SCREENING FOR MICROSATELLITES OF THE COCOA POD BORER *CONOPOMORPHA CRAMERELLA* (SNELLEN) (CPB); PRELIMINARY WORK WITH CPB FROM MALAYSIA

Roslina Binti Mohd Shah[1,] Marynold Purificacion[3],' Anisah Bintil Savantil[1] , Saripah Binti Bakar[2], Adly M.M. Abd-Alla[4]

[1] Centre for Cocoa Biotechnology Research, Malaysian Cocoa Board, Kota Kinabalu Industrial Park, Norowot Road 88460 Sabah, Malaysia

[2] Pusat Pembangunan Koko Bagan Datuk, Lembaga Koko Malaysia, Peti Surat 30, Jalan SG. Dulang 36307 SG. Sumun Perak, Malaysia

[3] Biological Control Research Unit, Center for Natural Science and Environmental Research, De La Salle University, 2401 Taft Avenue, 0922 Manila, Philippines

[4] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna International Centre, P.O. Box 100, 1400 Vienna, Austria

**Corresponding author: roslina@koko.gov.my*

Malaysian Cocoa J. 15 (1): 90-94 (2023)

ABSTRACT – *The cocoa pod borer (CPB) Conopomorpha cramerella (Snellen) (Lepidoptera: Gracillaridae) is one of the major constraints in cocoa production. In addition to the cultural and chemical control methods, biological control methods such as the Sterile Insect Technique (SIT) can be implemented for CPB control. However, SIT is species-specific dependent, and its successful implementation will mainly rely on the competitiveness of sterile males released in the targeted area and the presence of an isolated population to avoid reinvasion after the completion of the SIT programmes. Currently, no genetic markers are available for population genetics analyses of CPB. During the present work, we searched for suitable microsatellites in the partial genome sequence of CPB. Out of 28730 primer pairs found, 192 pairs were selected based on the amplicon size (180-300 nt) and the motif repeats (≥ 11) and synthesized and tested against C. cramerella DNA. Among those 192 pairs, a collection of 30 proves to be working well with CPB from Malaysia. This is encouraging for a preliminary investigation into finding appropriate microsatellites to use in a larger population of CPB from other ASEAN nations. Moths collected in Malaysia, Indonesia, the Philippines, and Vietnam will be analysed using the final twelve microsatellites that meet all criteria. A defined microsatellite loci will provide useful tools for the analysis of the population genetics of this pest to better understand its dynamics and ecology and to identify potential isolated populations that can be targeted with area-wide integrated pest management with SIT component.*

Key words: Sterile insect technique, microsatellites, primers , population genetics, amplicons

INTRODUCTION

Cocoa pod borer (CPB), *Conopomorpha cramerella* (Snellen), is the principal insect pest threatening cocoa production in South East Asia (Lepidoptera: Gracillaridae). It was hypothesised that CPB began with a shift from rambutan (*N. lappaceum L.*) and langsat (*L. parasiticum* (Osbeck) Sahn & Bennet) to cocoa as a host (Azhar & Long 1996). The cocoa pod, which contains the beans used in chocolate production, is attacked by CPB larvae. The cocoa pod is economically significant because it contains the beans used in chocolate production.

This pest is responsible for an average loss of 40 to 60% of cocoa bean production in Southeast Asia and up to 80% of farm losses in the absence of pest control (Posada *et al*., 2010). To reduce the CPB population, numerous control measures have been put in place. Protecting the cocoa pods with plastic bags yielded promising results but was labor-intensive and expensive.

Furthermore, biological control efforts such as the use of natural enemies (e.g., black ants, parasitoids, or entomopathogens) and the use of sex pheromones were ineffective or not economically viable (Niogret *et al*., 2022a,b). Pesticides remain the most prevalent method for controlling CPB (Zhang et al., 2008; Niogret et al., 2020). Adding a new control measure to these existing measures will increase their effectiveness.

Sterile Insect Technique (SIT) is a technique that has been successfully validated as part of an area-wide integrated pest management strategy (AW-IPM) to control insect pest populations in other crops. The efficacy of SIT is contingent on the mating competitiveness of the sterile male released into the targeted area.

In order to prevent reinvasion following the conclusion of SIT programmes, the effectiveness and viability of the SIT necessitate that targeted populations be isolated in some fashion (Dyck *et al*., 2021).

