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Microsatellite polymorphism for molecular characterization of pomelo (*Citrus maxima*) accessions from Indonesia

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Abstract. *Susandarini R, Subandiyah S, Daryono BS, Rugayah. 2020. Microsatellite polymorphism for molecular characterization of pomelo (Citrus maxima) accessions from Indonesia. Biodiversitas 21: 2390-2395. Citrus maxima* (Burm.) Merr. (pomelo) as a minor fruit crop deserve attention on its phenotypic and genotypic variability to avoid the risk of extinction. Previous study showed that pomelo from Indonesia has high morphological variability, and thus it is interesting to explore its genotypic variability using molecular markers. Microsatellite is a molecular marker widely used in *Citrus* taxonomy studies. This study aimed at revealing microsatellite polymorphism and its potential application in cultivar characterization of *C .maxima.* Eighty accessions of *C. maxima* consisting of registered cultivars and landraces from Indonesia were used in this study. Analysis of microsatellite sequences from genomic DNA amplified using DY296883 primer showed that *C. maxima* microsatellite has high polymorphism in the form of repeat length variation of $(GA)_n$, ranging from $(GA)_7$ to $(GA)_{19}$. This study proved the existence of high genotypic variability in *C. maxima*, and confirmed the role of microsatellite as a useful molecular marker for uncovering variability at intraspecific level. Observation of the microsatellite polymorphisms indicated that variability of $(GA)_n$ can be used to distinguish some pomelo cultivars.

Keywords: *Citrus maxima*, microsatellite, characterization, genotypic variability

INTRODUCTION

Pomelo (*Citrus maxima* (Burm.) Merr.) is a tropical fruit crop plant originating from Asia, with wide distribution areas covering Indo-China, Australia, Japan, India, Mediterranean regions, and tropical America (Sthapit et al. 2012). Pomelo has high phenotypic variability especially in fruits characters, which include shape, size, thickness of fruit skin, flesh color, and fruit flavor. In general, people recognize two variants of pomelo based on fruit color, namely white and red.

Pomelo as cultivated plant species has a risk of decreased variability as a result of the prolonged practice of artificial selection (Arora 2000). Cultivated plants undergoing selection during domestication process may experience a decrease in variation through the elimination of undesirable phenotypic characters, which results in a decrease genotypic variation (Smýkal et al. 2018). The decrease of genotypic and phenotypic variation might be caused by selection for desirable traits is a common phenomenon compared to its wild progenitor (Chinthiya and Bhavyasree 2019). It is, therefore, the risk of decreasing variability in pomelo warrants serious attention to prevent loss of biological resources with untapped potentials. An important step to overcome this problem is by documenting species variability through the inventory of pomelo cultivars and landraces, followed by characterization of their phenotype and genotype. Study on morphological variability on pomelo cultivars and landraces in Indonesia has been carried out along with the assessment of their taxonomic relationships (Susandarini et al. 2013). The present study was intended as initial step in exploring genotypic variability using molecular marker in an attempt to obtain comprehensive features of intraspecific variation on *C. maxima*.

The development of molecular systematic approach facilitates an effort in finding the most fundamental character as a basis for plant biodiversity assessment and classification. Microsatellite is a DNA-based molecular marker widely used in systematic studies on *Citrus*. Microsatellite, also known as simple sequence repeat (SSR) and short tandem repeat (STR), is a sequence of repetitive nucleotide motifs, composed of mono-, di-, tri-, tetra-, penta-, or hexa-nucleotide (Cristofani-Yaly et al. 2011). Microsatellite is a widely used molecular marker for plant germplasm characterization, cultivar identification, genetic diversity analysis, and phylogenetic analysis (Ijaz 2011, Rania et al. 2012, Sharma et al. 2015, Zhao et al. 2019). A number of studies using microsatellite reveal that microsatellites is a molecular marker with many advantages including high reproducibility, high degree of polymorphism, co-dominant, presents in large quantities and distributed in various plant genomes, and has high mutation rates (Lee et al. 2011, Abdul-Muneer 2014, Bilska and Szczecińska 2016, Vieira et al. 2016). This study reported the occurrence of microsatellite polymorphism in the form of repeat length variability as

molecular marker for characterization of *C. maxima* cultivars and landraces from Indonesia.

