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Determination of nuclear DNA content, ploidy, and FISH location of ribosomal DNA in *Hibiscus hamabo*

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ABSTRACT

Hibiscus hamabo Sieb.et Zucc, a deciduous shrub or tree, is an important semi-mangrove plant with golden yellow flowers. The plant is also cultivated for its fiber and grown as an ornamental. It is halophytic and considered one of the best afforestation trees used in reclamation programs in urban and coastal areas high in salt content. However, very little work has been done on its genetics; therefore, this study was undertaken to determine the genome size, the chromosome numbers, and rDNA (18S rDNA and 5S rDNA) loci in *H. hamabo* using flow cytometry, protoplast spread technique, and oligonucleotide fluorescence in situ hybridization (FISH), respectively. *H. hamabo* was found to have a 2C-value of 4.06 ± 0.08 pg (3973.08 ± 78.90 Mbp) and a chromosome number of $2n = 92$. We observed four 18S loci and one 5S rDNA locus on five different pairs of homologous chromosomes. The observed FISH signal intensities for the 18S rDNA varied from chromosome to chromosome, indicating that each locus had different rDNA repeat copy numbers. The pericentromeric regions of each chromosome observed were highly heterochromatic. The findings of this study will benefit future cytogenetics and genomic investigations as well as lay the groundwork for more efficient breeding programs aimed at developing improved *H. hamabo* hybrids.

1. Introduction

Hibiscus hamabo Siebold & Zuccarini, belonging to the Malvaceae family, is a deciduous shrub or tree that can reach 1 − 5 m in height. It grows in coastal areas in the warm temperate regions of the northwestern Pacific and is a hydrochorous species known to possess long-floating buoyant seeds (Nakanishi, 1985; Li et al., 2012; Yuan et al., 2019). It is a semi-mangrove plant (Nakanishi, 1979) native to Asia where its main distribution is in coastal sands of China, Japan, and Korea (Li et al., 2012). It is also cultivated in India and the Pacific islands (Wu et al., 1994). *H. hamabo* is halophytic and strives in habitats with NaCl concentrations ranging from 1.1 to 1.5 % (Yang et al., 2008), so it is considered one of the best afforestation plants used in reclamation areas, a shelterbelt tree species along the coasts plants used in reclamation areas, a shelterbelt tree species along the coasts, and a valuable plant in urban landscaping (Yang et al., 2008; Wang et al., 2019). *H. hamabo* is a good plant material to study the response mechanisms of salt-stressed woody plants (Li et al., 2012; Wang et al., 2019). The plant is also cultivated for its fiber. Because of its tolerance to saline soils, drought and, to some extent, cold, *H. hamabo* has been used as a rootstock for the grafting of other *Hibiscus* species such as *H. syriacus*.

In addition to its tolerance to the various abiotic stresses listed above, *H. hamabo* is attractive as an ornamental plant for its pale green to pale lemon-yellow flowers (Fig. 1a) in the summer and good fall color, but unfortunately its single flowers are sparse and short-lived. Improving the ornamental attributes of *H. hamabo* will require devising a breeding program aimed at hybridizing it with more ornamentally attractive *Hibiscus* species. An efficient plant breeding system requires knowing beforehand the chromosome numbers/ploidy of the species at hand. The genus *Hibiscus* consists of about 300 species, grouped in 10 sections, including a great variety of plants with complex interspecific relationships (Akpan, 2000). The genus is known for its varied chromosome number and complex polyploidy (Li et al., 2015). A recent

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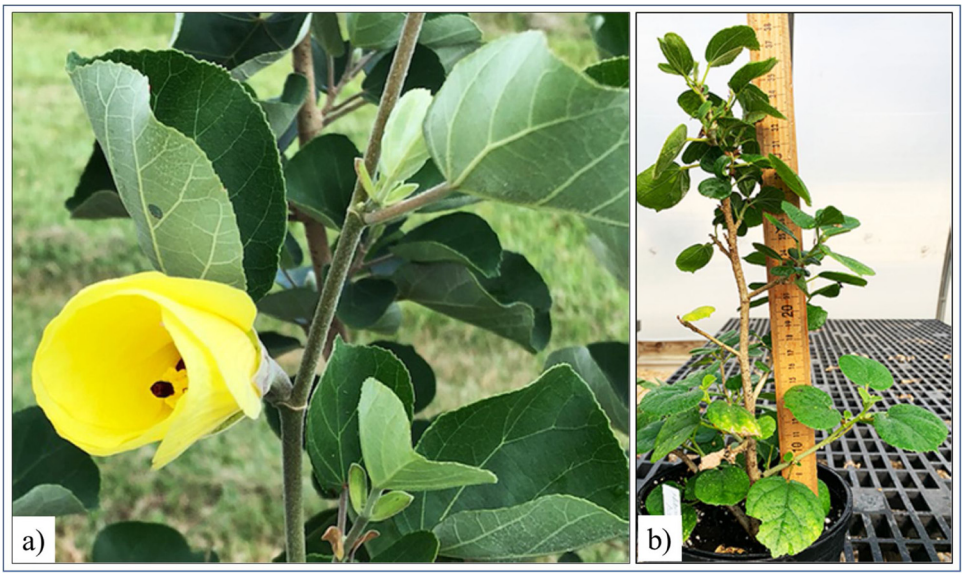


