TGGACGTGGCTGTTGAGTTCAAGACACTGAGGACTATCTTGC
HT14	GAG	1	0	0	0	0.001	0.000	0.000	0.000	GGAAGGCGGGCGTCACGATCACAGACAACTCTTTCCTGTACTCTCTGTGGTACT TCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCCGCTCATCTGTT GGACGTGGCTGTTGAGTTCAAGACCCTGAGGACTATCTTGC
HT15	GAG	0	0	0	1	0.000	0.000	0.000	0.002	GGAAGGCGGGCGTCACGATCACAGACAACTCTTTCCTCTACTCTCTATGGTACT TCTCGTTCTCTGTGATGGGCAACTTCAACCACTTCTTCTTCGCGGCTCATCTGTT GGACGTGGCCGTTGAGTTCAAGACTCTGAGGACTATCTTGC

†170 bp region flanking G4946E site (123-125bp)

Table S4

Frequency of haplotypes with A298S and/or G324A mutations of acetylcholinesterase1 (AChE1).

Haplotype ID	A298S site haplotype TCC(S), GCC(A)	G324A site haplotype GCA(A), GGA(G)	Count of haplotypes				Frequency of haplotypes				Sequence of haplotype 124 bp region (exon:1-33bp and intron: 34-124bp) flanking L1014F site (28-30bp)
			2016S	2016A	2017S	2017A	2016S	2016A	2017S	2017A	
HT1	TCC	GCA	748	429	337	425	0.846	0.855	0.840	0.855	ATATACAATACTTTGGCGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT2	GCC	GCA	56	32	18	29	0.063	0.064	0.045	0.058	ATATACAATACTTCGGCGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGGCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT3	GCC	GCA	49	25	22	33	0.055	0.050	0.055	0.066	ATATACAATACTTTGGCGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGGCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT4	TCC	GCA	12	7	0	7	0.014	0.014	0.000	0.014	ATATACAATACTTTGGCGGAAACCCTCATAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT5	TCC	GCA	0	0	20	0	0.000	0.000	0.050	0.000	ATATACAATACTTTGGCGGCAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT6	GCC	GCA	2	2	0	1	0.002	0.004	0.000	0.002	ATATACAATACTTTGGCGGAAACCCTCATAATGTCACATTGTTTGGAGAATCGGCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT7	GCC	GCA	0	0	4	0	0.000	0.000	0.010	0.000	ATATACAATACTTTGGCGGCAACCCTCACAATGTCACATTGTTTGGAGAATCGGCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT8	TCC	GCA	2	2	0	0	0.002	0.004	0.000	0.000	ATATACAATACTTTGGTGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT9	TCC	GCA	2	1	0	0	0.002	0.002	0.000	0.000	ATATACAATACTTTGGCGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCGAGAGA
HT10	GCC	GCA	1	0	0	1	0.001	0.000	0.000	0.002	ATATACAATACTTTGGCGGAAACCCTCACAATGTTACATTGTTTGGAGAATCGGCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT11	TCC	GGA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGGAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT12	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCATGGGCCATCATTTCCAGAGA
HT13	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAACCCTCACAATGTCACATTGTTCGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT14	GCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAACCCTCATAATGTCACATTGTTTGGAGAATCGGCCGGTGCGGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT15	TCC	GCA	0	1	0	0	0.000	0.002	0.000	0.000	ATATACAATACTTTGGCGGAAAATCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT16	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGGGGAAAATCCTCATAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT17	TCC	GCA	0	1	0	0	0.000	0.002	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCGCCTTGGGCCATCATTTCCAGAGA
HT18	TCC	GCA	0	1	0	0	0.000	0.002	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATTATTTCCAGAGA
HT19	GCC	GCA	0	1	0	0	0.000	0.002	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCTCATAATGTCACATTGTTTGGGGAATCGGCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT20	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCTCACAATGTTACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT21	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCAGCACCTTGGGCCATCATTTCGAGAGA
HT22	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATATTTTGGCGGAAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT23	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATATTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT24	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCCCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT25	GCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGCGGAAAACCCTCATAATGTCACATTGTTTGGAGAATCGGCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCTCTATCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT26	TCC	GCA	1	0	0	0	0.001	0.000	0.000	0.000	ATATACAATACTTTGGTGGAAAACCCTCATAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA
HT27	TCC	GCA	0	0	0	1	0.000	0.000	0.000	0.002	ATATACAATACTTCGGCGGAAACCCTCACAATGTCACATTGTTTGGAGAATCGTCCGGTGCAGTTTCCGTGTCATTACATTTACTGTCTCCGCTGTCAAGAAACATGTTTTCTCAAGCTATTATGCAATCTGCAGCCGCATCTGCACCTTGGGCCATCATTTCCAGAGA

