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Determination of the chromosome number and genome size of *Garcinia mangostana* L. via cytogenetics, flow cytometry and k-mer analyses

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ABSTRACT

Mangosteen (*Garcinia mangostana* L.) is one of the most popular tropical fruit of South-East Asia. It has considerable economic potential for local and export markets. This paper describes a research work to determine the number of chromosomes and genome size of *G. mangostana* through chromosome counting, flow cytometry and k-mer analyses. Chromosome count analysis revealed that the chromosome number of *G. mangostana* varied from 74 to 110. The high number observed could be due to the occurrence of mutation and aneuploidy in *G. mangostana*. Using flow cytometry with *Glycine max* cv. Polanka (2C = 2.5 pg) used as standard, *G. mangostana* genome size was found to be 2C = 6.00 ± 0.17 pg. Meanwhile, a genome survey of *G. mangostana* was performed using Illumina HiSeq 2000 DNA sequencing; k-mer analysis revealed that the genome size of *G. mangostana* was approximately 5.92 Gbp, or approximately 6.05 pg (1 pg DNA = 0.9780 × 10^9 bp). Based on the flow cytometry and genome survey, the study concludes that the genome size of *G. mangostana* is between 6.00 and 6.05 pg.

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Chromosome count; flow cytometry; genome size; *G. mangostana*; k-mer analysis

1. Introduction

Garcinia mangostana belongs to the Clusiaceae family. It is considered as an indigenous cultivated *Garcinia* species in Malaysia (Verheij 1991). The species was identified and named as *Garcinia mangostana* L. by Linnaeus (1753). It is sometimes referred to as "the queen of fruits" because of its delicious taste and attractive fruit shape and colour. The fruit contains white sweet pulp which is eaten fresh. *G. mangostana* has considerable economic potential both for the local and export market. It has been found to possess medicinal properties (Pedraza-Chaverri et al. 2008), and the tree of the non-fruit bearing type of *G. mangostana* provides timber for the furniture industry (Nakasone and Paull 1998; Yapwattanaphun et al. 2002). *G. mangostana* is cultivated mainly in South-East Asian countries such as Malaysia, Philippines, Thailand and Indonesia; and it is also planted in many other countries including Australia, USA and Brazil (Osman and Milan 2006).

Although most of the tropical fruit species are facultative apomicts, *G. mangostana* is considered as an obligate apomict hybrid (Ha et al. 1988; Richards 1990). As such, *G. mangostana* progenies are expected to have the same genotype as their mother plant with no or very little genetic variation within or between populations (Koltunow 1993; Abdullah et al. 2012). The lack of genetic variation poses great limitation to the breeding and selection for better varieties of *G. mangostana* for commercial purposes. *G. mangostana* has a long juvenile period and normally will only attain optimum fruiting 10 years after planting (Wieble et al. 1992; Abdullah et al. 2012). By comparing their morphological characteristics such as fruit colour, latex colour and possession of a sessile stigma, Richards (1990) reported that *G. mangostana* is an allotetraploid species, a hybrid from an interspecific cross between *G. malaccensis* and *G. hombroniana*.

In recent years, the interest in *G. mangostana* has increased due to its antiquity, attractiveness and good commercial value. Past studies conducted on *G. mangostana* were mostly associated with tissue culture, morphology and seed characteristics of the species such as the use of *in vitro* techniques for propagation (Normah et al. 1992, 1995), the characterisation of its flower and fruit (Richards 1990) and seed desiccation sensitivity (Normah et al. 1997, 2016). Very few studies were conducted on its genome size, chromosomal analysis and genome studies (Murugan et al. 2014). The study of nuclear genome size and chromosome number is important to understand the genetic aspects of *G. mangostana*. Data on genome size is especially important in works

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related to genome sequencing (Doležel and Greilhuber 2010).

For species having large number of chromosomes, chromosome count analysis is always difficult (Mallón et al. 2009). Therefore, high quality chromosome preparations with sufficient spread are required for a reliable chromosome count and karyotype analyses. Some individuals or species may have an unbalanced chromosome set. Namely, aneuploidy refers to the loss or gain of chromosomes relative to an established karyotype (Ramsey and Schemske 2002). This phenomenon can result from alteration in somatic division and irregularities in meiotic cell division (De Storme and Mason 2014).

Flow cytometry (FCM) is a powerful technique that is widely used in genome size analysis because the technique is fast and efficient (Galbraith et al. 1983; Arumuganathan and Earle 1991; Bennett and Leitch 2011; Vrána et al. 2014). In addition, with the fast development of next generation sequencing, k-mer analysis can be now utilised for genome size estimation (Liu et al. 2013).

