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Ecology, Behavior and Bionomics First Genotyping of Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) Progeny from Crosses between Bt-Resistant and Bt-Susceptible Populations, and 65-Locus Discrimination of Isofamilies

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Ecology, Behavior and Bionomics First Genotyping of Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) Progeny from Crosses between Bt-Resistant and Bt-Susceptible Populations, and 65-Locus Discrimination of Isofamilies

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Research Article

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ABSTRACT

Concerns exist that Bt-resistant populations of *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) found in Puerto Rico, could spread to continental United States, and about the lack of molecular tools to monitor potential crosses or distinguish populations. In this work, the feasibility of genotyping *S. frugiperda* crosses between *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) (Bt) resistant and susceptible populations using simple sequence repeats (SSRs, or microsatellites) was assessed. Parents and their corresponding progeny (five resistant, five susceptible phenotype) were genotyped using 192 SSRs on three reciprocate crosses alternating male and female from Bt-susceptible and Bt-resistant populations. Oviposition, mortality and fecundity were evaluated for five pairs of each of the crosses. Cluster analysis showed that progeny of each cross was associated with the paternal and not the maternal genotype, probably due to higher heterozygosity in male parents compared to females. Seven SSR markers had one or more alleles correlated ($p \leq 0.002$) with the Bt-resistant phenotype, and 18 SSRs showed uniparental inheritance. Analysis of additional samples showed genetic differences among isofamilies of *S. frugiperda* at 65 loci. The best 35 markers that discriminated isofamilies are reported, three of them related to components of the communication system in moths: pheromone, olfactory receptor and antennal esterase. This is the first report of: 1) a multi-locus genotyping in crosses of Bt-resistant and susceptible individuals of *S. frugiperda*, 2) seven codominant markers associated to Bt-resistance in this species, 3) discrimination of isofamilies by genetic differences at three loci that could affect the behavior of *S. frugiperda*.

INTRODUCTION

S. frugiperda is a polyphagous insect that causes significant losses in *Zea mays* (L.) (maize), *Arachis hypogaea* (L.) (peanut), *Gossypium spp.* (cotton), *Sorghum bicolor* (L.) (sorghum), *Oryza sativa* (L.) (rice), and other species [1,2]. *S. frugiperda* is endemic

to the American continent, and its control relies in certain areas on the use of transgenic crops expressing the Cry toxin of *Bacillus thuringiensis* (Berliner) [3]. In the year 2011, 75% of the acres planted with corn and 65% of the acres planted with cotton in the United States corresponded to Bt-toxin expressing crops according to the Economic Research Service (ERC) http://www.ers.usda.gov/Data/BiotechCropsD. In order to maintain the effectiveness of insect control by Bt toxins is important to implement management practices that minimize the development of Bt resistance in insect pests. Concerns that Bt-resistant populations found in Puerto Rico could spread to continental United States [4-6] made evident the lack of sufficient molecular tools to monitor their potential dispersal. Many studies were done to understand migration of *S. frugiperda* in the U.S.A., though, as pointed out by Nagoshi and Meagher [7] these studies sometimes had inconsistent results. Analysis of several loci were performed in order to characterize populations and find host associations in *S. frugiperda* [8,9]; but resolving issues of strain hybridization would require analysis of a large number of autosomal-codominant markers [10]. Microsatellites, also called SSRs (simple sequence repeats) or STRs (short tandem repeats), are codominant markers that could be suitable for that purpose. Few years ago, concerns were reported that transposable elements widespread throughout Lepidopteran genomes had rendered existing rnicrosatellites ineffective for their use in population studies of these insects [11]. In 2011, Arias et al. reported 192 microsatellite markers were for *S. frugiperda*, and shown their effectiveness in discriminating populations of this insect. Thus, the goal of the present work was to determine the feasibility of analyzing strain hybridization in *S. frugiperda* using a large number of microsatellites, as a further step towards their use in population genetics. For that purpose, crosses of Bt-resistant and Bt-susceptible populations of *S. frugiperda* were made: a) analyzed their biometrics and phenotyped the progeny for Bt resistance, b) genotyped parents and progeny using 192 microsatellites, c) explored the possible association between sex and heterozygocity at the studied loci, d) screened the microsatellite markers for their potential association to the Bt-resistant phenotype, and e) the potential discrimination of isofamilies using these markers. Some statistics on the abundance of transposon-related sequences in our microsatellite-enriched libraries generated from genomic DNA are also reported in this study.

MATERIALS AND METHODS

S. frugiperda colonies

For reciprocal crosses, two strains of *S. frugiperda* were used: colony FAW-512 which has resistance to Bt toxins Cry1Ac and Cry1Fa, collected from a maize field in Santa Isabella, Puerto Rico in 2008 and was previously reported [12]; and colony FAW-Mon (Bt-susceptible control), obtained from Monsanto Company, Memphis, TN, USA. FAW-512 has been reared for several generations by bioassay selection exposing the neonates to Bt-Cry proteins, while FAW-Mon has been cultured separately for few generations using regular diet [13]. Additional males and females from three colonies FAW-512, FAW-W and FAW-STV were also genotyped to determine their heterozygosity levels. The colony FAW-W was collected from a maize field in Washington County, MS; and FAW-STV was collected from maize expressing Bt-Cry1Ab in a field in Stoneville, both in MS, U.S.A. All colonies have been cultured by group-mated according to a method previously described [13] and were maintained at the Southern Insect Management Research Unit, USDA-ARS, in Stoneville, MS.

