Chromosome and Ploidy Analysis of Winter Hardy *Hibiscus* Species by FISH and Flow Cytometry

Deen Mohammad Deepo

Kyungpook National University

Islam MD Mazharul

Kyungpook National University

Yoon-Jung Hwang

Sahmyook University

Hong-Yul Kim

Kyungpook National University

Chang Kil Kim

Kyungpook National University

Ki-Byung Lim (✉ kblim@knu.ac.kr)

Kyungpook National University

Research Article

Keywords: Cytogenetics, Fluorescence in situ hybridization, Loci, 5S rDNA, 18S rDNA

Posted Date: April 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-404917/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Determination of nuclear DNA content, genome sizes, and ploidy level and, information on cytogenetic characteristics are all prerequisite of modern plant breeding. However, cytogenetic study of *Hibiscus species* is often hampered by the similarity in morphology and by the small size of mitotic chromosomes. The goal of the study was to ascertain the chromosome number, karyomorphology, distribution of 5S and 18S rDNA loci, chromosome length, and centromere positions as well as the ploidy level, genome sizes, 2C-DNA content of winter-hardy *Hibiscus*: *H. syriacus* 'Saejamyung', *H. sinosyriacus* 'Seobong', *H. moscheutos* 'Luna Red' and, *H. paramutabilis*. 5S rDNA and 18S rDNA loci were detected by fluorescence *in situ* hybridization (FISH). According to the FISH results, there are two 5S rDNA loci in *H. syriacus*, *H. sinosyriacus*, and *H. moscheutos*, and four 5S rDNA loci in *H. paramutabilis*. The range in length of somatic chromosomes in *H. syriacus*, *H. sinosyriacus*, *H. moscheutos*, and *H. paramutabilis* is 2.66- 7.06, 3.18-7.31, 2.91-5.23, and 4.75-7.60 μm , respectively. The 2C-DNA content of *H. syriacus*, *H. sinosyriacus*, and *H. moscheutos* are very similar, the amount was 4.06, 4.11, and, 2.06 pg, respectively whereas, *H. paramutabilis* has nearly double and the amount was 4.18 pg. These findings will contribute to the detailed cytogenetic assessment of *Hibiscus* and thus benefit plant breeding in this genus.

Introduction

Hibiscus a polymorphic genus of the Malvaceae family include approximately 300 species of trees, shrubs, and herbs growing in tropical, subtropical, and temperate regions around the world (Mohammad et al. 2020). There are as many as 350 varieties of the shrub *Hibiscus syriacus* also known as Rose of Sharon or Althea, which has a flowering period from 60 to 120 days usually blooming starts from last of June to mid of October (Lee 2013). They are categorized as single, semi double or double flowers depending on the number and arrangement of the petals. *H. sinosyriacus* is a deciduous, perennial, winter hardy shrub that is very similar to *H. syriacus*. The *H. sinosyriacus* cultivar 'Malmauve' cultivated in France in 2001, was introduced in Korea in 2003 and named as 'Seobong' (Jo et al, 2019). *H. moscheutos* L., often called swamp rose-mallow, is winter-hardy herbaceous, plant native to wetland areas in eastern North America. It has long been bred for its compact stature and large flowers in various in colors and shapes (Li and Ruter 2017). *H. paramutabilis* is a hardy, semi- woody, deciduous shrubs; compare with *H. syriacus* and *H. sinosyriacus*, it has larger, round leaves and more plentiful flowers (Bates 1965).

In order to carry out an efficient plant breeding system, it is important to know beforehand the chromosome number, ploidy level, and DNA contents of the species (Sakhanokho et al. 2020). However, contained in the *Hibiscus* genus are a wide variety of plants with complex interspecific relationships (Li et al. 2015). Basic chromosome number is one of the most important features of the taxonomic genus. Notwithstanding, *Hibiscus* species with varied basic chromosome numbers (7–44) have a wide range of ploidy levels, from diploid to sixteen-ploidy and total chromosome number from 22 to 180 (Fryxell 1988). Therefore, the basic chromosome number, ploidy levels, and total chromosome numbers of *Hibiscus species* are still in the disputes. Fluorescence *in situ* hybridization (FISH) and karyotyping are efficient techniques for cytogenetic studies (Anamthawat-Jónsson 2004). FISH results can provide basic

