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A Protocol for Mitotic Metaphase Chromosome Count Using Shoot Meristematic Tissues of Mulberry Tree Species

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Abstract

Studies on chromosomal status are a fundamental aspect of plant cytogenetics and breeding because changes in number, size, and shape of chromosomes determine plant physiology/performance. Despite its significance, the classical cytogenetic study is now frequently avoided because of its tedious job. In general, root meristems are used to study the mitotic chromosome number, even though the use of root tips was restricted because of sample availability, processing, and lack of standard protocols. Moreover, to date, a protocol using shoot tips to estimate chromosome number has not yet been achieved for tree species' germplasm with a large number of accessions, like mulberry (*Morus* spp.). Here, we provide a step-by-step, economically feasible protocol for the pretreatment, fixation, enzymatic treatment, staining, and squashing of meristematic shoot tips. The protocol is validated with worldwide collections of 200 core set accessions with a higher level of ploidy variation, namely diploid (2n = 2x = 28), triploid (2n = 3x = 42), tetraploid (2n = 4x = 56), hexaploid (2n = 6x = 84), and decosaploid (2n = 22x = 308) belonging to nine species of *Morus* spp. Furthermore, accession from each ploidy group was subjected to flow cytometry (FCM) analysis for confirmation. The present protocol will help to optimize metaphase plate preparation and estimation of chromosome number using meristematic shoot tips of tree species regardless of their sex, location, and/or resources.

Keywords: Chromosome number, Flow cytometry, *Morus* spp., Polyploidy, Shoot meristem

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Background

The plant genome is organized into chromosomes, preserving hereditary information and facilitating its replication, transcription, and transfer. One of the fundamental aspects of plant evolutionary biology is to understand the genome organization and its functional aspects that directly or indirectly act in concert with plant adaptability. Chromosomal studies imply a historical impression of natural consequences, for example, the patterns of chromosomal evolution influenced by factors like natural/artificial selection pressure or crop domestication (Tang et al., 2010). The evolution of chromosome size, structure, and number and the change in DNA composition suggest high plasticity of nuclear genomes at the chromosomal level (Guerra, 2008). In the recent past, integrated cytogenetics, chromosome-level sequencing, and comparative analysis were implemented to grasp the essential component of the evolutionary mechanisms of plant genomes. There are approximately 450,000 species of green plants, but only approximately 300 genome assemblies at the chromosome-scale, corresponding to 812 species (Arita et al., 2021; Kress et al., 2022). Additionally, polyploidy (or whole-genome duplication, WGD) is considered an evolutionary and ecological force in times of stress adaptation (Van de Peer et al., 2021). Hence, chromosomal information has significant importance in plant breeding, genetic, and biotechnological studies including chromosome-scale genome assembly, which can complement molecular phylogeny towards the understanding of complex evolutionary consequences.

Despite its significance, the estimation of chromosome numbers using the squash technique has been restricted (Goldblatt, 2007). For the majority of monocots, root tips are widely used to study the mitotic chromosome number, as the use of shoot tips is laborious, and squashing reduces the spread quality (Anamthawat-Jónsson, 2003). However, mitotic metaphase plate preparation using root tips or floral tissues present considerable disadvantages, specifically for tree plants: (a) living material with actively growing tissues, like meristems, are prerequisite (Guerra, 2008), and the collation of meristematic root tips from the mature tree plant is not easy (Sinha et al., 2016), (b) seed-derived root tips do not represent the same chromosome complements as their mother trees, because of introgressive hybridization (Anamthawat-Jónsson, 2003), (c) grafted plants do not comprise a true root system; hence, chromosomal study is difficult, and (d) the availability of floral tissues depends on the favorable season (Anamthawat-Jónsson, 2003). On the other hand, metaphase plate preparation using shoot meristematic tips present major advantages compared to root tips or floral tissues: (a) ease of collecting intact healthy explants from plants; (b) shoot tips tend to have less condensed chromosomes, and longer or extended chromosomes are desirable as the mapping resolution will be better (Anamthawat-Jónsson, 2003); further, for a highly heterozygous crop like mulberry, the chromosome size and number show huge variations (Datta et al., 1954), and shoot tips could be precious explants to estimate the chromosome number of higher ploidy plants; and (c) for recalcitrant seeds as well as endangered tree species, shoot tips of original mother plants may serve the purpose.

