Short Communications

Identification of ISSR-based Molecular Markers Associated with Ploidy Level of Orange Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai)

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ABSTRACT

Indonesia is an agrarian country whose territory is partly used as an agricultural sector, has especially watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai). At present, the development of watermelons in Indonesia still requires a variety of colors, superior watermelons need to be developed by making triploid orange watermelons. The new watermelon cultivar (F₁) is the result of crossing 'Jelita' (♀) and 'BallyBall' (♂) which are expected to provide a combination of characters that can produce large and sweet fruits. The purpose of this study was to identify ISSR markers that were associated with ploidy levels of horticultural crops, particularly watermelon. The watermelons were growns on the agricultural land of Kebondalem Hamlet, Madurejo Village, Prambanan District, D.I.Y. and analyzed at the Laboratory of Biotechnology PAU, UGM and Laboratory of Genetics and Breeding, Faculty of Biology UGM. Analysis of genetic variation was carried out using the PCR-ISSR method and the DNA bandwidth was calculated using *Paint Apps*, *Microsoft Excel* 2013, and *Microsoft Word* 2013. The results of crossing 'Jelita' and 'King Quality 'is sterile. The result of 'Jelita' with 'BallyBall' produces tillers until sterile harvest time. The results of the analysis of genetic variation using the PCR-ISSR method indicate that the ISSR BI, B3, B5, and CBTC 1 molecular markers cannot be used to distinguish ploidy from the tested watermelons.

Keywords: ISSR, Marker Assisted Breeding, orange watermelon, ploidy, triploid

INTRODUCTION

Watermelon is a fruit that is in great demand by the public. The peculiarity of the sweet, crunchy, and juicy taste of watermelon makes it attractive for consumption and cultivation. Moreover, watermelon contains high and complete nutrition (Khomsan 2009). In the health sector, watermelon has benefits as an antioxidant, anti-inflammatory, and vasodilating agent (Poduri et al. 2013) because of the vitamins, proteins, and minerals found in fruit skins, pulp, and seeds. Rapid technological advances make the quality and production power of superior watermelons continue to increase. Various shapes, levels of sweetness, color, and size of genetically engineered watermelons are cultivated rapidly both domestically and abroad. Unfortunately, watermelon, which has an attractive appearance and high economic value, comes from imported seeds. Therefore, it is necessary to make efforts to improve the quality of local watermelons. Faculty of Biology

UGM tries to contribute by crossing yellow watermelon 'maduri' with red watermelon 'princess pomegranate' to produce superior local melons with orange flesh fruit. Furthermore, this hybrid is being developed into a tetraploid orange watermelon by crossing several watermelon varieties.

As known, the selection of hybrids in conventional crosses takes a lot of time, money, and effort because it must wait until a certain plant age when the desired phenotype appears. Therefore, it is necessary to develop a molecular hybrid selection technique developed from the ISSR marker. Molecular selection in the breeding process is known to increase the success of breeding programs. Phenotype expression is closely related to the genetic diversity of an organism. The loci of genes of an agronomic trait of interest can be related to molecular marker loci so that they can be used for genotype selection. The use of molecular markers allows the selection of indirect traits at the seedling stage thereby accelerating the breeding process and facilitating the increase in difficult traits that cannot be easily selected using morphological markers (Varshney et al. 2009). Molecular markers can be used to eliminate inferior genotypes early so that breeders can only focus on the genotypes that have the greatest potential (Moose & Mumm 2008). Several previous studies have also demonstrated the successful use of molecular markers for genotype selection in the breeding process, such as breeding for resistance to soybean cyst nematodes (Cregan et al. 1999), resistance to cereal diseases (Varshney et al. 2006), and drought tolerance in maize (Ribaut & Ragot 2007).

Although possible, the development of molecular markers for ploidy selection has not been widely used. Research by Feng et al. (2018) attempted to develop EST-SSR markers for ploidy identification in *Misgurnus anguillicaudatus*. Schie et al. (2014) used a combination of several molecular markers for polyploid identification in garden dahlia. Therefore, the aims of this study were to identify ISSR markers that were associated with ploidy level of horticultural crops, particularly watermelon.