information on the ploidy level, and chromosome characteristics of each species. Moreover, the location of ribosomal DNA of 5S, 18S, 25S, and 45S loci has been widely used to study the genetic relationships among various plant species (Sakhanokho et al. 2020). Using FISH, oligos specific to a repetitive sequence or to a particular genomic region can be visualized (Islam et al. 2020). Karyotyping is one of the major cytological methods employed in empirical and theoretical research (Liang and Chen 2015). Karyotype analysis of plants with a high number of small chromosomes is labor-intensive and difficult due to the differences in the physical features of the homologues and similarities of chromosomes in a complement (Silva et al. 2018). Moreover, genome size is a significant character of living organisms and estimates of genome size have been useful in systematic and evolutionary studies (Knight et al. 2005). The change in DNA consistency and ploidy degree are positively correlated. Determination of genome size can be helpful in elucidating the relationship between the *Hibiscus* species, although very few studies of genome size or nuclear DNA contents of *Hibiscus* have been published. We therefore aimed to determine chromosome number, karyomorphology, and 5S and 18S rDNA loci mapping, nuclear DNA content, genome size, and ploidy level, of *H. syriacus* 'Saejamyung', *H. sinosyriacus* 'Seobong', *H. moscheutos* 'Luna Red' and, *H. paramutabilis*.

Materials And Methods

Plant materials

H. syriacus 'Saejamyung', *H. sinosyriacus* 'Seobong', *H. moscheutos* 'Luna Red' and, *H. paramutabilis* were used for this experiment. These plants are grown in greenhouse and field conditions at Kyungpook National University, Daegu, Republic of Korea.

Root collection and chromosome preparation

Early in the morning, healthy roots tips were collected and treated with an 8-hydroxyquinoline solution for 3–4 h at room temperature. The root tips were then washed with double-distilled water and transferred in an aceto-ethanol (1:3, v/v) solution to fix the roots overnight. After the roots were rinsed with distilled water, they were preserved in 70% ethanol at -20°C until further use. Chromosome preparation is the key step in all cytogenetic techniques. For slide preparation, the root tips were washed with distilled water to remove the ethanol solution and incubated with an enzyme mixture consisting of 0.3% pectolyase (Duchefa, Haarlem, The Netherlands), 0.3% cellulose (Duchefa), and 0.3% cytohelicase (Sigma, St. Louis, MO, USA) at 37°C for 40 min. The digested root tips were then transferred to a clean slide and compressed for viewing under a microscope. Finally, 20 µL acetic acid (60%) was added per slide to spread the cells, and the slides were air-dried.

Fluorescence *in situ* hybridization (FISH)

FISH analysis was conducted according to Lim et al. (2001) with a few modifications. Briefly, slides were pre-treated with 100 µg•mL⁻¹ RNase A at 37° C for 50 min. After rinsing with 2× SSC and fixing with 4% paraformaldehyde for 10 min, the slides were rinsed again with 2× SSC and dehydrated in an ethanol

series (70, 90, and 100%), followed by air-drying. The hybridization mixture contained formamide, 50% dextran sulfate, 20× SSC, 10% sodium dodecyl sulfate, herring sperm DNA, and rDNA probes. The mixture was placed in a water bath for DNA denaturation at 70° C for 5 min and then placed on ice for 15 min for fixation. Forty microliters of the mixture were added to each slide, and a cover slip was placed on the slide, while ensuring there were no bubbles. The slides were placed in a water bath for hybridization at 80° C for 5 min and then placed in a container with wet tissue paper and incubated in a humid chamber at 37° C for 16 h. The slides were then washed with 2× SSC buffer for 5 min followed by 0.1× SSC buffer for 30 min at 42° C with shaking and 2× SSC buffer for 5 min. The slides were then submerged in 1× detection buffer for 5 min. Streptavidin CY3 (Invitrogen, Carlsbad, CA, USA) and anti-digoxigenin fluorescein (Roche, Basel, Switzerland) were used to detect the labeled chromosomes. The slides with cover-slips were placed in a humid chamber to incubate at 37° C for 50 minutes. Three jars containing 1× detection buffer was incubated in the dark in a water bath at 37° C for 5 min followed by dehydration an ethanol series (70, 90, and 100%). The slides were then air-dried in the dark and counterstained with 4'6-diamidino-2-phenylindole (DAPI) and Vectashield at a 2:100 ratio (Vector Laboratories, Burlingame, CA, USA). The prepared slides were examined with a model Nikon BX 61 fluorescence microscope (Tokyo, Japan). Probe signals were analyzed using ultraviolet excitation filters. Cytovision and imaging software were used to acquire images of the chromosomes with 5S rDNA and 18S rDNA signals.

Karyotype analysis

For the karyotype analysis, five cells showing well-spread metaphase chromosomes were selected. The length of each chromosome was measured using software (Cytovision), and chromosome number was determined on the basis of short arm length order according to (Lim et al., 2001). Chromosome types were classified according to the ratio of the short arm to the long arm (Levan, 1964). The images of the chromosomes were captured with and 600× and 1,000× magnification.