Population genetics tools can be used to determine the degree of isolation of a population (Gooding and Krafsur, 2005). Molecular DNA

markers such as mitochondrial DNA (mtDNA) sequences, random amplified polymorphic DNA (RAPD), single nucleotide polymorphisms (SNPs), amplified fragment length polymorphism, and microsatellites are used to analyse population genetics (Abdul-Muneer, 2014; Behura, 2006). Microsatellite markers are abundant one-to-six nucleotide tandem repeats in the DNA that serve as genetic markers.

Due to the fact that they are highly polymorphic, species-specific, Mendelian, co-dominant, and easily amplified by polymerase chain reaction (PCR), they are widely used for population genetic analysis (Field and Wills, 1996). Recent efforts have been made to implement an SIT within the context of AW-IPM against CPB, which would significantly reduce this pest's direct and indirect economic impacts on cacao-cocoa production and the farmer's quality of life. Nevertheless, implementing the AW-IPM with SIT component would necessitate an accurate identification of the targeted insect species and the capability of identifying isolated populations in order to reduce the risk of reinvasion.

To date, no microsatellites have been developed for C. cramerella, and mitochondrial DNA has been utilised to analyse the population structure (Shapiro et al., 2008; Valenzuela et al., 2022). In this study, we developed and characterised C. cramerella microsatellite markers that can be used in future population genetics studies for SIT implementation.

MATERIALS AND METHODS

Insect collection and DNA extraction

Figure 1: CPB were collected from five different locations in Malaysia.

Conopomorpha cramerella cocoa pod borer (CPB) adults were collected from five different locations in Malaysia (Figure 1). Using a cylindrical plastic bag, CPB samples were collected manually from beneath infected cocoa branches. In order to ship samples to the Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Programme on Nuclear Techniques for Food and Agriculture, Seibersdorf, Austria, propylene glycol was substituted for ethanol. The propylene glycol was replaced with absolute ethanol upon arrival and stored at -20 degrees Celsius until DNA extraction. Using the Qiagen DNeasy® Blood & Tissue Kit (Qiagen Inc., Valencia, CA) as instructed by the manufacturer, total genomic DNA was extracted from one adult CPB. With a SynergyTM H1 microplate reader, the amount and quality of extracted DNA were evaluated (BioTek, Winooski, Vermont, USA).

Sequence analysis and microsatellite loci selection

The partial genome sequence of *C. cramerella* (Accession SJJU01000000, WGS SJJU01000001-

SJJU01073142) was utilised as a reference for microsatellite primer selection. Msatcommander 1.08 (Faircloth, 2008) was utilised to look for dinucleotide and trinucleotide motifs. On the basis of product size (180 – 380 nt) and the number of repeats (11 repeats), 192 primer pairs were chosen, synthesised, and tested for microsatellite amplification with PCR using the CPB extracted DNA.

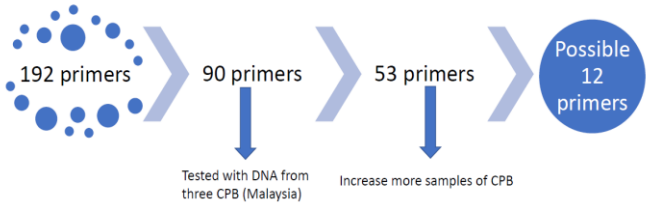

Figure 2: Gel picture of primers testing on CPB DNA from Malaysia

The 192 synthesized primer pairs were screened on the DNA extracted from individuals of *C. cramerella* from Malaysia. PCR amplifications were performed by mixing 12.5 µL of Qiagen *Taq* PCR Master Mix Kit (Qiagen Inc., Valencia, CA), 10 µL of nuclease-free H_2O (Qiagen Inc., Valencia, CA), 0.2 µM final concentration of each forward and reverse primer and 1.5 µL (4 ng) DNA). The PCR conditions were as follows: 94°C for 2 min; 34 cycles at 94°C for 15 s, 58°C for 15 s, and 68°C for 15 s; ending with a final extension for 5 min at 68°C. The PCR amplification was checked on 2% agarose E-gel™ stained with ethidium bromide (Invitrogen, ThermoFisher Scientific, Waltham, MA).

RESULTS AND DISCUSSIONS

Among the 192 primers, only 53 primers pair were successfully amplified the expected microsatellite fragments. These 53 primer pairs were then screened on tens more individuals of *C. cramerella* from Malaysia. The amplification patterns between samples were compared on 4% agarose E-gel™.