This study aims to determine the chromosome number and genome size of *G. mangostana*. The correct information on the chromosome number is crucial to understand the genetic aspects of *G. mangostana* and the congruency of analysis from both approaches will help to ascertain the genome size for ongoing efforts to assemble and annotate its genome.

2. Material and methods

2.1. Plant materials

For FCM analysis, young red-coloured leaf tissues of 10–12-month-old *G. mangostana* plants propagated from seeds (Figure 1) were obtained from the nursery of Universiti Kebangsaan Malaysia (UKM) and utilised for analysis. Young leaves were selected for nuclei suspension preparation as they were softer and easier to process and also contained fewer secondary metabolites (Jedrzejczyk and Sliwinska 2010).

For chromosome counting, young and whitish root tips were harvested from five 10–12-month-old plants. The root tips were harvested in the morning at 7.30–8.30 am because the cells are dividing actively during this period (Bakry and Shepherd 2008). The root tips were treated immediately with 2 mM 8-hydroxyquinoline (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1–2 h, 4°C for 1–2 h and fixed in Carnoy's fixative solution (absolute ethanol:glacial acetic acid 3:1) at room temperature for 18 h. The fixed root tips were then transferred into 70% ethanol solution and stored at –20°C until use.

2.2. Chromosome preparation

Chromosomal slides were prepared following the method of Schwarzacher (2016). The fixed root tips were rinsed three times for 10 min in enzyme buffer (0.01 M citric acid-sodium citrate, pH 4.6). The 1 mm end region of root tip meristematic cells was treated with enzyme mixture in an enzyme buffer (20% pectinase and 2% cellulose, Sigma-Aldrich) at 37°C for 60–90 min. The strength of enzyme and the time of digestion were optimised according to Schwarzacher et al. (1980), Madon (2000), Halfmann et al. (2007), Quen (2009) and Schwarzacher (2016) to ensure optimum removal of cell wall and allow good spreading of metaphase chromosomes. The mitotic chromosomes preparations of *G. mangostana* were prepared using the squashing technique, a technique reported to give higher quality

Figure 1. 10–12-month-old *G. mangostana* plants.

metaphase spreads for several plant species (Swedlund and Vasil 1985; Perry and Schrader 2004; Kaczmarek et al. 2009; Lucia et al. 2011). After removal from enzyme mixture, root tips with softened tissue were rinsed with enzyme buffer for at least 15 min and treated with 45% acetic acid for 1–5 min. Then, the root tips were placed on a glass slide with a drop of 60% acetic acid. The root tips were squeezed immediately with forceps to extract the protoplast of meristematic cells. The coverslips were applied on each glass slides to cover the protoplast suspension. Firm thumb pressure was applied on the glass slide to spread the protoplast nuclei and remove excess acetic acid. The coverslips were then sealed on each side with rubber solution to prevent drying. Each slide was screened under phase contrast using Carl Zeiss Axioplan^{R} microscope.

2.3. Image processing and chromosome counting

Twenty metaphase cells consisting of well-separated chromosomes with little overlapping were photographed using PAXcam^{R} camera and selected for chromosome count analysis. Chromosome counting was performed automatically by using ImageJ analysis software (https://imagej.nih.gov/ij/). All selected images were converted to greyscale (8-bit or 16-bit) using the menu command "Image/Type/8-bit". The chromosome structures were then highlighted by adjusting threshold value. Overlapping chromosomes were separated using the menu command "Process/Binary/Watershed" where a one-pixel thick line was added automatically. The menu command "Analyse/Analyse particles" was implemented to begin the counting analysis. To count all selected particles, the "size" and "circularity" options were adjusted at the default 0 to infinity and 0.00 to 1.00, respectively. The "outline" was then selected for "show" option. A copy of the image appeared automatically where all counted particles were then shown as numbered outlines. These numbers correspond to data for individual particles which are listed in the "Results" window. The result was displayed automatically after the analysis was completed.

