Chromosome and ploidy analysis of winter hardy *Hibiscus* species by FISH and flow cytometry

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**Abstract** Determination of nuclear DNA content, genome size, and ploidy level and, information on cytogenetic characteristics are all prerequisite of modern plant breeding. However, identification of individual chromosomes of *Hibiscus* species is extremely difficult due to high number, small size and similar shape of mitotic chromosomes. The goal of the study was to ascertain the chromosome number, karyomorphology, distribution of 5S and 18S rDNA signals, 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 signals were detected by fluorescence *in situ* hybridization (FISH). According to the FISH results, there are two 5S rDNA signals (green) in *H. syriacus*, *H. sinosyriacus*, and *H. moscheutos*, and four 5S rDNA signals 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, respectively. The 2C - DNA content of *H. syriacus*, *H. sinosyriacus*, and *H. paramutabilis* are very similar, the amount was 4.06, 4.11, and 4.18 pg, respectively whereas, *H. moscheutos* has nearly half and that amount was 2.06 pg. These findings will contribute to the detailed cytogenetic assessment of *Hibiscus* and thus benefit plant breeding in this genus.

Keywords *Hibiscus* · Cytogenetics · Fluorescence *in situ* hybridization · 5S rDNA · 18S rDNA

**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). *Hibiscus 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).

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; Liang and Chen 2015). 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). Karyotype analysis of plants with a high number of chromosomes and 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). In most plant species, complete and accurate genetic information is shared with the DNA content changed by ploidy level, while the change in DNA consistency and ploidy degree are positively correlated. In case of *H. syriacus*, DNA content was 4.63 pg/2C for tetraploid cultivar ‘Blue Bird’ whereas, it was 7.05 pg/2C for the hexaploid cultivar ‘Aprodite’ (Lattier et al. 2019). 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 5 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**

*Hibiscus 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**

Stem of *H. syriacus* ‘Saejamyung’, *H. sinosyriacus* ‘Seobong’, *H. moscheutos* ‘Luna Red’ and *H. paramutabilis* were cut keeping minimum two buds and planted in rockwool media using rooting hormone (Rhizopone AA). Cutting stems were nurtured in a greenhouse at a temperature of 25 °C during the day and 20 °C at night, with a humidity of 60%. After two and half months regular watering and observation, media allowed to spread roots. In early in the morning, healthy roots tips were collected and treated with 2m 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. 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 trans-
ferred to a clean slide one by one and only milky part
of roots were kept and other debris were removed
with the help of needles. 20 µL acetic acid (60%) was
added per slide to spread the cells, and the slides were
air-dried. Finally, slides were checked and location of
well spread chromosomes were recorded in top parts
of slides.

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 etha-
nol 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 dena-
turation at 70 °C for 5 min and then placed on ice
for 15 min for fixation. Forty microliters of the mix-
ture 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. Strepta-
vidin CY3 (Invitrogen, Carlsbad, CA, USA) and
anti-digoxigenin fluorescein (Roche, Basel, Switzer-
land) 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 min. 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 coun-
terstained 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 ana-
lyzed 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 soft-
ware (Cytovision), and chromosomes were ordered
and numbers assigned based on short arm length
(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 1000× magnification.

Flow cytometry analysis

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 approx-
imately 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 = DNA content of standard × mean fluores-
cence value of sample / mean fluorescence value of
standard (Greilhuber et al. 2005). Raphanus sativus
cv Tetra Ilowiecka (2C = 2.00 pg).

