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First Detection of Naturally Introgressed BT *Cry1Ab* in Asian Corn Borer [*Ostrinia furnacalis* (Guenée)] - Resistant Traditional Maize (Silangan) in the Philippines

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Resistance against insect pests is one of the most promising and highly favored characteristics of an economically important crop. In the Philippines, the Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée), is the most destructive insect pest of maize. The discovery of *Bacillus thuringiensis* and the Bt toxin, which led to the development of Bt corn, effectively controlled this pest. In this study, four traditional varieties of maize that showed resistance against ACB were tested for the presence of Bt *Cry1Ab* protein. Out of these four, a traditional variety from San Carlos City, Pangasinan, which was initially thought to have natural resistance against the pest, tested positive for the presence of the Cry protein. Confirmatory testing for the presence of the transgene was done through PCR, which also confirmed the locations in Pangasinan where introgressed Silangan are present. This is the first report of natural introgression of *cry1Ab* in a traditional maize variety in the Philippines. Results of this study can be used as basis to profile the existing CGUARD collection of traditional maize for the presence of transgenes, and in future corn germplasm conservation and utilization efforts. These can also be used to suggest improvement on the existing policy on biosafety measures to be implemented in the country.

Keywords: corn germplasm conservation, Bt corn, open-pollinated traditional corn variety, PCR-based detection, gene flow

Abbreviations: ACB – Asian corn borer, Bt – *Bacillus thuringiensis*, CTAB - cetyltrimethylammonium bromide, CGUARD – corn germplasm utilization through advanced research and development, DAP – days after planting, EDTA – ethylenediamine tetraacetic acid, GMO – genetically modified organism, IPB – Institute of Plant Breeding, OPV – open-pollinated variety, PCR – polymerase chain reaction, TAE – Tris-Acetate EDTA

INTRODUCTION

Maize (*Zea mays* L.) is the second most important crop in the Philippines and a major source of income for about 1.8 million people, and the primary source of feed for the poultry and livestock industry, with approximate production of 760 thousand metric tons in October 2020 alone (Gerpacio et al. 2004; Bequet 2020; PSA 2020). Diversity in maize is found in different parts of the country where it is a big part of the diet, income, and culture of the people (Salazar et al. 2016). These rich genetic resources may be tapped to improve yield of the country's existing maize traditional varieties towards food security while promoting agro-ecological diversity.

Pests still contribute a considerable loss in maize productivity. Pests globally feed on the amount of food crops that are estimated to sustain food consumption of an additional one billion people (Birch et al. 2015). The Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée), is the country's most destructive pest of maize, with economic loss of 20 to 80% (Morallo-Rejesus 2002; Javier et al. 2005; Caasi-Lit et al. 1989). The early control measure was mainly the use of chemical pesticides, but

this can result in adverse effects and hazards to humans and environment. Particularly, several systemic pesticides are widely used (Cornell CALS 2021), but this control measure was insufficient to fully contain the pest (Caasi-Lit et al. 1989).

The drawbacks of using pesticide drove the utilization of modern biotechnology in the development of genetically engineered MON 810 Bt maize. This corn hybrid is an alternative and a more effective way of controlling corn borer. It was developed by Monsanto to express the insecticidal *Cry1Ab* protein from *Bacillus thuringiensis*, a soil bacterium, which is toxic to specific group of insects particularly lepidopterans (He et al. 2003). Since its approval in December 2002, the biotech corn adoption in the country increased annually in terms of land area planted with Bt corn, from 10,000 hectares in 2003 to 560,000 hectares in 2018, along with several corn GM hybrids commercially introduced by different agricultural biotechnology companies (Aldemita et al. 2015; APAARI 2019). The Bt corn hybrids have provided benefits to Filipino corn farmers in the last 15 years since its commercialization. These include less dependency to insecticides, reduction in carbon footprints, and increase in agricultural productivity, thus, providing the farmers stable source of income. With the growing adoption of these novel crops, risk assessment studies are being required to ensure that biosafety standards are met by any released GM variety (Tsaftaris et al. 2000).

The Institute of Plant Breeding, University of the Philippines Los Baños (IPB-UPLB), in collaboration with the Department of Agriculture-Bureau of Agricultural Research (DA-BAR), spearheaded a program entitled "Corn Germplasm Utilization through Advanced Research and Development (CGUARD)" in 2015. One of the goals of this program was to conduct research involving initially 125 entries of traditional maize landraces from different provinces in the Philippines to conserve these germplasms for future breeding experiments. The resistance of these varieties against ACB was evaluated and the resistant entries were tested for the presence of GM transgenes and determine whether the resistance was purely inherent or due to natural pollination from Bt corn (Goggi et al. 2006).