2.4. Nuclei extraction and staining

For FCM analysis, leaf samples of *G. mangostana* plants were cut into small pieces and placed in a petri dish. In this study, nuclei suspensions were extracted from young leaves through manual chopping using a sharp scalpel. Three nuclear isolation buffers, namely LBO1 (Doležel et al. 1989), Tris-$MgCl_2$ (Pfosser et al. 1995) and Otto buffers (Otto 1992; Doležel and Gohde 1995) supplemented with RNaseA (Sigma-Aldrich), propidium iodide (PI) (Sigma-Aldrich) and reducing agents mercaptoethanol (Merck, Darmstadt, Germany) and polyvinylpyrrolidone (PVP-40) (Sigma-Aldrich) (Yokoya et al. 2000) were utilised. The suspension of released nuclei was filtered into a 10 ml falcon tube (Becton Dickinson, Franklin Lakes, NJ, USA) through 50 µm nylon mesh and incubated for 15 min at 4°C to allow optimum PI intercalation with the DNA strands. PI fluorochrome was selected for this analysis because it produced a precise fluorescent intensity histogram peak with lower coefficient of variation (CV) (Doležel and Bartos 2005) as well as intercalating the entire DNA region (Doležel et al. 2007).

2.5. Cytometric measurements

A FACSCalibur FCM (BD Biosciences, San Jose, CA, USA) equipped with 15 mW argon ion laser at 488 nm was used. Histograms were obtained over 1024 channels and 5000 events. Estimation of nuclear DNA content were based on soybean, *Glycine max* cv. Polanka (2C = 2.5 pg; seeds were provided by J Doležel, Olomouc) as an external reference standard (Doležel et al. 1994; Madon et al. 2008). Fluorescence intensity of all samples were compared with fluorescence intensity of *Glycine max* cv. Polanka to obtain the ratio, then multiplied by the genome size of soybean, 2C = 2.5 pg. The values of fluorescent intensity peaks and genome sizes of samples were analysed using CellQuest3^{R}.

2.6. Genome size estimation by k-mer analysis

DNA-sequencing data of *G. mangostana* were obtained from NCBI SRA database accession number SRX1426419 (Abu Bakar et al. 2016). Paired end raw reads of 101 bp from Illumina HiSeq 2000 sequencing were filtered to remove adapter sequences trimmed to Q-value of ≥25 using Trimmomatic (Bolger et al. 2014). Genome survey was performed via k-mer analysis using Jellyfish version 1.1.11 (Marçais and Kingsford 2011). Genome size was estimated using the formula: Genome size = (Total number of k-mers) / (Peak value of k-mer frequency distribution).

3. Results

3.1. Chromosome count analysis

Slides containing several metaphase spreads (Figure 2) were selected for chromosome counting. Images from chromosome count analysis are presented in Figure 3. The chromosome count analysis on 20 metaphase spreads each revealed the chromosome numbers varied from 74 to 110 (Table 1).

3.2. Flow cytometry analysis

The effect of buffer on the intensities of *G. mangostana* nuclei is presented in Figure 4. The results showed that LBO1 buffer produced no peak, which indicated that LBO1 buffer was not suitable for the *G. mangostana* sample. Tris-MgCl2 produced a broad peak with high CV, of >3% at channel 480–560 while the Otto buffer

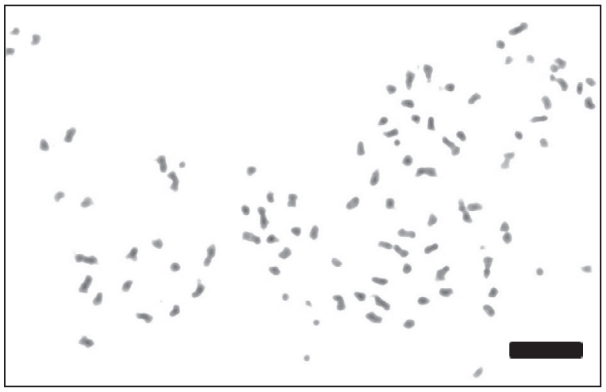

Figure 2. Microphotograph of well-spread mitotic metaphase plates of *G. mangostana* (bar indicates 20 µm).

produced symmetrical peak with low CV (<3%) at channel 400–450. Therefore, this study showed that Otto buffer supplemented with PVP-40 and mercaptoethanol produced the best quality nuclear suspension. The G0/G1 peak histogram revealed that Otto buffer was the most suitable buffer for nuclei isolation of *G. mangostana* as compared to Tris-MgCl2 and LB01 buffer hence was used for genome size estimation.

3.3. Nuclear genome size analysis

The fluorescence intensity peak in reference standard, *Glycine max* cv. Polanka as compared to *G. mangostana*, is presented in Figure 5. The figure shows that the 2C peak of *Glycine max* cv. Polanka was not overlapping with the 2C peak of *G. mangostana*. The 2C peak of *Glycine max* cv. Polanka was located on the channel 180–200 while the 2C peak of *G. mangostana* was located on the channel 420–440 (Figure 5). Therefore, in this study, *Glycine max* cv. Polanka (2C = 2.5 pg) was used as an external reference standard in order to estimate the nuclear genome size of *G. mangostana* due to the difference in 2C peak and easy handling of the *Glycine max* cv. Polanka leaves. The analyses were conducted in five replications. The results of the analysis showed that the genome size of *G. mangostana* was estimated to be 2C = 6.00 ± 0.17 pg (Table 2).