Fig. 1. *Hibiscus hamabo Hibiscus hamabo* Siebold & Zuccarini plants. a) *H. hamabo* flower; b) Representative of *H. hamabo* plants grown in the greenhouse and from which root tips were isolated for protoplast chromosome spreads and fluorescence in situ hybridization (FISH) rDNA analysis.

listing of *Hibiscus* chromosome numbers on the Chromosome Counts Database (CCDB) website shows 122 entries ranging from $2n = 22$ for *H. citrinus* (Fryxell, 1988) to $2n = 180$ for *H. maculatus* Lam. (Chromosome Counts Database (CCDB, 2019). *Hibiscus* genome complexity is illustrated by the existence of two or more cytotypes within some species such as *H. rosa-sinensis* L. for which chromosome numbers of $2n = 84$, 105, 138, 144, and 147 have been reported (Li et al., 2015). The genus includes diploids such as *H. phoeniceus* ($2n = 2x = 22$) and *H. pedunculatus* ($2n = 2x = 30$), and polyploids, *H. syriacus* ($2n = 4x = 80$) and *H. rosa-sinensis* ($2n = 16x = 144$) (Kim et al., 2017). To our knowledge, no report on chromosome number for *H. hamabo* is currently available. Genome size is a distinguishing character of living organisms, and estimates of genome size have been useful in systematic and evolutionary studies (Knight et al., 2005; Bancheva and Greilhber, 2006; Naganowska et al., 2006; Bainard et al., 2013; Nowicka et al., 2016). Determination of genome size can be helpful in elucidating the relationship between *H. hamabo* and other *Hibiscus* species, but no data on genome size of this species is available either. The localization of ribosomal DNA (rDNA) using fluorescence in situ hybridization (FISH) has been widely used to study genetic relationships among various plant species (Abirached-Darmency et al., 2005; Cerbah et al., 1998; Hanson et al., 1996; Hasterok et al., 2001; Robledo et al., 2009; Zoldos et al., 1999). However, except for *H. syriacus* (Lattier et al., 2019), no report of rDNA distribution on chromosomes exists for the genus *Hibiscus*. The objectives of this study were to determine the genome size, the chromosome number, and 18S rDNA and 5S rDNA loci number and their chromosomal locations in *H. hamabo*.

2. Materials and methods

2.1. Plant materials

The plants used for the flow cytometry analysis were from two sources. The first group of plants came from seed-derived plants grown and maintained at the USDA-ARS research station in Poplarville, Mississippi. A second batch of plants was acquired from Woodlanders Nursery, located in Aiken, South Carolina. Five plants were randomly selected from each source for a total of 10 plants for flow cytometry analysis.