Flow cytometry

DNA content and genome sizes were measured using flow cytometry (Partec PA, Ploidy Analyzer, Sysmex, Kobe, Japan) with five randomly collected recently expanded leaves. Leaves were cut to an area approximately 1 cm² and chopped in 500 µL of extraction buffer solution (Sysmex) in a petri dish and shaken for 30 s. The resulting solution was poured through a 30-µm nylon mesh filter into a 3-mL tube and 2 µL DAPI containing staining buffer was added. Finally, the nuclei suspension was injected into the flow cytometry analyzer. 2C genome sizes were calculated as $2C = \text{DNA content of standard} \times \text{mean fluorescence value of sample} / \text{mean fluorescence value of standard}$ (Greilhuber et al. 2005). *Raphanus sativus* cv Tetra Ilowiecka (2C = 2.00 pg) was used as internal standard of known genome size.

Results

Chromosome counting

Determination of chromosome number is a fundamental tool in cytogenetic study. However, detection of the chromosome number of *Hibiscus* species which have a high number of small chromosomes, is always challenging. Because the *Hibiscus* chromosomes are small, meticulous of a sample in a unifocal plane free of cell walls, nuclear membranes and, cytoplasmic debris is necessary for successful and accurate chromosomes counts. We performed photomicrographs of 45 well spread cells from each species to confirm the chromosome number. We clearly detected 88 chromosomes in *H. syriacus* 'Saejamyung' (Fig. 1) while *H. sinosyriacus* 'Seobong' has 80 chromosomes (Fig. 2). Among the four species, *H. moscheutos* 'Luna Red' had the least number of chromosomes 38 whereas, 82 chromosomes are found in *H. paramutabilis* (Fig. 2).

Chromosomal localization of 5S rDNA and 18S rDNA sites

In the metaphase chromosomes, the physical location of 5S rDNA and 18S rDNA was detected using dual color 5S and 18S FISH probes. Two 5S rDNA loci (green fluorescence) were found in *H. syriacus*, *H. sinosyriacus*, and *H. moscheutos*, and four 5S rDNA were detected in *H. paramutabilis* (Fig. 3). Six 18S rDNA loci (red fluorescence) were found in *H. sinosyriacus* and *H. moscheutos*, and five and ten loci were noticed in *H. syriacus* and *H. paramutabilis* respectively (Fig. 3). The distribution pattern of 5S rDNA loci for *H. syriacus*, *H. sinosyriacus*, and *H. moscheutos* *H. paramutabilis* were in the long arm of chromosome number #19 (Fig. 4a), the long arm of chromosome #6 (Fig. 4b), the short arm of chromosome number #10 (Fig. 4c) and, the long arm of chromosome number (Fig. 4d) respectively. In contrast, 18S rDNA loci were distributed in different locations of the chromosomes. Among five rDNA signals of *H. syriacus*, we found two signals in the short arm in chromosomes number #18 and #20, whereas two others were found in the long arm in chromosomes number #6 and #8 and, the rest of one signal was found in chromosome number #11, in the centromere positions (Fig. 4a). In *H. sinosyriacus*, we detected 5S and 18S rDNA signals in long arms of chromosome number # 6 (Fig. 4b). In the case of *H. moscheutos*, all 18S rDNA signals were in the long arm of the chromosome numbers #1, #4, #7, #9, #13 and, #18 (Fig. 4c). The highest number of 18 rDNA signals was found in *H. paramutabilis*, among ten signals, one was in the short arm and one in the centromere position in the chromosome numbers #5 and #3, respectively. The remaining eight red signals were located the long arm of the chromosome numbers #1, #7, #10, #12, #13, #15, #18, and #20 (Fig. 4d).

Karyomorphological analysis

The chromosomes were arranged according to decreasing order of short arm lengths. An ideogram of the four species is presented in Fig. 4. The number of chromosomes complements of are *H. syriacus* 88 (Fig. 4a), and *H. sinosyriacus* 80 (Fig. 4b), with metaphase chromosome lengths ranging from 2.66 ± 0.02 to $7.06 \pm 0.30 \mu\text{m}$ and from 3.18 ± 0.29 to $7.31 \pm 0.25 \mu\text{m}$, respectively. Among the four species, *H. moscheutos* $2n = 38$ (Fig. 4c) has the shortest range of chromosome length ($2.91 - 5.23 \mu\text{m}$) whereas, *H. paramutabilis* (Fig. 4d) has the longest-range chromosome length, from 4.75 ± 0.55 to $7.60 \pm 0.47 \mu\text{m}$. Moreover, we found that the last two chromosomes were in a pair and placed in chromosome #21 (Fig. 4d). Depending on the centromere positions, the homologous chromosomes are composed of

metacentric and submetacentric pairs, no telocentric or sub telocentric chromosome were detected. Most of the chromosomes' pair are metacentric in *H. syriacus*, *H. sinosyriacus* and *H. moscheutos* and the chromosome number is 13, 12, and 12 pairs respectively. In contrast, the chromosomes of *H. paramutabilis* are composed of 14 pairs in submetacentric and 7 pairs of metacentric chromosomes (Fig. 4 and Table 2).