Detection of transgenic pollen of modified crops in a nearby traditional field is not new, especially to angiosperms such as maize, and the polypoid nature of the endosperm makes the genetic background of angiosperm plants traceable (Lin and Pan 2016). In 2001, the first investigation on gene flow from GM to traditional landrace of maize was documented by Quist and Chapela (2001) in Mexico, which is the center of maize origin and diversity. The researchers sampled corn from an isolated region near Oaxaca, Mexico and analyzed for genetic markers. Their results indicated the presence of transgenic DNA in the traditional landrace, which fueled research to describe and elucidate this phenomenon.

This paper aims: (1) to screen traditional maize for ACB resistance; (2) to detect presence of *Cry1Ab* protein from screened landraces using ELISA strip; and (3) to determine presence of *cry1Ab* gene from collected variants of traditional maize. This also presents the findings on possible natural introgression event for a traditional variety from the province of Pangasinan. This is a pioneering work in the detection of transgenes in Philippine traditional maize. This may serve as reference on limited literature which report on the detection and monitoring of GM transgenes in the Philippine traditional maize varieties.

MATERIALS AND METHODS

Seed Collection and Storage

The 125 traditional landraces of maize were collected from different provinces by the Cereals Breeding Group of IPB through the CGUARD Program. Survey regarding the traditional maize "Silangan" (CGUARD N108) was done from January 2017 to March 2019 in different municipalities of Pangasinan, including San Carlos City, Aguilar, and Mangatarem. Six strains of Silangan were collected from Brgy. Salinap, San Carlos City (1 strain), Brgy. Bocboc, San Carlos City (2), and 3 from Brgy. Bocboc East, Aguilar. Seeds were collected from farmers who were currently planting the variety during the period indicated. The seeds were placed in resealable plastic bags to preserve quality during transport and were stored in an airconditioned room in the laboratory. Along with the control samples IPB Var 6, Bt/Gt, and IPB Var 13, the seeds were used as plant materials for molecular analysis such as DNA extraction and PCR analysis.

Test Plants

Seeds of collected corn and control varieties were planted in Tranca Experiment Station of IPB, Bay, Laguna on February 24, 2016. Each accession was planted to a plot area of 7.5 m^2 which consisted of three 5 meter rows of corn plants with distance of 0.25 m, using Alpha-Lattice Design with three replications. Standard agricultural practices were followed including basal fertilizer application, side-dressing, weeding, and irrigation.

ACB Resistance Screening

Varieties with resistance against corn borer evaluated from previous trials were included as part of current maize breeding program (Table 1). Field collection of leaf

Table 1. Maize varieties used for molecular analysis for the presence of *Bacillus thuringiensis cry1Ab* gene.

Sample #	Variety	Type	Origin of Seeds	Bt Gene Presence/Absence Using Molecular Detection
1	Silangan A	Traditional maize	Salinap, San Carlos City	Present
2	Silangan B	Traditional maize	Bocboc, San Carlos City	Present
3	Silangan C	Traditional maize	Bocboc, San Carlos City	Present
4	APN 120 S1	Traditional maize	Cereals Breeding Group, IPB-UPLB	Present
5	Silangan D-A	Traditional maize	Bocboc East, Aguilar	Present
6	Silangan D-B	Traditional maize	Bocboc East, Aguilar	Present
7	Silangan D-C	Traditional maize	Brgy.Bocboc East, Aguilar	Present
8	IPB Var 6	Open-pollinated	Cereals Breeding Group, IPB-UPLB	Absent
9	IPB Var 13	Open-pollinated	Cereals Breeding Group, IPB-UPLB	Absent
10	Bt/Gt	Bt corn hybrid	Syngenta Philippines, Inc.	Present

samples from the whorl of randomly sampled corn was done during the reproductive stage (40 to 45 days after planting). Leaf tissue samples were used with the assumption that the highest expression values for most proteins of maize were mostly obtained from the leaves.

Traditional varieties collected from various provinces which consistently showed least larval survival at 30 to 45 days after planting (DAP) were considered for confirmatory GM testing. Larval survival was obtained via leaf-disc assay using four-day old second-instar larvae in a no-choice test for seven days in the laboratory. Survival data was determined by the percentage of the number of surviving larvae over the total number of introduced larvae. The data were pooled from six successive trials from January 2014 to March 2016.

Testing for Presence of Bt Protein

Leaf samples from ten randomly selected plants in each row from the field were collected at 45 DAP and tested using a commercially available rapid enzyme-linked immunosorbent assay (ELISA) strip test kit (Envirologix®, Maine, USA, https://www.envirologix.com/) for the presence of Bt *Cry1Ab*. The instruction manual included in the kit was followed to prepare the samples. Five hundred (500) microliters of extraction buffer were added to each leaf sample (100 mg) from each accession. The leaf samples were macerated in microcentrifuge tubes and incubated for 5 minutes at 27°C. Bt strip was dipped into the extract and evaluated for presence of transgene. ELISA strips with two lines were treated as positive, while the strips with one line were negative. The whole procedure was repeated twice. Bt corn, which was acquired from Syngenta Philippines, Inc., was used as positive control for Bt protein while Philippine Supersweet from IPB was used as negative control.