Results

Chromosome counting

We performed photomicrographs of 45 well spread
cells from each species to confirm the chromosome
number. We clearly detected 88 chromosomes in H. syr-
iacus ‘Saejamyung’ while H. sinosyriacus ‘Seobong’
has 80 chromosomes (Figs. 1 and 2). Among the four
species, H. moscheutos ‘Luna Red’ had the least num-
ber of chromosomes 38 whereas, 82 chromosomes are
found in H. paramutabilis (Fig. 2).
Chromosomal localization of 5S rDNA and 18S rDNA sites

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; Table 1). Six 18S rDNA loci (red fluorescence) were found in *H. sinosyriacus* and *H. moscheutos*, and four and ten loci were noticed in *H. syriacus* and *H. paramutabilis* respectively (Fig. 3; Table 1). The distribution pattern of 5S rDNA loci for *H. syriacus*, *H. sinosyriacus*, *H. moscheutos* and *H. paramutabilis* were in the long arm of chromosome number #19 (Fig. 4a; Table 1), the long arm of chromosome #6 (Fig. 4b; Table 1), the short arm of chromosome number #10 (Fig. 4c; Table 1) and, the long arm of chromosome number (Fig. 4d; Table 1) respectively. In contrast, 18S rDNA loci were distributed in different locations of the chromosomes. Among four rDNA signals of *H. syriacus*, we found two signals in the long arm in chromosomes number #8 and two were found in the short arm in chromosome number #18 (Fig. 4a; Table 1). In *H. sinosyriacus*, we detected four 18S signals in long arms of chromosome number #6, #8 and two signals were in the short arm of chromosome number #11 (Fig. 4b; Table 1). In the case of *H. moscheutos*, all 18S rDNA signals were in the long arm of the chromosome numbers #1, #7, and #13 (Fig. 4c; Table 1). The highest number of 18 rDNA signals was found in *H. paramutabilis*; among ten signals, eight were in the short arm of chromosomes #5 and #13 and two are in the centromere position in the chromosome numbers #3 (Fig. 4d; Table 1).

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 ± 0.30 μm and from 3.18 ± 0.29 to 7.31 ± 0.25 μm, respectively. Among the four species, *H. moscheutos* 2n = 38 has the shortest range of chromosome length (2.91-5.23 μm) whereas, *H. paramutabilis* (Fig. 4d) has the longest-range chromosome length, from 4.75 ± 0.55 to 7.60 ± 0.47 μm. 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 sub metacentric pairs, no telocentric or sub telocentric chromosome were detected except *H. moscheutos*. 12 pairs of metacentric, 6 pairs of sub metacentric and one pair of sub telocentric chromosomes were detected except *H. moscheutos*. 12 pairs of metacentric, 6 pairs of sub metacentric and one pair of sub telocentric chromosomes were detected in *H. moscheutos*. In *H. syriacus* and *H. sinosyriacus*, the majority of chromosome pairs are metacentric, with numbers were 13 and 12, respectively, while 9 and 8 were sub metacentric accordingly. In contrast, the chromosomes of *H. paramutabilis* were composed of 14 sub metacentric and 7 of metacentric chromosomes (Fig. 4; Table 2).
Determination of nuclear DNA content

Based on the flow cytometry results in the study, 2 C-DNA contents of *H. syriacus*, *H. sinosyriacus*, and *H. paramutabilis* are nearly identical, being 4.06, 4.11, and 4.18 pg, respectively whereas, *H. moscheutos* has the amount at 2.06 pg. (Table 3). We found the same result in the 2 C 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).

**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 determined. The range in the basic chromosome number is wide 7/8/9/10/11/12/13/14/15/16/17/18/20/21/23/24/39/40/44. 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) (Skovsted 1941; Jo et al. 2019) however, not for *H. syriacus*. We noticed that there are over 150 cultivars of *H. syriacus*, with chromosomal numbers and ploidy levels that are highly varied (unpublished data). There is a lot of variation in Old World lupins (*Lupinus*), not only for chromosomal counts (2n = 32 – 52), but also for the basic chromosome number (x = 5 – 9, 13) (Susek et al. 2019). In the present study we report the chromosome count result of ‘Saejamyung’ for the first time. Pedigree of that cultivar is not determined. Its ploidy may term as hypertetraploid. It may happen for unreduced gamete or failure of chromosome pairing during meiosis (Karlov et al. 1999; Lee et al. 2020). *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 in each of *H. syriacus* ‘Saejamuyn’ *H. sinosyriacus* ‘Seobong’ and *H. moscheutos* ‘Luna Red’, whereas 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. sabdariffa* var. ‘sabdariffa’ (Mohammad et al. 2020) resulted