2.2. Flow cytometry analysis

Propidium iodide (PI)-stained nuclei isolated from young leaves were analyzed using a BD Accuri C6 flow cytometer and a BD Accuri C6 software version 1.0.264.21 (BD BioSciences, Ann Arbor, MI). A Sysmex Partec GmbH Cystain PI Absolute P (05–5022; Sysmex Partec Am Flugplatz, Germany) staining kit was used for nuclei extraction and PI DNA staining. The kit contained a pre-mixed extraction buffer and the components of the staining solution. The staining solution was prepared on the day of the flow cytometry procedure. The staining recipe was 20 mL of staining buffer per sample mixed with 120 µL of PI solution, 60 µL of RNAse solution (05–5022; Partec Am Flugplatz, Germany) and 5 % Polyvinylpyrrolidone-40,000 [(PVP-40), Sigma-Aldrich, St. Louis, Missouri, USA)]. Fresh leaves of *H. hamabo* and the internal standard *Solanum lycopersicum* L. cv. 'Stupicke' (2C 1.96 pg) were cut in equal size (~0.5 cm^2) and placed in a Petri dish before addition of 0.5 mL nuclei extraction buffer. The *H. hamabo* samples and the standard samples were co-chopped together for 30–60 seconds using 102 mm razor blades (Electron Microscopy Sciences, Hatfield, PA, USA), and the mixture was filtered through 50 µm nylon-mesh filters (Sysmex America, Inc., Lincolnshire, IL, USA) before addition of 2 mL of staining solution. The mixture was covered with aluminium foil to protect against light and incubated in a refrigerator at 4 °C for 15 min before flow cytometry analysis. In total, 10 randomly selected plants were used, and three replicates per plant were run. A minimum of 5000 events (nuclei) were gated for each run. The flow cytometer produced histograms for each of the sample and standard runs and included fluorescence intensity means, coefficients of variation (CV), and total cell counts. Only 2C DNA values with CV lower than 5 % were accepted and included in the data. Fluorescence ratios, calculated relative to the internal reference *Solanum lycopersicum* L. cv. 'Stupicke', were converted to DNA content values and expressed in picograms following the formula: Sample 2C -value (picograms) = reference 2C-value × [(Sample 2C mean peak)/(Reference 2C mean peak)]. The formula 1 pg = 978 Mbp was used to convert picograms into megabase pairs (Dolezel et al., 2003).

2.3. Chromosome spread preparation

Chromosome spreads were prepared from root tips of *H. hamabo* plants grown in potting soil in the greenhouse. Actively growing root

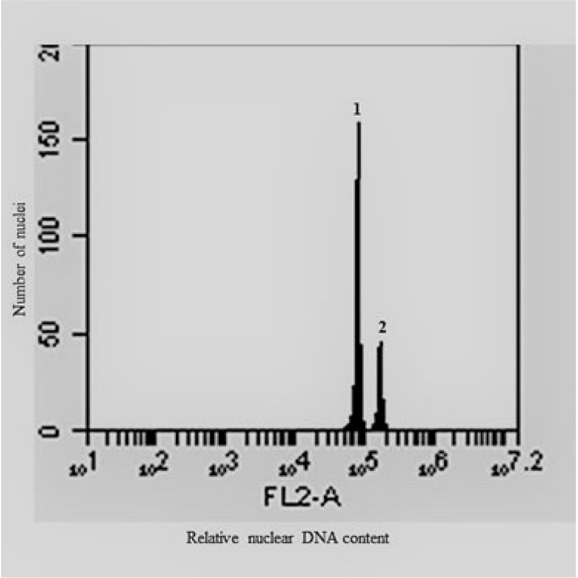


Fig. 2. Representative histogram of nuclear DNA content estimation of *Hibiscus hamabo* Siebold & Zuccarini using flow cytometry. Simultaneous analysis of nuclei isolated *Solanum lycopersicum* L. cv. 'Stupicke' (peak 1, 2C-DNA 1.96 pg) and *Hibiscus hamabo* ($2n = 92$, peak 2, 2C-DNA 4.06 $\pm$ 0.08 pg).

tips about 1.0 cm in length were harvested and immediately pre-treated with 0.04 % (w/v) 8-hydroxyquilonine for 3.75 h in the dark at room temperature, rinsed with ddH_2O and then fixed 4:1 (95 % ethanol: glacial acetic acid) and stored at room temperature until ready for enzyme digestion for chromosome spread. It is recommended to process the root samples sooner than later as the fresher the sample the better the chromosome spreads. Fixed root tips were rinsed with water (to remove the fixative), mildly hydrolyzed (0.2 N HCl at 60 °C for 5 min and then 10 min at RT), and rinsed with ddH_2O followed by 0.01 M cold (4 °C) citrate buffer before enzyme digestion. The enzyme mixture for this present work consisted of (2 % cellulase RS (w/v, Yakult Pharmaceutical Ind. Co., LTD, Japan), 1 % macerozyme R10 (w/v, Yakult Pharmaceutical Ind. Co., LTD, Japan), 2 % pectolyase Y23 (w/v, Kyowa Chemical Products, Co., LTD, Japan), 30 % cellulase (v/v, C2730, Sigma, USA), 30 % pectinase (v/v, P2611, Sigma, USA)] and 40 % 0.01 M Citrate buffer (pH 4.5). The enzyme digestion time varied (10–20 min) based on the thickness of root-tips. The chromosome spreads were prepared as described per Jewell and Islam-Faridi (1994).