3.4. Genome size estimation by k-mer analysis

The genome size estimation for *G. mangostana* using k-mer analysis is presented in Figure 6. The k-mer size of 41 was used for genome size estimation (Figure 6). The total number of k-mer and peak value of k-mer frequency distribution (predicted by Jellyfish) was 29,604,414,280 and 5 respectively. The genome size of *G. mangostana* was estimated at 5.92 Gbp. The genome size was then converted according to the following relationship: 1 pg DNA = 0.9780×10^9 (Doležel and Bartos 2005). The calculation gave approximately a genome size of 6.05 pg for *G. mangostana*.

4. Discussion

In this study, we conclude that *G. mangostana* has 2*n* = 74–110 chromosomes, which is in agreement with findings obtained by several previous researchers: 2*n*=76 (Krishnawary and Raman 1949); 2*n*=96 (Tixier 1960); 2*n*=110–120 (Ha 1978); 2*n*=88–90 (Richards 1990); and 2*n*=90 (Sarasmiyarti 2008). Chromosome count analysis of *G. mangostana* is difficult due to the presence of numerous small chromosomes (Richards 1990). Robson and Adams (1968) mentioned that it was not easy to count the chromosome number of *Garcinia* species due to their high number of chromosomes. The chromosome numbers of several *Garcinia* species were reported by previous researchers, e.g. 2*n*=48 for *G. benthamii* Pierre (Tixier 1960); 2*n*=44 for *G. hanburyi* Hook. f. (Tixier 1953); and for *G. indica* Choisy, Thombre (1964) found 2*n*=48, while Anerao et al. (2013) found 2*n*=54. For *G. indica* Choisy, Anerao et al. (2013) concluded that all populations of *G. indica* that they studied were diploid, 2*n* = 54. Since every precaution and optimisation has been made in the preparation of the chromosomes used in our study, we believe that our estimate of the chromosome number is thus by far the most reliable estimate. Furthermore, the use of automated image processing (ImageJ) for chromosome determination of *G. mangostana* further confirms the reliability of our results. This is important to ascertain the cytogenetics information on *G. mangostana* for future genome analysis or varietal comparative study. In addition, molecular cytogenetic analyses such as fluorescent *in situ* hybridisation (FISH) and genomic *in situ* hybridisation (GISH) will be useful in further study of *G. mangostana* and its relative species.

The most recent study proposed that *G. mangostana* of Jogorogo variety has 2*n* = 90 chromosomes (Sarasmiyarti 2008). However, our study found that the *G. mangostana* chromosome number reaches up to 110. Genetically, *G. mangostana* is considered as an obligate apomict species where progenies are expected to have the same genotype as their parents (Abdullah et al. 2012). However, genome mutations tend to accumulate in obligate apomictic species (Richards 1986) and could be a source of genetic variation in *G. mangostana* (Ramage et al. 2004). Ray (2002) and Sobir et al. (2011) reported that the presence of natural mutation in *G. mangostana*. The occurrence of gene mutations can impair chromosome segregation, which could cause aneuploidy (Zuzana 2012). Aneuploidy is an unbalanced change in chromosome number on a cellular or organismal level, where the total chromosome number is increased or reduced (Huettel et al. 2008). Previously, Richards (1990) reported that *G. mangostana* is an allotetraploid species, a hybrid from an interspecies cross between *G. malaccensis* and *G. hombroniana*. Regarding this, polyploidy phenomenon may also increase the chromosome number as well as complexity of their pairing and segregation, causing

abnormalities including aneuploidy during meiosis and mitosis (Comai 2005). Aneuploidy is frequently observed in nature, generally in plants with polyploid genomes (De Storme and Mason 2014). Chester et al. (2012) reported the occurrence of aneuploidy in natural allopolyploid species, *Tragopogon miscellus* (Asteraceae) via molecular cytogenetic analysis. Hence, natural mutation and chromosomal rearrangements may be the reasons why there are morphological variations observed in *G. mangostana* (Matra et al. 2016). In addition, Sarasmiyarti (2008) suggested that the variation of *G. mangostana* chromosome number might be due to the presence and variable number of B chromosomes (Bs). Genomic rearrangements following interspecies crosses offer another opportunity for Bs to arise (Jones et al. 2008).

