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Research Article

Selecting Primers for RAPD, Microsatellite and Mitochondrial Cytochrome Oxidase Subunit 1 for Genetic Variation Analysis of Asian Corn Borer (*Ostrinia furnacalis* Guenée) Population in Java, Indonesia

Ahmad Taufiq Arminudin[1], Y. Andi Trisyono[2]*, Arman Wijonarko[2], & Suputa[2]

[1]*Faculty of Agriculture and Animal Husbandry, Sultan Syarif Kasim State Islamic University*
Jln. H.R. Soebrantas Km. 18 Simpang Baru, Tampan, Pekanbaru, Riau 28293 Indonesia

[2]*Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada*
Jln. Flora No. 1, Bulaksumur, Sleman, Yogyakarta 55281 Indonesia

**Corresponding author. E-mail: anditrisyono@ugm.ac.id*

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ABSTRACT

Primer plays an important role in studying genetic diversity of an insect species. This research was aimed to select the suitable primers to visualize the genetic diversity of Asian corn borer (*Ostrinia furnacalis*) using Random Amplified Polymorphic DNA (RAPD), microsatellite, and mitochondrial cytochrome oxidase subunit 1 gene (mtCO1). Twenty four RAPD primers (OPA1, OPA4, OPA7,OPA8, OPA10, OPA11, OPA12, OPA13, OPB7, OPB10, OPB11, OPB12, OPB15, OPC4, OPC5, OPC14, OPC16, OPC18, OPC20, OPD3, OPD8, OPD10, OPD13, OPD14) and five microsatellite primers (T3, T4, T5, T81, D25) resulted high polymorphic informations of the genetics of *O. furnacalis* in Java Indonesia. Universal primers, Lep and Heb were appropriateto do molecular identification of *O. furnacalis* based on BLAST system on GenBank and BOLD systems.

Keywords: microsatellite, mtCO1, *Ostrinia furnacalis*, primer, RAPD

INTRODUCTION

Asian corn borer (ACB), *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae), is an important pest causes high losses in Indonesia (Abdullah & Rauf, 2011; Subiadi *et al*., 2014). The management techniques to control this pest are still developed, ecologically (Nonci, 2004) to genetically by producing *Bacillus thuringiensis* (Bt) transgenic crops which have been widely adopted by developing countries (ISAAA, 2017). The use of genetically modified crops (i.e. Bt transgenic crops) and the use of pesticides, lead to the pest resistance generation. Therefore, potential biotic and molecular studies to map genetic diversity are important to be conducted as baseline data to manage resistance of ACB population.

A PCR-based DNA analysis has been widely used by previous researches to analyze the genetic diversity of an insect (Pornkulwat *et al*., 1998; Xu *et al*., 1998; Kim & Sappington, 2004). Genetic diversity can be studied by DNA analysis in the nucleus using the Random Amplified Polymorphic DNA (RAPD) (William *et al*., 1990), microsatellite (Kim *et al*., 2008), and the diversity of nucleotides in the target of mitochondrial cytochrome oxidase subunit 1 gene (mtCO1) (Hebert *et al*., 2004; Li *et al*., 2014). The primers used to analyze DNA are different and need to be optimized to produce a good PCR product. The selection of RAPD primers is important to produce polymorphic DNA bands. Pornkulwat *et al.* (1998) used RAPD in studying the genetic diversity of *O. nubilalis*, whereas Xu *et al.* (1998) used 40 RAPD primers of Kit F (OPF1-OPF20) and W (OPW1-OPW20) to study genetic diversity of ACB in China. Yet, the study of ACB population in Indonesia has not been conducted.

More than 10 microsatellite primers were used in replicating of ACB DNA (Dalecky *et al*., 2006; Kim *et al*., 2008), while the recent study by Li *et al.* (2014) used 6 primers selected from Dalecky *et al.* (2006) and Kim *et al.* (2008) were able to visualize the genetic structure of ACB in China. Besides using the PCR technique, microsatellite also requires sequencing using color marker primers to show the homozygous and heterozygous characteristics of a certain species.

The selection of primer is needed to ensure that the microsatellite primer is able to duplicate DNA samples.

