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

RATNA SUSANDARINI1,*, SITI SUBANDIYAH2, BUDI S. DARYONO3, RUGAYAH3
1Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia.
Tel/fax. +62-274-580839, *email: ratna-susandarini@ugm.ac.id
2Faculty of Agriculture, Universitas Gadjah Mada. Jl. Agro, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia
3Research Center for Biology, Indonesian Institute of Sciences, Indonesia. Jl. Raya Jakarta-Bogor Km. 46, Cibinong Science Centre, Cibinong, Bogor 16911, West Java, Indonesia

Abstract. Susandarini R, Subandiyyah 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 (Shapit 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 (Smykal 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™ DNA Mite 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 MgCl₂ (Bioline™), 0.5 unit/μL of Taq polymerase (Mango™), and 0.5 μL DMSO. The primers used in this study were DY296883 (forward 5’-CCCCCTCTTTTTTCTTCCA-3’ and reverse 5’-TTCTGGGCCTGGTAGGTCAG-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 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.

| 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                      |

**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.

**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-3130xl 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).

![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](http://www.mesquiteproject.org)
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 (Vieira et al. 2016). This study indicated that microsatellite is a useful molecular marker for detecting genotypic variability within Citrus 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 aurantiun. 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 in pomelo. This is in line with previous studies which showed that microsatellite sequences were highly variable in pomelo. This is in line with previous studies which showed that microsatellite sequences were highly variable in pomelo. This is in line with previous studies which showed that microsatellite sequences were highly variable in pomelo. This is in line with previous studies which showed that microsatellite sequences were highly variable in pomelo.
Table 3. List of pomelo accessions, their morphological characters, and microsatellite repeat number (GA)n