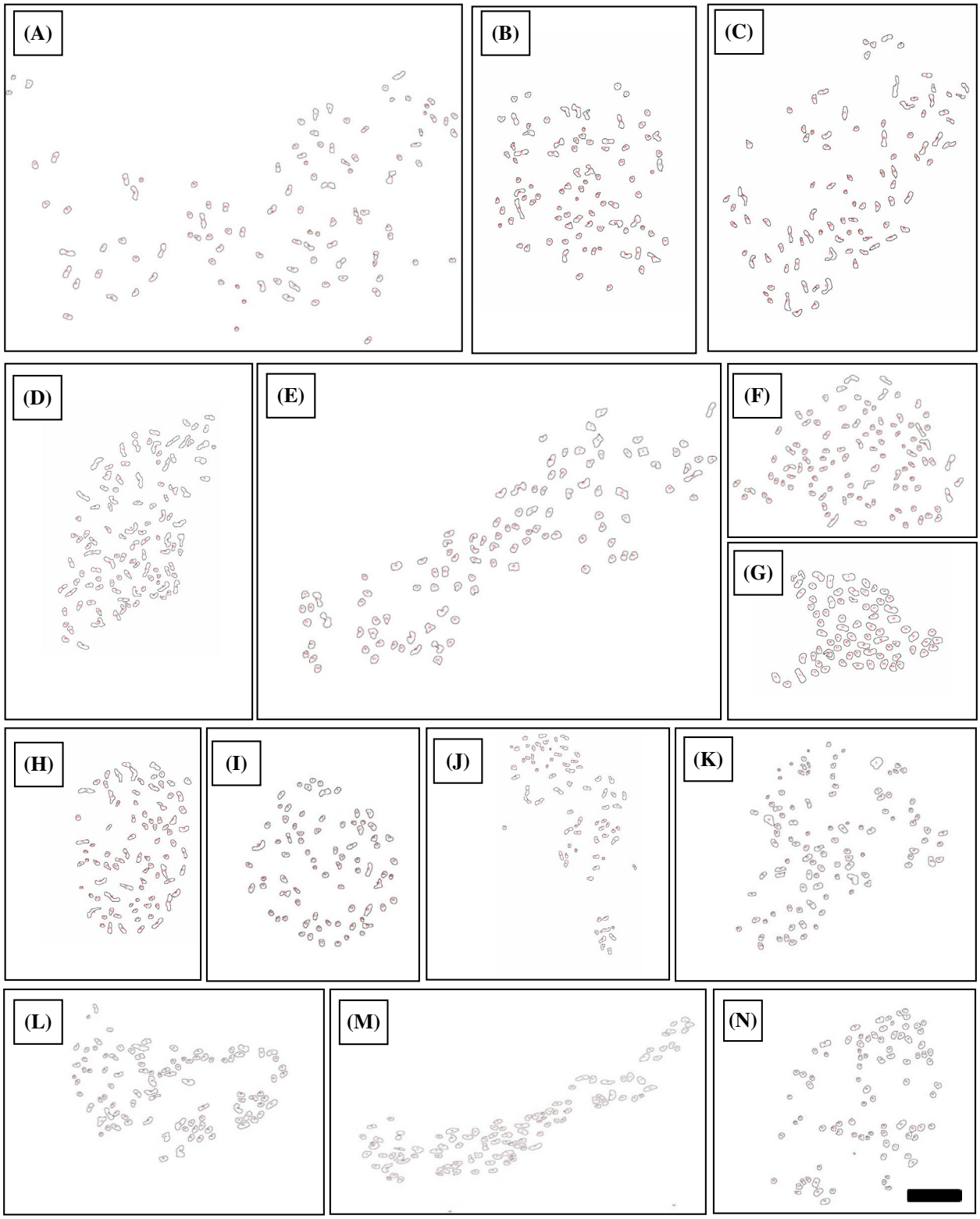

Figure 3. Chromosome count analysis on 20 metaphase spreads of *G. mangostana* was performed by ImageJ Software (bar indicates 20 µm).

