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Design, optimization, and validation of corn-strain-specific primers for fall armyworm (*Spodoptera frugiperda*) detection in the Philippines

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Abstract. *Labonete HJP, Fulgencio BKR, Abatay MP, Ampang MP, Ancheta DJ, Gumal SJR, Jimenez EA, Modina RMR, Yongco JE, Tabugo SRM. 2025. Design, optimization, and validation of corn-strain-specific primers for fall armyworm (*Spodoptera frugiperda*) detection in the Philippines. Asian J Agric 9: 844-853.* The fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is an invasive pest that threatens corn production in the Philippines. The presence of morphologically identical corn- and rice-strains, each with distinct host preferences, complicates accurate identification. Molecular diagnostics are significant in understudied regions of the country, such as Mindanao where *S. frugiperda* invasions are rapidly expanding, with primers offering reliable support in pest surveillance. This study developed and optimized corn-strain-specific primers (FAWC1–FAWC5) using ancestral Cytochrome Oxidase I (COI) haplotypes (GenBank U72974, U72976) and Primer3 in Geneious Prime (v2023.2.1). In silico PCR and specificity assessments confirmed optimal primer characteristics (20-22 bp, 57.5-60.3 °C Tm, 50-55% GC), amplicon sizes of 100-250 bp, and absence of secondary structures. The standardized annealing temperature for amplification is 53.7°C to ensure efficient primer binding and workflow consistency. The applied step-wise experimental validation ensured that all primer sets adhered rigorous technical standards supported by wet-lab testing which narrowed the best-performing candidates to FAWC3, FAWC4, and FAWC5. Verification using larval samples collected from corn and rice fields in Davao Oriental and corn fields in Iligan City demonstrated reliable amplification. It confirmed the dominance of the corn-strain across the tested sites, strengthening geographic validation and consistency in primer performance fit for community-level monitoring FAWC4 exhibited broad corn-strain detection capability making it suitable for initial screening, while FAWC5 provided strong lineage-specific resolution within Philippine populations. Together, these primers form a dual-function molecular toolkit that enables accurate strain-level identification, strengthens regional monitoring programs, and supports evidence-based fall armyworm management strategies in Philippine corn-growing regions.

Keywords: Annealing optimization, COI gene, corn strain, fall armyworm, pest management

INTRODUCTION

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a highly invasive moth species currently targeting staple cereal crops in the Philippines. First recorded in June 2019 in Piat, Cagayan Province (Navasero et al. 2019), the fall armyworm has rapidly spread throughout the country due to its extensive migratory ability and polyphagous feeding behavior. It feeds on up to 353 host plant species, mainly Poaceae, and has impacted farming communities in 70 provinces as of 2020 (Montezano et al. 2018; Cuaterno 2021). Corn, a staple crop for human consumption and livestock feed, has been significantly affected. As of August 2024, the Department of Agriculture (DA) reported infestations affecting over 5,200 farmers and 3,237 hectares, resulting in 734 metric tons of corn losses worth Php 57.03 million (Gomez 2024). By January 2025, the Philippine corn stockpile dropped by 45% to 328,400 metric tons as farmers shifted away from corn due to ongoing fall armyworm infestations (Lagare 2025). This decline has critical implications for food security, livestock industries, and farmer livelihoods.

The persistent fall armyworm infestation is driven mainly by the pest's highly polyphagous nature and the existence of two morphologically identical but genetically distinct strains, corn-strain and rice-strain (Pashley 1986; Nam et al. 2024). Identifying these strains is crucial for monitoring and control, yet it continuously remains challenging. Molecular identification tools offer a solution for *S. frugiperda* strain identification, as studies show that genetic markers remain heterogeneous, indicating genetic distinction with minimal gene flow (Kost et al. 2016; Tessnow et al. 2022). Molecular strain identification relies on polymorphisms in several genetic markers, such as the mitochondrial Cytochrome Oxidase I (COI) gene (Yousaf et al. 2021), the nuclear triosephosphate isomerase 1 (Tpi1) gene (Nagoshi 2010; Liang et al. 2024), and AFLP (Amplified Fragment Length Polymorphism) (Tessnow et al. 2024).

The Philippines, an archipelagic country, presents a complex landscape for understanding pest dynamics. Recent findings indicate a predominance of the rice strain in local *S. frugiperda* populations, contrasting with earlier observations that reported corn-strain dominance in Northern Luzon, Philippines (Navasero et al. 2023; Cabusas et al. 2024). Mindanao is the country's second-largest island and a major corn producer, with Bukidnon and Lanao del Norte leading in white and yellow corn production (Calalang et al. 2015). However, Mindanao remains an understudied region in terms of *S. frugiperda* distribution, particularly for strain-level identification, due to limited molecular surveillance and the lack of corn-strain-specific primers developed to date. Current diagnostic methods in the Philippines primarily rely on conventional COI barcoding using universal primers such as JM76 and JM77 (Navasero et al. 2023), which target conserved COI regions yet do not differentiate between the morphologically identical corn and rice strains. This limitation prevents early detection and accurate monitoring for region-specific Integrated Pest Management (IPM) strategies. Therefore, developing and validating corn-strain-specific molecular markers is critical for improving FAW diagnostics in Mindanao and strengthening nationwide surveillance and management programs.