Crosses, Biometrics and Challenge with Crylfa Toxin

FAW-512 and FAW-Mon colonies were used for these crosses. Five single pairs of virgin F10 FAW-512♀ × virgin F5 FAW-Mon♂ and five single pairs of virgin F10 FAW-512♂ × virgin F5-FAWMon♀ were confined for mating and oviposition in 500 ml containers (Model 42505LY, Consolidated® Plastics Co., www.consolidatedplastics.com) with an open top cover with Batist cloth (15 x 15 cm) (Zweigart®, Piscataway, NJ) and inner walls covered by thin wax paper (Reynolds Cut-Rite) to receive the eggs. A plastic cup of 37 rnl (T-125, Solo®, Urban, IL) containing approximately 15 ml of a 10% solution of sucrose-water and benzoic acid 1000 mg kg- 1 was provided to each single pair as a food source. For each pair, data of pre-oviposition period, oviposition period, number of eggs laid per day per female, and mortality were collected. One hundred neonates from the first group of eggs collected from each pair were bio-assayed by overlaying the artificial diet with 10 ng cm-2 of Cry1Fa purified proteins produced in Πσευδομονασ φλυορεσχενσ (Flügel) [13]. After seven days of exposure to Cry1Fa, surviving larvae were transferred to regular diet until 5111 instar (Bt-resistant). Bt-susceptible individuals were considered those larvae that died at 3rd instar, or remained at 3rd instar after transfer to normal diet. Two sets of parents with 10 progeny each (five Bt-susceptible and five Bt-resistant) were randomly selected for SSR-genotyping at 192 loci. The experiment was repeated at a later time using the next generation of each colony: five pairs of virgin F11 FAW-512♀ × virgin F6 FAW-Mon♂ and five pairs of virgin F11 FAW-512♂ × virgin F6 FAW-Mon♀. On the repeated experiment, the first crosses reproduced normally; however, no progeny were obtained from any of the five crosses of F11 FAW-512♂ × F6 FAW-Mon ♀. Thus, only 12 individuals (2 parents, 10 progeny) from one of these crosses were used for genotyping. Differences in pre-oviposition period, oviposition, fecundity and mortality between crosses were analyzed using PROC GLM on SAS program [14].

DNA Extractions, SSR Genotyping of *S. frugiperda* Parents and their Progeny

DNA was extracted from each individual using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Though the original microsatellites (SSRs) were developed using DNA from thorax tissue from adult individuals, the genotyping in the present work was done using DNA from entire individuals. Also is important to indicate that even from small-individual 3rd instar larvae, enough DNA was obtained (≥2 µg) to run all the markers. All 192 *S. frugiperda* SSR markers previously reported [15] and developed by the method of [16], were used to genotype each individual in the present work. Genotyping was done for 36 individuals from three crosses, that

is, two parents and 10 progeny (five with Bt-resistant phenotype and five with Bt-susceptible phenotype) per cross. To simplify the notation, samples were named as follows: a single letter for parents (A, B, C, D, E, F). Crosses were named AB, CD and EF, where the first letter corresponds to the female and bold/underlined means Bt-resistant. Thus, A and E were F10 and F11 females from the Bt-resistant population FAW-512, C was an F5 female from the Bt-susceptible population FAW-Mon, D was an F10 male from FAW-512, and B and F were F5 and F6 males of the FAW-Mon. The progeny was labeled with the name of the cross, followed by another letter and a number. The letter N (normal larvae, resistant to Bt toxin) or D (dead or delayed development, susceptible to Bt toxin) were added to the name of each cross, followed by the numbers 1 to 5 to identify each sample. Thus, parents A and B were crossed, and the 10 progeny analyzed corresponded to five normal/resistant larvae (ABN1 to ABN5) and five progeny dead or with delayed development (ABD1 to ABD5). Genotyping of the SSRs on the samples was done with the software GeneMapper 3.7 (Applied Biosystems, Foster City, CA) by visual examination of allele sizes in base pairs (bp) at each locus. DNA sequences containing SSRs were screened using NCBI Nucleotide Databases (BLASTnr and BLASTx) [17].

Cluster Analysis, Heterozygosity and Possible Association of Markers to Bt-resistance

To determine if the SSR genotyping would group samples by Bt-resistance, a cluster analysis was performed by Neighbor Joining [18] using the software NTSYSpc v. 2.2 (Exeter Software, Setauket, NY). The confidence levels for the dendrograms were assessed by bootstrap resampling (1000 replicates) [19,20] using WINBOOT [21]. Heterozygosity (H0) was also analyzed, defined here as the presence of more than one allele (amplicon) size per locus per individual, and it was calculated for each sample from crosses based on 100 SSR markers using the program UPIC [22]. Markers and phenotypes were analyzed for putative association as indicated by the Fisher's Exact Test using a 2-way contingency table to analyze allele frequencies as computed using the Proc Freq procedure of SAS ver. 9.2 [23,24].

Given the higher H0 observed in males that participated in the crosses, additional samples were analyzed to determine if indeed males are in general more heterozygous than females in this species; and also to determine if the SSRs were discriminating individuals by sex. Twelve additional samples, six females and six males of *S. frugiperda* from three isofamilies, FAW-512, FAW-W and FAW-STV, were genotyped with all 192 SSRs to determine if a gender bias existed in the level of heterozygosity. Cluster analysis and H0 were performed as indicated for crosses, using in this case 98 SSRs that amplified all samples.

RESULTS

Biometrics of FAW Crosses and Challenge with crylfa Toxin

Ovipositions started two days after the pairs were formed, and though for some pairs with Bt-susceptible females (F_{10} FAW-512♂ × F5FAW-Mon♀) ovipositions started a day later, the differences were not statistically significant. The total period of oviposition was 10 ± 1 days, slightly shorter for susceptible females, but again without significant differences between crosses, Figure 1. Fecundity was significantly higher (p 0.001) for Bt-resistant females (F_{10} FAW-512♀ × F_5 FAW-Mon♂). The cumulative number of eggs laid by five Bt-susceptible females (FAW-Mon♀) was <2000, whereas for five Bt-resistant females (FAW-512♀) was >4000, data are plotted in Figure 1. The average mortality in crosses of Bt-resistant females with susceptible males was 13%, whereas in crosses of susceptible females with Bt-resistant males the mortality was 11%, these differences were not statistically significant. In the repeated experiment, crosses with Bt-resistant females (FAW-512♀) resulted in progeny, but crosses with susceptible females (F_{11} FAW-512♂ × F_6 FAW-Mon♀) resulted in no progeny.