Table 1

FISH signals (5S rDNA and 18S rDNA) and their distribution in chromosomes of four *Hibiscus* species.

Species	No. of 5S rDNA	Location of 5S rDNA		Chromosome pair containing 5S rDNA	No. of 18S rDNA	Location of 18S rDNA		Chromosome pair containing 18S rDNA
		Long arm	Short arm			Long arm	Short arm	
<i>H. syriacus</i> 'Saejamyung'	2	2	0	Ch# 19	5	2	3	Ch# 6, 8, 11, 18, 20
<i>H. sinosyriacus</i> 'Seobong'	2	2	0	Ch# 6	6	3	3	Ch# 3, 6, 8, 9, 11, 14
<i>H. moschetous</i> 'Luna Red'	2	0	2	Ch# 10	6	6	0	Ch# 1, 4, 7, 9, 13, 18
<i>H. paramutabilis</i>	4	4	0	Ch# 4	10	8	2	Ch# 1, 3, 5, 7, 10, 12 13, 14, 18, 20

Table 2

The longest and shortest based on centromere positions in four *Hibiscus* species.

Species	Longest chromosome		Shortest chromosome		No. of chromosome based on Centromere position	
	Chr. no	Chr. Length (μm)	Chr. no	Chr. Length (μm)	Metacentric	Submetacentric
<i>H. syriacus</i> 'Saejamyung'	1	7.06 ± 0.30 ^z	21	2.66 ± 0.02	13	9
<i>H. sinosyriacus</i> 'Seobong'	1	7.31 ± 0.25	19	3.18 ± 0.29	12	8
<i>H. moschetous</i> 'Luna Red'	1	5.23 ± 0.13	19	2.91 ± 0.12	12	7
<i>H. paramutabilis</i>	1	7.60 ± 0.47	20	4.75 ± 0.55	7	14
^z Mean ± SE = 5						

Determination of nuclear DNA content

The flow cytometry analysis produces a histogram for each sample making possible the measurement of nuclear DNA and genome sizes in plants. The histogram peak channel for *H. moscheutos* was in 50 whereas, peak channel for *H. syriacus*, *H. sinosyriacus* and *H. paramutabilis* were in 100 (Fig. 5). Based on the flow cytometry results in the study, 2C-DNA contents of *H. syriacus*, *H. sinosyriacus*, and *H. paramutabilis* are nearly identical, being 4.06, 4.11, 4.18 pg, respectively whereas, *H. moscheutos* has the amount at 2.06 pg. (Table 3). We found the same result in the 2C genome, the amount was 4065.88, 4077, 2014.88, and 4096.41 Mbp for *H. syriacus*, *H. sinosyriacus*, *H. moscheutos* and *H. paramutabilis* respectively (Table 3).

Table 3

2C (pg) nuclear DNA content and genome size (Mbp) of four *Hibiscus* species were determined by flow cytometry

Species	Putative DNA content		Ploidy level
	2C Mbp	2C (pg)	
<i>H. syriacus</i> 'Saejamyung'	4065.88	4.06	2n = 4x = 88
<i>H. sinosyriacus</i> 'Seobong'	4077.04	4.11	2n = 4x = 80
<i>H. moscheutos</i> 'Luna Red'	2014.88	2.06	2n = 2x = 38
<i>H. paramutabilis</i>	4096.41	4.18	2n = 4x + 2 = 82