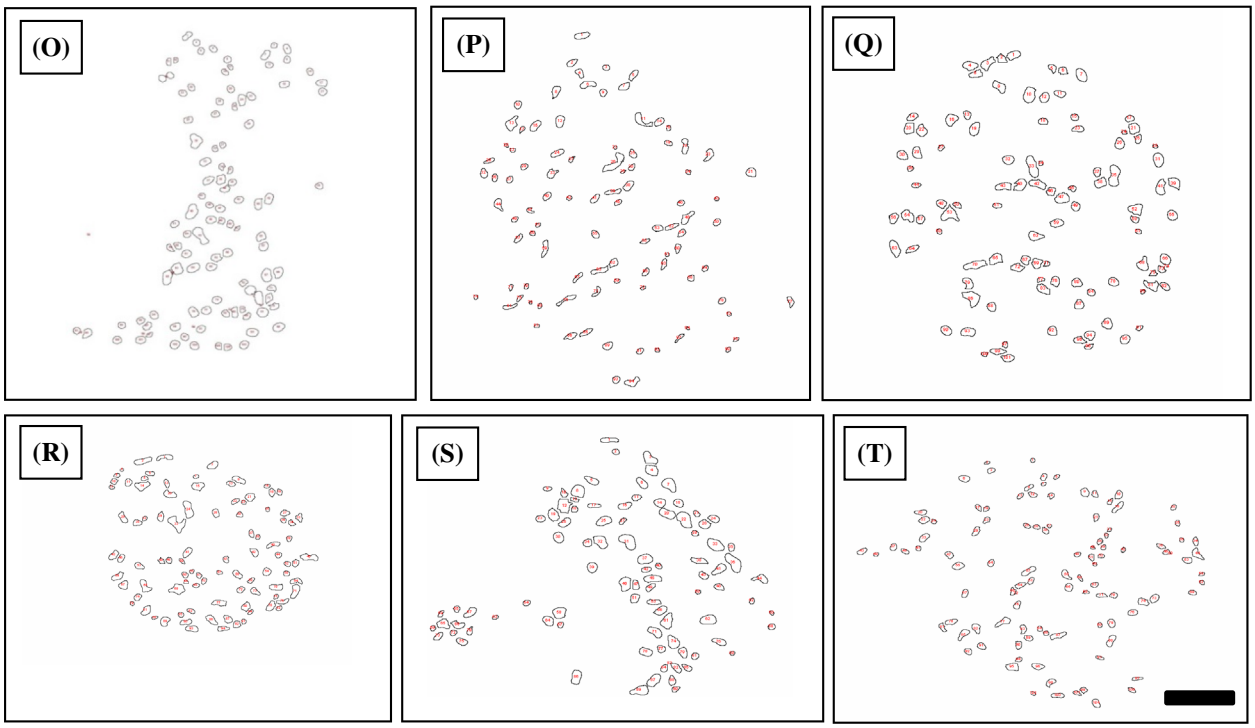

Figure 3. (*Continued*).

Table 1. Chromosome numbers of 20 metaphase spreads of *G. mangostana*.

Metaphase spread	Chromosome number (2*n*)
A	90
B	88
C	90
D	110
E	104
F	102
G	74
H	86
I	76
J	76
K	104
L	106
M	96
N	88
O	102
P	94
Q	100
R	94
S	90
T	102
Range	74–110
Mean±SD	93.6 ± 10.41

For FCM analysis, the addition of reducing agents such as mercaptoethanol and PVP-40 in Otto buffer counteracted the interference of phenolic compound with DNA staining, and thus decreased the CV value. However, different types of lysis buffer will produce different levels of resistance to phenolic compounds (Loureiro et al. 2006; Vrána et al. 2014). The quality of nuclei extraction depends on the selection of lysis buffer and fluorochrome. According to Doležel and Bartos (2005), the best G0/G1 peak should be in a symmetrical shape within low variation when expressed in CV. The value of CV is an important parameter in FCM technique (Prado et al. 2010). Low CV gives accurate measurement (Ulrich et al. 1988). Therefore, the fluorescent histogram peak data produced by Otto buffer was used in this study for genome estimation of *G. mangostana*. PI was used as DNA fluorochrome since it has been proved to give a precise G0/G1 histogram peak (Madon et al. 2008).

The presence of secondary metabolites in cytosol such as phenolic or cytosolic compounds during sample preparation may reduce the quality of cell suspension and cause an error during FCM analysis by reducing fluorescence level and increasing the level of CV (Loureiro et al. 2006; Bennett et al. 2008; Mallón et al. 2009). Phenolic compounds may cause some of the stoichiometric errors, especially in woody plants (Loureiro et al. 2006), and *G. mangostana* is one of them. Therefore, the selection of suitable isolation buffer and the addition of reducing agents including mercaptoethanol and PVP-40 in our FCM analysis may have suppressed the phenolic effect (Price 2000; Yokoya et al. 2000; Noirot et al. 2003; Loureiro et al. 2006). The unfavourable effects of secondary metabolite compounds may also have been reduced by reducing the amount of plant material and chopping intensity (Loureiro et al. 2006; Doležel et al. 2007). For genome size estimation, there are a few criteria to be observed in the selection of reference standard. Generally, the standard and target must be biologically similar, for example plant standards must be utilised for plant samples (Suda and Leitch 2010). Moreover, the genome size of standard should be well defined and genetically consistent, preferably not too close from the target sample