SSR genotyping of *S. frugiperda* reciprocal crosses

Parents, five progeny with Bt-resistant phenotype and five progeny with Bt-susceptible phenotype were genotyped with the SSR markers. A total of 36 *S. frugiperda* individuals corresponding to three pairs of parents with 10 progeny each were screened with 192 SSR markers previously developed for this species [15]. A total of 100 out of 192 markers (52%) amplified all 36 samples, 59 of these markers were polymorphic and 41 monomorphic, Table 1. Examples of the feasibility of genotyping parents and progeny of the crosses are shown in Figure 2a and 2b. Among the markers that amplified some but not all 36 samples: 55 did not show amplification in one or more samples, 17 had amplicons difficult to score, and 20 failed to amplify all samples. The specific markers within each group are listed in Table 1. The 59 SSR markers that were polymorphic and amplified all 36 samples were used for cluster analysis. This analysis showed that the progeny of each cross was closely associated to the paternal and not to the maternal genotype, and these clades were supported by 82 to 99% confidence using bootstrap resampling, Figure 3a. The three females from the crosses grouped in a separate clade that was supported by a 99% probability in bootstrap resampling, Figure 3a.

Heterozygosity in reciprocal crosses and additional samples.

The 100 SSR markers that amplified all the samples in the crosses were processed with UPIC software to calculate heterozygosity (H0) of each sample. The percentage of H0 of the parents in each cross was significantly lower for females ($p \leq 0.002$) A, C, E than for males B, D, F, independently of the population from which they originated, Table 2. Heterozygosity of each individual progeny was calculated and the average values for the Bt-susceptible and Bt- resistant phenotypes of each cross (AB, CD and EF) are shown on Table 2. The H0 of the progeny, whether they were resistant or susceptible, was much closer to the paternal H0 (±7%) than to the maternal H0 (±19%), Table 2. No significant differences in Ho were observed between Bt-resistant and susceptible progeny phenotypes within any of the crosses.

To determine if H0 was indeed higher for males as compared to females of this species, two males and two females from three isofamiles (FAW-512, FAW-W and FAW-STV) were fingerprinted using the 192 SSRs. The analysis showed that only in isofamily FAW-512 the H0 of the females was slightly higher than in males, whereas on the other isofamilies no gender bias was observed in the level of H0. Overall, the H0 of isofamily FAW-STV was significantly higher ($p \leq 0.05$) than for FAW-512 and FAW-W. Cluster analysis of the 98 markers that amplified all these 12 samples, indicated that the markers not necessarily distinguished the gender of the samples, though they clearly distinguished isofamilies, as shown by high confidence values of bootstrap resampling, Figure 3b. Genetic differences among isofamilies were observed at 65 loci. Table 1 indicates by a square box the best 35 of these markers that discriminated one isofamily or more, and some examples are shown in Figure 4.

Potential association of SSR markers to Bt-resistant phenotypes

Out of the 59 polymorphic SSR markers that amplified all samples in the crosses, seven had one or more alleles possibly linked to the Bt-resistant phenotype ($p \leq 0.002$) in one or more of the crosses, as shown by association tests. These were Stv_Spf858, Stv_Spfl125, Stv_Spf409, Stv_Spf1783, Stv_Spf1136, Stv_Spf1651, Stv_Spf1176. Examples of markers Stv_Spfl783, Stv_Spf858, and StvSpf406 for which between four and five progeny either Bt-resistant or susceptible carried the same allele/s, are shown in Figure 2c-2f.

Table 1: SSR markers used in the studies, organized by their efficiency in amplifying 36 samples in crosses of Bt-resistant and Bt-susceptible populations of *Spodoptera frugiperda*. Marker names indicated by a bordered cell, effectively discriminated isofamilies of this insect, and those in bold-italic font correspond to the 18 loci that showed some degree of uniparental inheritance. All marker sequences were published before [15].

Markers that amplified all 36 samples				Markers that amplified not all 36 samples			
Polymorphic		Monomorphic		One or some samples		Difficult scoring	No amplif.
Stv_Spf38	Stv_Spf967	Stv_Spf69	Stv_Spf978	Stv_Spf29	Stv_Spf1195	Stv_Spf20	Stv_Spf61
Stv_Spf103	Stv_Spf994	Stv_Spf85	Stv_Spf1050	Stv_Spf173	Stv_Spf1279	Stv_Spf73	Stv_Spf74
Stv_Spf120	Stv_Spf997	Stv_Spf102	Stv_Spf1058	Stv_Spf470	Stv_Spf1304	Stv_Spf143	Stv_Spf127
Stv_Spf147	Stv_Spf1106	Stv_Spf122	Stv_Spf1068	Stv_Spf658	Stv_Spf1363	Stv_Spf368	Stv_Spf151
Stv_Spf150	Stv_Spf1120	Stv_Spf148	Stv_Spf1079	Stv_Spf552	Stv_Spf1396	Stv_Spf407	Stv_Spf301
Stv_Spf187	Stv_Spf1125	Stv_Spf188	Stv_Spf1098	Stv_Spf664	Stv_Spf1401	Stv_Spf417	Stv_Spf587
Stv_Spf212	Stv_Spf1136	Stv_Spf255	Stv_Spf1170	Stv_Spf692	Stv_Spf1419	Stv_Spf452	Stv_Spf599
Stv_Spf240	Stv_Spf1168	Stv_Spf270	Stv_Spf1230	Stv_Spf746	Stv_Spf1435	Stv_Spf686	Stv_Spf695
Stv_Spf292	Stv_Spf1176	Stv_Spf306	Stv_Spf1245	Stv_Spf752	Stv_Spf1464	Stv_Spf688	Stv_Spf783
Stv_Spf305	Stv_Spf1221	Stv_Spf354	Stv_Spf1334	Stv_Spf807	Stv_Spf1469	Stv_Spf818	Stv_Spf929
Stv_Spf343	Stv_Spf1231	Stv_Spf413	Stv_Spf1349	Stv_Spf823	Stv_Spf1471	Stv_Spf1102	Stv_Spf950
Stv_Spf406	Stv_Spf1260	Stv_Spf488	Stv_Spf1460	Stv_Spf824	Stv_Spf1486	Stv_Spf1187	Stv_Spf1057
Stv_Spf462	Stv_Spf1264	Stv_Spf526	Stv_Spf1461	Stv_Spf835	Stv_Spf1552	Stv_Spf1387	Stv_Spf1147
Stv_Spf467	Stv_Spf1268	Stv_Spf636	Stv_Spf1466	Stv_Spf868	Stv_Spf1573	Stv_Spf1473	Stv_Spf1161
Stv_Spf538	Stv_Spf1315	Stv_Spf653	Stv_Spf1539	Stv_Spf869	Stv_Spf1576	Stv_Spf1797	Stv_Spf1192
Stv_Spf544	Stv_Spf1382	Stv_Spf717	Stv_Spf1561	Stv_Spf904	Stv_Spf1673	Stv_Spf1860	Stv_Spf1243
Stv_Spf52	Stv_Spf1406	Stv_Spf728	Stv_Spf1582	Stv_Spf914	Stv_Spf1680	Stv_Spf1863	Stv_Spf1600
Stv_Spf559	Stv_Spf1409	Stv_Spf747	Stv_Spf1604	Stv_Spf941	Stv_Spf1683		Stv_Spf1634
Stv_Spf578	Stv_Spf1432	Stv_Spf751	Stv_Spf1712	Stv_Spf975	Stv_Spf1698		Stv_Spf1690
Stv_Spf581	Stv_Spf1447	Stv_Spf756	Stv_Spf1890	Stv_Spf988	Stv_Spf1706		Stv_Spf1754
Stv_Spf615	Stv_Spf1502	Stv_Spf828		Stv_Spf1000	Stv_Spf1713		
Stv_Spf662	Stv_Spf1529			Stv_Spf1036	Stv_Spf1723		
Stv_Spf670	Stv_Spf1587			Stv_Spf1054	Stv_Spf1728		
Stv_Spf738	Stv_Spf1592			Stv_Spf1075	Stv_Spf1747		
Stv_Spf743	Stv_Spf1651			Stv_Spf1128	Stv_Spf1758		
Stv_Spf764	Stv_Spf1685			Stv_Spf1134	Stv_Spf1844		
Stv_Spf789	Stv_Spf1707			Stv_Spf1171	Stv_Spf606a		
Stv_Spf858	Stv_Spf1783				Stv_Spf606b		
Stv_Spf908	Stv_Spf1856						
Stv_Spf918							