The characteristics of mtCO1 primers need to be studied to match the DNA target in ACB, to find out the relationship between samples, and to determine genetic diversity between populations based on the nucleotide diversity in the mtCO1 gene. The mtCO1 primers commonly used are Heb or LCO-HCO for relationship analysis of Orthoptera and Coleoptera (Folmer *et al.*, 1994; Tokuda *et al.*, 2010; Setyolaksono *et al.*, 2017); and Lep or LepF-LepR primers for Lepidoptera, *Arctornis* moth (Hebert *et al.,* 2004; Sutrisno, 2015). COI primer designed from the complete genome in ACB mitochondria is used to identify the diversity of nucleotides and genetic structures of ACB in China (Li *et al.,* 2014). Specific DNA analysis for ACB population in Indonesia has never been conducted yet. Therefore, the study of suitable primers to analyze the genetic variation of ACB population from central production corn in Java using RAPD, microsatellite, and mtCO1 are necessary to be conducted. The high genetic diversity of primers is important to identify the ACB genetic variation hence become a baseline data in molecular studies to manage ACB population.

MATERIALS AND METHODS

Insect Sampling

Four locations of central production corn in Central Java [Brebes (LS 07 1′47.42″, BT 10856′39. 61″) and Grobogan (LS 071′29.65″, BT 110 55′57.59″)] and East Java [Kediri (LS 07 45′44.89″, BT 112 4′55.78″) and Tuban (LS 07 4′37.35″, BT 111 58′16.14″)], Indonesia, was selected to collect the ACB samples on October−November 2016,with a distance of more than 100 KM between locations. Five female adults of ACB were collected from each location. Larval and pupal stadia were reared until becoming adults in plastic vials fed using artificial diet (formulated from the Laboratory of Control Technology, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta) under the regimes of 26−27ºC and 65−80% RH.

DNA Isolation

The thorax of ACB adult was taken for isolating DNA (Coates *et al*., 2005) and other parts were stored in a 1.5 ml Eppendorf tube contained 95% absolute ethanol. The thorax was extracted according to the GT100 mini isolation kit DNA protocol (Geneaid Biotech Ltd. Taiwan) to obtain the ACB DNA genome. The DNA concentration was determined by a NanoVue Plus Spectrophotometer (GE Healthcare Life Sciences Canada).

RAPD PCR Procedure

Seventy-three RAPD primers were used to run PCR, each with 10 nucleotide bases, i.e. kit A (OPA1-OPA13), kit B (OPB1-OPB20), kit C (OPC1-OPC20), and kit D (OPD1-OPD20) based on Operon Technologies (Xu *et al*., 1998). One sample from each location was tested. The PCR procedure was carried out using T100 Thermal Cycler (Bio-Rad USA) according to a method from the Laboratory of Genetic and Breeding, Faculty of Agriculture, UGM, modified a method by William *et al*. (1990): annealing stage at 37ºC for 45 seconds with a PCR reaction composition of 5 µl Gotaq Green (Promega USA), 0.5 µl RAPD primer, 2.5 µl ACB DNA (30 ng/µl), and 2 µl Nucleus Free Water (NFW), thus a total mixture is 10 µl in a 0.2 ml PCR tube. The PCR result was visualized using 1.5% agarose gel in 1X TBE solution, added a color marker of GelRed (Biotium-USA) 1:10.000X, at a voltage of 100V 400 mA for 30 minutes then the gel was placed in a UV transilluminator and visible DNA bands were photographed.

Microsatellite PCR Procedure

Table 1 showed five microsatellite primers (T3, T4, T5, T81, and D25) used with the basic characteristics of each primer. PCR was conducted using T100 Thermal Cycler (Bio-Rad USA) according to Li *et al*. (2014). DNA from Tuban were 5 samples, with a total mixture per PCR reaction was 10 µl [5 µl Gotaq Green (Promega USA), 0.25 µl forward primer, 0.25 µl reverse primer, 2.5 µl DNA samples, 2 µl NFW) by modifying formula from the Laboratory of Genetic and Breeding, Faculty of Agriculture, UGM, Yogyakarta. The PCR results were visualized using 8% Polyacrylamide Gel Electrophoresis (PAGE) at a voltage of 120 V for 90 minutes. Electrophoresis results were visualized by immersing PAGE in DNA staining solution from GelRed (Biotium-USA) 1:10.000X for 15 minutes without sequencing, then the gel was placed in a UV transilluminator and photographed.

mtCO1 PCR Procedure

ACB DNA from Tuban was duplicated using mtCO1 gene using three types of primers (Table 2).