The materials used in this study were triploid watermelon leaves 'Jelita' and 'King Quality' and diploid red watermelon 'BallyBall' which had been grown in greenhouses and open planting areas, Prambanan, DI Yogyakarta, ice gel, Phytopure DNA isolation kit 1 chloroform, isopropanol, 70% ethanol, TE buffer 1x, PCR Kit (Taq HS Red Mix 2X Bioline), ISSR Primer (Table 1), sterile ddH2O, MgCl2, agarose, TBE 1X, fluorosafe DNA staining, aquades, and 100 bp Geneaid DNA marker.

Table 1. The sequence of ISSR markers used in this research (Vinoth & Ravindhran 2016).

Primer	Primer Sequence 5' – 3'	The DNA band length (bp)
CBTC 1	(AG)8-T	200 - 2000
B1	(AG)8-C	400 - 2200
B3	(GA)8-A	400 - 1900
B5	(GA)8-T	400 - 1600

Watermelon samples were grown in four beds (mounds). Leaf samples were taken from 15 plants for each bed randomly. A total of 45 leaf samples were used in the molecular analysis of this study. On average, 3 to 5 leaf samples were taken for each watermelon cultivar. Leaves taken were eaves fifth to ninth from the base of the leaves with a healthy appearance (green, smooth, and do not have infection symptoms). The leaf samples were then put into plastics with a certain code in the icebox. After arriving at the laboratory, the sample was then put in a freezer or cooler so that the DNA on the leaves is not damaged.

As much as 0.3 g leaf samples were used for DNA isolation as described by Daryono and Natsuaki (2002) with little modifications. The leaf samples (without leaf bones) were crushed with a pestle and mortar. The crushed samples were transferred into a 1.5 mL tube then added with 500 µL of Phyto pure reagents I and 200 µL of reagent II, continued with incubation at 65 °C for 20 minutes. Then, the samples were incubated at room temperature for 5 minutes followed by incubation in the freezer for 5 minutes. After that, the samples were added with 400 µL of cold chloroform and 20 µL of resin then shake for 30 minutes. Furthermore, the sample solution was centrifuged at 1,300 rpm for 10 minutes. The supernatant obtained was transferred to a new tube and added with cold isopropanol through the tube wall as much as the volume of the supernatant. The sample solution was shaken gently by hand for 10 minutes and centrifuged at 10,000 rpm for 10 minutes. DNA pellets were washed and purified with 100 µL of 70% ethanol 3 times. As a storage buffer, 50 µL 1X TE buffer was added. The isolated DNA was stored at -20 °C and was checked for purity using Nanovue Plus Nanodrop spectrophotometer at absorbance 260/280 nm.

For PCR analysis, we only selected three DNA isolates of the highest quality from each cultivar. In this research, DNA samples were analysed using four ISSR primers as mentioned in Table 1. The targeted DNA bands were varying from 200 – 2200 bp. As much as 25 µL PCR solution containing 12.5 µL PCR kit Bioline 2x MyTaq™ HS RedMix (Bioline, United Kingdom), 1.5 µL primer (10 pmol), 2 µL DNA samples (200 ng), 1 µL MgCl$_2$, and 8 µL ddH$_2$O was used. The PCR reaction was conducted with an initial denaturation at 95 °C for 5 min; continued with 35 cycles of 95 °C for 45 s, 47.9 °C for 45 s, and 72 °C for 60 s; and then ended with a final extension at 72 °C for 5 min. The PCR results were analyzed using 60 µL of 2% agarose gel stained with a 6 µL floroSafe DNA stain (First BASE, Singapore).