Discussion

Chromosome counts

Basic chromosome number is one of the key features of cytogenetic characteristics of any genus. However, *Hibiscus* species have a high number of small chromosomes and the basic number of chromosomes of most species are not yet fixed. The range in the basic chromosome number is wide 7 / 8 / 9 / 10 / 11 / 12 / 13 / 14 / 15 / 16 / 17 / 18 / 19 / 20 / 21 / 23 / 24 / 39 / 40 / 44. Ploidy levels in this genus from diploid to sixteen-ploidy and total chromosome number from 22 to 180 (Fryxell 1988; Lattier et al. 2019). In our experiment, we found 88 chromosomes of *H. syriacus* 'Saejamyung' and 80 chromosomes of *H. sinosyriacus* 'Seobong', which is consistent with a previous result for *H. sinosyriacus* (80 chromosome) however, Skovsted (194) reported, *H. syriacus* mostly possess chromosome number 80. We note that there are more than 150 cultivars of *H. syriacus* and both chromosome number and ploidy level vary from one cultivar to another. In the present study, we report the chromosome count (88) result of 'Saejamyung' for the first time. *H. moscheutos* and, *H. paramutabilis* were found to have a chromosome number of 38 and 82 respectively which is consistent with reports in the literature (Barrios and Ruter 2019; Van Laere et al. 2010). Song (2001) reported the following chromosome number in the genus *Hibiscus*: *H. schizopetalus* 2n = 42, *H. mutabilis*; 2n = 92, *H. rosa-sinensis*; 2n = 4x = 84, *H. rosa-*

sinensis, "Double Rainbow" $2n = 5x = 105$, *H. rosa-sinensis*, 'Flavo-plenus' $2n = 6x = 138$, and *H. rosa-sinensis*, 'Carminatus' $2n = 7x = 147$. Clearly, in the genus *Hibiscus*, chromosome number varies depending on ploidy and cultivar.

Chromosomal localization of 5S rDNA and 18S rDNA loci

FISH is a molecular cytogenetic technique in which fluorescent-label probes are used to classify complementary DNA sequence in nuclei (Speicher and Carter 2005); the technique has been shown to be an efficient technique for cytogenetic study of woody angiosperms (Prado et al. 1996). The use of rDNA signals, combined with flow cytometry, has proved meaningful for confirmation of ploidy levels in *Hibiscus* a genus characterized by numerous small chromosomes and tolerance of high level of polyploids (Lattier et al. 2019). In our results, two 5S rDNA loci were *H. moscheutos* 'Luna Red' and four 5S rDNA loci were seen in *H. paramutabilis*. A study of 5S rDNA in cotton plants (close relatives to *Hibiscus*) revealed that most diploid had two 5S rDNA signals and all allotetraploid species had four 5S rDNA signals (Gan et al. 2013); the same result was found in woody species of genus *Rubus* (Wang et al. 2015). A recent FISH analysis of tetraploid *H. sabdaiffa* 'sabdariffa' (Mohammad et al. 2020) resulted in the identification of four 5S rDNA loci. The discovery of only two 5S rDNA loci in tetraploid *H. syriacus* and *H. sinosyriacus* could provide evidence being diploid however, recent genome analysis confirms their polyploid status 'Tetraploid' after multiple occurrences of whole-genome duplication followed by diploidization after speciation (Kim et al. 2017). In addition, among-ploidy level variation in rDNA loci, it is possible to have variation within the same ploidy for a species. Species with the same chromosome number have been found to have up to a five-fold difference in rDNA loci in a comparative analysis of species in *Brassicaceae* (Hasterok et al. 2006). Our findings showing *H. moscheutos* 'Luna Red' is diploid and *H. syriacus*, *H. sinosyriacus* and *H. paramutabilis* are tetraploid which follow the results of previous studies (Chen et al. 2019; Jo et al. 2019; Li and Ruter 2017; Sakhanokho and Kelley 2009).

Karyomorphology of chromosomes

Karyotyping is a useful tool for detecting chromosome variation and is also helpful to interpret in constructing of genetic map (Chung et al. 2018). Karyotype is considered to be the prime cytological trait employed in empirical and theoretical research (Liang and Chen 2015). In karyotype analysis, the difference within each species or cultivar may indicate changes in chromosomes arrangement. According to the finding in the present study, the average length range of *Hibiscus*'s chromosome (2 to 8 μm) is shorter than that *Lilium* spp.; for example, the range of chromosome length in *Lilium triginum* is 15.92 to 30.18 μm (Hwang et al. 2011). Depending upon the position of the centromere, the chromosomes of *Lilium triginum* can be categorized as metacentric, submetacentric, telocentric, and subtelocentric; whereas, in four species of *Hibiscus* (*H. syriacus*, *H. sinosyriacus*, *H. moscheutos*, and *H. paramutabilis*) metacentric and submetacentric type of chromosomes were detected, but no telemetric chromosomes were found. Compared with simple shape classification, the application of karyotype data can address the difficulties of interpreting traditional chromosome shape and classification (Zhu and Wang 2008). To the best of our knowledge, the karyomorphology of *H. paramutabilis* ($2n = 4x + 2 = 82$) in the present

study is the first, to be reported; a further research is needed for a detailed explanation of the last set of chromosomes.