Table 1: OD reading of some of the CPB from Malaysia

Sample Read#	Name	260	280	260/280	ng/µL	Mean	CV (%)
1	M1	0.113	0.053	2.13	112.62	102.932	13.311
	M1	0.093	0.043	2.177	93.243		
2	M2	0.114	0.053	2.129	113.879	111.499	3.019
	M2	0.109	0.051	2.136	109.119		
3	M3	0.074	0.035	2.145	74.47	81.249	11.798
	M3	0.088	0.041	2.137	88.027		
4	M4	0.142	0.065	2.19	142.497	137.488	5.153
	M4	0.132	0.061	2.173	132.479		
5	M5	0.194	0.089	2.182	193.794	215.247	14.095
	M5	0.237	0.108	2.193	236.7		
6	M6	0.073	0.033	2.191	72.646	70.168	4.994
	M6	0.068	0.031	2.168	67.69		
7	M7	0.063	0.03	2.054	62.552	59.732	6.677
	M7	0.057	0.027	2.086	56.911		
8	M8	0.186	0.085	2.2	186.369	271.29	44.268
	M8	0.356	0.163	2.189	356.21		
9	M9	0.071	0.034	2.083	70.518	61.818	19.903
	M9	0.053	0.024	2.212	53.119		
10	M10	0.094	0.043	2.172	93.887	102.488	11.868
	M10	0.111	0.052	2.157	111.088		