Table 1. Microsatellite primers for DNA analysis of *Ostrinia furnacalis* from Tuban, East Java, Indonesia

Primer	Sequence (5'-3')	Repeat Motif	Target Size (bp)	Reference
T3	F: GGCAGCTATGGAGGCTAAGA R: CTGGCCCTGCATCTGTTG	GCA	151−160	Kim *et al*. (2008), Li *et al*. (2014)
T4	F: CTACGAGCCGCACTTGTACC R: CGTGAGAAGCGTCCTACCTG	AGC, ACC	175−184	Kim *et al*. (2008), Li *et al*. (2014)
T5	F: CCACAATCCTGCTCTGTAAAA R: AGGAGCAGCAGTTCCTCA	G(T/C)T	214−220	Kim *et al*. (2008), Li *et al*. (2014)
T81	F: AGTGGTTTGGGTTGTGCGTTGATAG R: GCACTTTATACTCGGGCATGGGTAAT	ACTG	112	Dalecky *et al*.(2006), Li *et al*. (2014)
D25	F: GAAGAAAACTCTCCATCGGCACTCT R: AGTCGGGGAATGGCAATCTATTAGTAAA	AG	79	Dalecky *et al*.(2006), Li *et al*. (2014)

Remarks: bp=base pair; repeat motif= the repetition motif of the DNA target duplicated by the primer.

Table 2. Primers of mtCO1 gene and PCR stages for DNA replication of *Ostrinia furnacalis* from Tuban, East Java, Indonesia

Primer	Tm (ºC)	Sequence (5'-3')	Target (bp)	Reference
Lep-F	49.8	ATTCAACCAATCATAAAGATATTGG	648	Hebert *et al*. (2004)
Lep-R	53.4	TAAACTTCTGGATGTCCAAAAAATCA		
Heb-F	46.3	GGTCAACAAATCATAAAGATATTGG	710	Folmer *et al*. (1994)
Heb-R	55.3	TAAACTTCAGGGTGACCAAAAAATCA		
COI-F	54.9	CAAGAAGAATCGTTGAAAATGGAGC	1187	Li *et al*. (2014)
COI-R	55.5	TGGAAGTTCGTTATATGAATGTTCTGC		

Remark: Tm= melting temperature

The PCR procedure was carried out according to Hebert *et al*. (2004), Folmer *et al.* (1994), and Li *et al.* (2014) using T100 Thermal Cycler (Bio–Rad USA) with the composition of the PCR reaction based on the protocol from Gotaq Green (Promega USA) with modification: every 0.2 ml PCR tube contained 22.5 µl Gotaq Green, 1.8 ml for each forward and reverse primers (each primer dissolved first in NFW with a ratio of 1:9), and 6 ml DNA samples from Tuban (T). The PCR product (6 µl per sample) was visualized using 1% agarose gel with 1X TBE solution and 1 kb ladder as a DNA size comparator at a voltage of 100 V 400 mA for 35 minutes. Electrophoresis results were visualized by UV transilluminator and photographed. The remaining samples showing DNA bands according to the targets (Table 2) were sent to 1st Base Singapore (http://www.base-asia.com) for DNA sequencing.

Data Analysis

Photographs of agarose gel from RAPD were analyzed by scoring 1 (visible DNA bands) and 0 (unvisible DNA bands) to calculate the number of the visible band, the quality of the band, the monomorphic and polymorphic DNA bands, and the Polymorphic Information Content (PIC) with allele frequency data from GenAlex ver. 6.05 (Nagy *et al.,* 2012; Peakall & Smouse, 2012; Chesnokov & Artemyeva, 2015). PAGE gel photo for microsatellite primers was used to determine the doubling target according to the reference using the marker (ladder). The DNA samples sequenced for the mitochondrial cytochrome oxidase subunit 1 gene from 1st Base Singapore were analyzed by MEGA 6 (Tamura *et al*., 2013) and alignment using BioEdit ver. 7.0.5.3 (Hall, 1999). Sequence consensus was carried out by the BLAST method in http://ncbi.nlm.nih.gov/ to determine the similarity of sample sequences to the species of ACB in GenBank. The alignment was carried out by comparison of the ACB complete genome in GenBank accession numbered AF467260 (Coates *et al.,*2005) to determine the position of the doubling target by each primer.