Loci with uniparental inheritance

The analysis of SSRs in crosses of fall armyworm revealed that at 18 loci either the maternal or the paternal alleles were absent in some of the progeny of the crosses. The maximum absence of maternal alleles was observed on Stv_Spf406, for which

only paternal alleles were present in all 30 progeny analyzed from three crosses. Interestingly, BLAST results showed that the DNA sequence corresponding to Stv_Spf406 had certain homology to a protocadherin. The other extreme case was Stv_Spf1315, for which only maternal alleles were present in 29 out of 30 progeny on the three crosses. Sequences of three markers (Stv_Spf1502, Stv_Spf578 and Stv_Spf967) that showed absence of paternal alleles (in 4, 7, and 12 out of 30 samples, respectively) had short regions of homology to mitochondrial DNA. An interesting marker in this group was Stv_Spf764, because in all 30 individuals, progeny from three crosses, each individual received either the paternal or the maternal allele, but not both.

DISCUSSION

Fecundity and fitness cost

Oviposition started earlier in crosses between Bt-resistant females and susceptible males than in reciprocate crosses, and the oviposition period was slightly longer when the resistant parent was a female, Figure 1, but these differences were not significant. However, the fecundity of Bt-resistant females in crosses with susceptible males was significantly higher, Figure 1. There are examples of Lepidoptera in which no fitness cost was associated to acquiring Bt resistance, i.e., acquiring resistance to Cry2Ab in Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae) [25], and in Pectinophora gossypiella (pink bollworm) [26]. However, a fitness cost such as reduced fecundity is expected in most insects acquiring Bt resistance [27]. This has been observed in other Lepidoptera, Helicoverpa armigera, when a different toxin was used (Cry1Ac) [28,29]. In fall armyworm, *S. frugiperda*, growth rate of F1 hybrids of Bt-resistant females by susceptible males was significantly higher from those obtained in F1 hybrids from resistant males by susceptible females (Portilla, unpublished). This change in fecundity could have contributed to the absence of eggs during our replicated experiment, in which crosses of resistant males (FAW-512) with susceptible females (FAW-Mon) resulted in no progeny.

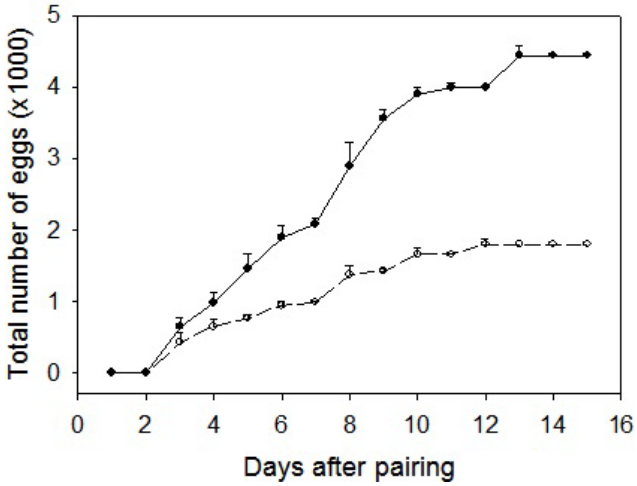

Figure 1: Cumulative number of eggs on daily oviposition of *S. frugiperda* crosses between a Bt- resistant (FAW-512) and a Bt-susceptible (FAW-Mon) population. Filled circles and solid lines correspond to F5 FAW-512♀ x F to FAW-Mon♂; empty circles and dash lines correspond to F10 FAW-Mon♀ x F5 FAW-512♂. Data points are the total cumulative number of eggs of five crosses with their corresponding standard errors of the means. Arrows indicate the last day of oviposition period.