| Accession code | Selected morphological characters* | (GA)n |
|----------------|------------------------------------|-------|
| ACH 1          | Ovate, Oblongate, Spherical, Medium, Pink, Soft, Few | 17    |
| ACH 2          | Ovate, Oblongate, Spherical, Medium, Pink, Soft, Seedless | 17    |
| ACH 3          | Ovate, Oblongate, Spherical, Medium, Pink, Soft, Seedless | 17    |
| ACH 4          | Linear, Elliptical, Medium, Pink, Medium, Few | 16    |
| ACH 5          | Linear, Elliptical, Medium, Pink, Soft, Few | 16    |
| ACH 6          | Ovate, Obovate, Spherical, Medium, Pink, Soft, Few | 17    |
| JTG 1          | Ovate, Obovate, Spherical, Smooth, Red, Soft, Numerous | 19    |
| JTG 2          | Ovate, Obovate, Elliptical, Medium, Yellowish white, Medium, Numerous | 19    |
| JTG 3          | Ellips, Obovate, Spherical, Smooth, Yellowish white, Medium, Medium | 17    |
| JTG 4          | Ovate, Obovate, Elliptical, Medium, Pink, Medium, Numerous | 17    |
| JTG 5          | Ellips, Obovate, Spherical, Smooth, Pink, Medium, Numerous | 10    |
| JTG 6          | Ovate, Obovate, Spherical, Smooth, Pink, Medium, Few | 7     |
| JTG 7          | Ovate, Obovate, Elliptical, Medium, Yellowish white, Medium, Few | 13    |
| JTG 8          | Ovate, Obovate, Elliptical, Medium, Yellowish white, Firm, Few | 12    |
| JTG 9          | Ellips, Obovate, Spherical, Smooth, Pink, Medium, Numerous | 16    |
| JTG 10         | Ovate, Obovate, Spherical, Smooth, Pink, Medium, Few | 12    |
| JTG 11         | Ovate, Obovate, Elliptical, Medium, Yellowish white, Medium, Numerous | 16    |
| JGY 1          | Ovate, Obovate, Elliptical, Rough, Red, Firm, Few | 17    |
| JGY 2          | Ovate, Obovate, Elliptical, Rough, Red, Medium, Medium | 16    |
| JGY 3          | Ovate, Obovate, Spherical, Medium, Pink, Medium, Medium | 16    |
| JGY 4          | Ovate, Obovate, Oblongate, Rough, White, Medium, Medium | 15    |
| JGY 5          | Ovate, Obovate, Spherical, Smooth, Red, Firm, Numerous | 13    |
| JGY 6          | Ovate, Obovate, Spherical, Smooth, Pink, Firm, Medium | 13    |
| JGY 7          | Ovate, Obovate, Elliptical, Spherical, Medium, Pink, Medium, Few | 10    |
| JGY 8          | Ovate, Obovate, Spherical, Smooth, Pink, Medium, Firm | 10    |
| JGY 9          | Ovate, Obovate, Elliptical, Rough, Pink, Soft, Medium | 12    |
| JGY 10         | Ovate, Obovate, Spherical, Smooth, Pink, Medium, Few | 17    |
| JGY 11         | Ovate, Obovate, Oblongate, Medium, Pink, Soft, Seedless | 12    |
| JGY 12         | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 10    |
| JGY 13         | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 10    |
| JGY 14         | Ovate, Obovate, Elliptical, Medium, Pink, Firm, Medium | 17    |
| JTM 1          | Ovate, Obovate, Spherical, Medium, Pink, Soft, Few | 13    |
| JTM 2          | Ovate, Obovate, Spherical, Medium, Pink, Soft, Medium | 13    |
| JTM 3          | Ovate, Obovate, Spherical, Medium, Pink, Medium, Medium | 13    |
| JTM 4          | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 13    |
| JTM 5          | Ovate, Obovate, Spherical, Rough, Pink, Medium, Few | 13    |
| JTM 6          | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 13    |
| JTM 7          | Ovate, Obovate, Spherical, Medium, Pink, Medium, Numerous | 7     |
| JTM 8          | Ovate, Obovate, Spherical, Medium, Pink, Soft, Seedless | 13    |
| NTT 1          | Ovate, Obovate, Spherical, Smooth, Pink, Medium, Medium | 12    |
| NTT 2          | Ellips, Obovate, Spherical, Medium, Pink, Medium, Seedless | 16    |
| NTT 3          | Ovate, Obovate, Spherical, Smooth, Pink, Medium, Medium | 17    |
| NTT 4          | Ovate, Obovate, Spherical, Medium, Yellowish white, Medium, Numerous | 10    |
| NTT 5          | Ovate, Obovate, Spherical, Medium, White, Soft, Few | 13    |
| NTT 6          | Ovate, Obovate, Spherical, Medium, Pink, Medium, Medium | 12    |
| NTT 7          | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 16    |
| NTT 8          | Ovate, Obovate, Spherical, Medium, Yellowish white, Medium, Medium | 10    |
| NTT 9          | Ellips, Obovate, Spherical, Medium, Pink, Medium, Medium | 17    |
| NTT 10         | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 16    |
| NTT 11         | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 17    |
| NTT 12         | Ovate, Obovate, Elliptical, Rough, Pink, Medium, Numerous | 17    |
| NTT 13         | Ellips, Obovate, Spherical, Medium, Pink, Medium, Few | 16    |
| NTT 14         | Ovate, Obovate, Elliptical, Medium, Red, Soft, Seedless | 17    |
| NTT 15         | Ovate, Obovate, Elliptical, Medium, Yellowish white, Medium, Seedless | 13    |
| NTT 16         | Ovate, Obovate, Spherical, Smooth, Yellowish white, Medium, Medium | 17    |
| NTT 17         | Ovate, Obovate, Spherical, Medium, Pink, Medium, Few | 10    |
| NTT 18         | Ovate, Obovate, Elliptical, Medium, Pink, Medium, Seedless | 13    |
| NTT 19         | Ovate, Obovate, Pyriform, Medium, Pink, Firm, Few | 16    |
| NTT 20         | Ovate, Obovate, Elliptical, Medium, Pink, Medium, Medium | 13    |
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 \((GA)_n\). These four accessions belonged to the same cultivar-group based on phenetic analysis of their morphology \(\text{(Susandarini et al. 2013).}\) 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 \((GA)_n\), 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 \((\text{EST-SSR})\), to be developed into a specific marker, as mentioned by Dillon et al. \(\text{(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 \(C. \text{maxima}\) 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. \(\text{(2007) which found that microsatellite sequences in the form of perfect microsatellite repeats, such as \((GA)_{17}\), 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. \(\text{(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. \(\text{(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. \(\text{(2015) on the Citrus paradisi, which showed that there was a correlation between genetic diversity detected by microsatellites and morphological data even though the correlation was at a low level.}\)}

The use of EST-SSR in Citrus systematic studies has been mentioned by Shahzadi et al. \(\text{(2014) who noted that microsatellite is very informative molecular marker for genetic diversity analysis. In addition, Vieira et al. \(\text{(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 \(\text{(Biswas et al. 2015).}\)} This fact emphasized the importance

| NTT 21 | Orbicular | Obcordate | Elliptical | Medium | Pink | Medium | Medium | 17 |
| NTT 22 | Ovate | Obdeltate | Elliptical | Medium | Pink | Medium | Numerous | 17 |
| NTT 23 | Ovate | Obdeltate | Spherical | Medium | Pink | Medium | Numerous | 12 |
| NTT 24 | Ovate | Obdeltate | Spherical | Medium | Pink | Firm | Medium | 17 |
| NTT 25 | Ovate | Obdeltate | Elliptical | Medium | Pink | Medium | Medium | 18 |
| SLW 1 | Ovate | Obdeltate | 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 | Obdeltate | 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 \(\text{(Susandarini et al. 2013).}\) LS: leaf shape, PW: petiole wing shape; FS: fruit shape, PT: peel texture, FC: flesh color, FT: flesh texture, SN: seed number \(\text{(determination of character states referred to Descriptors for Citrus - IPGRI 1999 with some modifications).}\) ACH: Nangroe Aceh Darussalam, JTG: Central Java, JGY: Special Province of Yogyakarta, JTM: East Java, NTT: East Nusa Tenggara, SLW: South Sulawesi.
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)\(^n\) to (GA)\(^{19}\) found in this study, were largely due to insertions and deletions on microsatellite sequences.

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