So far, published articles/protocols using shoot tips are very limited; cytogenetics cannot be applied in population studies unless samples are obtained from actual plants in the field, because chromosome number varies among the progeny (Anamthawat-Jónsson, 2003). Recently, Chang et al. (2018) suggested that the small size of chromosomes of mulberry also limits distinguishing euploid/aneuploid, and karyotypic analysis could help identifying different ploidy. It is, therefore, necessary to develop a standard, economically feasible base protocol for the screening and characterization of large-scale germplasm accessions. In addition, high-cost flow cytometry (FCM) analysis for large-scale accessions of germplasm may not be affordable for all researchers (Windham et al., 2020). This barrier is exacerbated by a lack of sufficient details on critical aspects of the protocol like tissue choice, maceration, and squashing (Windham et al., 2020).

Mulberry (*Morus* spp.) has been commercially exploited as the host of the monophagous pest silkworm (*Bombyx mori* L.). It belongs to the Moraceae family, which comprises 37 genera, with more than 1,100 species (Clement and Weiblen, 2009). The genus *Morus* has over 10 species with more than 1,000 cultivated varieties spanning Asia, Europe, Africa, and the United States (He et al., 2013). Efforts were made to classify *Morus* species; however, to date, taxonomic nomenclature remains doubtful (Zeng et al., 2015). Besides, genetics of inheritance are also complicated in *Morus* species due to the higher level of heterozygosity as well as WGD (Jain et al., 2022). Mulberry has a wide range of polyploidy; for example, *M. notabilis* was reported as a haploid, having a chromosome complement of 2n = x = 14 (He et al., 2013), while *M. alba*, *M. atropurpurea*, *M. bombycis*, *M. indica*, *M. latifolia,* and *M. rotundiloba* were considered diploids, having 2n = 2x = 28 (Datta, 1954). The majority

of triploids (2n = 3x = 42) and tetraploids (2n = 4x = 56) have been identified in *M. laevigata* (Das, 1961). Hexaploid species (2n = 6x = 84), such as thick leaf *M. serrata* (Basavaiah et al., 1989) and *M. tiliaefolia* (Seki, 1952), are also recognized; the ploidy can extend up to decosaploid (2n = 22x = 308), as in *M. nigra* (Basavaiah et al., 1990).

The generation of chromosomal/ploidy-related information of non-model tree plants like mulberry, where the occurrence of polyploidization is common, can be a logistic strategy to create a foundation for future molecular cytogenetics and next-generation sequencing–based work, toward potential crop development and conservation. In this context, the metaphase chromosome number of 200 germplasm accessions of different accessible *Morus* spp. was counted using shoot meristematic tissue. Accessions were obtained from the Central Sericultural Germplasm Resources Centre (CSGRC), Hosur, India. Hopefully, the present protocol will help to optimize metaphase plate preparation and chromosome number estimation using meristematic shoot tips of tree species regardless of sex, location, and/or resources.

Materials and reagents

Consumables

1. Eppendorf tubes, 1.5 mL (Tarsons, India)
2. Reagent bottles, amber 25 mL (Borosil, catalog number: 1519009)
3. Glass slides, 76 mm × 26 mm (Borosil, Product code 9100P02)
4. Coverslip, rectangular, 24 mm × 60 mm (Blue Star, India)
5. Aluminum foil, 72 m (Century, India)
6. Dissecting needle (Labkafe, India Product code: LKBI 008/1)

Plant Material

Tree plants approximately 15 years old, maintained at CSGRC's field gene bank, of different cytotypes as diploids (MI-0014 and MI-0308), triploids (MI-0173 and MI-0799), tetraploids (K2-4X), hexaploids (ME-0126, MI-0426, and MI-0571), and decosaploid (ME-0241) were selected for this study. In the present protocol, for metaphase chromosome count, apical shoot meristematic tips were collected after 20 days of pruning.