Plant Materials for Molecular Analysis

Collected seeds from different sites in Pangasinan were used as test materials for molecular analysis. The batch of seeds collected from a specific site was treated as separate sample or variants (Fig. S1). S1 lines of CGUARD N108 were provided by the Cereals Breeding Group of IPB-UPLB. Control checks include IPB Var 6 from Cereals Breeding Group as negative control and Bt/Gt as positive control from Syngenta Philippines Inc. IPB Var 13 was also included in the analysis to provide information on the possible outcrossing of transgenes in open-pollinated varieties. Summary of the samples used for molecular analysis is presented in Table 1.

Five treatments were used to prepare the tissue samples for genomic DNA extraction (Fig. S2). For Treatments 1 to 3, seeds were sown in pots under greenhouse condition, and leaves were collected at 7 (T1), 14 (T2), and 21 DAP (T3) respectively. For Treatment 4 (T4), 15 seeds per sample were germinated in Petri dish lined with damp tissue paper, with two replicates per variety to make a total of 30 seedlings. The shoot tip was cut after seven days. Seeds soaked overnight in distilled water were used for Treatment 5 (T5).

Genomic DNA Extraction

Genomic DNA of maize varieties were extracted for each treatment using cetyltrimethylammonium bromide (CTAB) method modified from Dellaporta et al. (1983). Two hundred fifty milligrams plant sample was macerated with liquid nitrogen using mortar and pestle. Pre-heated extraction buffer of 2 mL volume was added to the homogenate containing the following reagents: 2% (w/v) CTAB; 1.5% (w/v) polyvinylpyrrolidone (PVP); 1.4 M sodium chloride; 100 mM Tris hydrochloride, *pH* 8.0; and 20 mM ethylenediaminetetraacetic acid (EDTA), *pH* 8.0.

Under the fume hood, 4 µL of 2-mercaptoethanol was added to the samples. The homogenates were transferred to microcentrifuge tubes divided into 500 µL per tube and incubated in hot water bath at 65°C for 1 hour. After addition of 500 µL 24:1 chloroform:isoamyl alcohol solution, the samples were vortexed and centrifuged at 12,000 rpm for 10 mins. Supernatant of 400 µL was collected, transferred into fresh microcentrifuge tubes,

added with 320 μL (0.8X) cold isopropanol, and incubated in freezer overnight (-20°C). DNA pellets were collected by decanting the alcohol, spinning down at 10,000 rpm for 10 minutes, and successive washing with 320 μL cold 70% ethanol twice. The pellets were dried until the alcohol evaporated. These were resuspended in Tris-EDTA (TE) buffer to preserve the quality. RNA was removed by incubating with RNase A at 37°C for one hour.

Quality of the extracted DNA was determined through agarose gel electrophoresis. One percent agarose gel was prepared by combining molecular-grade agarose, 0.5X Tris-Acetate EDTA (TAE) Buffer, and GelRed® Nucleic Acid Stain (Biotium, Inc., USA). DNA samples were mixed with 6X loading dye containing xylene cyanol and bromophenol blue. After electrophoresis at 150V for 45 minutes or until the dye reached 80% of the gel length, the bands were viewed under UV light using Gel Doc XR+ TransIlluminator (Bio-Rad Laboratories, Inc., USA) (Fig. S3). Absorbances at 260 and 280 nm as well as the concentration of the extracted DNA were determined using Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., USA). Upon determination of the concentration (Table S1), the samples were diluted to final concentration of 50 ng/μL with sterile nanopure water. Samples with lower concentrations were not diluted.

PCR Analysis

Detection of Bt transgene in Silangan strains collected was done through polymerase chain reaction (PCR) using Bio-Rad T100™. The primers used targetted *cry1Ab* in event Bt11, and were adopted from Rønning et al. (2003). The primer pairs were Bt11-97-F (GCG GAA CCC CTA TTT GTT TA) and Bt11-97-R (TCC AAG AAT CCC TCC ATG AG) with an expected PCR product length of 70 kb. PCR was performed in 25 μL reaction using the mix: 1X PCR buffer; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.2 μM each of forward and reverse primers; 0.2 U *Taq* DNA Polymerase; and 50 ng DNA sample. The PCR reagents used were obtained from Vivantis DNA Amplification Kit, including the DNA ladder, and loading dye. The PCR profile was initial denaturation at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 1 minute, annealing at 51°C for 45 seconds, and extension at 72°C for 1 minute; final extension at 72°C for 3 minutes; and final hold at 4°C.

After PCR, the PCR products were loaded in 2% agarose gel (components: agarose, 0.5X TAE Buffer, GelRed® Nucleic Acid Stain) submerged in 0.5X TAE running buffer. The gel was viewed under UV light and transgene was visualized through the presence of bands with reference to the expected product size.