MATERIALS AND METHODS

Plant samples for DNA analysis

A total of 80 pomelo accessions were used in this study. Plant samples were collected from six provinces in Indonesia representing both registered cultivars and landraces (Table 1). Fully expanded leaves from mature individual plants were used as materials for DNA isolation.

DNA isolation, PCR, Electrophoresis, and Purification of PCR product

Genomic DNA isolation was done using Microzone™ *DNAMite* extraction kit (Microzone, UK) according to manufacturer's protocol. PCR reaction to amplify microsatellite was performed in a 25 µL volume consisted of 25 ng DNA, 1x buffer (Mango™), 0.2 mM of dNTPs (Scientific™), 0.6 µM of primer (each for forward and reverse primers), 2.0 mM of $MgCl_2$ (Bioline™), 0.5 unit/µL of *Taq polymerase* (Mango™), and 0.5 µL DMSO. The primers used in this study were DY296883 (forward 5'-CCCCCTCTTTTTCTCTTCCA-3' and reverse 5'-TTCTGGGCTGGTAGGTTCAG-3') developed by Luro et al. (2008) that has been tested for its transferability among *Citrus* species. PCR reaction was done on Biorad™ thermal cycler using the following program: initial denaturation at 94°C for 3 minutes, followed by 35 amplification cycles of denaturation at 94°C for 50 seconds, annealing at 50°C for 2 minutes, elongation at 72°C for 90 seconds, and final extension at 72°C for 6 minutes.

The amplification product was visualized on 1% agarose gel in TAE buffer and stained with *GelRed* nucleic acid stain (Biotium™). Two microliters of PCR product was mixed with 1 µL loading dye (0.25% bromophenol blue and 40% (w/v) sucrose). At each gel, a 1 Kb or 100 bp DNA ladder (Bioline™) was loaded as a standard for measurement of DNA fragments. The electrophoresis was run at constant voltage of 100 volts for 30 minutes. Electrophoresis results were viewed and documented using *Kodak Gel Logic 100 Digital Imaging System* (Kodak Inc. USA) and saved as JPEG file. Purification of PCR product was done using DNA purification kit (Viogen™) according to manufacturer's protocol. Purification result was checked on agarose gel using the same protocol as described for visualization of PCR product.

Table 1. Collection sites and sample number

Collection sites (Province)	Accession code	Number of plant samples
Nangroe Aceh Darussalam	ACH	6
Central Java	JTG	11
Yogyakarta	DIY	14
East Java	JTM	8
East Nusa Tenggara	NTT	25
South Sulawesi	SLW	16

Reaction for sequence cycle and precipitation of reaction product

Sequence cycle to amplify purified DNA was carried out in a volume of 20 µL consisted of 25 ng DNA, 0.6 µM of primer (each for forward and reverse primer), 1.5 µL *Big Dye Terminator (BDT)*, 3.5 µL *BDT* buffer, and 10.9 µL sterile water. The sequence cycle was run as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of amplification program consisted of denaturation at 94°C for 10 seconds, annealing at 50°C for 5 seconds, and elongation at 60°C for 4 minutes. The amplification products from sequence cycle reactions were precipitated using ammonium acetate. The precipitated DNA was then resuspended by adding 10 µL Hi-Di Formamide before being applied to DNA sequencer. DNA sequencing process was performed using ABI-3130*xl* Genetic Analyzer (Applied Biosystems, USA).

Data analysis

The microsatellite sequences were read using *SeqMan* and *EditSeq* tools in *Lasergene* program of DNASTAR software version 9.0 (DNASTAR, Inc.). Sequence alignment was done using ClustalW program on Mesquite software version 2.75 (Mesquite Project, http://www.mesquiteproject.org).

RESULTS AND DISCUSSION

Results

DNA amplification on target regions containing microsatellite of $(GA)_n$ repeats using SSR primer DY296883 resulted in a single fragment of 215 bp as z microsatellite $(GA)_n$ on 80 accessions of *C. maxima* showed a high variation in repeat length, or repeat number of dinucleotide unit (GA), from 7 to 19. The microsatellite was found on the 106th to 155th nucleotide positions. Microsatellite polymorphism observed in this study reflected a high genotypic variability. There were nine types of $(GA)_n$ found in this study, namely $(GA)_7$, $(GA)_{10}$, $(GA)_{12}$, $(GA)_{13}$, $(GA)_{15}$, $(GA)_{16}$, $(GA)_{17}$, $(GA)_{18}$, and $(GA)_{19}$.