†169 bp region flanking A298S site (54-56bp) and G324A site (132-134bp)

Table S5

Frequency of haplotypes with L1014F mutation of voltage-gated sodium channel.

Haplotype ID	L1014F site haplotype TTT(F)	Count of haplotypes				Frequency of haplotypes				sequence of haplotype † 124 bp region (exon:1-33bp and intron: 34-124bp) flanking L1014F site (28-30bp)
		2016S	2016A	2017S	2017A	2016S	2016A	2017S	2017A	
HT1	TTT	785	189	296	497	0.937	0.931	0.703	0.910	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTGCCCTGGTT TGAGTATTAAACGA
HT2	TTT	0	0	68	13	0	0	0.162	0.024	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTGCCCTGGTT TGAGTATTAAACGT
HT3	TTT	40	12	8	19	0.048	0.059	0.019	0.035	TTCTTGGCCACCGTCGTCATTGGCAATTTTGTGGTAAGCTGTTGTCTCTTTGCTA TAATCTTAGTTGTTACCTTCTTTGGATTCGGTATTTAAAAGTGTTTGCCCTGGTT TGAGTATTAAAAGA
HT4	TTT	8	2	20	9	0.010	0.010	0.048	0.016	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTCTCCTGGTT TGGGTATAATTAGT
HT5	TTT	1	0	12	4	0.001	0	0.029	0.007	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGCTGTGTCTTTGCT ATCATCTGAGTTGTTACCTTCTTTGGATTTTGTATTTAAAAGTGTTTGCCCTGGT TTAGGTATTAAAAGA
HT6	TTT	4	0	3	2	0.005	0	0.007	0.004	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTTCTGTCTCTTTGCTA TCATCTGAGTTGTTACCTTCTTTAGATTTTTTATTTAAAAGTGTTTGCCCTGGTT TGGGTATTAAAACA
HT7	TTT	0	0	6	1	0	0	0.014	0.002	TTCTTGGCCACCGTCGTCATTGGCAATTTTGTGGTAAGCTGTTGTCTCTTTGCTA TAATCTTAGTTGTTACCTTCTTTGGATTCGGTATTTAAAAGTGTTTGCCCTGGTT TGAGTATTAAACGA
HT8	TTT	0	0	2	0	0	0	0.005	0	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTGCCCTGGTT TGAGTATTAAAAGA
HT9	TTT	0	0	2	0	0	0	0.005	0	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGCTGTGTCTTTGCT ATCATCTGAGTTGTTACCTTCTTTGGATTTTGTATTTAAAAGTGTTTGCCCTGGT TTGAGTATTAAACGA
HT10	TTT	0	0	1	0	0	0	0.002	0	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTGCCCTGGTT TGTGTATTAAACGA
HT11	TTT	0	0	1	0	0	0	0.002	0	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTGCTCTGGTT TGAGTATTAAACGA
HT12	TTT	0	0	0	1	0	0	0	0.002	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGACCTCAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTGCCCTGGTT TGAGTATTAAACAA
HT13	TTT	0	0	1	0	0	0	0.002	0	TTCTTGGCCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAATGTTTGCCCTGGTT TGAGTATTAAACGA
HT14	TTT	0	0	1	0	0	0	0.002	0	TTCTTGACCACCGTCGTCATTGGCAACTTTGTGGTAAGCTGTTGTTTCTTTGCTA TGATCTTAGTTGTTACCTTCTTTAGATTTTGTATTTAAAAGTGTTTGCCCTGGTT TGAGTATTAAACGA

†124 bp region (exon:1-33bp and intron: 34-124bp) flanking L1014F site (28-30bp)

Table S6

Results of the bioassay for various insecticides of practical dose.