Feasibility of genotyping parents and progeny in crosses between Bt-resistant and Bt-susceptible populations

In 2004, Prowell suggested that a large number of co-dominant markers would be necessary to study strain hybridization in *S. frugiperda*. Since then, ten microsatellites for Spodoptera exigua [30] and six for *S. frugiperda* [31] have been reported and successfully used for the genus Spodoptera, though this number of markers would still be 12 insufficient to study strain hybridization in this insect. From the 192 used microsatellites for genotyping parents and progeny of *S. frugiperda* crosses between Bt-resistant and Bt-susceptible populations, 100 markers (59 polymorphic, 41 monomorphic) amplified all 36 samples, and the majority were easy to score, examples in Figure 2a and 2b. Transposon-related elements were believed to be present throughout Lepidoptera genomes, and were considered a limiting factor for the use of microsatellites in population studies of these insects [32]. In the present work with crosses of individuals from two isofamilies, the feasibility of genotyping parents and progeny using the previously reported microsatellites were tested [15]. From the prepared microsatellite-enriched libraries of *S. frugiperda*, no particular abundance of transposon-related sequences was observed. BLASTn and BLASTx analysis of 1904 contigs containing microsatellites detected only three sequences related to retrotransposons, these corresponded to markers Stv_Spf69, Stv_Spfl582 and Stv_Spf1713. Two of these markers (Stv_Spf69 and Stv_Spf1582) were monomorphic across all the samples analyzed (36 in the crosses and 12 additional males and females) and were easy to score. Marker Stv_Spf1713, though it failed to amplify some samples in the crosses, it was actually effective discriminating isofamilies.

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Table 2: Heterozygosity observed in samples analyzed in crosses of Bt-resistant and Bt-susceptible *Spodoptera frugiperda* across 100 loci. The crosses were AB, CD and EF, where A, B, C, D, E, F are parents, the first letter in the pair corresponds to the female, and bold-underlined font indicates Bt-resistant parent. The progeny were named with the two letters of the corresponding parents followed by N (for Bt-resistant) or D (for Bt-susceptible phenotype), and the numbers 1 to 5 to identify each sample. S.E.: standard error of the mean. Additional samples are 6 males and 6 females from three isofamilies (FAW-512, FAW-W and FAW-STV).

Parents & progeny	Number of loci	Average H_0 (%)	S.E.
A (FAW-512 ♀)	100	21.0	
B (FAW-Mon ♂)	100	35.0	
ABN1-5 (Bt-resistant)	100	31.4	0.45
ABD1-5 (Bt-susceptible)	100	32.0	0.18
C (FAW-Mon ♀)	100	23.0	
D (FAW-512 ♂)	100	38.0	
CDN1-5(Bt-resistant)	100	32.8	0.54
CDD1-5 (Bt-susceptible)	100	31.8	0.33
E (FAW-512 ♀)	100	38.0	
F (FAW-Mon ♂)	100	53.0	
EFN1-5 (Bt-resistant)	100	37.2	0.57
EFD1-5 (Bt-susceptible)	100	35.0	0.25
Additional samples of males and females			
F512-1 ♀	98	29.6	
F512-2 ♀	98	27.6	
M512-1 ♂	98	25.5	
M512-2 ♂	98	17.3	
FW-1 ♀	98	23.5	
FW-2 ♀	98	28.6	
MW-1 ♂	98	29.6	
MW-2 ♂	98	23.5	
FSTV-1 ♀	98	40.8	
FSTV-2 ♀	98	35.7	
MSTV-1 ♂	98	41.8	
MSTV-2 ♂	98	39.8	

Ten percent of the 192 SSR markers did not amplify any of the samples within crosses of *S. frugiperda*. In addition to possible null alleles, many factors can affect amplification, including DNA quality, genetic diversity of the samples and robotic liquid handling. Using the same method of SSR development for other species, the percentage of SSR markers that failed to amplify all samples was found with 3% in Chionanthus [33], 6% in Macrophomina [34], 12% in Rotylenchulus [35] and 29% in Cyperus [36]. Thus, the 10% of markers without amplification in *S. frugiperda* was not necessarily high compared to other species. Overall, the microsatellites had a good quality with not limitations for their use.

Dominant or recessive Cry resistance

In this study 7 (Stv_Spf858, Stv_Spfl125, Stv_Spfl409, Stv_Spfl783, Stv_Spfl136, Stv_Spfl651, Stv_Spf1176) out of 60 polymorphic-SSR markers tested had alleles significantly correlated with Bt-resistance (p 0.02), some examples are shown in Figure 2c-2f. In general, high levels of resistance to Bt toxins in Lepidoptera have been interpreted as the result of one or few autosomal recessive or incompletely recessive genes [4]. In contraposition, low levels of resistance have been interpreted as the result of dominant or co-dominant genes [37-39]. Since SSR markers are inherently co-dominant, the list of seven markers associated to Bt-resistance provided in the present work could be used as a starting point to study low levels of Bt- resistance in *S. frugiperda*.

Cluster analysis and heterozygosity

It was expected that cluster analysis of parents and progeny of reciprocal crosses using microsatellites would group Bt-resistant and Bt-susceptible individuals; instead, the analysis showed association of progeny to the male parent, and grouped the three females from the crosses in a separate clade, Figure 3a. Two hypotheses were explored: a) that males were more heterozygous than females; or b) that the progeny were not randomly expected 50% males and 50% females, but probably they were all males and the markers were grouping samples by sex. Since heterozygosity (H0) level is usually considered an indicator of environmental fitness in organisms (Livshits & Kobyliansky 1985), levels H0 of parents and progeny as possible explanation to the results of cluster analysis were explored. Females in the crosses had a H0 between 27-38%, whereas Hoof their male counterparts was significantly higher, between 35-53%. In *S. frugiperda*, the female is heterozygous carrying the chromosomes ZW, whereas the homozygous is the male, which carries the chromosomes ZZ [40]. Thus, the next question was, if H0 in *S. frugiperda* was gender

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specific, as this could have consequences in the biology of the insect. So, another 12 individuals were fingerprinted, 6 females and 6 males, from three isofamilies; the results showed no significant differences in H0 for males vs. females (Table 2), and that the microsatellites did not discriminate by sex, but showed a clear distinction by isofamilies, Figures 3b and 4.