RESULTS AND DISCUSSION

At present, few or no undertaking has been done to determine the presence of GM transgenes in the traditional maize since the adoption of Bt corn in the Philippines 15 years ago. It was only recently that the potential of Philippine traditional corn varieties towards the development of maize varieties at par in terms of biotic and abiotic stresses caught the interests of scientists and researchers. As a precautionary approach to address the concern on the genetic purity of the maize being tested as breeding materials, we subjected initial promising germplasms against ACB to preliminary detection of GMO using commercially available protein strip test sensitive to *Cry1Ab*/Ac. The *Cry1Ab* protein of *Bacillus thuringiensis* still represents the key Bt toxin in GM-based insect control strategies.

ACB Resistance Screening

From the 125 total maize accessions used for ACB resistance screening, four traditional varieties showed the highest and most promising ACB resistance: one from the province of Pangasinan (CGUARD N108), and three from Bukidnon (CGUARD N33, CGUARD N32, CGUARD N36). The percent larval survival from the 2014-2016 resistance screening and the Bt strip test result of the four varieties using ELISA are summarized in Fig. 1. Among the tested varieties, the "Silangan" variety (CGUARD N108) from Pangasinan showed the lowest percentage of larval survival, with mean larval survival of 22.49% and standard error of 4.15. This indicates the effectiveness of Silangan variety against corn borer larvae. However, it also tested positive for the presence of *Cry1Ab* protein for three trials, along with the resistant check Bt/Gt, which suggests that the source of resistance of the traditional variety was the presence of *Cry1Ab* protein in its genome, and not natural resistance.

Genomic DNA Extraction

Extraction of genomic DNA of the maize samples CTAB extraction method was successfully done. In Fig. S3, the intact genomic DNA can be visualized from the high molecular weight bands at around 10 kb marker. Among the five treatments, majority of the samples in T1 have either faint or no visible bands at all, indicating very low concentrations of extracted DNA, which is validated through spectrophotometry (Table S1). The other four treatments provided good quality of extracted genomic DNA with an approximate size of 10,000 bp, with reference to 1 kb+ DNA ladder. However, compared to T4 and T5, extracted DNA samples using T2 and T3 are of lower yield.

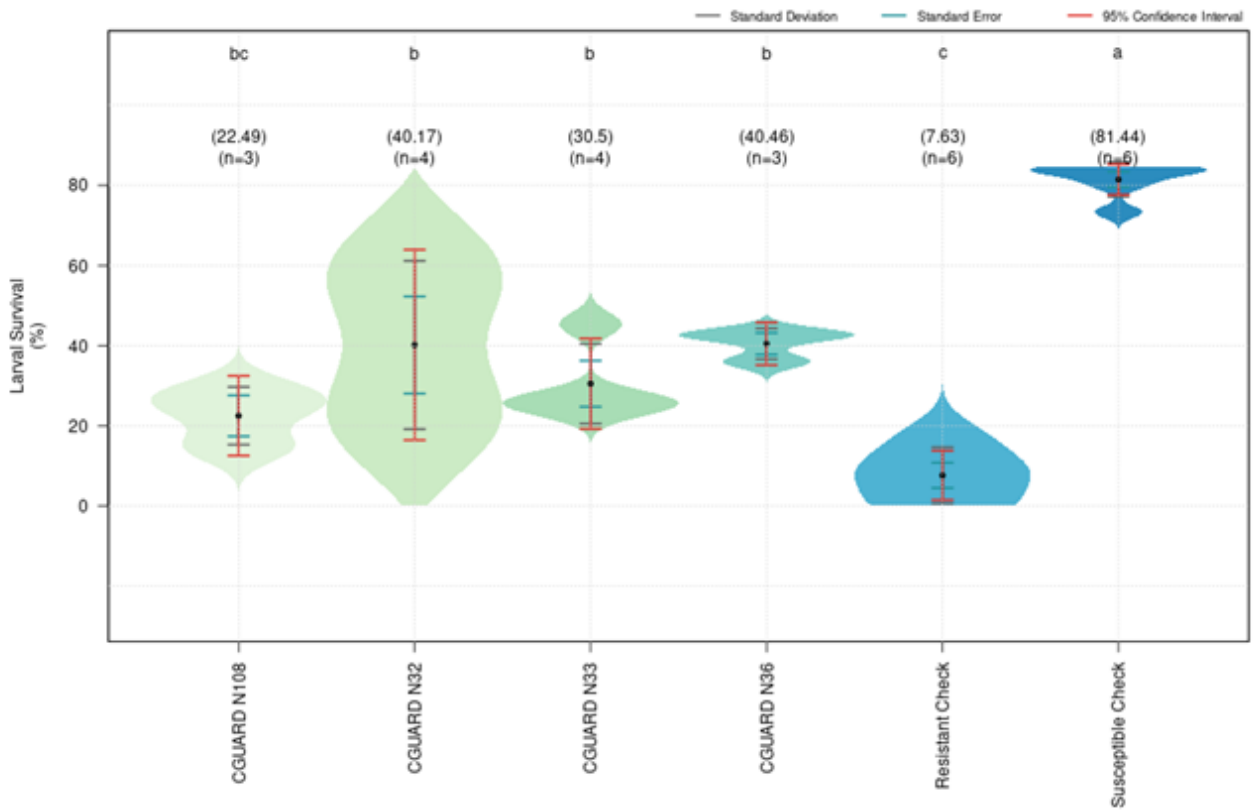

Fig. 1. Survival of Asian corn borer larvae (second instar) in promising corn borer-resistant Philippine traditional maize varieties.