Nuclear DNA content analysis

Flow cytometry offers a quick, precise and simple way to determine nuclear DNA content (C-value) of plants (Galbraith, 2009). The technique facilitates the characterization of plants species in natural and agricultural settings, allows easy identification of ploidy level, and is responsive to the environment in term of evolutionary fitness. Despite, the feasibility of flow cytometry methods, C-values have been calculated for only approximately 2% of the described angiosperm species (Galbraith 2009). Our search of Plant DNA C-values database uncovered only one *Hibiscus species* for which there is a recorded DNA C-value (*H. cannabinus*, 2C DNA = 3 pg). In present study, we determined the 2C nuclear DNA content of *H. syriacus* 'Saejamyung' and *H. sinosyriacus* 'Seobong' were found 4.06 pg and 4.11 pg respectively, which are nearly to the tetraploid cultivar of *H. syriacus* 'Diana' (2C-DNA = 4.58 pg) (Lattier et al, 2019; Van Huylenbroeck et al. 2000). *H. moscheutos* 'Luna Red' have nuclear DNA contents of 2.06 pg, that value is 15-fold larger than that of the angiosperm, with the smallest recorded genome, *Genlisea margaretae* Hutch (2C = 0.1296 pg) (Sakhanokho et al. 2020) and, 125- fold smaller than that of the monocot *Fritillaria assyriaca* (2C = 254.6 pg) (Bennett and Leitch 2005). We estimated the genome size of *H. paramutabilis* (2C = 4.18 pg) that's value is consistent with the previous genome sizes values of tetraploid *H. sabdariffa* and, tetraploid species of *Gossypium* (Mohammad et al. 2020; Hendrix and Stewart 2005).

Conclusion

Chromosome number, karyotype, nuclear DNA content, and rDNA distribution (5S and 18S rDNA) of *H. syriacus* 'Saejamuyng' *H. sinosyriacus* 'Seobong', *H. moscheutos* 'Luna Red' and *H. paramutabilis* were determined in this study. Our results will help to minimize the disputes regarding chromosome number and ploidy level of these four species. Specially the chromosome number of Saejamyung (88) was determined for the first time. The detailed karyotyping of *Hibiscus* will aid further research in this genus and lay the groundwork for an efficient hybridization scheme aimed at improving targeted traits.

Declarations

Acknowledgements

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (iPET) through the Agri-Bio Industry Technology Development Program (or Project), funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (Grant no. iPET318021-4) and Agri-Bioindustry Technology Development Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea. (No. iPET 112129-5).

Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution statement

Deen Mohammad Deepo, Islam MD Mazharul designed the manuscript. Yoon-Jung Hwang, Hong-Yul Kim, Chang Kil Kim and Ki-Byung Lim verified every steps of experiment. Lim Ki-Byung supervised and encouraged throughout the work. All authors discussed the results and contributed to write a final manuscript.

References

1. Anamthawat-Jónsson K (2004) Preparation of chromosomes from plant leaf meristems for karyotype analysis and *in situ* hybridization. *Method cell sci* 25:91–95. <https://doi.org/10.1007/s11022-004-5620-y>
2. Barrios K, Ruter JM (2019) Developing Triploid Hardy *Hibiscus* (*Hibiscus moscheutos*). *HortScience* 52:676–681
3. Bates DM (1965) Notes on the cultivated Malvaceae: *Hibiscus*
4. Bennett MD, Leitch IJ (2005) Plant genome size research: a field in focus. *Ann Bot* 95:1–6. <https://doi.org/10.1093/aob/mci001>
5. Chen H, Xue L, Li T, Contreras RN (2019) Quantile Regression Facilitates Simultaneous Selection of Negatively Correlated Floral Traits among BC1F1 Progeny of Male-fertile Hybrid *Hibiscus* Cultivars Lohengrin and Resi (*H. syriacus* × *H. paramutabilis*). *J Am Soc Hortic Sci* 144:70–76. <https://doi.org/10.21273/jashs04569-18>
6. Chung YS, Jun TH, Lee YG, Jung JA, Won SY, Hwang YJ, Silva RR, Choi SC, Kim CJ (2018) A genetic linkage map of wild Chrysanthemum species indigenous to Korea and its challenges. *Int J Agric Biol* 20:2708–2716. DOI:10.17957/IJAB/15.0818
7. Fryxell PJ (1988) Malvaceae of México. *Monogr Syst Bot*. <https://doi.org/10.2307/25027717>
8. Galbraith D (2009) Simultaneous flow cytometric quantification of plant nuclear DNA contents over the full range of described angiosperm 2C values. *Cytometry A* 75:692–698. <https://doi.org/10.1002/cyto.a.20760>
9. Gan Y, Liu F, Chen D, Wu Q, Qin Q, Wang C, Li S, Zhang X, Wang Y, Wang KJ (2013) Chromosomal locations of 5S and 45S rDNA in *Gossypium* genus and its phylogenetic implications revealed by FISH. *PloS one* 8:e68207. <https://doi.org/10.1371/journal.pone.0068207>
10. Greilhuber J, Doležal J, Lysak MA, Bennett MD (2005) The origin, evolution and proposed stabilization of the terms ‘genome size’ and ‘C-value’ to describe nuclear DNA contents. *Ann Bot* 95:255–260. doi:10.1093/aob/mci019
11. Hasterok RE, Wolny M, Hosiawa M, Kowalczyk S, KulakKsiazczyk T, Ksiazczyk WK, Heneen J, Maluszynska (2006) Comparative analysis of rDNA distribution in chromosomes of various species of Brassicaceae. *Ann Bot* 97: 205–216. <https://doi.org/10.1093/aob/mcj031>