Genetic markers are molecular markers that can describe certain traits so that they can be used to assist the plant selection process in the plant breeding process. This research was conducted to obtain specific DNA band candidates that can distinguish triploid and haploid watermelon plants. The method that was used in this research was the PCR-ISSR method because it does not require genome sequence information (Arifiyanti 2015). ISSR is a multi-locus molecular that amplified regions between both ends of microsatellite. ISSR was suitable for asses genetic diversity because of its high genetic variability and ability to generate multi-locus data even though there was limited sequence information. Moreover, ISSRs are more reproducible than RAPDs and cheaper dan faster than AFLP (Ng & Tan 2015).

In some PCR visualization results, smears or bands that look faint are seen, so an electropherogram was made to facilitate analysis (Figure 1b – 4b). According to Azizah (2009), poor amplification results can be caused by mis-priming, efficiency, and optimization of the PCR process. In practice, mis-priming can occur because primers may anneal at incorrect sites. The occurrence may depend on the difference between the melting rates of primers at correct and incorrect sites. Failure in the PCR process can produce many non-specific DNA products of various sizes that appear as ladders or smears on agarose gels and sometimes even no product at all. Apart from mis-priming, mutations that are accidentally introduced into the amplicon can lead to heterogeneous or non-target PCR products. resulting in a heterogeneous population of PCR products (Mamedov et al. 2008).

Based on Table 2, B1 has the highest polymorphic bands than other primers. The percentage of polymorphisms that were obtained from this primer was 80%. From Figure 1, it was known that there was a DNA band measuring 770 bp which was only amplified in the triploid sample. These

Table 2. Primer used in molecular analysis with corresponding bands scored with polymorphic bands, observed in samples.

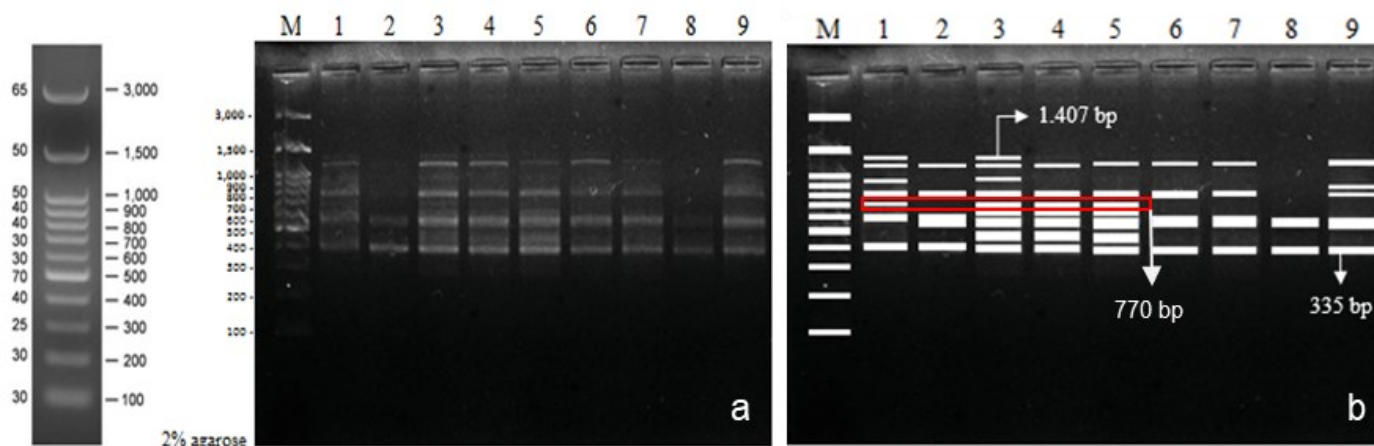| Primer	DNA bands size	Amplified bands	Monomorphic bands	Polymorphic bands	Percent of Polymorphism (%)
B1	335-1407 bp	10	2	8	80
B3	170-943 bp	9	6	3	33.3
B5	222-985 bp	7	4	3	42.8
CBTC1	228-1472 bp	10	7	3	30

Figure 1. The PCR-ISSR molecular test results of watermelon using B1 primer (a) visualization of gel electrophoresis (b) electrophoregram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').