Fig. 3 FISH results of a *H. syriacus* ‘Saejamuyn’ $2n = 88$; 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 1000x. Size bar 5 μm. (Color figure online)
in the identification of four 5S rDNA loci. The study of only two 5S rDNA loci in tetraploid *H. syriacus* and *H. sinosyriacus* could provide evidence being diploid however, recent genome analysis confirmed their polyploidy status after multiple occurrence of whole-genome duplication followed by

Fig. 4 FISH karyotype detail of 5S rDNA and 18S rDNA loci on chromosomes of *a* *H. syriacus* ‘Saejamyung’; *b* *H. sino syriacus* ‘Seobong’; *c* *H. moscheutos* ‘Luna Red’; and *d* *H. para- mutabilis*, where green fluorescence indicate 5S rDNA and red fluorescence indicates 18S rDNA. (Color figure online)
polyploidization 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. The reduced copy number and interstitial of 5S rDNA often observed in flowering plants (Nguyen et al. 2021). 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 chromosomes length of Hibiscus spp. is 2 to 8 μm. The chromosome length of H. mutabilis f. mutabilis were recorded 1.24 to 10.89 μm which were slightly higher than our studies (Li et al. 2015). Moreover, small chromosome studies such as chromosomes length of the investigated Chrysanthemum species were 9.70 to 12.24 μm, and 9.02 to 13.37 μm for Chrysanthemum boreale, C. makinoi respectively (Hoang et al. 2020). The karyotype studies of Populus species (woody plants having small chromosomes) found that relative chromosome length of P. trichocarpa is 4.30 to 11.02 and P. euphratica is 4.10 to 10.28 (Xin et al. 2020). Depending upon the position of the centromere, the chromosomes

| Species                  | No. of 5S rDNA | Location of 5S rDNA | No. of 18S rDNA | Location of 18S rDNA |
|--------------------------|----------------|---------------------|----------------|---------------------|
| Hibiscus syriacus        | 2              | Ch# 19              | 4              | Ch# 1, 7, 13        |
| ‘Saejamyung’             |                |                     |                |                     |
| H. sinosyriacus          | 2              | Ch# 6               | 6              | Ch# 6, 8, 11        |
| ‘Seobong’                |                |                     |                |                     |
| H. moscheutos ‘Luna Red’| 2              | Ch# 10              | 6              | Ch# 3, 5, 1, 13     |
| H. paramutabilis         | 4              | Ch# 4               | 10             |                     |
of H. mutabilis can be categorized as metacentric, sub metacentric, whereas, among four species of H. moscheutos has metacentric (m), sub metacentric (sm) and sub telocentric (st) type of chromosome and three other species (H. syriacus, H. sinosyriacus and H. paramutabilis) were detected with metacentric and sub metacentric type of chromosomes. 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 karyotype formula of H. syriacus ‘Saejamuyung’ (2n = 52 m + 36 sm = 88), H. paramutabilis (2n = 28 m + 54sm = 82), H. sinosyriacus ‘Seobong’ (2n = 48 m + 32sm = 80), and H. moscheutos ‘Luna Red’ (2n = 24 m + 12 sm + 2st = 38) in the present study is the first, to be reported. The reported karyotype formula of H. mutabilis f. mutabilis were 2n = 86 m + 6sm (Li et al. 2015).