Reagents

1. Sterile, double-distilled water (ddH_2O)
2. 100% glacial acetic acid (Rankem, catalog number: A0031)
3. 0.5 M ethylenediaminetetraacetic acid (EDTA) (GeNei, catalog number: FC43)
4. Ethanol (HiMedia, catalog number: MB106)
5. Potassium chloride (KCl) (HiMedia, catalog number: PCT0012)
6. 2.5 (w/v) pectinase (HiMedia, catalog number: PCT1519)
7. 2.5 (w/v) pectolyase (HiMedia, catalog number: PCT1520)
8. 1:1 (v/v) cellulase (HiMedia, catalog number: RM3331)
9. 1% acetocarmine (HiMedia, catalog number: PCT1304)
10. 1% Orcein (HiMedia, catalog number: RM277)
11. Saturated *para*-dichlorobenzene (PDB) (HiMedia, catalog number: GRM6907) (see Recipes)
12. 0.002 M 8-hydroxyquinoline (HQ) (HiMedia, catalog number: GRM7135) (see Recipes)
13. 45% GAA (glacial acetic acid) (see Recipes)
14. 70% ethanol (see Recipes)
15. 3EtOH:1GAA (see Recipes)
16. 75 mM KCl (see Recipes)
17. Enzyme cocktail (see Recipes)

18. 1% aceto-orcein (see Recipes)

Equipment

1. Surgical blade, size 22 (Surgeon, India, REF 10122)
2. Personal protection equipment (Oriley, model: ORPPE6), including gloves (model: KSN30) and safety glasses (Augen, model: safety glass-SG-03)
3. Ice flaker (PAREX, PSW-130)
4. Squeeze bottle (Borosil, model: 0166024)
5. Highly absorbent blotting paper (Swastik, India)
6. Forceps, pointed, 5" (Borosil, model: LAFP8888005)
7. Minicooler (Tarson, model: 525060)
8. Incubator (ESCO, model: CCL-050B-8)
9. Micropipette (Eppendorf)
10. Freezer (Whirlpool, model: ICEMAGIC FF-350)
11. Stereo zoom microscope (Leica, model: Wild M8-308700)
12. Portable digital microscope (Medprime, model: BT-E2020) with iPad (AppleInc.)
13. iMac 27'' M1 chip-macOS Monterey (Apple Inc.)
14. Sankalp immersion oil (Oil LV, model: 1017)
15. Spirit lamp (HiMedia, model: LA275)
16. Sealing wax (Alpha Chemika, model: AL2934)

Software

1. Cilika (Version 1.30), Medprime Technology Pvt. Ltd. (www.medprimetech.com), image capture software
2. Microsoft Excel, Microsoft
3. Keynote presentation software (Apple Inc. Version 10)
4. Floreada.io (https://floreada.io/flow-cytometry-software)

Procedure

A. Collections of samples and pre-treatment

1. Select 3–5 young healthy shoots and use needle and forceps to dissect fresh apical shoot meristematic tips, approximately from 0.5 to 1.0 cm, between 9:00 and 10:00 am. Immediately transfer the collected samples to the pre-fixative solution, i.e., 1 mL of PDB with 20 µL of HQ in a 1.5 mL Eppendorf tube (Figure 1).
   Note: For pretreatment of shoot apical meristematic tips, a minimum of three (for large size tetraploid accession) and a maximum of five (small size apical tips specifically for diploid accession) samples per Eppendorf tube can be used. The sample should be transferred immediately to the pre-fixative solution, to enhance the metaphase arrest stage. PDB and HQ stock solutions should be kept separately in amber reagent bottles at room temperature (RT) and mixed properly by gently inverting five times before collection of the sample.