RESULTS AND DISCUSSION

ACB samples from Brebes, Grobogan, Kediri, and Tuban were extracted with genomic DNA concentration of 27.31 ng/µl (17.5−51 ng/µl). DNA genome concentration for RAPD-PCR was 0.5−50 ng/µl (William *et al.,* 1990), for doubling of the target mitochondrial genes was 10−50 ng in 50 µl reaction mixture (Hebert *et al*., 2004), whereas based on the protocol of Gotaq Green (Promega-USA) was less than 250 ng for each reaction mixture. The concentration of DNA samples affected the results of genetic analysis based on PCR techniques. The accuracy of the DNA concentration of the sample to be reacted is very important. If the concentration is less than it was required, the duplication process was not optimal, hence the visualization of DNA bands would be difficult, either resulted from electrophoresis (Samal *et al*., 2003) or sequencing (Hebert *et al*., 2004; Rukhsana & Sebastian, 2016).

RAPD Primer for Analysing the Genetic Variations of* Ostrinia furnacalis *Population in Java

The fragmentation of 73 primers RAPD Kit A (13 primers), B (20 primers), C (20 primers), and D (20 primers) using 4 ACB samples originated from Brebes, Grobogan, Kediri, and Tuban showed 67 primers succeeded in duplicating ACB DNA, while 6 primers did not succeed in duplicating DNA samples (OPB14, OPB16, OPB19, OPC3, OPC6, and OPD17). The failure of multiplication may be caused by unoptimized annealing temperatures of the primer (William *et al*., 1990; Samal *et al*., 2003), while the temperature used in this study (37ºC) was suitable for most primers. The G-C base content of 73 primers was similar to William *et al.* (1990) stated that high-quality primers usually contain 60-70% of G-C base composition.

Fragmentation of 73 RAPD primers from the 4 kits produced 1–15 DNA bands, with 24 primers capable of producing 10 or more DNA bands with a number of 0−4 monomorphic bands, and 7–15 bands polymorphic bands (Table 3). On the other hand, Xu *et al.* (1998) used primers from Kit F and Kit W to produce high polymorphic bands (8–35 DNA bands). Furthermore, Pornkulwat *et al.* (1998) used 10 selected primers from Kit A, Kit B, Kit C, Kit D, and Kit F to distinguish the genetic diversity of *O. nubilalis*, European Corn Borer (ECB), a similar species to ACB. Primers that produce high polymorphic bands are usually used as primers

Table 3. RAPD primers with Polymorphic Information Content values close to 0.5 of the *Ostrinia furnacalis* DNA bands fragmentation from Brebes, Grobogan, Kediri, and Tuban

Primer	Sequence (5'-3')	% GC	Number of DNA Band	Monomorphic Band	Polymorphic Band	PIC Value
OPA 1	CAGGCCCTTC	70	10	0	10	0.44
OPA 4	AATCGGGCTG	60	10	2	8	0.34
OPA 7	GAAACGGGTG	60	11	3	8	0.31
OPA 8	GTGACGTAGG	60	10	2	8	0.34
OPA 10	GTGATCGCAG	60	11	2	9	0.34
OPA 11	CAATCGCCGT	60	15	0	15	0.44
OPA 12	TCGGCGATAG	60	11	4	7	0.30
OPA 13	CAGCACCCAC	70	15	4	11	0.31
OPB 7	GGTGACGCAG	70	15	2	13	0.36
OPB 10	CTGCTGGGAC	70	10	0	10	0.41
OPB 11	GTAGACCCGT	60	11	2	9	0.35
OPB 12	CCTTGACGCA	60	14	3	11	0.33
OPB 15	GGAGGGTGTT	60	11	2	9	0.32
OPC 4	CCGCATCTAC	60	14	1	13	0.38
OPC 5	GATGACCGCC	70	14	4	10	0.30
OPC 14	TGCGTGCTTG	60	13	2	11	0.38
OPC 16	CACACTCCAG	60	12	3	9	0.31
OPC 18	TGAGTGGGTG	60	10	1	9	0.38
OPC 20	ACTTCGCCAC	60	14	4	10	0.30
OPD 3	GTCGCCGTCA	70	10	1	9	0.38
OPD 8	GTGTGCCCCA	70	15	2	13	0.35
OPD 10	GGTCTACACC	60	12	2	10	0.35
OPD 13	GGGGTGACGA	70	11	2	9	0.35
OPD 14	CTTCCCCAAG	60	10	0	10	0.45

Remark: PIC= polymorphic information content (Chesnokov & Artemyeva, 2015) for DNA analysis (Samal *et al*., 2003; Kim & Sappington, 2004) to visualize the high genetic diversity, thus is able to distinguish the certain ACB population.