Product name-Type † ® (Manufacturer)	IRAC code ‡	Common name	Dilution ratio	Survivorship (%) §														
				2015 (from Jun - Sept) ¶							2016 (Jun - Sept)				2017(Jun - Sept) ¶			
				NNO	NEM	NEL	NEC	NCS	NSX	NST	NNI	NNA	NCS	NSX	NEL	NCL	NCS	NSX
Prince F®(BASF Japan, Tokyo, Japan)	2	Fipronil	2000	59	57	43	37	31	53	80	-	-	-	-	24	26	33	18
Scout F® (Nippon Soda Co., Ltd., Tokyo, Japan)	3A	Tralomethrin	2000	97	90	89	100	88	100	100	-	-	-	-	-	-	-	-
Mospilan WDG® (Nippon Soda Co., Ltd., Tokyo, Japan)	4A	Acetamiprid	2000	96	56	93	80	55	100	80	-	-	-	-	-	-	-	-
Spinoace WDG® (Corteva Agriscience, Wilmington, Delaware)	5	Spinosad	5000	0	0	0	0	1	30	0	0	0	0	0	0	0	0	0
Affirm EC® (Syngenta Japan Co., Ltd., Tokyo, Japan)	6	Emamectin benzoate	2000	18	1	0	0	0	47	27	0	0	0	0	2	0	0	0
XenTari WDS® (Valent BioScience LLC, Libertyville, Illinois)	11A	Cry IAa, Cry IAb, Cry IC, Cry ID	2000	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
Tune-Up WDS® (Agro-Kanesho Co., Ltd., Tokyo, Japan)	11A	Cry IAb	2000	-	-	-	-	-	-	-	0	0	0	0	-	-	-	-
ToarowCT WP® (Otsuka chemical Co., Ltd., Tokyo, Japan)	11A	Cry IAb	1000	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0
Kotetsu F® (Nippon Soda Co. Ltd., Tokyo, Japan)	13	Chlorfenapyr	2000	48	28	79	50	22	83	50	10	0	22	37	17	50	40	25
Padan SP® (Sumitomo Chemical Co., Ltd., Tokyo, Japan)	14	Cartap hydrochloride	1500	11	5	11	10	0	0	0	0	0	0	7	1	6	8	18
Cascade EC® (BASF Japan, Tokyo, Japan)	15	Flufenoxuron	4000	98	65	43	93	51	92	70	0	3	0	0	-	-	-	-
Falcon F® (Corteva Agriscience, Wilmington, Delaware)	18	Methoxy fenozide	1000	-	-	-	-	-	-	-	53	0	56	3	-	-	-	-
Hachi-Hachi® (Nihon Nohyaku Co., Ltd., Tokyo, Japan)	21A	Tolfenpyrad	1000	49	56	7	17	43	30	53	-	-	-	-	-	-	-	-
TornadeAce DF® (FMC, Tokyo, Japan)	22A	Indoxacarb	2000	42	8	0	7	10	13	0	-	-	-	-	10	7	5	13
Axel F® (Nihon Nohyaku Co., Ltd., Tokyo, Japan)	22B	Metaflumizone	1000	72	27	7	12	3	83	17	-	-	-	-	27	23	5	25
Phoenix WDG® (Nihon Nohyaku Co. Ltd., Tokyo, Japan)	28	Flubendiamide	2000	46	61	0	65	50	3	93	53	0	45	50	57	83	47	93
Prebason® (FMC, Tokyo, Japan)	28	Chlorantraniliprole	2000	43	98	93	62	76	7	80	0	0	56	70	72	60	11	71
Vermark® (FMC, Tokyo, Japan)	28	Cyantraniliprole	4000	0	5	0	7	3	10	0	0	0	0	0	-	-	-	-
Benevia® (FMC, Tokyo, Japan)	28	Cyantraniliprole	4000	-	-	-	-	-	-	-	0	0	1	0	1	6	8	7
Pleo F® (Sumitomo Chemical Co., Ltd., Tokyo, Japan)	UN	Pyridalyl	1000	3	26	46	10	3	67	43	7	0	7	3	12	15	27	0
FineSave F® (Nissan Chemical Corporation, Tokyo, Japan)	UN	Flometoquin	1000	-	-	-	-	-	-	-	3	0	2	17	9	15	12	11