2.4. Fluorescence in situ hybridization (FISH) with oligonucleotide 18S rDNA and 5S probes

We have used four different types each of 18S rDNA and 5S rDNA oligonucleotides sequences (Table 2) for detecting the respective rDNA sites on *H. hamabo* chromosomes. These oligonucleotides sequences were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). Oligonucleotide FISH is very fast and reliable with little to no background noise (Lan et al., 2018; Waminal et al., 2018). Freshly prepared (about a week-old preparation), slides with well-separated chromosome spreads were treated with RNase-A (30 µg/ml) in 2X SSC in a coupling jar in a water bath at 37 °C for 45 min followed by two washes in 2x SSC at 37 °C, 5 min each. The slides were dehydrated through an ethanol series, 5 min each (70 %, 85 %, 95 % and 100 %) at room temperature and air-dried over-night. The hybridization mixture (25 µl/slide) consisted of 50 % deionized formamide (12.5 µl of di-formamide), 10 % dextran sulfate (5 µl of 50 % ds), 5.0 µg of *E. coli* DNA (used as blocking DNA), 25 ng of each 18S rDNA (total 100 ng/slide) and 25 ng of 5S rDNA (total 100 ng/slide) oligo probes in 2X SSC (2.5 µl of 20X SSC), and the final volume was adjusted with TE buffer. Afterwards, 25 µl of hybridization mixture was placed on each slide, covered with a glass-cover slip (22 × 30 mm), and sealed with rubber cement (Elmer's Products, Inc., OH, USA). The slides were then placed in a humidity chamber, and the chromosomal DNA and probe DNAs were denatured together at 80 °C in a convection oven for 5 min. After denaturation, the slides were cooled down for about 2–3 min at RT and then placed at 37 °C for incubation for 2 h. After incubation, the rubber cement was carefully removed using fine forceps and the cover-glass washed off using a 2X SSC squeeze bottle. Slides were immediately washed in 2X SSC at RT for 5 min followed by two washes in 0.1X SSC for 5 min each at 40 °C and washed in 2X SSC at RT for 5 min followed by a quick rinse in di-H2O. The slides were dried with forced air using a benchtop vacuum pump (GE Commercial Motor, G608GX, Sold by Fisher Scientific INC., USA). Chromosomes were counter-stained by adding a small drop (10 µl) of Vectashield containing DAPI (Vector Laboratories, USA) to the preparation and adding a glass cover-slip (24 × 50 mm) to prevent photo-bleaching of the fluorochromes and over-flow of immersion oil when checking the FISH results.

2.5. Digital image capture and processing

FISH images were viewed under a 63X plan apo-chromatic oil-immersion objective and digital images were recorded using an epi-fluorescence microscope (AxioImager M2, Carl Zeiss Inc., Germany) with suitable filter sets (Chroma Technology, Bellows Falls, VT, USA) and captured with a Cool Cube 1 (MetaSystems Group Inc., Boston, MA, USA) high performance charge-coupled device (CCD) camera. Images were pre-processed with Ikaros and ISIS v5.1 (MetaSystems Inc.) and then further processed with Adobe Photoshop CS v8 (Adobe Systems Inc., Broadway, NY, USA).

3. Results

3.1. Genome estimation

The flow cytometer produced histograms for each of the sample and standard runs and included fluorescence intensity means, coefficients of variation (CV), and total cell counts. A representative histogram of the relative DNA content with two peaks corresponding to the G0/G1 nuclei of *H. hamabo* and *Solanum lycopersicu m* L. cv. 'Stupicke' is shown in Fig. 2. The 2C nuclear DNA content and 2C genome of *H. hamabo* were 4.06 $\pm$ 0.08 pg and 3973.08 $\pm$ 78.90 Mbp, respectively (Table 1).

4. Chromosome number (ploidy) determination

Well separated somatic chromosome spreads at prophase and metaphase without cover glass squashes were prepared as described in the protocol. We photomicrographed 22 *H. hamabo* chromosome spreads, of these 10 were from metaphase and 12 were from prophase stages. All but one showed a clear count with 92 well-separated chromosomes (Fig. 3: Fig. S1). To our knowledge, this is the first chromosome count report in *H. hamabo*. Morphologically, the *H. hamabo* chromosomes are small but spread uniquely and mostly in a unifocal plane free of cell walls, nuclear membranes, and cytoplasmic debris – all critical pre-requisites for successful FISH analysis and accurate chromosome counts.