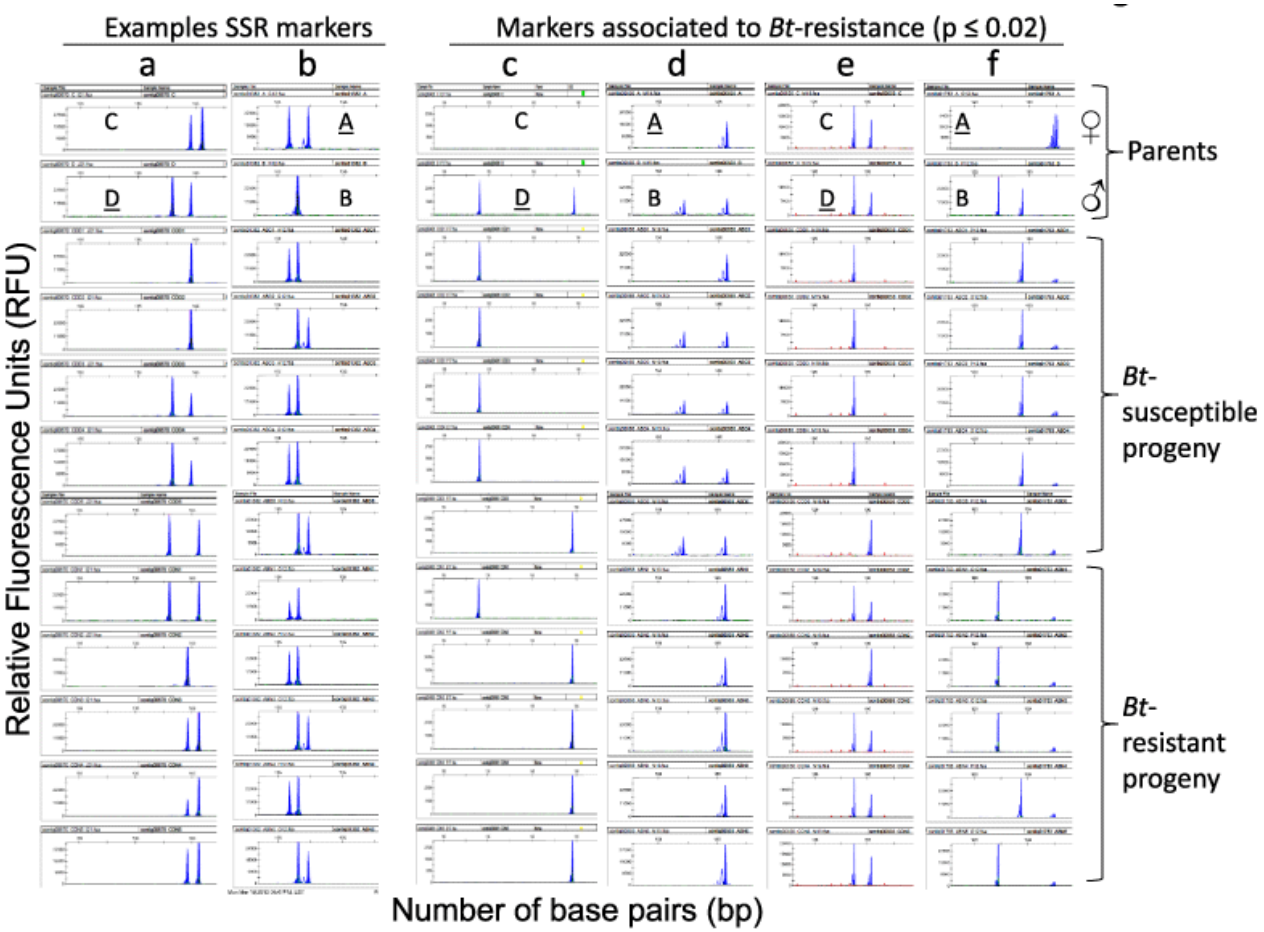

Figure 2: Examples of SSR genotyping of parents and progeny in crosses between Bt-resistant and Bt-susceptible *Spodoptera frugiperda* populations. Panels a and b show examples of good quality markers. Panels c, d, e, f, correspond to marker Stv_Spf406 (in cross CD), Stv_Spf858 (in crosses AB and CD), and Stv_Spf1783 (in cross AB), where A, B, C, D are parents, the first letter in the pair corresponds to the female, and bold-underlined font indicates Bt-resistant parent. Alleles observed for parents and their corresponding Bt-susceptible progeny (ABD1 to ABD5) and Bt-resistant progeny (ABN1 to ABN5).

Isofamilies

A total of 65 microsatellite markers showed some distinction of isofamilies. In this study are reported the best 35 microsatellites that discriminated one isofamily or more of *S. frugiperda* on the 12 samples of males and females from three isofamilies (FAW-512, FAW-W and FAW-STV) tested. These markers are indicated by bordered cells in Table 1, and examples shown in Figure 4. Within this group of markers, one was Stv_Spf1221 related to a protein-folding calnexin, but the most interesting markers were: Stv_Spf173, Stv_Spf544 and Stv_Spf292, whose DNA sequences had homology to a pheromone, and olfactory receptor and an antennal esterase, respectively. All three of these components interact with each other and are essential in the social life of moths. Pheromones are released by female moths to indicate their readiness to mate [41], and are detected by olfactory receptors located in the male antennae where the odor guides them in flight toward the females [42]. In order to maintain high sensitivity to the signal during flight, pheromones are rapidly inactivated [43], what is achieved in microseconds by antennal esterases [44]. This means that the polymorphism observed in markers Stv_Spfl73, Stv_Spf544 and Stv_Spf292 is evidence of genetic variations in three important components of the communication system in *S. frugiperda*. Even more so, the pheromone/olfactory receptor is an extremely sensitive and selective system, "tuned to its key compound" and used for intra-specific communication [45], where the selectivity of the pheromone receptor neurons determine the chemical response specificity [46]. Given the high specificity of the system, would not be surprising that the simultaneous genetic variations on pheromone, olfactory receptor and antennal esterease was detected by the three microsatellites, may impact the "intra-strain" or "intra-colony" behavior in *S. frugiperda*. Thus, markers Stv_Spfl73, Stv_Spf544 and Stv_Spf292, may not only distinguish populations, but also provide valuable information regarding the social life of this insect, and deserve further investigation.