† DF: dry flowable, EC: emulsifiable concentrate, F: flowable, OD: oil dispensable solution, SP: water-soluble powder, WDG: water-dispensable granule, WP: water powder

‡ Insecticide Resistance Action Committee (IRAC) Mode of action classification (accessed November 2020)

§10 individuals of third instar larvae, which were the first to third generations of the field-collected larvae, were tested using each insecticide product three times (30 individuals in total). Middle (>10%) and high survivorship (>50%) after 96 h of insecticide treatment are shaded in light and dark grey, respectively. The geographic locations of the codes can be found in Figure S3.

¶The data in 2015 and 2017 (except those of Vermark) have already been published (Kitabayashi & Sato 2020).

Supporting Information1 (Library construction)

Six libraries of multiplexed amplicon sequences for 2213 individuals collected in the four seasons were constructed to identify genotypes of the mutation sites related to insecticide resistance in Table S1 in each individual. Each library included 384 individuals, except for the sixth run, which included 293 individuals. The method used to construct the libraries is described below.

Each male diamondback moth used for the amplicon sequencing in Figure S1 was placed in a 96-well PCR plate (0.2 mL). DNA was extracted from each sample by adding 200 μL of DNA extraction buffer (10 mM Tris-HCl pH 9.0, 1 mM EDTA, 0.1% Triton-X100) to each well, incubating in a thermal cycler for 15 min at 99.9 °C, and centrifuging for 5 min at 750 × g. The forward primers for amplicon PCR (8 μM for each primer) in Table S7 were 5'-phosphorylated by mixing with 1 × T4 polynucleotide kinase buffer, 1 mM ATP, and 10 U T4 polynucleotide kinase (Takara Bio, Japan) in a reaction volume of 400 μL for 90 min at 37 °C. They were then mixed with reverse primers of amplicon PCR (Table S7) in a final concentration of 1 μM per primer. The first PCR (multiplex PCR of target DNA fragments including SNP/INDEL sites in Table S8) was then performed with the extracted DNA using the primer mix. The volume of the PCR mixture was 6 μL, including 3 μL of 2 × Multiplex PCR buffer in Multiplex PCR Assay Kit Ver.2 (Takara Bio, Japan), 1.2 μL of the primer mix (1 μM for each primer of amplicon PCR), 0.03 μL of Multiplex PCR enzyme mix in the Multiplex PCR Assay Kit Ver.2, 1.2 μL of template DNA, and 0.57 μL of distilled water. The PCR conditions were as follows: 94 °C for 60 s; 30 cycles at 94 °C for 30 s, and 60 °C for 60 s; and 72 °C for 10 min.