Traditional PCR, which includes denaturation, primer annealing, and DNA extension, is widely used for species strain identification due to its simplicity, cost-effectiveness, and rapid turnaround time (Thomassen et al. 2021; Kurmanov et al. 2022). A key step in PCR is proper primer design, as it determines the specific DNA region to be amplified, directly impacting the accuracy and efficiency of gene expression analysis and, ultimately, the PCR success (Freeland 2017; Thaenkham et al. 2022). Moreover, annealing temperature critically affects amplification, balancing specificity and efficiency (Tripathi and Rathinam 2025). Optimizing annealing temperature is essential for developing corn-strain-specific primers for *S. frugiperda*, yet such approaches remain underutilized in the Philippines, especially in Mindanao, despite existing studies (Tsai et al. 2020; Cabusas et al. 2024).

The Cytochrome Oxidase I (COI) gene is widely utilized in molecular studies for insect strain differentiation due to its mutation rate, maternal inheritance, and suitability for DNA barcoding (Wilson-Wilde et al. 2010; Liu et al. 2016; Kurata et al. 2024). COI gene polymorphisms have been instrumental in distinguishing *S. frugiperda* strains (Acharya et al. 2021; Yousaf et al. 2021). Building on this, the present study designed, optimized, and validated five novel corn-strain-specific COI primers for precise molecular identification of *S. frugiperda* populations in Mindanao, Philippines. Primer design and in silico testing were performed using Geneious Prime, followed by laboratory validation through a step-wise PCR assay by initially screening all primers using a corn-reared colony from Davao Oriental and subsequently confirming performance on the top three primers on field-collected larvae from Iligan City. This combined computational and empirical validation enhances the reliability of the primers. It strengthens their potential as diagnostic tools for strain-level monitoring of *S. frugiperda* in Mindanao, ultimately supporting targeted IPM strategies nationwide.

MATERIALS AND METHODS

Sample collection and maintenance

Fall armyworm larvae were obtained from two sources. The first was a 6th-generation corn-reared culture maintained by Prof. Cyril L. Tura of the Department of Agriculture at Davao Oriental State University, Philippines. The first colony was established from 50 larval individuals collected from corn and rice fields in Baon, Mati, San Isidro, and Banaybanay, Davao Oriental, on February 22, 2025. The second colony was derived from twenty larval individuals collected from a corn field along Pugaan–Mandulog Road, Iligan City, Lanao del Norte, on April 11, 2025. All larvae were transported to the Center of Integrative Health, the Premier Research Institute of Science and Mathematics (PRISM) at Mindanao State University–Iligan Institute of Technology (MSU-IIT). They were reared in the laboratory on castor plant leaves (*Ricinus communis*) (Dahi et al. 2020) under controlled conditions of 26±2°C, 75-80% relative humidity, and a 16:8 h (L:D) photoperiod (Pavana et al. 2023). A subset of fourth instar larvae was used for DNA extraction, as their softer tissues and lower chitin content facilitate more efficient isolation (Huanca-Mamani et al. 2015).

Larvae DNA extraction

Genomic DNA was extracted from *S. frugiperda* 4th instar larvae using the NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's protocol for animal tissue. Approximately 25 mg of larval tissue was homogenized in 75 µL Phosphate-Buffered Saline (PBS). Pre-lysis was initiated by adding 180 µL Buffer T1 and 25 µL Proteinase K, followed by incubation at 56°C for 1-2 h to ensure complete tissue breakdown. Following pre-lysis, 200 µL Buffer B3 was added to each tube, and the samples were vortexed and incubated at 70°C for 10 min to facilitate cell lysis. The resulting lysate was transferred to a new microcentrifuge tube, mixed with 210 µL of 96-100% ethanol, and applied to a NucleoSpin® Tissue Column seated in a Collection Tube. Samples were centrifuged at 11,000× g for 1 min, and the flow-through was discarded. The DNA-bound columns were washed sequentially: first with 500 µL Buffer BW (centrifuged at 11,000× g for 1 min), then with 600 µL Buffer B5 (centrifuged under the same conditions). To remove residual ethanol, a final dry spin was performed at 11,000× g for 1 min. Columns were transferred to clean 1.5 mL microcentrifuge tubes, and DNA was eluted by adding 100 µL Buffer BE directly to the membrane, incubating at room temperature for 1 min, and centrifuging at 11,000× g for 1 min. The concentration and purity of the extracted DNA were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific). DNA yields ranged from 200 to 1500 ng/µL, with A260/280 ratios of 1.5-2.0, indicating generally acceptable purity for PCR amplification (Lucena-Aguilar et al. 2016). A total of fourteen representative biological samples were processed. The first set consisted of seven

larvae from the Davao Oriental colony and was used for the initial screening of all five primer sets. The second set included seven larvae from the Iligan City colony, which were used for independent validation of the top three performing primers.

Primer design, and in silico validation

Corn strain sequences of the COI gene (accession numbers U72974 and U72976) were obtained from the NCBI primary database (https://www.ncbi.nlm.nih.gov). These partial COI gene sequences were selected as they are considered the most likely ancestral haplotypes of *S. frugiperda* (Malekera et al. 2023). PCR primer design and in silico analysis were carried out by utilizing software and web-based informatics tools. The Primer design was performed using Primer3 version 2.3.7, integrated into Geneious Prime (version 2023.2.1; https://www.geneious.com). The designed primers were evaluated for specificity using NCBI's Primer-BLAST tool to ensure accurate detection of *S. frugiperda*. Initially, 10 forward primers and 10 reverse primers (10 primer sets) were obtained, each for *S. frugiperda* strains from corn (Table 1). Among these, five primers were selected that have met the specific criteria for good primers, namely with base lengths of 20 to 22 base pairs (bp), Tm values between 57.5°C and 60.3°C, and GC contents from 50% to 55%, ideal or recommended amplicon length of 100-250 bp, and have no secondary structure. To ensure novelty and assess performance, our primer sets were compared with established COI primers for *S. frugiperda* used in Asia and the Americas (Table 2).