Some representatives of microsatellite-containing sequences showing variability of microsatellite $(GA)_n$ repeat number on was shown in Table 2. Complete data showing variability in microsatellite repeat numbers of 80 accessions used in this study, accompanied by 7 selected morphological characters were shown in Table 3. The seven morphological characters displayed here were those most prominent and easily recognizable in the field.

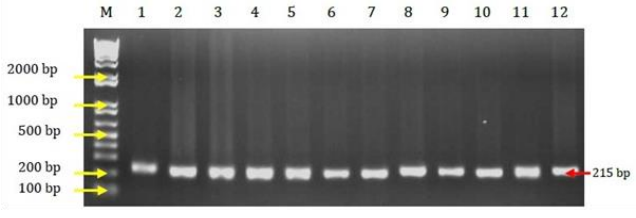

Figure 1. Amplification products of primer DY296883 showing microsatellite fragment at 215 bp. M: 1 Kb DNA ladder; number 1-12: amplification results of *C. maxima* samples

Table 2. Representatives of multiple sequence alignment showing polymorphism in microsatellite repeat number (GA)n. The polymorphic SSR region is typed in bold

Accession	Nucleotide sequence on 106th to 155th position	Number of (GA)n
ACH 1	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**------CTTCGG	17
ACH 2	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**------CTTCGG	17
ACH 3	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**------CTTCGG	17
ACH 4	GCCC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**AC------TTCCCG	16
ACH 5	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**CA------CTTCGG	16
ACH 6	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**------CTTCGG	17
JTG 1	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**CCCTTCGG	19
JTG 2	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**CCCTCCGG	19
JTG 3	GGCC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**------CTTCCG	17
JTG 4	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**------CTTCGG	17
JTG 5	GCTC**GAGAGAGAGAGAGAGAGA**----------CTTCGG	10
JTG 6	GCTC**GAGAGAGA**----------**GAGAGA**------CTTCGG	7
JTG 7	GCCC**GAGAGAGAGAGAGAGAGAGAGAGA**----------CTTCCG	13
JTG 8	GCTC**GAGAGAGAGAGAGAGA**----------**GAGAGAGA**------CTTCGG	12
JTG 9	GCTC**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA**CA------CTTCGG	16
JTG 10	GCCC**GAGAGAGAGAGAGAGAGAGAGAGA**CC----------TTCCG	12

Discussion

In this study, the detection of microsatellite polymorphisms to determine genotypic variations were done through analysis of DNA sequences where microsatellites are located. Analysis of microsatellite polymorphisms by examining variations of repeat units performed by sequencing method has advantages over the detection of polymorphisms based on amplicon size variations generated through capillary gel electrophoresis (Viruel et al. 2018). Examination of sequences containing microsatellite revealed high variability in repeat number of $(GA)_n$, with a range of $(GA)_7$ to $(GA)_{19}$, which clearly showed the evidence of microsatellite polymorphisms in pomelo. It was widely recognized that microsatellites polymorphism as indicated by variability in repeat number of dinucleotide unit resulted from mutations (Putman and Carbone 2014). In such a case, Sonah et al. (2011) mentioned that variability on microsatellites was caused by selection pressures during its evolutionary process, resulting in a particular type of repeat number that is dominant over the others. In this study, the most frequent repeat number found in pomelo was $(GA)_{17}$.

Microsatellite polymorphism obtained in this study was in the form of reduction or addition of repeat units among the individual plants studied. This is in line with the results of Vieira et al. (2016), that different individuals showed variations of microsatellites in the form of differences in repeat numbers. Based on the alignment of 80 microsatellite sequences, the differences in microsatellite length was due to substitution, deletion, and insertion at various positions. Among these three causes of variation, insertions and deletions (indels) were more common than nucleotide substitutions. Indels occurred at the ends of microsatellites, especially at the 3' end. In this regard, Kelkar et al. (2010) and Putman and Carbone (2014) noted that changes in microsatellite length were generally caused by slippage, i.e., transient dissociation on DNA strands during replication process. DNA slippage was followed by misaligned during the formation of double-stranded DNA. These processes may lead to insertion or deletion of microsatellite repeat units, and thus causing variations in microsatellite length. Most of the slippage during DNA replication events will be corrected through the mismatch-repair system and only in certain cases when the natural repair process did not take place there will be mutations in microsatellites (Mondini et al. 2009).