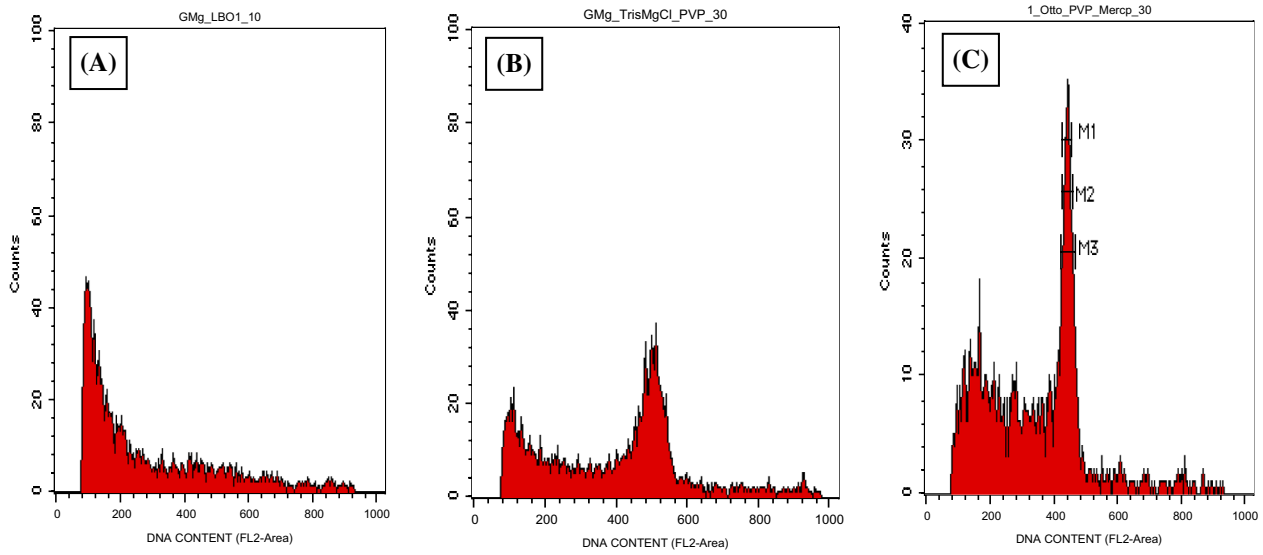

Figure 4. Histogram of fluorescence intensities in *G. mangostana* nuclei isolated from leaves using three different nuclei isolation buffers: (a) LBO1, (b) Tris-MgCl2, (c) Otto.

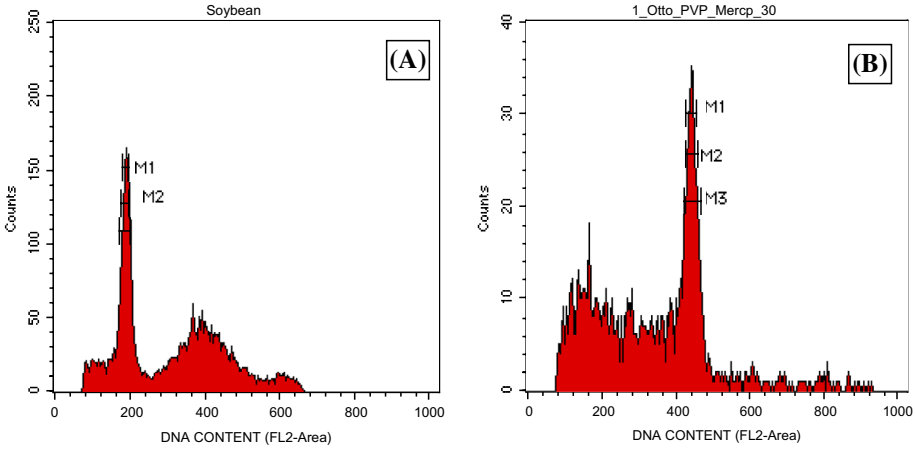

Figure 5. Histogram of fluorescence intensity peak in reference standard, (a) *Glycine max* cv. Polanka and (b) *G. mangostana*.

in order to avoid the G1 peaks of sample and standard nuclei from overlapping (Doležel and Bartos 2005; Greilhuber et al. 2007).