*Resistant check is NK8840 (commercial grain corn variety), and susceptible check is Philippine Super Sweet (commercial sweetcorn variety).
**Letters on top of each density plot refers to the grouping based on Tukey's Honest significant difference at α=5%.
***Numbers inside parenthesis are the mean percent larval survival, and below that with "n=" is the number of replicates referring to the number distinct environmental locations and conditions the tests were conducted, i.e. ^{1}1st: Tranca Experimental Station (TES) (01-25-2014); 2nd: TES (05-04-2015); 3rd: IPB Sampaloc Area (ISA) (8-17-2015); 4th: ISA (10-02-2015); 5th: TES (12-22-2015); and 6th: TES (03-01-2016);

Quality and Quantity Check of Extracted DNA

The use of five treatments for preparation of tissue source is compared (Table S2). In T1, the DNA pellets were observed to have brown discoloration, which suggests phenolic contamination. This is usually observed if younger leaf samples are used in DNA extraction since these have higher phenolic content than mature leaves, which affects the quality of DNA if to be used for downstream applications such as PCR (Nantitanon et al. 2010). The absorbance ratio of the genomic DNA plays an important role in determining the quality of the extracted DNA. In general, a good quality DNA has an absorbance ratio at 260 nm and 280 nm of 1.8 to 2.0 (Sinden 1994). DNA absorbs UV light maximally at 260 nm while protein absorbs UV light at 280 nm. Lower absorbance ratio indicates protein contamination while higher absorbance ratio indicates high phenolic content of the extracted DNA. Majority of the samples in T1 have either very low or very high absorbance ratio than the acceptable range. The samples in T2 and T3 have good absorbance ratios, as well as in T4 and T5, although few samples have high absorbance reading.

Using seed samples as tissue source for genomic DNA extraction was explored in this study as one method to develop a rapid technique of transgene detection, as seen from the comparison of the tissue samples used (Table S2). In both treatments (T4 and T5), the cost of input was reduced since the seeds were not planted in pots and the use of fertilizer and water were eliminated. It also required very low maintenance and space and can be monitored with minimal efforts inside the laboratory. Also, compared to the leaf samples, the length of time needed to acquire tissue sample is shorter. However, in T4, one problem noticed was the development of molds on the seeds 3 to 4 days after setting up. Nevertheless, this did not affect the quality of the extracted DNA since only the shoot tips were collected and used for the experiment. Generally, among the treatments used, T4 was observed to be the best treatment to be used in obtaining good quality DNA at short period of time. Samples under this treatment were used in further molecular analysis.

Since the Philippine government's approval of the commercialization and importation of GM crops, various stakeholders have responded differently. The protein strip test which was used in this study is a simple, fast, cheap, and reliable way of GMO detection; however, protein denaturation during the process is a disadvantage (Van Dujin et al. 2002). Also, the commercially available ELISA strip test kits have limits of detection and might provide false negative results if the GM protein level in the sample is lower than the detection limit, as concentrations of Bt lower than 1% are usually not detected by the strip test (Ma et al. 2005). On the other hand, DNA-based methods, such as PCR analysis, are more advantageous since DNA molecules are relatively stable, although the method is costly and will require highly trained personnel (Van den Bulcke et al. 2005). Upon detection through PCR, more information can be generated, and more analysis can be done from the DNA sequences of the transgenes detected.

Detection of *Cry1Ab* Through PCR

PCR analysis was conducted upon checking the quality of the extracted DNA. From Fig. 2, the *cry1Ab* gene was detected in all the strains of Silangan collected in different areas in Pangasinan, as well as in S1 lines generated by the Cereals Breeding Group, with an estimated molecular size of 75 bp, indicated by the arrow and with reference to the positive control, Bt/Gt (Sample 10). Only Samples 8 and 9 are negative for the presence of transgene, indicating that IPB Var 6 and IPB Var 13 remain pure from possible introgression of *cry1Ab*.

Majority of the corn fields in the province of Pangasinan are being planted with Bt corn, except in areas where fishing is the main livelihood such as Dagupan (Table S3). Pioneer-Hibred Philippines Inc. (now Corteva Agriscience), Monsanto Philippines Inc. (now Bayer) and Syngenta Philippines, Inc. are the three main agrochemical companies operating and distributing Bt corn seeds in the province. The municipality of San Carlos City contributes a big percentage in the production of Bt corn of about 6.07 to 9 tons per hectare. Approximately 95% of the corn fields in this area is planted with Bt corn, which started in 2003. Majority of the areas in San Carlos City planted with traditional corn varieties are also being planted with Bt corn (Fig. 3). The 'Silangan' strains used in this experiment were mainly collected from Aguilar and San Carlos City.