Uniparental inheritance, and gender effect on resistance

In addition to the issue of dominant or recessive inheritance of Bt-resistance, the gender of the resistant parent has also been reported as a determinant factor in the level of resistance of the progeny. In Helicoverpa armigera resistance to Cry1Ac was

suggested as dominant and maternally enhanced inheritance [47]. In Heliothis virescens Fabricius and in *S. frugiperda* isofamily 456, the susceptibility to Bt-toxin was dependent on the gender of the susceptible parent, suggesting that resistance was linked to sex-genes [4,12]. In the present work, SSR analysis showed that at 18 loci either the maternal or the paternal alleles were absent in one or more samples, markers indicated in bold-italics in Table 1. Three of these loci (Stv_Spf967, Stv_Spf578, Stv_Spfl502) had some homology to mitochondrial DNA. That could explain absence of paternal alleles if the SSR marker amplified maternal mitochondria; or absence of maternal alleles if the mitochondria were paternally inherited. In insects, paternal mitochondria can be inherited [48]. Among the 18 loci with various levels of uniparental inheritance, an interesting marker was Stv_Spf406 for which maternal alleles were absent in all 10 progeny from each of the three crosses (30 individuals). The DNA sequence of marker Stv_Spf406 had some homology to a protocadherin. Cadherin-like proteins confer susceptibility to Bt- toxins in vitro by binding to the toxin, whereas disruption of a cadherin gene confers resistance to Bt-toxin [27,49,50]. Thus, if a protocadherin associated to marker Stv_Spf406 is only paternally inherited, it could explain the Bt-resistance linked to sex genes previously observed in this insect [4]. Another particularly interesting locus in the group of 18loci was marker Stv_Spf764. This marker was monoallelic, both in parents and progeny, showing either the maternal or the paternal allele but not both, in all 36 samples from three crosses. BLAST analysis did not link the DNA sequence of this marker to any known sequence.

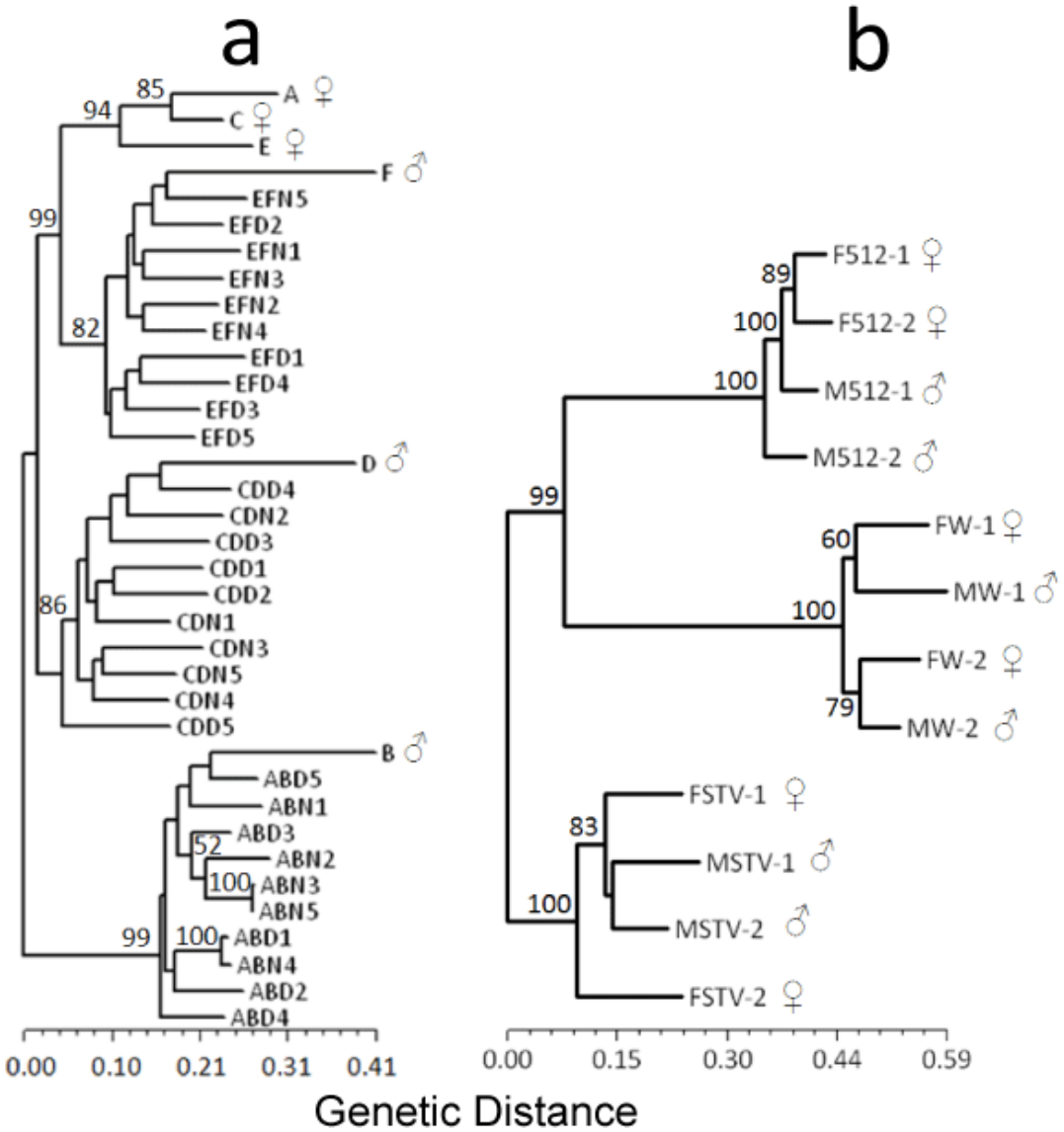

Figure 3: (a) Cluster analysis of three *Spodoptera frugiperda* crosses between Bt-resistant (FAW-512) and Bt-susceptible (FAW-Mon) populations. Neighbor Joining calculated using data from 59 polymorphic-SSR markers that showed amplification in all 36 individuals - three pairs of parents with 10 progeny each. Crosses were AB, CD and EF, where A, B, C, D, E, F are parents, the first letter in the pair corresponds to the female, and bold-underlined font indicates Bt-resistant parent. The progeny were named with the two letters of the corresponding parents followed by N (for Bt-resistant) or D (for Bt-susceptible phenotype), and the numbers 1 to 5 to identify each sample**. (b)** Cluster analysis by Neighbor Joining of male and female individuals from three isofamilies (FAW-512, FAW-W, and FAW-STV) using 98 SSRs; markers clearly distinguish isofamilies and do not group samples by sex. Bootstrap coefficients of 1000 resampling are indicated at the nodes for values higher than 50%.