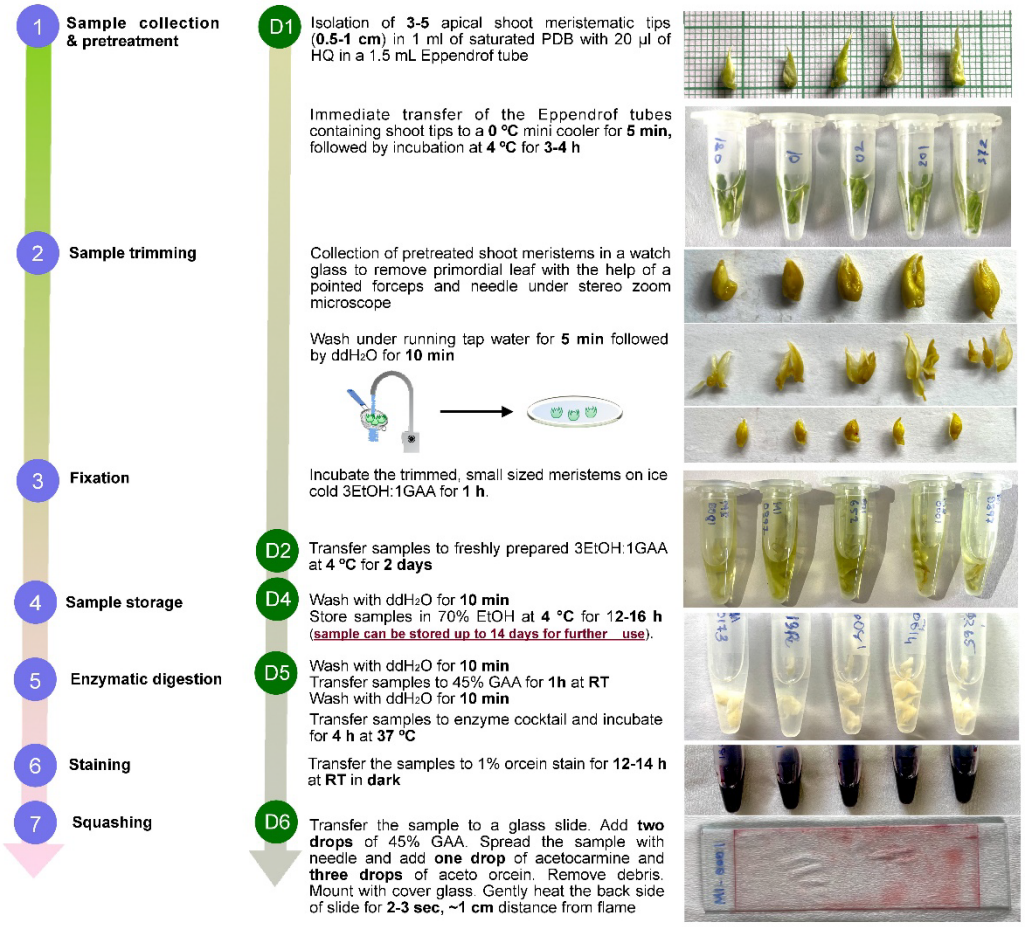


Figure 1. Detailed steps and daily activity for mitotic metaphase plate preparation using shoot meristematic tissues of *Morus* spp.

2. Transfer the pretreated samples to a mini cooler at 0 °C for 5 min, followed by 4 °C for 4 h.
   Note: Place the mini cooler in the ice bucket before conducting sample collection for the maintenance of the mini cooler's temperature.
3. Discard the pre-fixative (PDB+HQ) solution and remove young leaf primordia using pointed forceps and a needle under a stereo zoom microscope. Transfer the trimmed apical shoot tips to a strainer and wash thoroughly under running tap water for 5 min, followed by 5 mL of ddH_2O for 10 min in a watch glass (Figure 2).
   Note: Removal of the young leaf primordia (usually 3–4 numbers) is useful to enhance enzymatic treatment (Step C9). Thorough washing is necessary to remove PDB+HQ residues.