DNA bands could be used as candidates for the development of molecular markers linked to ploidy levels on watermelons. Safaei-Chaeikar & Rahimi (2017) reported that ISSR marker can be used to develop molecular markers that associated with agronomical traits in the medicinal plant lemon balm. The ISSR marker had high polymorphic bands indicating its high efficiency in differentiating targeted traits.

Based on Table 2, it is known that B3 primer produces only 33% of polymorphic bands. The existence of 6 identical DNA fragment patterns in all samples showed the genetic stability of watermelon plants tested at a certain length of DNA fragments. From Figure 2, we also know that the primer B-3 produces fewer polymorphic bands than other primers.
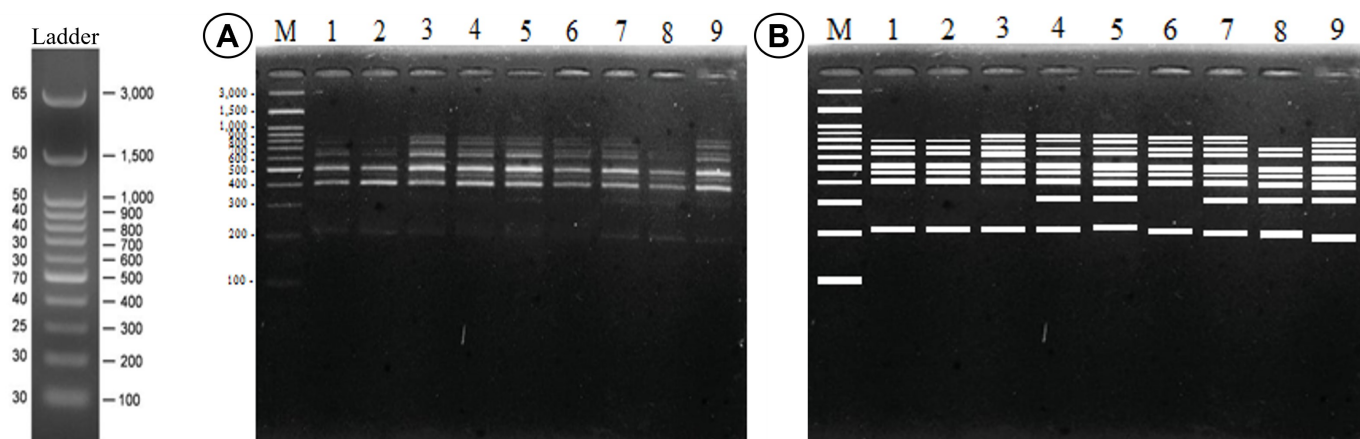