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, 2 C DNA = 3pg). In present study, we determined the 2 C nuclear DNA content of H. syriacus ‘Saejamuyung’ and H. sinosyriacus ‘Seobong’ were found 4.06 pg and 4.11 pg respectively, which were nearly to the tetraploid cultivar of H. syriacus ‘Diana’ Lucy, Woodbridge and Blue Bird (2 C-DNA = 4.58, 4.62, 4.48 and 4.63 pg, respectively) (Lattier et al., 2019; Van Huylenbroeck et al. 2000). H. moscheutos ‘Luna Red’ have nuclear DNA contents of

| Species                  | Putative DNA content | Ploidy level |
|--------------------------|----------------------|--------------|
|                         | 2 C Mbp   | 2 C (pg)    |
| H. syriacus ‘Saejamuyung’| 4065.88  | 4.06        |
| H. sinosyriacus ‘Seobong’| 4077.04  | 4.11        |
| H. moscheutos ‘Luna Red’ | 2014.88  | 2.06        |
| H. paramutabilis         | 4096.41  | 4.18        |

Table 2 The longest and shortest based on centromere positions in four Hibiscus species

| Species                  | Longest chromosome Chr. no. | Length (µm)         | Shortest chromosome Chr. no. | Length (µm)         | No. of chromosome based on Centromere position Meta-centric | Submetacentric + Subtelocentric |
|--------------------------|------------------------------|---------------------|-----------------------------|---------------------|-----------------------------------------------------------|--------------------------------|
| H. syriacus ‘Saejamuyung’| 1                            | 7.06 ± 0.30²        | 21                          | 2.66 ± 0.02         | 13                                                        | 9                              |
| H. sinosyriacus ‘Seobong’| 1                            | 7.31 ± 0.25         | 19                          | 3.18 ± 0.29         | 12                                                        | 8                              |
| H. moscheutos ‘Luna Red’ | 1                            | 5.23 ± 0.13         | 19                          | 2.91 ± 0.12         | 12                                                        | 6+1                           |
| H. paramutabilis         | 1                            | 7.60 ± 0.47         | 20                          | 4.75 ± 0.55         | 7                                                         | 14                             |

²Mean ± SE = 5

Table 3 2 C (pg) nuclear DNA content and genome size (Mbp) of four Hibiscus species were determined by flow cytometry

of H. mutabilis f. mutabilis can be categorized as metacentric, sub metacentric, whereas, among four species of H. moscheutos has metacentric (m), sub metacentric (sm) and sub telocentric (st) type of chromosome and three other species (H. syriacus, H. sinosyriacus and H. paramutabilis) were detected with metacentric and sub metacentric type of chromosomes. 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 karyotype formula of H. syriacus ‘Saejamuyung’ (2n = 52 m + 36 sm = 88), H. paramutabilis (2n = 28 m + 54sm = 82), H. sinosyriacus ‘Seobong’ (2n = 48 m + 32sm = 80), and H. moscheutos ‘Luna Red’ (2n = 24 m +12sm + 2st = 38) in the present study is the first, to be reported. The reported karyotype formula of H. mutabilis f. mutabilis were 2n = 86 m + 6sm (Li et al. 2015).

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2.06 pg which was almost half than that of tetraploid *Hibiscus syriacus* cultivars. We estimated the genome size of *H. paramutabilis* (2C = 4.18 pg) that’s value was consistent with the previous genome sizes values of tetraploid *H. sabdariffa* and, tetraploid species of *Gossypium* (Mohammad et al. 2020; Hendrix and Stewart 2005) (Fig. 5).

**Conclusions**

Chromosome number, karyotype, nuclear DNA content, and rDNA distribution (5S and 18S rDNA) of *H. syriacus* ‘Saejamuyung’, *H. sinosyriacus* ‘Seobong’, *H. moscheutos* ‘Luna Red’ and *H. paramutabilis* were determined in this study. The detailed karyotyping of *Hibiscus* will assist further research in this genus and lay the groundwork for an efficient hybridization scheme aimed at improving targeted traits.

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**Author contributions**  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.
Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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