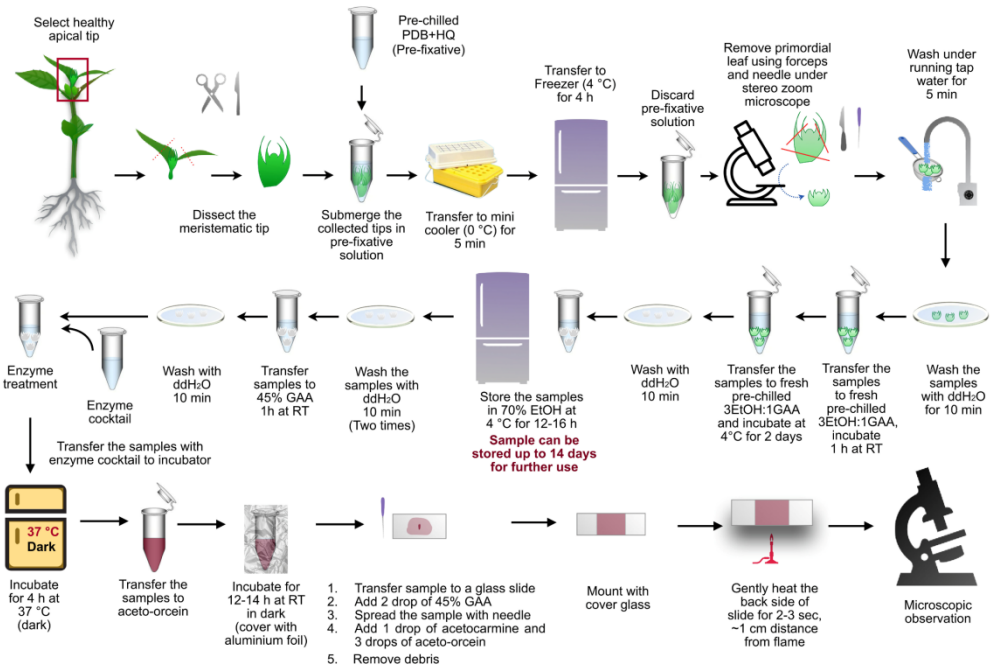


Figure 2. Step-by-step flowchart of sample collection, pretreatment, fixation, staining, and squash for microscopic observation

B. Fixation of meristems

4. Transfer the washed samples to 1 mL of ice-cold 3EtOH:1GAA in a 1.5 mL Eppendorf tube and incubate for 1 h at RT (24 ± 2 °C).
   Note: Prepare 3EtOH:1GAA fresh before fixation.
5. Discard the 3EtOH:1GAA solution and replace with freshly prepared ice-cold 3EtOH:1GAA solution. Incubate the samples at 4 °C for a minimum of two days. Replace with fresh 3EtOH:1GAA solution every 12 h.
6. Discard the 3EtOH:1GAA solution and wash thoroughly in 5 mL of ddH_2O for 10 min; then, store the samples in 1 mL of 70% ethanol in a 1.5 mL Eppendorf tube at 4 °C for further use.
7. Wash the samples in 5 mL of ddH_2O twice in a watch glass for 10 min each. Transfer the samples to 1 mL of 45% GAA in a 1.5 mL Eppendorf tube and incubate for 1 h at RT.
8. Wash thoroughly in 5 mL of ddH_2O for 10 min in a watch glass and remove water from the sample with a blotting paper.

C. Enzymatic digestion

9. Treat the samples with an enzyme cocktail comprised of cellulase (2%), pectinase (2.5%), and pectolyase (1%) for 4 h at 37 °C in the dark using an incubator.

D. Staining

10. Transfer enzyme-treated samples to 500 µL of 1% aceto-orcein in a 1.5 mL Eppendorf tube covered with aluminum foil (to maintain dark conditions) and incubate the sample-containing tubes for 12–14 h at RT.

E. Squashing (see Video 1)

Video 1. Squash technique for chromosome study of *Morus* spp.