Figure 2. The PCR-ISSR molecular test results of watermelon using B3 primer (a) visualization of gel electrophoresis (b) electrophoreram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').
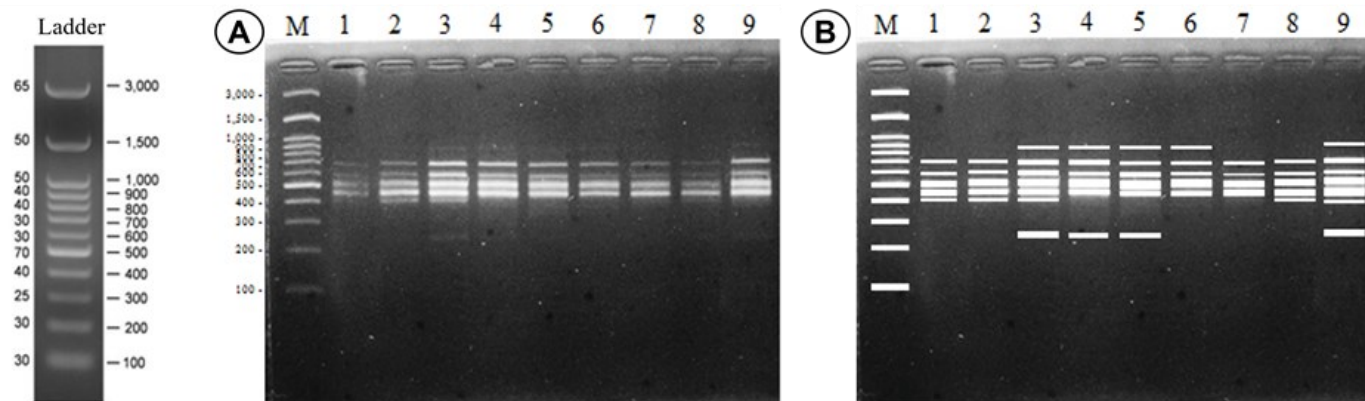
Figure 3. The PCR-ISSR molecular test results of watermelon using B5 primer (a) visualization of gel electrophoresis (b) electrophoregram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').
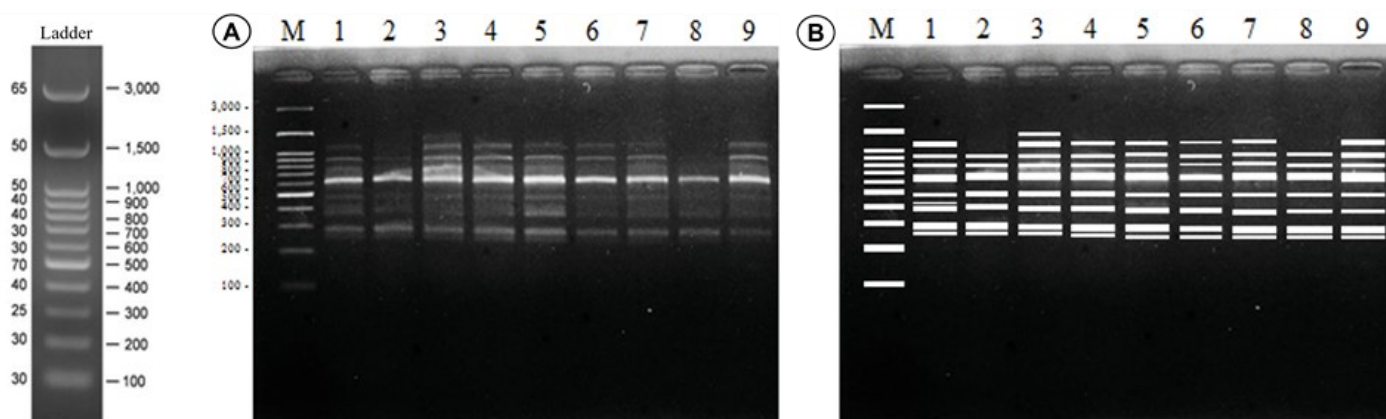

Figure 4. The PCR-ISSR molecular test results of watermelon using CBTC1 primer (a) visualization of gel electrophoresis (b) electrophoregram (Note: M = DNA ladder; 1 - 3 = Triploid watermelon 'King Quality'; 4 - 6 = Triploid watermelon 'Jelita'; 7 - 9 = Diploid watermelon 'Bally Ball').

PCR-ISSR using primer B5 (Figure 3) produced 7 DNA fragments measuring from 222 - 985 bp. There were four monomorphic DNA fragments and three polymorphic DNA fragments. The pattern of obtained DNA fragments showed genetic variation with a percentage of 42%. There was a similar pattern of DNA fragments in wells 1 - 6 because these plants are non-seed or triploid plants with the same number of ploidies so they have many similarities even though the colour of the flesh fruit is different. Based on this result, it was known that primer B1 (AG) 8 cannot be used as a molecular marker to distinguish watermelon ploidy.

PCR-ISSR results using CBTC 1 primer (Figure 4) produced the lowest polymorphic band among the used primer. A similar pattern was found on the three plant varieties, both diploid and triploid watermelon samples. It is also known that CBTC 1 primer did not produce specific DNA bands that were different in diploid and triploid watermelons so that it cannot be developed further as a molecular marker linked to the ploidy level of watermelon.