Though absence of maternal or paternal alleles was observed at 18 loci, the total number of genotypes with absent maternal alleles (137) was similar to the total number of genotypes with absent paternal alleles (147). Therefore, uniparental inheritance per se cannot explain the close association of the progeny with their paternal genotype observed during cluster analysis Figure 3a. It was assumed that the progeny in the crosses AB, CD and EF had 50% probability of being males or females. Additional male and female samples are shown that the microsatellites did not group the individuals by sex, Figure 3b, and that the number of samples with uniparental maternal or paternal inheritance was not significantly different. Then, other than the higher heterozygosity of the males involved in the crosses, there was not an explanation for the association of progeny and male parent.

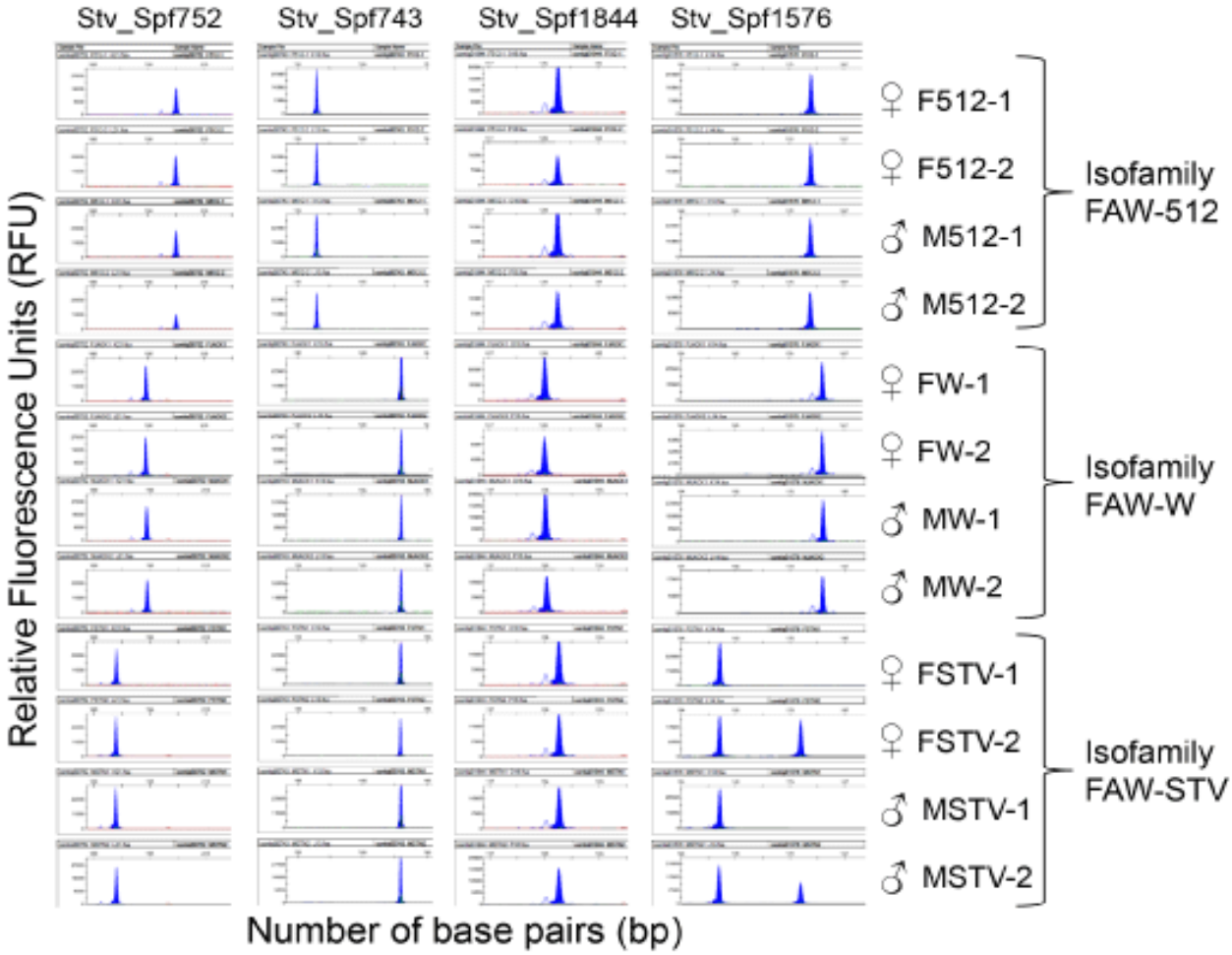

Figure 4: Examples of SSRs that discriminate isofamilies of *Spodoptera frugiperda*. Panels show alleles that characterized three isofamilies of this insect (FAW-512, FAW-W and FAW-STV), independently of the gender of the samples.

CONCLUSIONS

In this work was best demonstrated that it is feasible to genotype parents and progeny in crosses between Bt-resistant and Bt-susceptible *S. frugiperda* individuals, and 100 SSRs out of 192 were effective for this purpose. Though higher heterozygosity was observed in males during the crosses, this was not a general trend when additional samples from more isofamilies were analyzed. Seven microsatellites were found associated to the Bt-resistant phenotype of the progeny in one or more crosses; these could be the starting point to study low levels of Bt-resistance, which are generally associated to dominant or codominant genes. SSR analysis also showed alleles characteristic of individual isofamilies at 65 loci, the best 35 that showed those genetic differences are reported in this study, including three markers with DNA sequence homology to a pheromone, an olfactory receptor and an antennal esterase. This is the first report of such genetic differences at the isofamily level for this insect, what could have significant implications on the behavior and communication of *S. frugiperda*.

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DATA ACCESSIBILITY

Data outputs from ABI 3730 are several hundred Mb, and will be provided upon request.

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AUTHOR CONTRIBUTIONS

Arias RS: conceived the ideas, wrote the entire manuscript, did the fingerprinting analysis, cluster analyses, boostrapping, graphics, tables, designed additional experiments.

Portilla M: made the insect crosses and evaluated their biometrics and phenotypes, did statistical analysis of biometrics

Ray JD: performed the statistical analysis of association of markers to Bt-resistance

Simpson SA: performed all the fingerprinting reactions and contributed to discussions on the experimental design.

Blanco CA: started and maintained the insect colonies, and provided the Bt-toxin for the experiments.

Schemer BE: contributed the equipment, reagents and discussion

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