The five primer pairs generated using Geneious Prime software were analyzed and evaluated using PrimerStat (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) for sensitivity and suitability towards the target sequences. Each primer pair was assessed individually to ensure compliance with key parameters, including GC content, melting temperature (Tm), and absence of secondary structures or self-dimers. To evaluate the strain specificity of the designed corn-strain primers, an in silico binding analysis was performed using Primer3. A total of 213 *S. frugiperda* COI gene sequences were retrieved from the NCBI through Geneious' integrated search, comprising 83 annotated as corn-strain isolates and 130 as rice-strain isolates. Sequence retrieval was conducted using a targeted search query that filtered for records explicitly labeled with the host strain designation ("corn" or "rice"). The five corn-specific primer sets candidate were assessed using the "Test Saved Primers" function in Geneious, which evaluates binding performance across multiple input sequences. Primer-template alignment was based on default stringency parameters to avoid non-specific binding. Each primer set was tested for binding against all 213 sequences to determine amplification selectivity. The resulting binding profiles were tabulated according to strain classification (Table 4). Additionally, 10 COI sequences from closely related noctuid species were included to assess cross-species amplification. To provide a benchmark, these published COI primers (Table 2) were also analyzed to confirm that our primers were distinct from previously reported COI primers, thereby ensuring novelty. The first binding was assessed with a zero-mismatch tolerance to ensure specificity. Validated primers were synthesized and ordered from Integrated DNA Technologies (IDT). Upon arrival, the primers were received as a lyophilized pellet. Each primer was reconstituted in nuclease-free water. Microcentrifuge tubes were clearly labeled and stored as follows: stock solutions (100 µM) at -20°C for long-term storage and working solutions (10 µM) at 4°C for short-term use.

Table 1. Specification of COI gene primer candidates (corn strain) of *S. frugiperda*

Set	Primer name	Sequence (5' > 3')	Tm	%GC	Amplicon size (bp)
1	101 F	TTCGAGCTGAATTAGGGACTCC	59.6	59.6	178
	278 R	CGTGGGAAAGCTATATCAGGGG	50.0	50.0	
2	102 F	TCGAGCTGAATTAGGGACTCC	60.3	60.3	175
	276 R	TGGGAAAGCTATATCAGGGGC	54.5	54.5	
3	104F	GAGCTGAATTAGGAACTCCAGG	50.0	58.2	270
	373R	CTGTTCATCCAGTTCCTGCTC	52.4	58.6	
4	103F	CGAGCTGAATTAGGAACTCCAG	50.0	58.5	269
	371R	GTTCATCCAGTTCCTGCTCC	55.0	58.3	
5	105 F	AGCTGAATTAGGGACTCCAGG	60.2	60.2	171
	275 R	GGGAAAGCTATATCAGGGGCTC	54.5	54.5	
6	253 F	GGAGCCCCTGATATAGCTTTCC	57.8	57.8	168
	420 R	TACTGAACTACCGCCATGAGC	50.0	50.0	
7	254 F	GAGCCCCTGATATAGCTTTCCC	59.9	59.9	121
	374 R	ACTGTTCATCCAGTTCCTGCTC	54.5	54.5	
8	255 F	AGCCCCTGATATAGCTTTCCC	60.0	60.0	118
	372 R	TGTTCATCCAGTTCCTGCTCC	54.5	54.5	
9	256 F	GCCCCTGATATAGCTTTCCC	58.9	58.9	118
	373 R	CTGTTCATCCAGTTCCTGCTC	52.4	52.4	
10	257 F	CCCCTGATATAGCTTTCCCACG	60.0	60.0	118
	374 R	ACTGTTCATCCAGTTCCTGCTC	54.5	54.5	

Note: Five primers (FAWC1–FAWC5) were selected for further testing based on optimal Tm (57.5-60.3°C), GC content (50-55%), primer length (20-22 bp), and absence of self-complementarity

Table 2. Existing COI gene primers of *Spodoptera frugiperda* utilized in various studies

Primer name	Type	Primer sequence (5' to 3')	Source	Study area(s) applied (Author/s)
LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994	India (Maruthadurai and Ramesh 2021) Europe (Mavridis et al. 2025) Indonesia (Lestari et al. 2020)
HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA		
C1J2195	Forward	TTGATTTTTTGGTCATCCAGAAGT	Frohlich et al. 1999	Pakistan (Yousaf et al. 2021)
TL2N3014	Reverse	TCCAATGCACTAATCTGCCATATTA		
JM76 F	Forward	GAGCTGAATTAGGRACTCCAGG	Nagoshi et al. 2007	Brazil, Texas, Florida (Nagoshi et al. 2007)
1058R R	Reverse	ACACCTGTTAATCCTCCTACAG		
924F	Forward	TTATTGCTGTACCAACAGGT	Nagoshi et al. 2020	Myanmar, southern China, Africa and India (Nagoshi et al. 2020)
1303R	Reverse	CAGGATAGTCAGAATATCGACG		
891F	Forward	TACACGAGCATATTTTACATC		
1472R	Reverse	GCTGGTGGTAAATTTTGATATC		