The primary election will be strongly supported by considering the value of Polymorphic Information Content (PIC). The PIC values of 73 primers in this study indicated that 24 primers with PIC values of 0.3−0.45 (Table 3). This value was near 0.5 which is the highest value for PIC in primers with dominant targets, such as RAPD (Chesnokov & Artemyeva, 2015). A PIC value of 0.5 was used by Zothansangi *et al.* (2011) using different species (genus *Cirrochroa*, subfamily Heliconiinae), which showed a primer ability to produce high polymorphic bands hence it suitable to be used for the study of genetic diversity (Nagy *et al*., 2012; Chesnokov & Artemyeva, 2015). Visual observation for DNA bands needs to be developed continuously, though the primary PIC value is high. These observations are to ensure clarity and to ease the visualization of DNA bands. In this study, although OPD14 has the highest PIC value, however some bands were unclear, hence it was difficult to score the DNA bands. These results were different with OPD13 with a clearer band and the PIC value also approaches 0.5 (Figure 1).

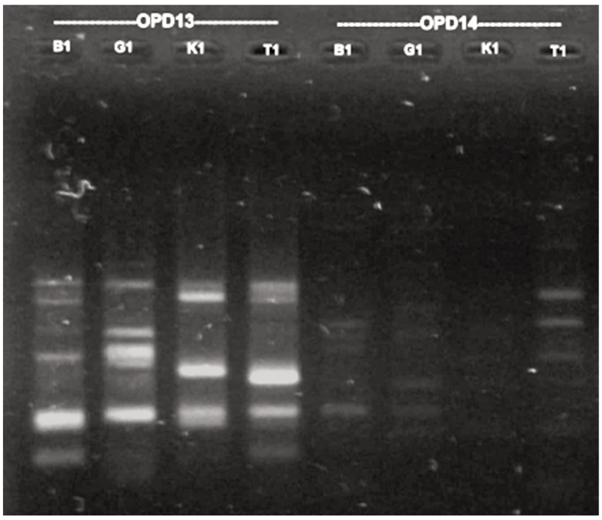

Figure 1. Electrogram of *Ostrinia furnacalis* PCR using OPD13 and OPD14 primers; visible bands of OPD13 are clearer than OPD14; B1 = sample from Brebes, G1 = sample from Grobogan, K1 = sample from Kediri, T1 = sample from Tuban

Microsatellite Primer for Analysing the Genetic Variation of **Ostrinia furnacalis** *Population in Java*

Microsatellite can be used to analyze the cell nucleus DNA, in fact, this method is considered to be better because of the consistency of the amplification and can be co-dominant to identify homozygotes and heterozygotes (Kim *et al*., 2008). Amplification of DNA samples using 5 microsatellite primers (Table 4) according to Li *et al.* (2014) produced DNA size ranges similar with the findings of Dalecky *et al.* (2006) and Kim *et al.* (2008) (Figure 2). The annealing temperature in each primer is suitable for amplification, this was proved by the number of visible alleles from the amplification, the highest produced by T81 primer and the lowest by D25. However, there is a slight difference in PCR product sizes of the two primers in PAGE compared to study by Dalecky *et al.* (2006). This size difference may be influenced by different DNA samples, the ACB sample used by Dalecky *et al.* (2006) originated from China, therefore genetic diversity would be different. The use of PAGE for the visualization of PCR products using microsatellite is useful as an initial study of genetic diversity before using genetic analyzer and sequencing as conducted by Li *et al.* (2014), which requires higher costs. Moreover, PAGE can be used to design multiplex PCR methods as study conducted by Dalecky *et al.* (2006) using several microsatellite primers, to reduce the costs and time.