To add unique indices to the first PCR product of each individual, the combination of internal barcodes and Illumina p5/p7 indices (384 combinations in total) were added to each individual in turn in the following steps. Hybridization of barcoded Illumina adapters (internal barcode) in Table S7 was performed to prepare a 4 μM working adapter stock in hybridization buffer (0.1 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8,0) with the following hybridization program in a thermal cycler: 75 °C for 5 min, ramping down to 25 °C at 1 °C per second for 50 min, and 25 °C for 30 min. The first PCR product was then mixed with the working adapter in a total volume of 25 μL, including 6 μL of the first PCR product, 2 μL of the 4 μM working adapter, 2.5 μL of 10 × Ligase buffer, and 0.1 μL of T4 DNA Ligase (NIPPON GENE, Japan), 3 μL of ethanol, and 11.6 μL of distilled water followed by incubating for 30 min at 20 °C. The adapter-ligated DNA product was then purified by mixing with 16 μL of AMPureXP (Beckman Coulter, USA), 8 μL of 20% PEG8000, 2.5 M NaCl, and 7 μL of ethanol. Samples were then incubated for 5 min at room temperature. The magnetic beads were separated with a SPRIPlate 96 Ring Super magnet plate, washed twice with 75

The second PCR was then performed with the product to add p5/p7 indices (Table S9). The volume of the PCR mixture was 6 μL, including 1 μL of the adapter-ligated DNA product, 0.48 μL of 2.5 mM dNTP, 1.2 μL of 5 × Q5 reaction buffer, 0.03 μL of Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, USA), 1.2 pmoles of each p5/p7 index primer, and 6 μL of distilled water. The PCR conditions were as follows: 98 °C for 30 s; 16 cycles of 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 5 min. All PCR products were combined and concentrated by ethanol precipitation. The final product was purified by removing the primers using AMPureXP beads, and eluted with 50 μL of 10 mM Tris-HCl at pH 8.5.

Table S7

Amplicon PCR primers used for multiplexed amplicon sequencing.

Name	Target gene	Target SNP/INDEL	Orientation	Sequence (5'-3')	Product size (bp)	Target scaffold ID
Primers used for all samples						
px_ryr_4946_F	Ryanodine receptor (RyR)	G4946E	forward	AGACTGGCGCTACCAAGTGT	190	unitig_16226
px_ryr_4946_R			reverse	CCCGTTATGCGTGACAGACT		
px_ryr_1338_F	Ryanodine receptor (RyR)	E1338D	forward	GCTGGGTGGAGATCAAGGA	105	unitig_16226
px_ryr_1338_R			reverse	TCATAGTGAACCCGCTCCTC		
px_ryr_4790_F	Ryanodine receptor (RyR)	I4790M I4790K	forward	AAGAYCCSATAGAGCTGGTG	125	unitig_16226
px_ryr_4790_R			reverse	CTTCAAATGGTAGTAYCCGATC		
px_ache1_298-324_F	acetylcholinesterase1 (AChE1)	A298S G324A	forward	GTTGCAGTGGGTGAAAGACA	209	unitig_1919
px_ache1_298-324_R			reverse	TGCCCCTTATCACACTCTCC		
px_abcc2_525_F	ABC transporter C2 (ABCC2)	Q525*	forward	DAARGAGCTTCCGGTGTCKT	209	unitig_1130
px_abcc2_525_R	ABC transporter C2 (ABCC2)		reverse	CCRAAGAGGATGTTRTCCMG		
Primers used for all samples except for 293 samples collected in 2017 (sequenced by the first to the fifth MiSeq runs)						
px_sc_918-929_F1	voltage gated sodium channel (Nav)	M918I T929I	forward	TCGAGTATTCAAATTGGCAAAG	152	unitig_16115
px_sc_918-929_R1			reverse	TTTTCCCGAATAGTTGCATACC		
px_sc_1014_F1	Nav	L1014F	forward	GGACTTCATGCACAGCTTCA	352	unitig_16115
px_sc_1014_R1			reverse	TGAAGGCTTCCGCTATTTTG		
px_sc_1101_F1	Nav	A1101T	forward	TTAAAGGCGGCTCTCTGTG	307	unitig_16115
px_sc_1101_R1			reverse	CCCTATGATTGTCCGTTATGG		
Primers used for 293 samples collected in 2017 (sequenced by the sixth MiSeq run))						
px_sc_918-929_F1	Nav	M918I T929I	forward	CAAAGTCATGGCCGACAC	132	unitig_16115
px_sc_918-929_R1			reverse	TCCCGAATAGTTGCATACCC		
px_sc_1014_F1	Nav	L1014F	forward	ACGTCTCCTGTATCCCCTTC	184	unitig_16115
px_sc_1014_R1			reverse	GGCCAAGAAAAGGTTAAGTACC		
px_sc_1101_F1	Nav	A1101T	forward	CAGACCGAGTGGACAACGAG	103	unitig_16115
px_sc_1101_R1			reverse	TCGCCTATGGCTACTTCGAC		
Primers used for samples collected in 2017 (sequenced by the fourth to sixth MiSeq runs))						
px_nAChR_a6_3del_F	nicotinic acetylcholine receptor α6 subunit (nAChR α6)	3aa deletion at TM4	forward	GTTTTGCCTGTTCGTGTTCA	189	unitig_14226
px_nAChR_a6_3del_R			reverse	ATGAATGGGCGTGATCTAAG		