DNA amplification, PCR optimization, and DNA visualization

PCR reactions were optimized using gradient PCR to find the best annealing temperature for each primer pair. Each 10 µL reaction included 1 µL of template DNA extracted from *S. frugiperda* larvae, 0.5 µL of each primer (forward and reverse), 5 µL of PCR mix (1×PCR buffer with 1.5-2.5 mM of $MgCl_2$, 200 µM of each dNTP), and 0.1 µL of Taq DNA polymerase or a high-fidelity alternative. Nuclease-free water (2.9 µL) was added to bring the final volume to 10 µL. There were no positive or negative controls in this study. Future work should incorporate universal COI primers as positive controls and no-template reactions as negative controls to rigorously establish diagnostic sensitivity, specificity, and reproducibility. The reactions were run in a thermal cycler with the following settings: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 49°C, 50.2°C, 51.4°C, 52.5°C, 53.7°C, 54.9°C, 56.1°C, 57.3°C, 58.5°C, 59.6°C, 60.8°C, 62°C (gradient range) for 1 minute, and extension at 72°C for 1 minute. A final extension was done at 72°C for 5 minutes. Based on the gradient results, an annealing temperature of 53.7°C was selected for subsequent PCR reactions, as it yielded the most specific and efficient amplification. Seven genomic DNA samples extracted from *S. frugiperda* larvae, each with unknown host strain identity, were subjected to PCR amplification using five primer pairs designed for strain-specific detection. Each DNA sample was individually amplified with all five primers to determine which primer(s) produced specific amplicons, thereby inferring the potential host strain for each sample. PCR products were analyzed using 1.5% agarose gel electrophoresis in a 1× TBE buffer. Agarose (0.35 g) was dissolved in 25 ml of 1× TBE buffer using a microwave oven, then 2 µL of Gel Green was added. The gel was cast in a tray with a comb and allowed to solidify. After solidification, the gel was placed in a tank and submerged in a 1× TBE buffer. A DNA ladder (100 bp plus) was loaded into the first well. Each PCR product, mixed with loading dye (1 µl dye + 2 µl DNA), was loaded into separate wells. Electrophoresis was run at 100 V and 100 mA for 5 minutes using a Nanopac-300P machine. DNA bands were visualized under a UV transilluminator and compared with the 100bp plus ladder to confirm product size. Following the initial PCR validation of corn-strain primers (FAWC1–FAWC5) on larval samples from corn and rice fields in Davao Oriental, the three most effective primers underwent step-wise validation using separate larval samples collected from corn fields in Iligan City to assess their reliability for strain identification further.

RESULTS AND DISCUSSION

Primer design and in silico testing

Workflow and rationale

To design corn-strain-specific primers for the molecular identification of *S. frugiperda*, we followed a systematic pipeline to ensure accuracy and specificity (Figure 1). The workflow included sequence selection, in silico primer design, structural evaluation, and experimental validation. Several COI primers for *S. frugiperda* have been developed globally (Table 2). However, most are intended for species-level detection, lack strain-level resolution, and produce long amplicons (>600 bp), limiting their use for degraded or field samples. This gap provided the rationale for developing shorter, corn-strain-specific primers.

The reference sequences selected for primer design were GenBank accession numbers U72974 and U72976, due to their well-established status as ancestral corn-strain haplotypes. These sequences were identified initially from native U.S. populations and are consistently cited in the literature as standard markers for strain differentiation (Levy et al. 2002). U72974 has been shown to represent the predominant COI haplotype in invasive corn-strain populations globally, including those in Asia and Africa. It is presumed to be one of the ancestral lineages introduced during the species' spread (Nayyar et al. 2021). Their central position in phylogenetic and haplotype network analyses reflects their evolutionary stability and makes them ideal reference points for distinguishing between the corn and rice strains.

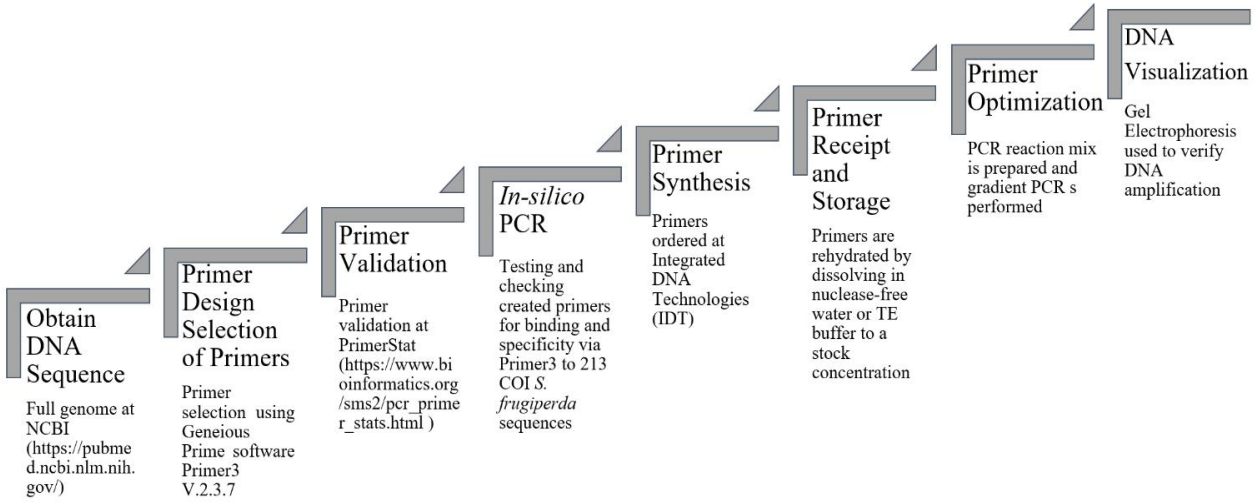

Figure 1. Pipeline for PCR design and in silico PCR analysis utilizing integrative software and web-based bioinformatic tools