11. Transfer one of the processed (stained) samples from the aceto-orcein stain to a glass slide.
    Note: Remove the excess stain with the help of blotting paper if required.
12. Add two drops of 45% GAA to the sample and gently dissolve the tissue with the back side of the needle.
13. Gently mix the sample with the addition of one drop of acetocarmine and three drops of aceto-orcein stain; subsequently, remove the debris with a dissecting needle.
14. Place a coverslip over the slide. Keep the slide inside a folded blotting paper and gently press with your thumb to remove the excess stain and to spread uniformly.
15. Gently tap over the coverslip using the backside of the needle to obtain an optimum spread of cells as well as chromosomes.
    Note: To enhance the spreading quality, continuous tapping is required until the clump of cells spread over the slide as a thin layer of cells. Ensure that no air bubbles remain.
16. Apply flame heat (using spirit lamp) on the bottom side of the mounted slide for 2–3 s and gently tap for precise chromosome spreading.
17. Seal the mounted slide with wax (optional).
18. Place the slide and visualize the chromosome under the microscope (Portable digital microscope, Medprime, model: BT-E2020). Any compound microscope with 40× or 100× objectives with oil immersion can be used. Microscopic images of representative accessions from each ploidy group are represented in Figure 3.

F. Flow cytometry (FCM) analysis

To confirm the ploidy level cytotypes, genome size was estimated by FCM of selected ploidy (2x, 3x, 4x, 6x, and 22x) accessions, which were identified through chromosome number count (Figure 4). A dual laser FACSCalibur™ (BD Biosciences, United States) was used to estimate genome size with some modifications to the protocol described by Galbraith et al. (1983). In brief, young mulberry leaves (5–6 days old) of approximately 0.5 cm^2 were collected between 8:30 and 9:00 am. With a razor blade, the leaf sample was chopped in 2 mL of nuclear isolation buffer [hypotonic propidium iodide (50 µg/mL), trisodium citrate dihydride (3 g/L), 0.05% (v/v) Nonidet P-40, and RNase A (2 mg/mL)]; filtered (30 µm nylon mesh) nucleus suspensions were collected in tubes. The tubes were capped and kept at 37 °C for 30 min. Then, the samples were subjected to FCM analysis. *Pisum sativum* was used as the standard reference and measurements were made in triplicates.