Table 1
Mean 2C (pg) nuclear DNA content and genome size (Mbp) of *Hibiscus hamabo* Siebold & Zuccarini determined by flow cytometry.*.

Variable	Mean $\pm$ Std	Minimum	Maximum	Median
2C-DNA (pg)	4.06 $\pm$ 0.08	3.93	4.21	4.06
2C genome size (Mbp)	3973.08 $\pm$ 78.90	3847.88	4120.76	3968.47

* The mean is the average of 30 runs from 10 *H. hamabo* plants (3 runs/plant).

Table 2
List of oligonucleotide probes used in this experiment for rDNA analysis.*.

No.	Probe	Sequence	Length	Modification
1	18SrDNA_UniOP_1	CCGGAGAGGGAGCCTGAGAAACGGCTAC	28	5'AlexaFluor488
2	18SrDNA_UniOP_2	ATCCAAGGAAGGCAGCAGGCGCGCAA	26	5'AlexaFluor488
3	18SrDNA_UniOP_3	GGGCAAGTCTGGTGCCAGCAGCCGCGGT	28	5'AlexaFluor488
4	18SrDNA_UniOP_4	TCGAAGACGATYAGATACCGTCSTAGT	27	5'AlexaFluor488
5	5SrDNA_ang_1	GGATGCGATCATACCAGCACTAAAGCACCG	30	5'Cy3
6	5SrDNA_ang_2	CCCATCAGAACTCCGAAGTTAAGCGTGCT	29	5'Cy3
7	5SrDNA_ang_3	GCGAGAGTAGTACTAGGATGGGTG	24	5'Cy3
8	5SrDNA_ang_4	CCTGGGAAGTMCTCGTGTTGCAYYCC	26	5'Cy3

* For details see Waminal et al. (2018).

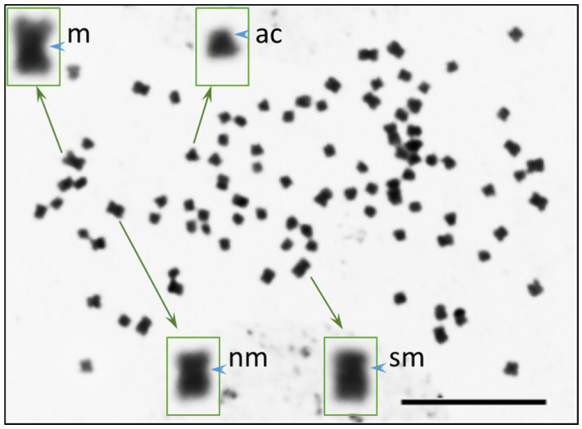


Fig. 3. A complete metaphase chromosome spread of *Hibiscus hamabo* using protoplast technique stained with Azure-B. The enlarged images of four chromosomes represent/example of metacentric (m), near metacentric (nm), submetacentric (sm) and acrocentric (ac) chromosomes. Scale bar = 10 μm.

The *H. hamabo* chromosomes are mostly metacentric and sub-metacentric and a few are acro-centric as revealed by sharp primary constrictions (Fig. 3). The peri-centromeric regions of nearly all prophase chromosomes are highly heterochromatic, and the distal regions of each chromosome arms are euchromatic containing DNA (Islam-Faridi et al., 2002; Kim et al., 2005) as illustrated by dark and light Azure blue staining, respectively (Fig. S1.a–c).