Results of this study support the use of microsatellite in *Citrus* taxonomic studies. Variation on repeat numbers of microsatellite (GA)n sequences indicates high genotypic variation within *C. maxima*. In taxonomic context, knowledge on genotypic variation is very important for the development of cultivar databases and the utilization of plant germplasm resources, as mentioned by Shahzadi et al. (2014) in a study on sixteen *Citrus* cultivars. Previous studies in *Citrus* taxonomy also have successfully employed microsatellite as molecular marker. Analysis of microsatellite characterization and polymorphism was reported by Singh et al. (2011) in 30 *Citrus* genotypes and by Polat et al. (2012) in *Citrus aurantium*. Furthermore, Shahzadi et al. (2014) and Sharma et al. (2015) also noted that variations in microsatellite were useful to study genetic diversity in *Citrus*. From taxonomic point of view, Sonah et al. (2011) argued that microsatellite polymorphisms in terms of sequence lengths due to insertion or deletion indicated the process of molecular evolution.

Results of this study indicated that microsatellite is a useful molecular marker for detecting genotypic variability in pomelo. This is in line with previous studies which showed that microsatellite sequences were highly variable molecular markers and were useful for molecular characterization of cultivars (Ijaz 2011, Rania et al. 2012), and for hybrid identification (Ahmad et al. 2012). Previous study indicated that microsatellite serves as polymorphic and informative molecular marker in detecting genetic diversity and useful for identification of cultivars and clones in *Citrus sinensis* (Mahjbi et al. 2016).

Table 3. List of pomelo accessions, their morphological characters, and microsatellite repeat number $(GA)_n$