mtCO1 Primer for Molecular Identification of **Ostrinia furnacalis** *in Indonesia*

The PCR electrophoresis results of three mtCO1 primers of two ACB samples from Tuban population has the the same size with the study by Hebert *et al.* (2004) for Lep (648 bp), Folmer *et al.* (1994) for Heb (710 bp), and Li *et al.* (2014) for COI (1187 bp) (Figure 3). Alignment sequence product from Lep primers is relatively similar to Heb (1479−2179 nt), whereas the product sequencing results from COI primer was 1800−2934 nt (Table 5). These conditions were obtained by multiple alignments using the BioEdit software version 7.0.5.3 compared with the complete ACB genome of GenBank accession number AF467260 (Coates *et al.,* 2005). The composition of nucleotides for the three primers is different. That is related to the nucleotide diversity of the species and

Table 4. Characteristics of the DNA amplification of *Ostrinia furnacalis* Tuban population with five microsatellite primers

Primer	Tm (ºC) F−R	Ta (ºC)	Number of Samples	Amplified Sample	Number of DNA Band	Number of Allele	Product Size Range (bp)	*DNA Size Range by Reference (bp)
T3	56.5−56.6	55	5	5	8	5	150−280	151−160
T4	57.9−57.4	57	5	5	9	5	170−190	175−184
T5	52.9−56.0	55	5	5	5	2	210−220	214−220
T81	59.7−58.8	58	5	5	10	4	110−190	112
D25	58.7−57.7	57	5	5	5	3	70−85	79

Remarks: F = forward, R = reverse, Tm = melting temperature , Ta = annealing temperature, bp = base pair, *reference to Table 1.

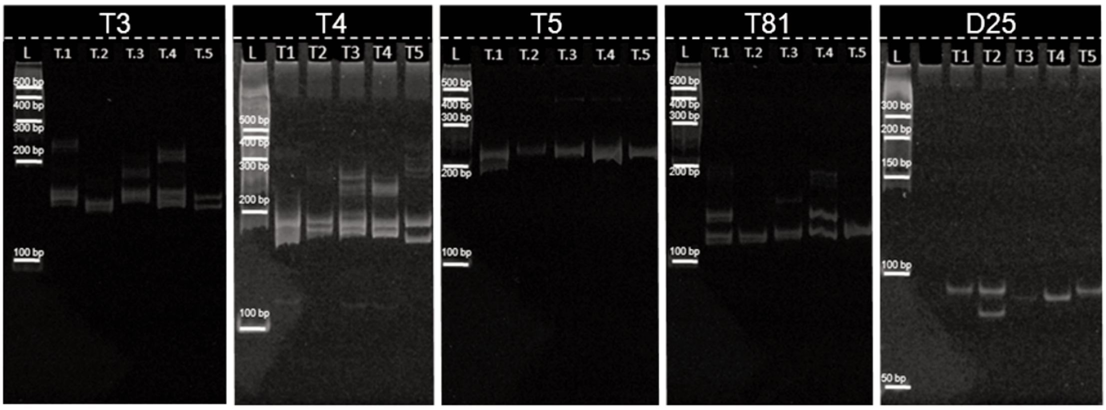

Figure 2. PAGE electrogram of five microsatellite primers (T3, T4, T5, T81, and D25); L = ladder (T3-T81 with 100 bp, D25 with 50 bp); T.1−T.5 = sample from Tuban