Transgene Introgression in Traditional Maize

Since maize is an open-pollinated crop, the possibility of the introgression of transgenic pollen from the surrounding Bt corn fields into the traditional maize is very high (Sulewska et al. 2014; Goggi et al. 2006). This provides evidence that the possible source of transgene detected in the traditional 'Silangan' is likely due to the introgression from nearby Bt corn fields. Riesgo et al. (2010) stated that a field distance of 40 meters would be sufficient to provide reduction in the cross-pollination of corn hybrids. This was not observed in the surveyed field in Pangasinan, and the presence of the transgene in the six strains collected and the S1s can extrapolate the presence of Bt transgene in majority of 'Silangan' maize planted.

Despite the wide acceptance of Bt corn in the province, farmers still plant open-pollinated varieties (OPVs). Glutinous corn or "Lagkitan", a non-GM maize,

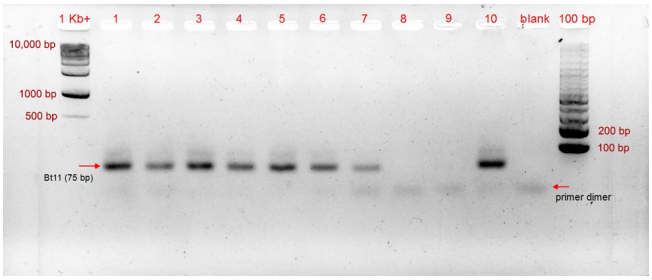

Fig. 2. Gel electrophoretogram of amplified PCR products using primers targeting the event Bt11 in CGUARD N108 (Lanes 1-7), negative controls (Lanes 7-8), and positive control (Lane 10).

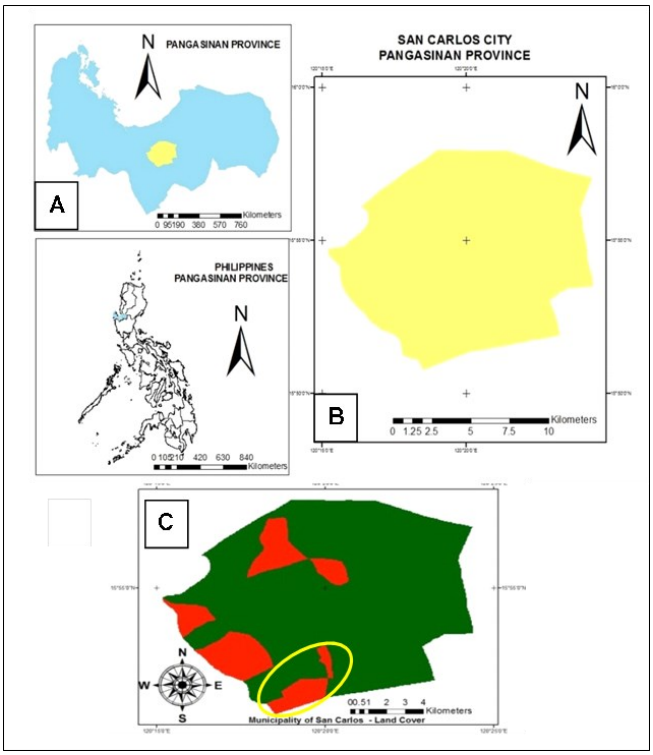

Fig. 3. Map of (A) Pangasinan and (B) San Carlos City and (C) areas (red) planted with both Bt corn and traditional corn. Area planted to traditional corn is encircled in yellow.

is still preferred by the local people. Traditional maize and other OPVs which usually do not have names are consumed as food source due to their soft kernels. Compared to Bt corn hybrid, the cost of production is less, and the area planted is quite smaller. 'Silangan' maize is planted mainly for personal consumption only.

Based on our research in the province and survey among the local people of Pangasinan, 'Silangan' is widely popular in San Carlos City, Aguilar and Mangatarem mainly due to its palatability. The estimated average yield is 1.5 to 2 tons/ha. It is known that this variety has a radiant yellow to orange color with resemblance to the rising sun, hence, the name "Silangan". Few farmers claim that it was originally brought in the province in the 1930s by a native from Isabela which is located on the east side of Pangasinan. Before the proliferation of hybrid varieties in Pangasinan, majority of the corn fields were planted with Silangan. This traditional maize became popular and useful substitute among the people of Pangasinan during the rice shortage in the 1970s. With the introduction of Bt corn in 2003, most of the original 'Silangan' farmers shifted to the use of corn hybrids.

Due to the limited knowledge on the possible effect of transgenic pollen being incorporated in the traditional maize, the local corn farmers continued to plant 'Silangan' amidst the Bt corn fields. Two corn farmers from the province that have been planting 'Silangan' maize until now stated that planting of this variety started from their parents. Unknowingly, through time, 'Silangan' improved its quality due to generations of selection by the farmers. For more than 15 years prior to the initial investigation of possible introgression event, this variety has been constantly consumed as food.