Based on the results of the PCR-ISSR study, the four primers used produced 36 DNA fragments consisting of 17 polymorphic DNA and 19 monomorphic DNA. The highest polymorphism was obtained using primer B1, namely 80%. Dje et.al. (2009) reported that primers BI (AG) 8-C, B3 (GA) 8-A, and B5 (GA) 8-T when used for genetic analysis of watermelons, produced a polymorphism percentage of 100% while the CBTC 1 (AG) primers 8 -T is 92.3%. The difference in the percentage of this polymorphism occurred in previous studies using different watermelon

varieties grown in planting media with different rainfall so that they had higher genetic variations. The low percentage of polymorphisms causes the possibility to obtain molecular markers that are linked to certain properties to become smaller. This is because the more polymorphic fragments are produced, the greater the chances of finding variations. The high number of polymorphic bands indicates that the used primers amplified genomic regions that have high genetic variation between samples (Sivaprakash et al. 2004; Jena & Chand 2021). The research of Vinoth and Ravindhran (2016) was known to use the same type of ISSR primer as this study to analyse the genetic diversity of watermelons resulting from somatic embryogenesis. The four primers were reported to produce DNA bands of 2-11, were reproducible, and were able to show genetic stability. These results are like those obtained in this study which the amount of obtained DNA bands ranged from 7-10 bands. Based on this, it could be said that this primer amplifies the relatively conserved regions in the watermelon genome.

The molecular markers expected because of this study were specific DNA fragments that can only be found in triploid individuals but cannot be found in diploid individuals and vice versa. These fragments can be used as the basis for molecular selection of diploid and triploid watermelons without having to wait for phenotypic expressions to appear at a certain plant age. The Marker Assisted Selection (MAS) also makes the phenotypic ploidy hybrid selection more effective because it does not have to use other analyzes such as flow cytometry or karyotyping. MAS development for ploidy analysis has been carried out several times, such as in Dahlia (Schie et al. 2014) and blueberries (Oliviera et al. 2020). The use of MAS for plant ploidy selection has not been widely used. MAS is generally used for the selection of disease resistance, resistance to environmental stress, color, size, and the number of fruit or flowers (Ibitoye & Akin-Idowu 2011).

The results of this study, a DNA band measuring 770 bp amplified only on triploid watermelons (Figure 1), need to be developed further before being used as molecular markers in watermelon ploidy selection. This is because the ISSR primer was still universal and random, so if it produces a lot of DNA bands when used in a selection, thus it will become less specific. The molecular markers for genetic selection must be reproducible and robust enough to tag specific traits not only in one progeny line of the crop. Generally, molecular links to specific traits can be SCAR markers, allele-specific markers, or SNP associated markers (Collard & Mackill 2008; Ibitoye & Akin-Idowu 2011). The success of MAS development was influenced by three main factors: (1) Genetic map with a number of polymorphic markers with sufficient uniform space for marking quantitative trait locus (QTL) or major genes accurately, (2) A close relationship between adjacent markers with QTL or the targeted main genes, and (3) Adequate recombination between markers and the rest of the genome (Ibitoye & Akin-Idowu 2011).

The ISSR primers B1 (AG) 8-C, have the potential to be developed as a molecular marker to differentiate ploidy in watermelon plants to increase the effectiveness of conventional plant breeding. Development can be done by constructing a SCAR marker from a specific 770 bp DNA band sequence amplified from primer B1.

AUTHORS CONTRIBUTION
A.J.S. carried out laboratory works (samples collection, DNA extraction, PCR amplification, agarose gel electrophoresis, data analysis, and drafted the manuscript). A.S.S. translated and revised the final manuscript as well as supervised the laboratory works and publication process. B.S.D. designed the research and supervised all of the processes from laboratory works until

publication. All authors contributed to this research and approved the final manuscript.

CONFLICT OF INTEREST
The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock 0wnership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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