12. Hendrix B, Stewart, J. M., 2005. Estimation of the nuclear DNA content of *Gossypium* species. Ann. Bot. 95, 789-797. <https://doi.org/10.1093/aob/mci078>
13. Hwang YJ, Kim HH, Kim JB, Lim KB (2011) Karyotype analysis of *Lilium tigrinum* by FISH. Hortic Environ Biotechnol 52:292–297. <https://doi.org/10.1007/s13580-011-0225-2>
14. Islam MM, Yesmin R, Jung MJ, Kim HY, Kim CK, Lim KB (2020) Investigation of the morphological and cytogenetic variations of an intraspecific Asiatic lily hybrid using 5S and 18S rDNA probes. Hortic Environ Biotechnol 1–8. <https://doi.org/10.1007/s13580-019-00216-7>
15. Jo YK, Mazharu IM, Kim CK, Kim HY, Lim KB (2019) Morphological Characteristics and FISH Analysis of *Hibiscus* F1 Hybrids and Parental Lines. Hortic Sci Technol 37:630–639. <https://doi.org/10.17660/actahortic.2019.1262.28>
16. Kim YM, Kim S, Koo N, Shin AY, Yeom SI, Seo E, Park SJ, Kang WH, Kim MS, Park J, Jang I (2017) Genome analysis of *Hibiscus syriacus* provides insights of polyploidization and indeterminate flowering in woody plants. DNA Res 24:71–80. <https://doi.org/10.1093/dnares/dsw049>
17. Knight CA, Molinari NA, Petrov DA (2005) The large genome constraint hypothesis: evolution, ecology and phenotype. Ann Bot 95:177–190. <https://doi.org/10.1093/aob/mci011>
18. Lattier JD, Chen H, Contreras RN (2019) Variation in genome size, ploidy, stomata, and rDNA signals in *Althea*. J Am Soc Hortic Sci 144:130–140. <https://doi.org/10.21273/jashs04618-18>
19. Lee J (2013) Distribution and status of *Hibiscus syriacus* of the world, Proceedings of the 5th symposium of *Hibiscus syriacus* research society. Seoul, Korea 47–50
20. Levan AJ (1964) Nomenclature for centromeric position on chromosomes. 52: 201–220 <https://doi.org/10.1111/j.1601-5223.1964.tb01953.x>
21. Li YP, Zhang X, Wu WT, Miao SX, Chang J (2015) Chromosome and karyotype analysis of *Hibiscus mutabilis* f. *mutabilis*. Hereditas 8:300–304. <https://doi.org/10.1080/21553769.2015.1041166>
22. Li Z, Ruter JM (2017) Development and Evaluation of diploid and polyploid *Hibiscus moscheutos*. HortScience 52:676–681. <https://doi.org/10.21273/hortsci11630-16>
23. Liang G, Chen HJ (2015) Scaling chromosomes for an evolutionary karyotype: a chromosomal tradeoff between size and number across woody species. PloS one 10:e0144669. <https://doi.org/10.1371/journal.pone.0144669>
24. Lim KB, Wennekes J, Jong JH, Jacobsen E, Van Tuyl JM (2001) Karyotype analysis of *Lilium longiflorum* and *Lilium rubellum* by chromosome banding and fluorescence *in situ* hybridisation. Genome 44:911–918. <https://doi.org/10.1139/gen-44-5-911>
25. Mohammad DD, Mazharul IM, Ann TC, Kim HY, Lim KB (2020) Phenotypic Characteristics and Karyotype Analysis of *Hibiscus sabdariffa* var. *sabdariffa* by Fluorescence *in situ* Hybridization (FISH). Hortic Sci Technol 38:695–704. <https://doi.org/10.7235/HORT.20200063>
26. Prado E, Faivre-Rampant P, Schneider C, Darmency MJ (1996) Detection of a variable number of ribosomal DNA loci by fluorescent *in situ* hybridization in *Populus* species. Genome 39:1020–1026. <https://doi.org/10.1139/g96-127>