Development and evaluation

Primer development was carried out using Geneious Prime 2023.2.1 with the integrated Primer3 tool, which enabled alignment of multiple COI sequences and identification of corn-strain *S. frugiperda* with high specificity and theoretical efficiency (Kearse et al. 2012; Untergasser et al. 2012; Aulia et al. 2024). Using the "Test Saved Primers" feature and validated ancestral haplotypes, primer sets were efficiently designed and evaluated in silico for strain-specific amplification potential. Out of an initial pool of ten primer candidates (Table 1), five primer sets were shortlisted for further testing based on their predicted thermodynamic quality and structural stability. These primers ranged from 20-22 bp in length, with melting temperatures (Tm) between 57.5-60.3°C and GC contents from 50-55%, all within ideal parameters for PCR primer design (Dieffenbach et al. 1993; Sharma et al. 2023) (Table 3). Gradient PCR identified 53.7°C as the optimal annealing temperature, approximately 4-6 °C below the Tm of each primer, consistent with accepted design practices to enhance specificity.

All five primers incorporated a GC clamp at the 3′ end, which improves binding stability and extension efficiency (Dieffenbach et al. 1993; Yang et al. 2022). The 3′-end stability is an important consideration in primer design. Excessively stable base pairing at the 3′ terminus increases the risk of mispriming and primer-dimer formation, especially in complex samples (Asif et al. 2021). This was minimized across all sets by avoiding overly strong 3′-end motifs, thus reducing non-specific interactions while preserving high binding efficiency at intended target sites. This refinement is especially critical in diagnostic applications where false positives must be avoided. In silico structural evaluation using PrimerStat showed that none of the five primer sets formed stable hairpins, self-dimers, or cross-dimers at the annealing temperature. This absence of secondary structure is critical for preventing primer sequestration and non-productive binding, which can reduce PCR efficiency (Rianti et al. 2021; Sari et al. 2024).

Among the candidates, FAWC3 and FAWC4 exhibited the most favorable characteristics, including closely matched forward and reverse Tm values, balanced GC content, and no structural issues. These attributes suggest that FAWC3 and FAWC4 are the strongest candidates for selective and reliable amplification of the corn strain. These parameters are hallmarks of well-designed primers and are likely to yield strong and specific amplicons in PCR. By combining ancestral haplotype references (U72974, U72976) with a robust primer design environment, these primers conform to classical PCR design principles while providing enhanced strain-level specificity compared with previously reported COI primers (Levy et al. 2002; Kearse et al. 2012; Untergasser et al. 2012; Nayyar et al. 2021; Sharma et al. 2023; Aulia et al. 2024). Overall, these parameters and design refinements are likely to yield strong, specific amplicons suitable for both laboratory and field diagnostic applications.

In silico validation

In silico validation of the five designed primer sets was performed against a curated dataset of 213 global *S. frugiperda* COI sequences, comprising 83 corn-strain and 130 rice-strain accessions. To avoid circular validation, the reference sequences used for primer design (U72974 and U72976) were excluded from the dataset. Under zero-mismatch conditions, FAWC3 and FAWC4 exhibited the highest binding efficiency, successfully amplifying 52 corn-strain sequences each, with no binding to rice-strain sequences (Table 4). FAWC1 and FAWC2 amplified 15 corn-strain sequences each, also showing no cross-reactivity with rice-strain sequences. In contrast, FAWC5 did not bind any sequences in the global dataset under these strict parameters. Species-level specificity testing was conducted using COI sequences from ten non-target lepidopteran species, including seven closely related noctuids (*Spodoptera litura, S. exigua, Helicoverpa armigera, Mythimna separata, Sesamia inferens, Leucania loreyi,* and *Chrysodeixis includens*), one erebid (*Hydrillodes lentalis*), and two other agricultural moths

(*Plutella xylostella* and *Ostrinia furnacalis*). None of the primer sets showed binding to these non-target sequences under zero-mismatch conditions, confirming their taxonomic specificity and minimizing the likelihood of false-positive amplification in field samples.

The in silico results indicate that FAWC3 and FAWC4 are the most robust primers for global corn-strain detection, given their high amplification rates and absence of cross-reactivity with rice-strain sequences. FAWC1 and FAWC2 also exhibit strain specificity, although their lower binding coverage suggests a more limited diagnostic reach. FAWC5, despite failing to amplify sequences in the global dataset, successfully amplified six out of seven Philippine-derived corn-strain samples in wet-lab experiments (Figure 3). This result suggests that FAWC5 targets a conserved motif present in local corn-strain populations that may be underrepresented in public COI databases. FAWC5 was designed based on U72976, a variant of the canonical corn-strain haplotype U72974, recognized in global studies as a distinct yet evolutionarily stable lineage (Levy et al. 2002; Nayyar et al. 2021). Collectively, these results demonstrate that the combination of FAWC3, FAWC4, and FAWC5 provides comprehensive coverage, capturing both globally distributed and region-specific corn-strain haplotypes while maintaining high specificity and minimizing off-target amplification.