4.1. Chromosomal localization of 18S rDNA and 5S rDNA sites

We used oligonucleotide FISH probes (Waminal et al., 2018) to determine number and chromosomal locations of 18S and 5S rDNA on *H. hamabo* chromosome spreads and observed eight 18S and two 5S rDNA FISH signals on 10 chromosomes, i.e. four sites of 18S loci and one site of a 5S locus located on five different pairs of homologous chromosomes (Fig. 4). The same number of 18S and 5S rDNA FISH signals were also clearly observed in prophase chromosome spreads. The pericentromeric heterochromatic regions of nearly all prophase chromosomes appeared very bright DAPI images (Fig. S2) as they are A–T rich (Schweizer, 1980). The homologous pairs for the 18S sites are numbered as 1-1′, 2-2′, 3-3′ and 4-4′ based on their respective FISH signal intensities in descending order and categorized as two “major” (1-1′ and 2-2′ pairs), one “intermediate” (3-3′) and one “minor" (4-4′) loci (Fig. 4A.a). An enlarged image of each homologous pair for 18S rDNA (AlexaFlour-488 (green) with DAPI and only DAPI) and 5S rDNA FISH signals (Cy3 (spectrum-orange with DAPI and only DAPI) is presented in Fig. 4B where the centromere position of each pair can easily be identified in DAPI stained chromosomes (Fig. 4B 1a-1a', 2a-2a' and so on). By convention, the “p-arm” and “q-arm” stand for short and long arms of a chromosome, respectively. Henceforth, we will follow this convention throughout the paper. The first major 18S site is located interstitially on the q-arm of the 1-1′ pair, the 2nd major 18S site is located towards the end of the p-arm of the 2-2′ pair, the intermediate 18S site is located towards the end of the p-arm of the 3-3′ pair, and the minor 18S site is located interstitially on the p-arm of the 4-4′ pair. The homologues 1-1′ is a sub-metacentric chromosome, 2-2′ and 3-3′ are near metacentric chromosomes and the 4-4′ is a metacentric chromosome as revealed by the DAPI images (Fig. 4B) where the centromere positions clearly stand out. The 5S site is located proximally on the q-arm on a pair of near sub-metacentric chromosome (Figs. 4B.5-5' and 5a-5a'), and based on FISH signal intensity, this locus can be referred to as an intermediate site. A clear structural image of 5S rDNA chromosome from two different cells of each metaphase clearly showed that the 5S signals are on the q-arm (Fig. S3).