27. Sakhanokho HF, Islam-Faridi N, Babiker EM, Nelson CD, Stringer SJ, Adamczyk J Jr (2020) Determination of nuclear DNA content, ploidy, and FISH location of ribosomal DNA in *Hibiscus hamabo*. *Sci Hortic* 264:109167. <https://doi.org/10.1016/j.scienta.2019.109167>
28. Sakhanokho HF, Kelley RY (2009) Influence of salicylic acid on in vitro propagation and salt tolerance in *Hibiscus acetosella* and *Hibiscus moscheutos* (cv 'Luna Red'). *Afr J Biotechnol* 8
29. Silva GS, Souza MM, de Melo CA, Urdampilleta JD, Forni-Martins ER (2018) Identification and characterization of karyotype in Passiflora hybrids using FISH and GISH. *BMC Genet* 19:26. <https://doi.org/10.1186/s12863-018-0612-0>
30. Skovsted AJ (1941) Chromosome numbers in the Malvaceae. II. *Compt. Rend. Trav. Lab. Carlsberg Ser Physiol* 23:195–242. <https://doi.org/10.1007/bf02982344>
31. Song J (2001) Studies on the Chromosome Number, Ploidy Relationship and Relative Relationship of Several Plants in *Hibiscus*. Shantou University
32. Speicher MR, Carter NP (2005) The new cytogenetics: blurring the boundaries with molecular biology. *Nat Rev Genet* 6:782–792. <https://doi.org/10.1038/nrg1692>
33. Van Huylenbroeck J, De Riek J, De Loose MJ (2000) Genetic relationships among *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis* revealed by AFLP, morphology and ploidy analysis. *Genet Resour Crop Evol* 47:335–334. <https://doi.org/10.1007/s10681-006-9328-8>
34. Van Laere K, Khrustaleva L, Van Huylenbroeck J, Van Bockstaele EJ (2010) Application of GISH to characterize woody ornamental hybrids with small genomes and chromosomes. *Plant Breed* 129:442–447. <https://doi.org/10.1111/j.1439-0523.2009.01692.x>
35. Wang Y, Wang X, Chen Q, Zhang L, Tang H, Luo Y, Liu ZJ (2015) Phylogenetic insight into subgenera *Idaeobatus* and *Malachobatus* (*Rubus*, *Rosaceae*) inferring from ISH analysis. *Mol Cytogenet* 8:11. <https://doi.org/10.1186/s13039-015-0114-y>
36. Zhu WY, Wang C (2008) A Review on Chromosomal Segment Substitution Lines in Crops Jiangsu J. of Agr. Sci 24:963–968

Figures

Figure 1

Photomicrograph of metaphase chromosome from root tip cell of *H. syriacus* 'Saejamyung' ($2n = 88$). Viewed at 1,000× Size bar 5 μm .

Figure 2

Chromosomes counts in (a) *H. syriacus* 'Saejamyung' $2n = 88$; (b) *H. sinosyriacus* 'Seobong' $2n = 80$; (c) *H. moscheutos* 'Luna Red' $2n = 38$; and (d) *H. paramutabilis* $2n = 82$. Viewed at $1,000\times$ Size bar $5\ \mu\text{m}$.

Figure 3

FISH results of (a) *H. syriacus* 'Saejamyung' $2n = 88$; (b) *H. sinosyriacus* 'Seobong' $2n = 80$; (c) *H. moscheutos* 'Luna Red' $2n = 38$; and (d) *H. paramutabilis* $2n = 82$, 5S rDNA and 18S rDNA loci are indicated by green and red fluorescence respectively. Viewed at 1,000 $\times$ Size bar 5 μm .

Figure 4

FISH karyotype detail of 5S rDNA and 18S rDNA loci on chromosomes of (a) *H. syriacus* 'Saejamyung'; (b) *H. sinosyracus* 'Seobong'; (c) *H. moscheutos* 'Luna Red'; and, (d) *H. paramutabilis*, where green fluorescence indicate 5S rDNA and red fluorescence indicates 18S rDNA.

Figure 5

Histogram of nuclear DNA content of (a) *H. syriacus* 'Saejamyung', (b) *H. sinosyriacus* 'Seobong', (c) *H. moscheutos* 'Luna Red' and, (d) *H. paramutabilis*.