PCR optimization and validation

Optimization

To identify the optimal annealing temperature for each primer set, a gradient PCR was conducted. The temperature range was selected based on the average melting temperatures (Tm) of the forward and reverse primers. Two primer sets, FAWC2 and FAWC4, were chosen for initial gradient PCR due to their favorable thermodynamic properties, including optimal Tm values (~58-60°C), minimal secondary structure formation, and suitable GC content. These characteristics suggested high amplification potential and primer stability, making them suitable representatives for experimental validation despite differences in predicted binding efficiency from the in silico analysis (Table 4).

The gradient PCR results, shown in Figure 2, revealed that the optimal annealing temperatures were 58.5°C for FAWC2 and 53.7°C for FAWC4. FAWC2 exhibited the strongest amplification at 58.5°C (lane a R9), with diminished product yield at both lower and higher temperatures, indicating a relatively narrow optimal window. In contrast, FAWC4 produced optimal amplification at 53.7°C (lane b R5) and demonstrated relatively stable amplification across a broader temperature range. These observations are consistent with the general principle that annealing temperatures slightly below the primer Tm enhance binding efficiency while preventing non-specific amplification (Asif et al. 2021).

Based on these findings, an annealing temperature of 53.7°C was selected for all five primer sets (FAWC1-FAWC5) to ensure workflow efficiency, assay reproducibility, and compatibility across primers during multiplex testing. While this temperature represents a compromise for FAWC2, it allowed consistent amplification for all primer sets and aligned with best practices for standardized PCR assays (Asif et al. 2021; Sharma et al. 2023).

Table 4. In silico binding results (zero mismatch) of five designed primer sets against a curated dataset of 213 global *S. frugiperda* COI sequences (83 corn-strain, 130 rice-strain). Reference sequences used for primer design (U72974 and U72976) were excluded

Primers	COI corn sequences (83)	COI rice sequences (130)
FAWC1	15	0
FAWC2	15	0
FAWC3	52	0
FAWC4	52	0
FAWC5	0	0

Note: FAWC5 did not bind any global sequences under zero-mismatch conditions, but was retained because it showed strong, specific amplification of Philippine-derived samples in wet-lab experiments (see Figure 3)

Table 3. The top five corn strain PCR primer sets were generated and used in this study

Reference sequence (Acc. no.)	Primer set	Primer name	Direction	Primer sequence (5'- 3')	Length (bp)	GC (%)	Amplicon size (bp)	Hairpin Tm	Self-Dimer Tm	Tm
U72974.1	FAWC1	104 F	Forward	GAGCTGAATTAGGAACTCCAGG	22	50	270	41.8	None	58.2
		373 R	Reverse	CTGTTCATCCAGTTCCTGCTC	21	52.4		None	None	58.6
U72976.1	FAWC2	103 F	Forward	CGAGCTGAATTAGGAACTCCAG	22	50	269	41.8	None	58.5
		371R	Reverse	GTTCATCCAGTTCCTGCTCC	20	55		None	None	58.3
U72974.1	FAWC3	256 F	Forward	GCCCCTGATATAGCTTTCCC	20	55	118	None	None	57.5
		373 R	Reverse	CTGTTCATCCAGTTCCTGCTC	21	52.4		None	None	58.6
U72974.1	FAWC4	254 F	Forward	GAGCCCCTGATATAGCTTTCCC	22	54.5	121	35.2	None	60
		374 R	Reverse	ACTGTTCATCCAGTTCCTGCTC	22	50		43	None	60.3
U72976.1	FAWC5	253 F	Forward	GGAGCCCCTGATATAGCTTTCC	22	54.5	168	35.2	None	60
		420 R	Reverse	TACTGAACTACCGCCATGAGC	21	52.4		38.5	None	59.9

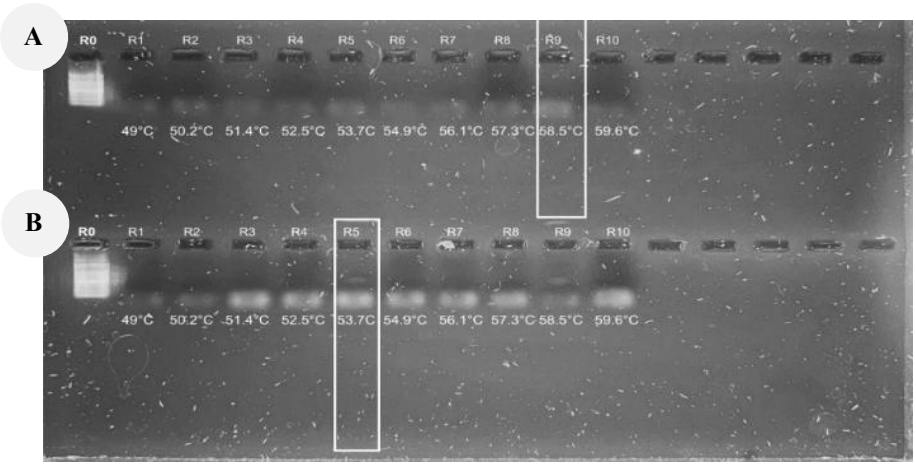

Figure 2. Gradient PCR optimization of the top two primer sets designed in this study. Amplification results are shown for COI primer sets A. FAWC2 and B. FAWC4 using DNA from Specimen 1 (Sp1) of *Spodoptera frugiperda* (fall armyworm) larvae. Lanes labeled R0 contain 100 bp molecular weight markers (Dongsheng Biotech), while lanes R1-R10 contain PCR products generated at different annealing temperatures. Boxed lanes indicate the optimal annealing temperature for each primer set