Accession code	Selected morphological characters*							(GA)n
	LS	PW	FS	PT	FC	FT	SN	
ACH 1	Ovate	Obdeltate	Spherical	Medium	Pink	Soft	Few	17
ACH 2	Ovate	Obdeltate	Spherical	Medium	Pink	Soft	Seedless	17
ACH 3	Ovate	Obdeltate	Spherical	Medium	Pink	Soft	Seedless	17
ACH 4	Ovate	Linear	Elliptical	Medium	Pink	Medium	Few	16
ACH 5	Ovate	Linear	Elliptical	Medium	Pink	Soft	Few	16
ACH 6	Ovate	Obovate	Spherical	Medium	Pink	Soft	Few	17
JTG 1	Ovate	Obcordate	Spherical	Smooth	Red	Soft	Numerous	19
JTG 2	Ovate	Obdeltate	Elliptical	Medium	Yellowish white	Medium	Numerous	19
JTG 3	Ellips	Obcordate	Spherical	Smooth	Yellowish white	Medium	Medium	17
JTG 4	Ovate	Obcordate	Elliptical	Medium	Pink	Medium	Numerous	17
JTG 5	Ellips	Obcordate	Spherical	Smooth	Pink	Medium	Numerous	10
JTG 6	Ovate	Obcordate	Spherical	Smooth	Pink	Medium	Few	7
JTG 7	Ovate	Obdeltate	Spherical	Medium	Yellowish white	Medium	Few	13
JTG 8	Ovate	Obdeltate	Spherical	Smooth	Yellowish white	Firm	Few	12
JTG 9	Ellips	Obcordate	Spherical	Smooth	Pink	Medium	Numerous	16
JTG 10	Ovate	Obcordate	Spherical	Smooth	Pink	Medium	Few	12
JTG 11	Ovate	Obdeltate	Spherical	Medium	Yellowish white	Medium	Numerous	16
JGY 1	Ovate	Obdeltate	Elliptical	Rough	Red	Firm	Few	17
JGY 2	Ovate	Obdeltate	Elliptical	Rough	Red	Medium	Medium	16
JGY 3	Ovate	Obovate	Spherical	Medium	Pink	Medium	Medium	16
JGY 4	Ovate	Obdeltate	Obloid	Rough	White	Medium	Medium	15
JGY 5	Ovate	Obdeltate	Spherical	Smooth	Red	Firm	Numerous	13
JGY 6	Ovate	Obdeltate	Spherical	Smooth	Pink	Firm	Medium	13
JGY 7	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	10
JGY 8	Ovate	Obcordate	Spherical	Smooth	Pink	Medium	Few	10
JGY 9	Ovate	Obdeltate	Elliptical	Rough	Pink	Soft	Medium	12
JGY 10	Ovate	Obcordate	Spherical	Smooth	Pink	Medium	Few	17
JGY 11	Orbicular	Obdeltate	Obloid	Medium	White	Soft	Seedless	12
JGY 12	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	10
JGY 13	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	10
JGY 14	Ovate	Obcordate	Elliptical	Medium	Pink	Firm	Medium	17
JTM 1	Ovate	Obcordate	Spherical	Medium	Pink	Soft	Few	13
JTM 2	Ovate	Obdeltate	Spherical	Medium	Pink	Soft	Medium	13
JTM 3	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Medium	13
JTM 4	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	13
JTM 5	Ovate	Obcordate	Spherical	Rough	Pink	Medium	Few	13
JTM 6	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	13
JTM 7	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Numerous	7
JTM 8	Ovate	Obdeltate	Spherical	Medium	Pink	Soft	Seedless	13
NTT 1	Ovate	Obcordate	Spherical	Smooth	Pink	Medium	Medium	12
NTT 2	Ellips	Obcordate	Spherical	Medium	Pink	Medium	Seedless	16
NTT 3	Ovate	Obdeltate	Spherical	Smooth	Pink	Medium	Medium	17
NTT 4	Ovate	Obdeltate	Spherical	Medium	Yellowish white	Medium	Numerous	10
NTT 5	Ovate	Obcordate	Spherical	Medium	White	Soft	Few	13
NTT 6	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Medium	12
NTT 7	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	16
NTT 8	Ovate	Obdeltate	Spherical	Medium	Yellowish white	Medium	Medium	10
NTT 9	Ellips	Obcordate	Spherical	Medium	Pink	Medium	Medium	12
NTT 10	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Few	16
NTT 11	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	17
NTT 12	Orbicular	Obdeltate	Elliptical	Rough	Pink	Medium	Numerous	17
NTT 13	Ellips	Obdeltate	Spherical	Medium	Pink	Medium	Few	16
NTT 14	Ovate	Obdeltate	Spherical	Medium	Red	Soft	Seedless	17
NTT 15	Ovate	Obdeltate	Elliptical	Medium	Yellowish white	Medium	Seedless	13
NTT 16	Ovate	Obcordate	Spherical	Smooth	Yellowish white	Medium	Medium	17
NTT 17	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	10
NTT 18	Ovate	Obcordate	Elliptical	Medium	Pink	Medium	Seedless	13
NTT 19	Ovate	Obcordate	Pyriform	Medium	Pink	Firm	Few	16
NTT 20	Ovate	Obcordate	Elliptical	Medium	Pink	Medium	Medium	13

NTT 21	Orbicular	Obcordate	Elliptical	Medium	Pink	Medium	Medium	17
NTT 22	Ovate	Obdeltate	Elliptical	Medium	Pink	Medium	Numerous	17
NTT 23	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Numerous	12
NTT 24	Ovate	Obdeltate	Spherical	Medium	Pink	Firm	Medium	17
NTT 25	Ovate	Obcordate	Elliptical	Medium	Pink	Medium	Medium	18
SLW 1	Ovate	Obcordate	Spherical	Medium	Yellowish white	Medium	Medium	12
SLW 2	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Few	12
SLW 3	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Few	12
SLW 4	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Medium	12
SLW 5	Ovate	Obdeltate	Spherical	Medium	White	Medium	Medium	17
SLW 6	Ovate	Obdeltate	Spherical	Medium	White	Medium	Medium	17
SLW 7	Ovate	Obdeltate	Spherical	Medium	White	Medium	Few	17
SLW 8	Ovate	Obdeltate	Spherical	Medium	White	Medium	Medium	17
SLW 9	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Few	16
SLW 10	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Medium	16
SLW 11	Ovate	Obdeltate	Spherical	Medium	Yellowish white	Firm	Medium	10
SLW 12	Ovate	Obdeltate	Spherical	Medium	Pink	Medium	Few	13
SLW 13	Obovate	Obcordate	Elliptical	Medium	Pink	Medium	Medium	18
SLW 14	Ovate	Obcordate	Spherical	Medium	Pink	Medium	Few	17
SLW 15	Obovate	Obcordate	Spherical	Medium	Pink	Medium	Medium	13
SLW 16	Obovate	Obcordate	Spherical	Medium	Pink	Medium	Medium	13