The product size of each primer pair is based on the mapped position on the reference genome assembly of *Plutella xylostella* available at Lepbase.

Table S8

Barcoded Illumina adapter primers used for multiplexed amplicon sequencing.

Name	Barcode	Orientation	Sequence
Primers used for samples collected in 2016			
AGCTA_TA_P1.1	AGCTA	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTAT
AGCTA_TA_P1.2n		reverse	TAGCTAGATCGGAAGAGCACACGTCTGAACTCCAG
ATACG_TA_P1.1	ATACG	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATACGT
ATACG_TA_P1.2n		reverse	CGTATAGATCGGAAGAGCACACGTCTGAACTCCAG
GAGAT_TA_P1.1	GAGAT	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGATT
GAGAT_TA_P1.2n		reverse	ATACTAGATCGGAAGAGCACACGTCTGAACTCCAG
GAGTC_TA_P1.1	GAGTC	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGTCT
GAGTC_TA_P1.2n		reverse	GACTCAGATCGGAAGAGCACACGTCTGAACTCCAG
Primers used for samples collected in 2017			
CTGCG_TA_P1.1	CTGCG	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGCGT
CTGCG_TA_P1.2n		reverse	CGCAGAGATCGGAAGAGCACACGTCTGAACTCCAG
CTGTC_TA_P1.1	CTGTC	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTCT
CTGTC_TA_P1.2n		reverse	GACAGAGATCGGAAGAGCACACGTCTGAACTCCAG
GACAC_TA_P1.1	GACAC	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACACT
CTGTC_TA_P1.2n		reverse	GTGTRCAGATCGGAAGAGCACACGTCTGAACTCCAG
TACTG_TA_P1.1	TACTG	forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTACTGT
TACTG_TA_P1.2n		reverse	CAGTAAGATCGGAAGAGCACACGTCTGAACTCCAG

Internal barcodes (five bp) in sequences are coloured in red. The barcode is detected as the first five bp of each sequenced read. The four internal barcode combinations were used to demultiplex sequenced reads (up to 384 samples) with the combination of Illumina p5/p7 indices (96 combinations) in Table S7.

Table S9

Illumina index primers used for multiplexed amplicon sequencing.