This molecular analysis in the detection of *cry1Ab* in 'Silangan' may be a basis in the development of a reliable system of detection of other transgenes in the remaining traditional varieties, and thus will provide information in the possible introgression events occurring in our maize varieties. This system may be in the form of PCR-based molecular markers, which is a rapid and highly discriminating method of detecting the target genes in the genome of an organism.

CONCLUSION

Maize is known to outcross or cross-pollinate readily to other maize plants, and pollination can even occur at long distance, making it as one of the most critical GM crops to contain. The detection using protein-based approach and the consequent molecular analysis through PCR merely indicate the presence of Bt transgene in the traditional 'Silangan' maize from Pangasinan province, which was originally thought to have natural resistance against ACB. Genetic analysis is underway to confirm this result and a highly specific way of detecting transgene introgression in the traditional and non-Bt varieties is needed, including development of molecular markers. Data that will then be generated can be used in formulation of traceability strategies in larger geographic range. The quantification of GM genes in the food and agroecosystems, especially on traditional varieties and other non-target species is crucial and necessary. Since traditional maize are often utilized as food and feed, undetected GM content in these germplasms can affect the ecosystem. Furthermore, we believe that the information that will then be gathered can strongly influence the potential for adequate implementation and maintenance of legislation requirements of GM products in the country.

No technology or human activity is completely risk-free (Center for Biological Diversity and United Nation Environmental Program 2003). While modern biotechnology may have great potential, it must be developed and used with adequate safety measures, particularly for the environment. Around 70 percent of the maize being planted in the Philippines is GM corn. Hence, unintended escape and introgression of GM transgene can never be ruled out, especially in maize. Thus, this will serve as a guide to make sure that biosafety measures are properly implemented in the country with regards to GM maize and other crops. Likewise, this measure will ensure that genetic diversity of agricultural crops will be conserved.

RECOMMENDATION

All maize accessions stored in the collection of the Breeding Group, and from the collections that will be done in the future, must be screened for the presence of transgenes and for possible events of introgression. Field screening can be done in the field first, and if resistance to corn borer is observed, then molecular detection will follow only on those showing resistance. Same with rapid screening for herbicide tolerance, only those plants that survive will undergo molecular analysis for the presence of transgene. In this study, only the gene *cry1Ab* was detected. There is a need for the other transgenes to be detected as well in all traditional maize before storage to conserve these germplasms. Development of molecular marker for this detection system is highly preferred to rapidly screen accessions available in the collection. To confirm the identity of the detected *cry1Ab* and other possible transgenes, sequencing the PCR products is also suggested.

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Supplementary Figures and Tables

Fig. S1. Sample picture of Silangan seeds used for molecular analysis.

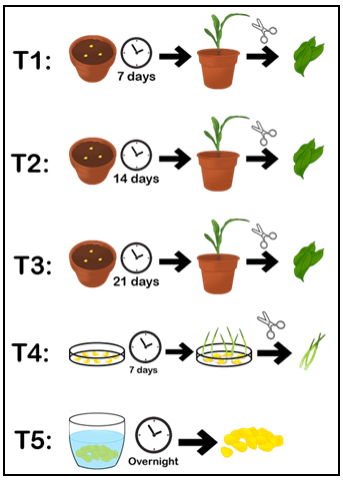

Fig. S2. Treatments used for plant sample in genomic DNA extraction.