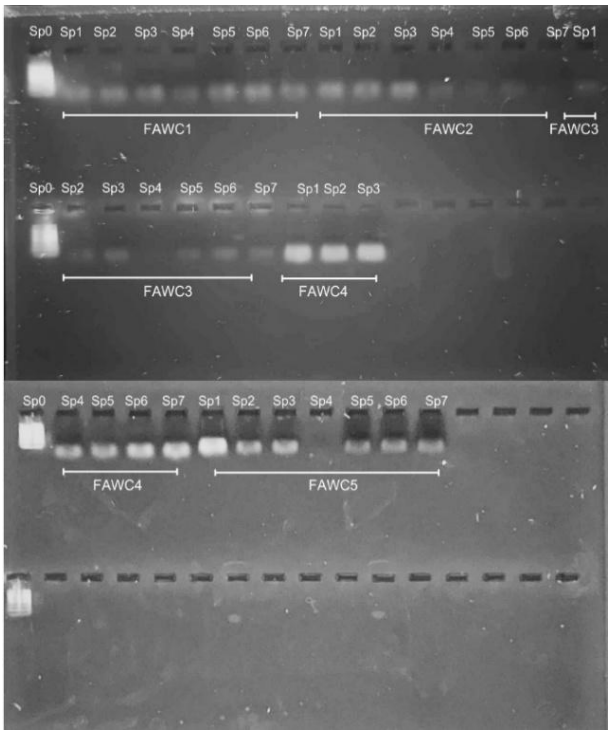

Figure 3. PCR amplification results of primer sets targeting the corn strain (FAWC1, FAWC2, FAWC3, FAWC4, FAWC5) in seven *Spodoptera frugiperda* specimens from Davao Oriental. Lane Sp0 represents the 100 bp molecular weight marker (Dongsheng Biotech). At the same time, lanes Sp1-Sp7 correspond to individual *S. frugiperda* specimens tested. The presence of a clear amplicon band indicates successful amplification of the corn-strain-specific COI region, confirming the sample's corn-strain identity

Initial validation

PCR amplification using the five corn-strain-specific primer sets was tested on seven Philippine laboratory-reared *S. frugiperda* samples (Sp1-Sp7), and the results are shown in Figure 3. The 100 bp plus DNA ladder (Sp0) provided molecular size references, confirming that visible bands corresponded to the expected COI amplicon sizes. Among the primer sets, FAWC4 produced strong, consistent amplification across most samples, whereas FAWC5 selectively amplified only specific individuals. FAWC1 and FAWC2 yielded moderate amplification in some samples, whereas FAWC3 showed faint or undetectable bands across all individuals. Specifically, FAWC2, optimized at 58.5°C, performed reasonably at 53.7°C, with Sp1-Sp3 showing moderate amplification, but Sp4-Sp6 showed little or no amplification. This highlights the inherent trade-off between maintaining assay uniformity for operational simplicity and optimizing primer-specific efficiency for maximal sensitivity. FAWC1 produced moderate amplification across several individuals. FAWC4, targeting a broadly conserved corn-strain motif, consistently amplified most samples, while FAWC5 successfully amplified six out of seven specimens, demonstrating its selectivity for specific lineages. Examination of individual responses revealed haplotype variation: Sp4 showed strong amplification with FAWC4, moderate bands with FAWC1, faint detection with FAWC2, and no amplification with FAWC3 or FAWC5. Conversely, Sp1 showed strong amplification with FAWC4 and FAWC5 but weak or absent bands with FAWC1, FAWC2, and FAWC3.

The observed amplification patterns highlight several important points. First, while in silico predictions identified FAWC3 and FAWC4 as the most promising primers for global corn-strain detection, FAWC3's poor experimental performance indicates that computational predictions may not fully account for template mismatches, secondary structures, or regional haplotype diversity. Experimental factors such as salt concentration, DNA quality, inhibitors, or secondary structures can further influence amplification success (Sidstedt et al. 2020; Jäger 2024; Kalendar et al. 2024).

FAWC2, although thermodynamically optimized, showed variable amplification across samples, reflecting sensitivity to both thermal parameters and haplotype

diversity. FAWC1 displayed moderate stability, but FAWC4 consistently amplified multiple individuals, confirming its ability to target a conserved motif across diverse corn-strain lineages. FAWC5, despite limited global in silico binding, successfully amplified Philippine-derived samples, supporting its utility as a regionally adapted diagnostic primer. Differences between individual samples, such as those observed in Sp1 and Sp4, illustrate mitochondrial haplotype variation within the Philippine population and underscore the need for complementary primers to capture both broadly conserved and lineage-specific variants.

Overall, these results support a combined approach: using FAWC4 for broad corn-strain detection and FAWC5 for lineage-specific confirmation. This strategy balances sensitivity and specificity, accommodates local haplotype diversity, and reinforces the importance of pairing in silico analysis with experimental validation in local populations. The Philippine colony used in this study, derived from mixed corn- and rice-field sources and maintained on corn for multiple generations, likely retains diverse corn-strain haplotypes. The complementary performance of FAWC4 and FAWC5 confirms the presence of multiple lineages and provides a robust framework for localized strain monitoring.