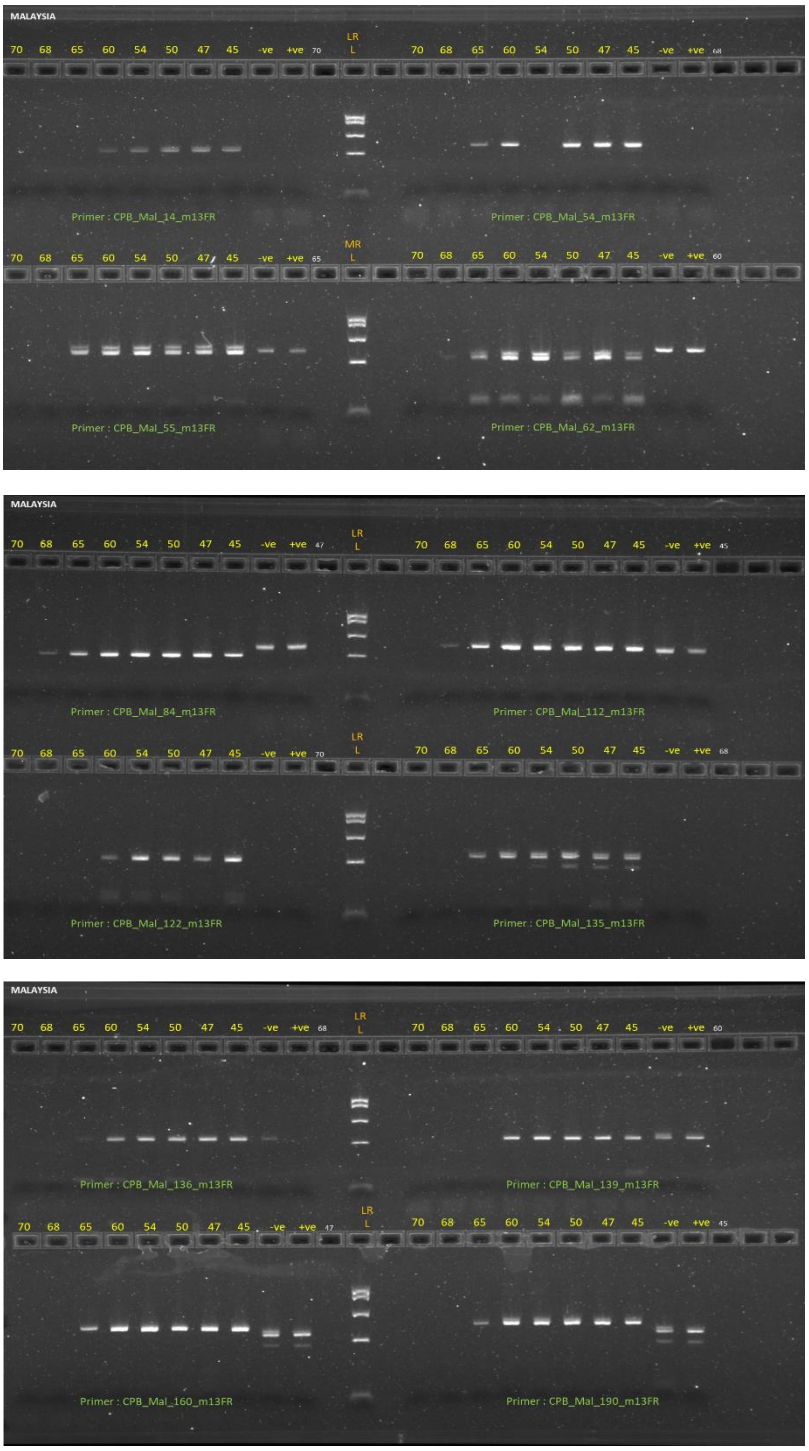

Figure 2: Gel picture of primers testing on CPB DNA from Malaysia

CONCLUSION

Among the 192 pairs of microsatellite primers tested as part of the preliminary examination of CPB DNA from Malaysia, 30 pairs yielded encouraging results. As part of future research, these microsatellites will be tested on a more diverse population of CPB from Indonesia, the Philippines, and Vietnam. The results will be more accurate because they will represent a larger and genetically diverse population, and screening these microsatellites will eventually reduce the number of primers to twelve or fewer.

ACKNOWLEDGEMENT

The study was financially supported under the 12th Malaysian Plan Development Fund (P20001001210005 – Development of Cocoa Agri-Biotechnology Innovation Programme) and Collaboration with the International Atomic Energy Agency (IAEA) in Vienna under the RAS5086 project with the Malaysian Cocoa Board. The authors would like to express unique appreciation to the Director General of the Malaysian Cocoa Board, the Deputy Director General of the Research and Development, and the Director of the Biotechnology Division for their continuous support and kind approval to conduct this research.

REFERENCES

Abdul-Muneer, P.M., 2014. Application of Microsatellite Markers in Conservation Genetics and Fisheries Management: Recent *Advances in Population Structure Analysis and Conservation Strategies. Genet. Res. Int*. 2014, 11. https://doi.org/10.1155/2014/691759

Azhar I & Long GE (1996) Effect of cocoa pod age on egg distribution and egg parasitism of the cocoa pod borer in Malaysia. *Entomologia Experimentalis et Applicata* **81**: 81–89.

Dyck, V.A., Hendrichs, J., Robinson, A.S. (Eds.), 2021. Sterile Insect Technique: *Principles and Practice in Area-Wide Integrated Pest Management, 2nd ed. CRC Press, Boca Raton,* FL. https://doi.org/10.1201/9781003035572

Field, D., Wills, C., 1996. Long, polymorphic microsatellites in simple organisms. *Proc. R. Soc. B Biol. Sci.* **263**, 209–15. https://doi.org/10.1098/rspb.1996.0033

Gooding, R.H., Krafsur, E.S., 2005. Tsetse genetics: Contributions to biology, systematics, and control of tsetse flies. *Annu. Rev. Entomol*. **50**, 101–123.

Niogret J., Ekayanti A., Zhang A. 2022a. Sex pheromone of cocoa pod borer, Conopomorpha cramerella: Field activity evaluation of pheromone formulations in Indonesian plantation. *Insects*, **13**, 663. doi.org/10.3390/insects13080663

Niogret J., Kendra P.E., Ekayanti A., Zhang A., Marelli J-P., Tabanca N., Epsky N. **2022b**. Development of a Kairomone-Based Attractant as a Monitoring Tool for the Cocoa Pod Borer, Conopomorpha cramerella (Snellen) (Lepidoptera: Gracillariidae). *Insects*, **13**, 813. doi.org/10.3390/insects13090813

Posada FJ, Virdiana I, Navies M, Pava-Ripoll M & Hebbar P (2010) Sexual dimorphism of pupae and adults of the cocoa pod borer, Conopomorpha cramerella. *Journal of Insect Science* **52**: 1–8

Shapiro, L.H., Scheffer, S.J., Maisin, N., Lambert, S., Purung, H., Sulistyowati, E., Vega, F.E., Gende, P., Laup, S., Rosmana, A., Djam, S., 2008. Conopomorpha cramerella (Lepidoptera: Gracillariidae) in the Malay Archipelago: genetic signature of a bottlenecked population?. . 2008 Sep 1;101(5):930-8. 2008, *Annals of the Entomological Society of America* **101**, 930–938. https://doi.org/10.1603/0013-8746(2008)101[930:CCLGIT]2.0.CO;2

Zhang, A., Kuang, L.F., Maisin, N., Karumuru, B., Hall, D.R., Virdiana, I., Lambert, S., Purung, H.B., Wang, S., Hebbar, P., 2008. Activity Evaluation of Cocoa Pod Borer Sex Pheromone in Cocoa Fields. *Environ. Entomol.* **37**, 719–724. https://doi.org/10.1603/0046-225X(2008)37[719:AEOCPB]2.0.CO;2