5. Discussion

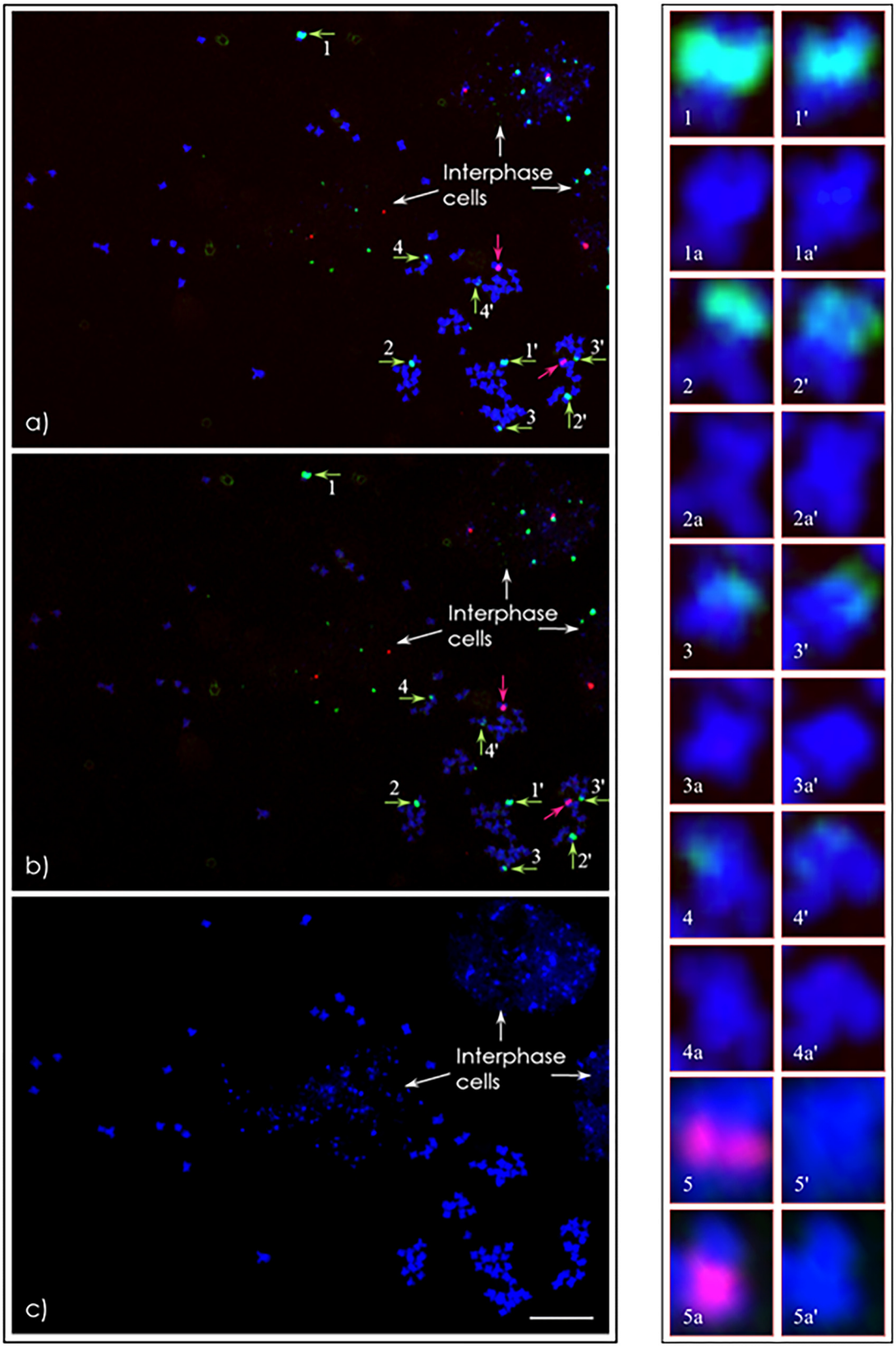
5.1. Nuclear DNA content analysis

We determined the 2C nuclear DNA content of *H. hamabo* to be 4.06 pg, which is about 31-fold larger than that of the angiosperm *Genlisea margaretae* Hutch with the smallest genome size (2C = 0.1296 pg) but nowhere close to that of *Paris japonica* (Franch. & Sav.) Franch., which is 1C = 152.23 pg, extending the range of genome sizes 2400-fold across angiosperms (Pellicer et al., 2010). Surprisingly, despite its large size, wide distribution across the world (tropical, sub-tropical, and, to a lesser extent, temperate regions), and economic importance, very little data exists on nuclear DNA content of the *Hibiscus* genus. Recently, Silva et al. (2017) reported 2C = 5.12 pg for the hexaploid (2n = 6*x* = 72) species *H. sabdariffa* L. while Lattier et al. (2019) found 2C-DNA values ranging from 4.55 to 8.98 pg for various cytotypes of *H. syriacus*, including tetraploid, pentaploid, hexaploid, and octoploid taxa. The 2C-DNA value of *H. hamabo* reported here is closer to those of the tetraploid cytotypes of *H. syriacus* (Lattier et al., 2019) and to those of several *Gossypium* species (Malvaceae) (Hendrix and Stewart, 2005). Phylogenetically, *H. hamabo*, *H. glaber* Matsum. (2*n* = 82), and *H. tiliaceus* L. (2*n* = 96) are reported to belong to a sub-clade in the section Azanza (Takayama et al., 2005). Tammoy et al. (2015) also found *H. hamabo* to form a sub-clade with *H. macrophyllus* Roxb. However, we are not aware of any reports on genome size data for *H. glaber*, *H. tiliaceus*, or *H. macrophyllus*, so comparison of the genome sizes of these species with that of *H. hamabo* is not presently possible.

5.2. Chromosome counts

Protoplast technique was found to be exceptionally superior to traditional squashing technique to prepare plant chromosome spreads as evidenced by the sharpness of the primary constriction (centromere position) of most chromosomes in Fig. 3 and Fig. S1d. In general, the pericentromeric regions of plant chromosomes are highly hetrochromatic, and this is true for *H. hamabo* as illustrated in all stages of prophases and pro-metaphase where the euchromatic regions stained lightly with Azure blue (Fig. S1a–c).

Twenty-two intact chromosome spreads (12 prophases and 10



(caption on next page)

Fig. 4. Fluorescence in situ hybridization (FISH) probed with 18S and 5S rDNA probes on *H. hamabo* somatic metaphase chromosomes; A.a) a superimposed image from blue (DAPI), green (FITC) and spectrum orange (Cy3) filters. The 18S rDNA FISH signals are marked as green arrows and the homologous pairs are numbered as 1/1′, 2/2′, 3/3′ and 4/4′, and the 5S rDNA signals are marked as red arrows; A.b) the same cell as "A.a", but the blue chromosomes background image was recorded or captured with minimal exposure time for better understanding of accurate location of green signals (and/or signal intensity and right location) on chromosomes because the blue chromosome background sometimes masked the signals, the scattered red and green signals are from interphase cells as marked with white arrows; A.c) The image was taken under DAPI filter (blue chromosome background) and the interphase cells were further enhanced using AdobePhotoshop (CS 8.0). Scale bar = 10 μm. B) An enlarged image of each of the four homologous pairs those contained the sites (loci) of 18S rDNA and 5S rDNA oligo probes, the DAPI image for each pair shown below of each as 1a and 1a' and so on (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