Note: * major distinguishing characters between accessions; detail morphological analysis has been published (Susandarini et al. 2013). LS: leaf shape, PW: petiole wing shape; FS: fruit shape, PT: peel texture, FC: flesh color, FT: flesh texture, SN: seed number (determination of character states referred to Descriptors for Citrus - IPGRI 1999 with some modifications). ACH: Nangroe Aceh Darussalam, JTG: Central Java, JGY: Special Province of Yogyakarta, JTM: East Java, NTT: East Nusa Tenggara, SLW: South Sulawesi

Further observations on results of this study indicated that variability on microsatellite sequences has the potential to be developed as intraspecific molecular marker. This is particularly applicable for distinguishing between cultivars or cultivar-groups of pomelo. This potential could be observed in the consistency of $(GA)_n$ types on some of cultivars used in this study. The cultivars "Adas Nambangan" (JTM 1, JTM 2) and "Adas Duku" (JTM 4, JTM 5) from East Java consistently had microsatellite type of $(GA)_{13}$. These four accessions belonged to the same cultivar-group based on phenetic analysis of their morphology (Susandarini et al. 2013). The same case was found for pomelo cultivars from South Sulawesi, in which "Pangkajene Merah" (SLW 1, SLW 2, SLW 3) with microsatellite type of $(GA)_{12}$, was clearly different from "Pangkajene Putih" cultivar (SLW 6, SLW 7, SLW 8) with $(GA)_{17}$ type. Studies on several plant species indicated the potential of microsatellite, especially the expressed sequence tags-simple sequence repeat (EST-SSR), to be developed into a specific marker, as mentioned by Dillon et al. (2014) for *Mangifera indica*, Biswas et al. (2015) for *Poncirus trifoliata*, and Zhang et al. (2019) for *Elymus* species. A study by Ramadugu et al. (2015) indicated that microsatellite is molecular marker that has the ability to discriminate among accessions within a species, and thus could be developed as species-specific marker, as reported on *Citrus medica*. The potential of microsatellite $(GA)_n$ to be developed as a specific marker for pomelo cultivars deserve further studies. Thus, results of this study offered an opportunity to test the consistency and reliability of $(GA)_n$ for molecular characterization of *C. maxima* by using more cultivars collected from different regions.

The DNA target region used in this study is part of the EST. This result confirmed the study of Palmieri et al. (2007) which found that microsatellite sequences in the form of perfect microsatellite repeats, such as $(GA)_n$, were the most common type of EST-SSR. Results of this study also provide an opportunity for a deeper examination of the possible linkages between types of $(GA)_n$ with particular phenotypes, given that the microsatellite used in this study is EST-SSR, or part of a coding region on ribosomal DNA. This is in line with the statement from Victoria et al. (2011) that the EST-SSR has the potential as a functional marker to detect associations between a gene and a particular phenotype. The same opinion was expressed by Zhao et al. (2013) by referring to the potential of EST-SSR as a gene-related marker that can carry gene function information, particularly related to the phenotype characteristics of a cultivar. Similar result was reported by Sharma et al. (2015) on the *Citrus paradisi*, which showed that there was a correlation between genetic diversity detected by microsatellites and morphological data even though the correlation was at a low level.

The use of EST-SSR in *Citrus* systematic studies has been mentioned by Shahzadi et al. (2014) who noted that microsatellite is very informative molecular marker for genetic diversity analysis. In addition, Vieira et al. (2016) noted that the nature of microsatellite mutations makes it as informative molecular markers, and argued that microsatellite was even more informative than SNPs. The application of EST-SSR in plant systematic studies is also supported by a fairly high level of transferability among species, and even among genera within a family, which reached 56% among *Poncirus*, *Fortunella*, and *Citrus* (Biswas et al. 2015). This fact emphasized the importance

of EST-SSR in plant systematics studies, particularly in studying the origin, relationships between species, and the evolution of *Citrus* and their relatives.

Based on the results of this study it could be concluded that microsatellite polymorphism in the forms of repeat length variations has the potential to be developed as molecular markers for the characterization of *C. maxima* cultivars. The repeat length variations on *C. maxima* microsatellites, ranging from $(GA)_7$ to $(GA)_{19}$ found in this study, were largely due to insertions and deletions on microsatellite sequences.

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