Step-wise validation

To further corroborate these observations, step-wise validation using independent DNA samples (Figure 4) confirmed the differential performance of the top three primers (FAWC3, FAWC4, FAWC5). FAWC4 consistently produced strong, uniform bands across most lanes (B2–B7), reinforcing its suitability as a primary diagnostic primer for corn-strain *S. frugiperda*. FAWC5 demonstrated selective amplification, with bands appearing in specific lanes (C3-C6) while slightly faint in one (C7), consistent with its lineage-specific detection of Philippine haplotypes. In contrast, FAWC3 exhibited faint and inconsistent amplification across most lanes (A2-A7) and no amplification in one lane (A1), suggesting limited reliability in practical applications. These results, consistent with earlier observations from the laboratory colony, indicate that FAWC3 likely suffers from 3′-end mismatches and potential secondary structures that reduce annealing efficiency, underscoring the importance of validating primers across multiple experimental conditions and integrating in silico with empirical approaches for robust strain-specific primer development. Collectively, the step-wise validation confirms that FAWC4 and FAWC5 function effectively as complementary primers: FAWC4 for broad corn-strain detection and FAWC5 for regionally adapted, lineage-specific monitoring. Together, this dual-primer strategy achieves greater strain discrimination than existing general COI primers, which typically detect species but not intra-species variation. This approach ensures accurate identification across diverse mitochondrial haplotypes while minimizing false negatives, providing a robust framework for local surveillance and management of *S. frugiperda* populations.

Limitations and future work

The discrepancies between computational predictions and laboratory outcomes, particularly with FAWC3 and FAWC5, underscore both the strengths and constraints of in silico primer evaluation. Factors such as template mismatches, secondary structures, and sample quality can significantly influence amplification success. Despite these challenges, the primers, especially FAWC4 and FAWC5, demonstrated greater precision in strain identification than existing COI-based diagnostics, validating the novelty of this study's contribution. However, as this was a preliminary validation, the absence of comparison with other COI-based primers as positive controls and no-template reactions as negative controls limits the complete assessment of diagnostic sensitivity and specificity. Future work should address these gaps and further refine primer performance under optimized assay conditions to enhance both reliability and broader applicability in field diagnostics.

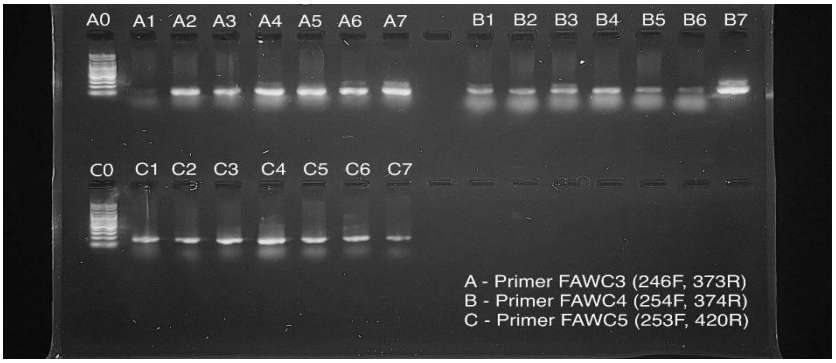

Figure 4. Step-wise validation of corn-strain-specific primers (FAWC3, FAWC4, and FAWC5) using DNA from a separate population of *Spodoptera frugiperda* specimens collected from Iligan City. Lanes A1-A7, B1-B7, and C1-C7 represent amplification using FAWC3, FAWC4, and FAWC5, respectively. Lane A0 and C0 show the 100 bp DNA ladder (Dongsheng Biotech) as a molecular size reference. FAWC4 shows consistent amplification across samples, while FAWC5 demonstrates selective lineage-specific amplification. FAWC3 exhibits faint and inconsistent amplification. The presence of a visible band indicates successful amplification of the corn-strain-specific COI target

In conclusion, this study successfully designed and validated corn-strain-specific COI primers for the molecular identification of *S. frugiperda*, addressing a key limitation in current diagnostic tools in Mindanao, Philippines. The newly developed primers (FAWC1–FAWC5) produce short, strain-specific amplicons suitable for both laboratory and field-collected samples. To enhance reproducibility and streamline diagnostic workflows, a standardized annealing temperature of 53.7°C was implemented across all primer sets as a practical compromise that ensured reliable amplification under both laboratory and field conditions. Among these, FAWC4 demonstrated consistent and strong amplification across diverse populations, establishing it as the most reliable primer for broad detection of corn-strain FAW. FAWC5, while more lineage-specific, showed high sensitivity to Philippine-derived haplotypes, making it valuable for regional monitoring where local genetic diversity may differ from global references. The complementary use of FAWC4 and FAWC5 thus provides a practical dual-primer strategy. FAWC4 is best used for routine field surveillance, and FAWC5 for refined lineage tracking specific to Philippine-derived samples, enhancing diagnostic accuracy and guiding targeted control measures. Overall, this study demonstrates that integrating global bioinformatic analyses with local experimental validation produces more comprehensive and practical diagnostic solutions for sustainable agricultural practices in the Philippines. By enabling early and precise strain detection, these primers support more informed pesticide application, minimize chemical overuse, and contribute to sustainable and region-specific Integrated Pest Management (IPM) strategies.

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