metaphases) of *H. hamabo* were selected to analyze the chromosome morphology and determine the chromosome count or ploidy. All but one of the 22 spreads each clearly showed 92 chromosomes, most of which are metacentric, near metacentric, or sub-metacentric with a few acrocentric or near acrocentric chromosomes as revealed by their sharp primary constrictions (centromeres) (Fig. 3; Fig. S1d). *Hibiscus* is a complex genome with about 300 species divided into 10–12 different sections (Akpan, 2000), and there are both auto and allopolyploid forms with various basic chromosome numbers. A chromosome number of $2n = 92$ has been reported in *H. mutabilis* L. (Song and Zhuan, 2001). Li et al. (2015) found that *H. mutabilis f. mutabilis* also has a chromosome number of $2n = 92$ and concluded that *H. mutabilis f. mutabilis* was a diploid ($2n = 2x = 92$), thus the basic chromosome number for that species would be $x = 46$. The basic chromosome number of *H. hamabo* analyzed in this study is not known. *H. hamabo* is generally propagated by seeds, and with $2n = 92$, *H. hamabo* could be either diploid ($x = 46$) or diploidized tetraploid ($x = 23$) species.

5.3. Chromosomal location of 18S and 5S rDNA

The 45S and 5S rDNA genes along with chromamycin A3 (CMA3) have been used to develop karyotypes in many plant species with moderate to large chromosomes such as pine (Doudrick et al., 1995), oak (Zoldos et al., 1999), peanut (Robledo et al., 2009), and tulip (Lan et al., 2018).The 45S and 5S rDNA genes along with CMA3 (chromamycin A3) have been used to discriminate a set of chromosome compliments to develop karyotype in many plant species, including pine (Doudrick et al., 1995), oak (Zoldos et al., 1999), peanut (Robledo et al., 2009), and tulip (Lan et al., 2018), with moderate to large size chromosomes. The *H. hamabo* chromosomes are fairly small, so it is, at present, impractical to develop a karyotype until chromosome-specific FISH DNA sequence markers are available; for examples, see sorghum (Kim et al., 2002) and maize (Kato et al., 2004). However, we have clearly structurally characterized five homologous chromosomes of *H. hamabo* using 18S and 5S rDNA oligonucleotide probes and primary constrictions (i.e., centromere) observed in DAPI image (Fig. 4.B). These are one sub-metacentric (1-1′ pair), two near metacentric (2-2′ and 3-3′ pairs) and one metacentric (4-4′ pair) chromosomes for 18S site and a sub-metacentric chromosome for 5S rDNA site. No two homologous pairs for the 18S rDNA site were observed to be similar in length, suggesting that *H. hamabo* may be a diploid species ($2n = 2x = 92$).

Oligonucleotide FISH is reliable and fast, and no difference was observed between oligonucleotide and plasmid probes FISH (Lan et al., 2018). The DNA:DNA hybridization time (incubation at 37 °C) can be reduced to one to two hours compared to over-night or two-day incubation. We have observed that the signal to background noise ratio was very low, i.e. the target specificity is accurate and sharp. The four 18S sites of *H. hamabo* are marked as two "major", one "intermediate" and one "minor" loci as reflected by their specific FISH signal intensities. Strong or heavy FISH signals were registered for the homologue pair 1-1′ and 2-2′, medium for 3-3′ and minor signal for 4-4′ pair ranked as 1-1′ > 2-2′ > 3-3′ > 4-4′ in respect to 18S rDNA copy number (Zoldos et al., 1999).

6. Conclusion

Genome size, chromosome, and rDNA distribution on *H. hamabo* chromosomes were determined for the first time. Our results help understand the basic genetics of *H. hamabo*, and will be useful for future genetic, genomic, and phylogenetic investigations of this species. Tolerance to abiotic stresses (salt, drought, moderate cold hardiness) of *H. hamabo* is an attractive and desired trait; however, improving floral longevity, number, and frequency will be necessary to increase its ornamental value. Our results, in particular the determination of the chromosome number, lays the groundwork for an efficient hybridization scheme aimed at improving these traits. Interspecific hybridization has been used extensively to combine traits from different *Hibiscus* species. To this end, knowledge of chromosome number will facilitate the introgression of adaptation traits from *H. hamabo* into existing *Hibiscus* cultivars.

Credit authorship contribution

All the authors contributed significantly to the paper. Specifically, HFS and NIF conceived and conducted experiments. EMB, CDN, SJS, and JJA helped write, review, and edit the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2019.109167.

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