In this study, FCM analysis revealed that the genome size of *G. mangostana* to be 6.00 pg. This value, however, differs from Matra et al. (2014). They reported that the genome size of *G. mangostana* was 2C = 7.42 pg based on FCM analysis. The difference may be due to the different DNA fluorochrome and plant material used for FCM analysis. Matra et al. (2014) utilised 4′6-diamidino-2-phenylindole (DAPI) while we used PI. DAPI intercalates preferentially on a specific region of DNA, which is AT-selective (Doležel et al. 1992). In addition, the material utilised by Matra et al. (2014) may have a higher genome size due to the larger chromosome number as compared to material analysed in this study. Regarding this, k-mer analysis has been utilised to support our FCM result. Based on the k-mer analysis, genome size of *G. mangostana* was estimated at 5.92 Gbp (6.05 pg). Therefore, we conclude the genome size of *G. mangostana* to be between 6.00 to 6.05 pg via FCM and k-mer analyses.

Table 2. Flow cytometric estimation of genome size of *G. mangostana* using *Glycine max* cv. Polanka (2C=2.5 pg) as external reference standard.

	Replicate	DNA content (pg)
G. mangostana	1	6.04
	2	6.09
	3	6.08
	4	5.70
	5	6.10
	Mean±SD	6.00±0.17

The genome size data is important for many fields of research including ecology, evolution and taxonomy (Albach and Greilhuber 2004; Bennett and Leitch 2005; Chneeweiss et al. 2006; Kron et al. 2007; Vrána et al. 2014). The correct information on genome size is crucial for the understanding of genome sequencing projects (Bennett et al. 2000). In the structuring of genome sequencing projects, it is necessary to have accurate

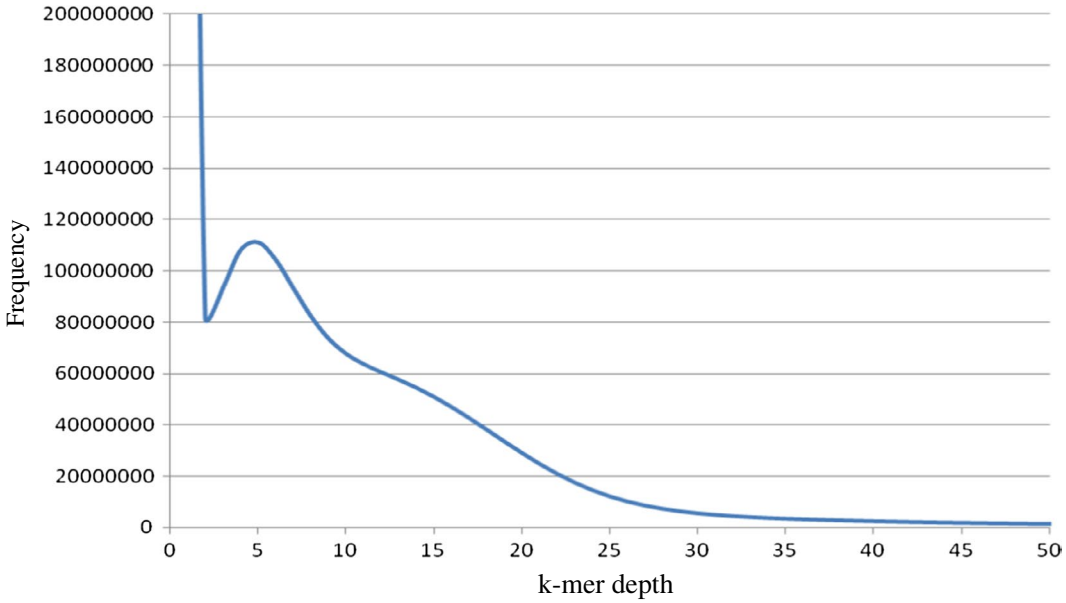

Figure 6. k-mer 41 depth distribution of whole-genome Illumina reads. A peak at 5 was identified.

knowledge on genome size since the projects' scale and cost depend on the genome size (Doležel and Greilhuber 2010; Cardoso et al. 2012).

5. Conclusion

To date, this is the first report on the genome size of *G. mangostana* by using both FCM and k-mer analyses. Both estimation methods were highly consistent. From this study it was proposed that the genome size of *G. mangostana* is around 6.00–6.05 pg, which can be used as a reference for future genome sequencing projects. For chromosome count analysis, it was concluded that *G. mangostana* has $2n = 74–110$ chromosomes, using automated image processing. This report gives important information needed for further genomic studies of this economically important fruit species.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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