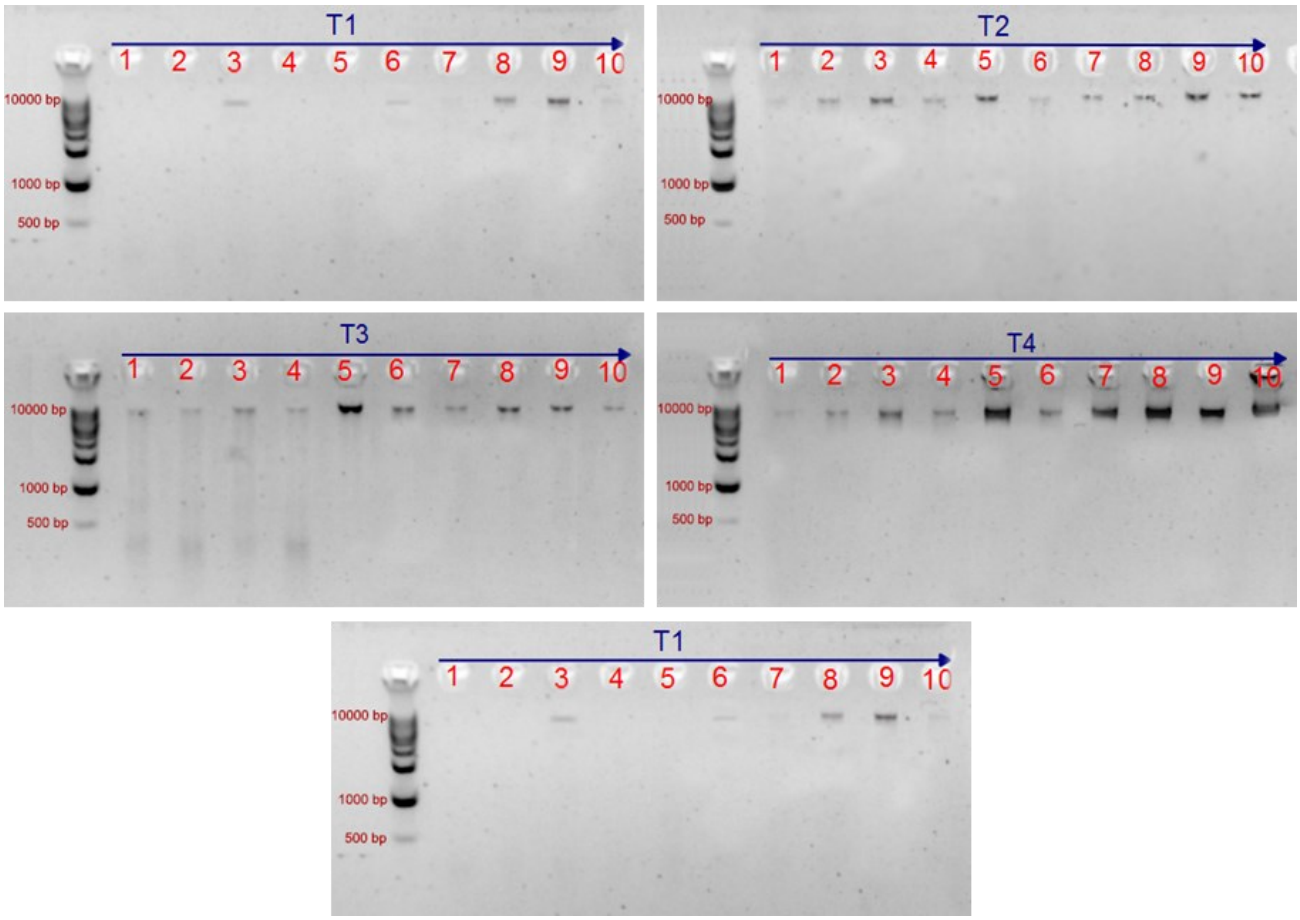

Fig. S3. Gel electrophoretogram of the extracted genomic DNA using CTAB extraction method.

Table S1. Concentration and absorbance ratio of the extracted DNA using five treatments.

Sample	Concentration (ng/µL)					Absorbance Ratio (A_{260}:A_{280})				
	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
1	4.56	47.40	35.02	20.65	9.30	2.438	2.012	2.025	2.658	2.045
2	10.63	39.40	33.77	22.24	16.75	1.140	2.033	2.039	1.820	1.663
3	18.96	80.71	68.34	50.65	25.79	1.561	2.034	2.029	2.196	1.986
4	6.55	49.34	44.76	34.53	14.98	2.917	2.045	2.025	2.310	2.097
5	7.90	75.69	41.95	217.21	88.20	2.150	1.998	2.036	2.013	2.109
6	12.50	43.41	48.89	63.68	15.77	1.971	2.011	1.955	2.194	1.803
7	8.97	36.05	59.07	172.05	152.43	2.227	1.966	1.976	2.009	1.731
8	13.16	48.48	43.93	489.31	159.88	1.614	2.022	2.047	2.051	2.066
9	16.63	125.88	169.73	265.62	137.21	2.068	2.038	2.049	2.121	1.912
10	4.62	103.66	137.91	814.27	215.63	2.083	2.058	2.006	2.006	1.972

Table S2. Comparison of the five treatments used for preparation of tissue sample for genomic DNA extraction.

Condition	T1	T2	T3	T4	T5
Tissue used	Leaf	Leaf	Leaf	Shoot tip	Seed
Duration to acquire tissue	7 days	14 days	21 days	7 days	Overnight
Growth medium	Soil	Soil	Soil	Wet tissue	Water
Water requirement	Yes	Yes	Yes	No	No
Fertilizer requirement	Yes	Yes	Yes	No	No
Space required	Large	Large	Large	Small	Small
Plants sampled	Multiple	Multiple	Multiple	Multiple	Single
Ease of homogenization	Soft	Soft	Soft	Soft	Hard
Contaminant	High phenolics	-	-	Molds	High starch

Table S3. Areas in Pangasinan with present Bt corn production.

Municipality/City/Area	Corn Farming
Asingan	Green corn, *Bt* corn
Malasiqui	*Bt* corn
Bayambang	*Bt* corn
Mangatarem	*Bt* corn
Sta. Maria	*Bt* corn
San Carlos	*Bt* corn*

*high production of *Bt* corn, contributing an average of 6.07 to 9 tons/ha