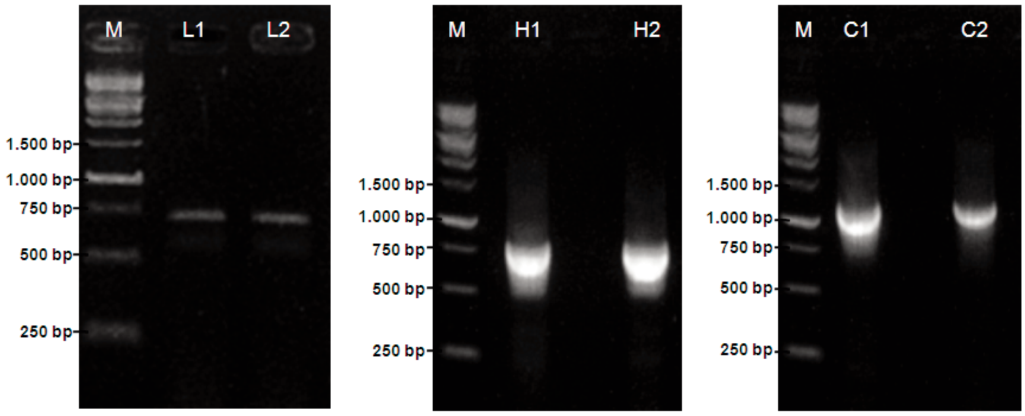

Figure 3. PCR electrophoresis visualization of *Ostrinia furnacalis* Tuban population with primers Lep, Heb, and COI; L1−L2 = Lep primer, H1−H2 = Heb primer, C1−C2 = COI primer, and M = 1 Kb ladder/marker

translation of codons to amino acids (Rukhsana & Sebastian, 2016). All sequences showed similarity to ACB species using the BLAST method in GenBank, with a value of 99% (Table 5).

The sequencing results (Table 5) showed that the three primers can be used for the molecular identification of ACB up to species-level with the target gene in mtCO1 because a similarity was close to 100% indicated a very close relationship between samples and sequences from GenBank. The characteristics of Lep and Heb primers were different in the percentage of arginine (A) and guanine (G) nucleotides which affect the % GC and% AT. This results showed the difference in the same sample with different primers. These results were similar with ACB sequence information

Table 5. Sequence characteristics of *Ostrinia furnacalis* Tuban population with three primers of mitochondrial cytochrome oxidase subunit 1 gene (mtCO1)

Primer	Ta (ºC)	The Length Sequence Total (bp) (F-R)	Consensus Size (bp)	%A	%T	%G	%C	%GC	%AT	*Similarity (%)	**Sequence Position (nt)
Lep	51	689−694	660	32.1	38.9	14.4	14.6	28.9	71.1	99	1479−2139
Heb	47	681−683	659	31.9	38.9	14.7	14.6	29.3	70.7	99	1479−2139
COI	58	1164−1188	1134	31.9	39.4	13.6	15.1	28.7	71.3	99	1800−2934

Remarks: F = forward, R = reverse, Ta = annealing temperature, bp = base pair, nt = nucleotide
*similarity (based on the BLAST system); the results of the Lep and Heb primers are similar to the accession number KF491966 and COI primer to the accession number DQ2048781 in GenBank
**multiple alignments compared to the complete genome of accession number AF467260 (14.536 bp) in GenBank

in GenBank and the Barcode of Life Data System (BOLD) website (http://www.boldsystems.org) showed that Lep primer was more widely used by researchers (more than five accessions) than Heb primer. Heb primer has only one accession in GenBank combined with Lep (the accession number KX862807 of an ACB sample from Pakistan). These conditions indicate that Lep primer is more informative to be used analyzing ACB than Heb because more information is available in GenBank and BOLD system. Furthermore, the studies of Lepidoptera order mostly use Lep primer (Hebert *et al*., 2004; Hajibabaei *et al.,* 2006; Sutrisno, 2015). Nevertheless, the two universal primers are suitable for molecular identification of ACB species based on the same similarity value (99%) in GenBank.

CONCLUSION

The suitable RAPD primers to analyse the Asian corn borer DNA (ACB), *Ostrinia furnacalis* in Indonesia are OPA1, OPA4, OPA7, OPA8, OPA10, OPA11, OPA12, OPA13, OPB7, OPB10, OPB11, OPB12, OPB15, OPA4, OPA8, OPA10, OPA11, OPA12, OPA13, OPB7, OPB10, OPB11, OPB12, OPB15, OPA4, OPA8, OPA10, OPA11, OPA12, OPA13, OPB7, OPB10, OPB11, OPB12, OPB15, OPA4, OPA8, OPA10, OPA11, OPA12, OPA13, OPB7, OPB10, OPB11, OPB12, OPB15, OPA4, OPA8 OPC5, OPC14, OPC16, OPC18, OPC20, OPD3, OPD8, OPD10, OPD13, and OPD14. Microsatellite primers of T3, T4, T5, T81, and D25 are suitable to support the study of genetic variation of ACB in Indonesia. Universal primers of Lep and Heb can be used to analyze the relationship of ACB species as a molecular identification with the mitochondrial cytochrome oxidase subunit 1 (mtCO1) gene target.

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