Name	Index	Sequence
p5 index		
D501	TATAGCCT	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACG
D502	ATAGAGGC	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACG
D503	CCTATCCT	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGACG
D504	GGCTCTGA	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGACG
D505	AGGCGAAG	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACG
D506	TAATCTTA	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACG
D507	CAGGACGT	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGACG
D508	GTACTGAC	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGACG
p5 index		
D701	ATTACTCG	CAAGCAGAAGACGGCATACGAGATATTACTCGGTGACTGGAGTTCAGACGTGTGC
D702	TCCGGAGA	CAAGCAGAAGACGGCATACGAGATTCCGGAGAGTGACTGGAGTTCAGACGTGTGC
D703	CGCTCATT	CAAGCAGAAGACGGCATACGAGATCGCTCATTGTGACTGGAGTTCAGACGTGTGC
D704	GAGATTCC	CAAGCAGAAGACGGCATACGAGATGAGATTCCGTGACTGGAGTTCAGACGTGTGC
D705	ATTCAGAA	CAAGCAGAAGACGGCATACGAGATATTCAGAAGTGACTGGAGTTCAGACGTGTGC
D706	GAATTCGT	CAAGCAGAAGACGGCATACGAGATGAATTCGTGTGACTGGAGTTCAGACGTGTGC
D707	CTGAAGCT	CAAGCAGAAGACGGCATACGAGATCTGAAGCTGTGACTGGAGTTCAGACGTGTGC
D708	TAATGCGC	CAAGCAGAAGACGGCATACGAGATTAATGCGCGTGACTGGAGTTCAGACGTGTGC
D709	CGGCTATG	CAAGCAGAAGACGGCATACGAGATCGGCTATGGTGACTGGAGTTCAGACGTGTGC
D710	TCCGCGAA	CAAGCAGAAGACGGCATACGAGATTCCGCGAAGTGACTGGAGTTCAGACGTGTGC
D711	TCTCGCGC	CAAGCAGAAGACGGCATACGAGATTCTCGCGCGTGACTGGAGTTCAGACGTGTGC
D712	AGCGATAG	CAAGCAGAAGACGGCATACGAGATAGCGATAGGTGACTGGAGTTCAGACGTGTGC

Eight bp index in sequences are coloured with red. The combination of eight p5 indexes and 12 p7 indexes gave 96 combinations. Combing the p5/p7 indices with the internal barcodes (four combinations) in Table S8 gives 384 (96 × 4) different combinations.

Supporting Information2 (Genetic mutation of ABC transporters for Bt (Cry-1A) resistance)

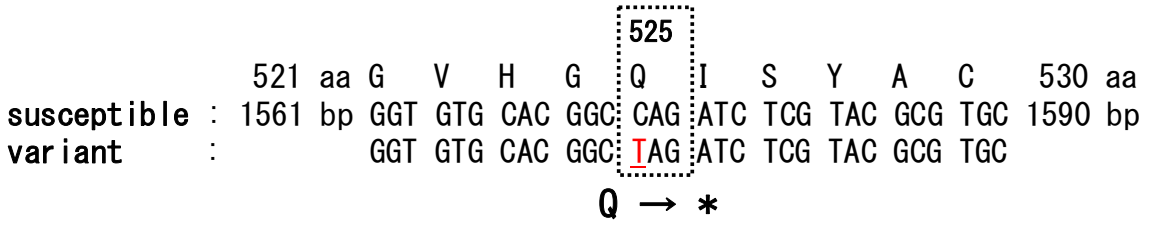

Figure S4. A single nucleotide markers introduced a premature stop-codon in the ABC transporter C2 (ABCC2) gene of *Plutella xylostella*

A premature stop-codon caused by a point mutation (C > T) in the ABCC2 gene at 525 aa (Q525*) was observed in a small percentage of individuals collected in Nagano prefecture in 2016 and 2017. Previously, we first identified the Q525* mutation in the ABCC2 gene of the diamondback moth individuals (purchased from a commercial supplier in Japan) survived after treated with 100 ppm of Cry1Ac toxin (GuardJet WP®, Kubota Corp., Osaka, Japan) by sequencing cDNA of the ABCC2 gene derived from the individuals (not published yet). The Q525* mutation was not identified in other individuals (purchased from the same supplier) susceptible to Cry1Ac toxin. The ABCC2 gene of the diamondback moth encodes a receptor for Bt Cry1Ac toxin. A 30 bp (10 aa) deletion between 1032 and 1041 aa in the ABCC2 gene of the diamondback moth is related to Cry1Ac resistance (Baxter et al. 2010). Although functional evidence is needed to confirm that the Q525* mutation is related to Cry1Ac resistance, the Q525* mutation may introduce a destructive effect on the receptor function for the Cry1Ac toxin, similar to the 10 aa deletion. The accession number of the ABCC2 gene sequence of the susceptible strain is QLH55670 (GenBank).