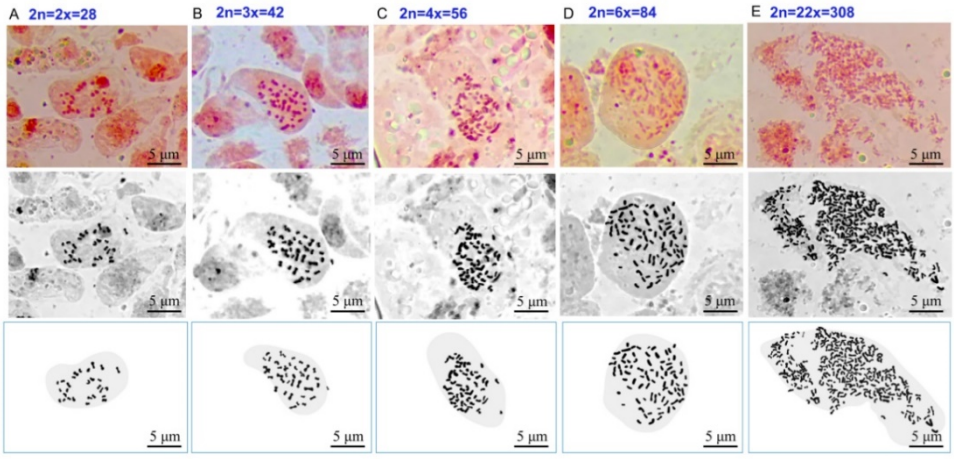
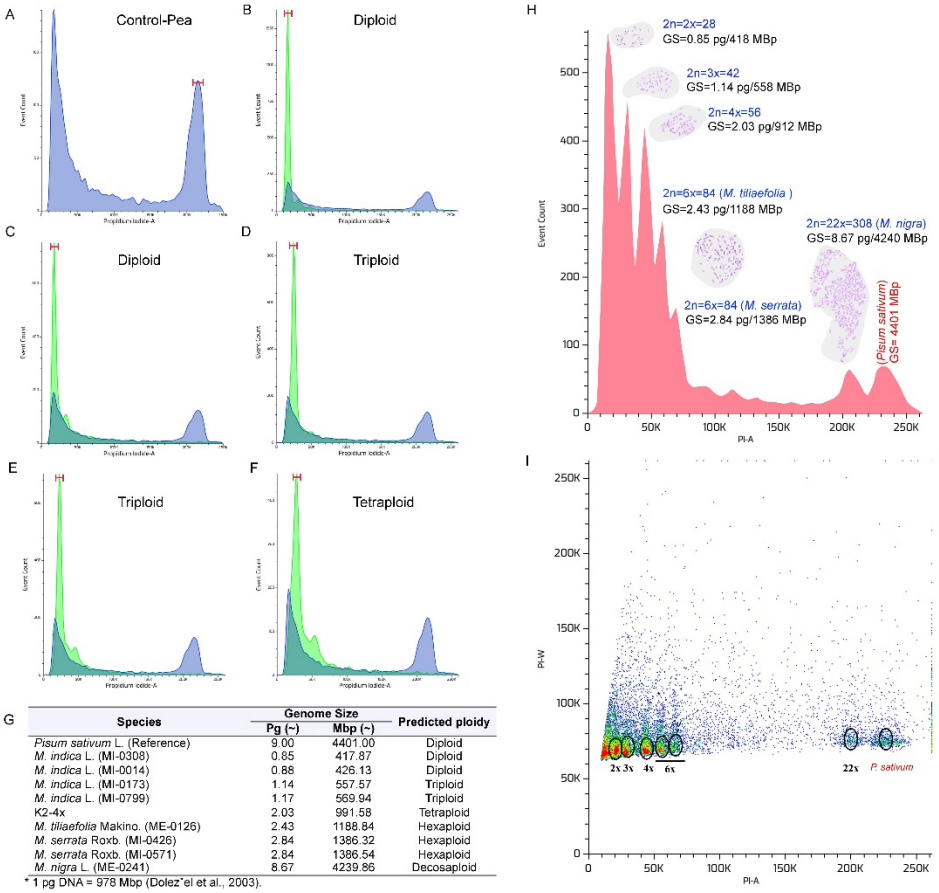


Figure 3. Metaphase plates and estimated chromosome number of different cytotypes of *Morus* spp. (A) diploid V1 (2n = 2x = 28), (B) triploid AR12 (2n = 3x = 42), (C) tetraploid *M. laevigata* L. (2n = 4x = 28), (D) hexaploid *M. Serrata* Roxb. (2n = 6x = 84), and (E) decosaploid *M. nigra* L. (2n = 22x = 308). Scale bar = 5 µm.



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Species	Genome Size		Predicted ploidy
	Pg (~)	Mbp (~)	
Pisum sativum L. (Reference)	9.00	4401.00	Diploid
M. indica L. (MI-0308)	0.85	417.87	Diploid
M. indica L. (MI-0014)	0.88	426.13	Diploid
M. indica L. (MI-0173)	1.14	557.57	Triploid
M. indica L. (MI-0799)	1.17	569.94	Triploid
K2-4x	2.03	991.58	Tetraploid
M. tiliaefolia Makino. (ME-0126)	2.43	1188.84	Hexaploid
M. serrata Roxb. (MI-0426)	2.84	1386.32	Hexaploid
M. serrata Roxb. (MI-0571)	2.84	1386.54	Hexaploid
M. nigra L. (ME-0241)	8.67	4239.86	Decosaploid

* 1 pg DNA = 978 Mbp (Dolež˘el et al., 2003).

Figure 4. Confirmation of different mulberry cytotypes using flow cytometry analysis. Histogram of propidium-iodine-A (PI-A) fluorescence intensity of (A) reference of *Pisum sativum,* (B–C) diploid, (D–E)

triploid, and (F) tetraploid. (G) List of accessions studied, genome size [in mega base pairs (Mbp) and pictogram (pg)], and predicted ploidy level. (H) Histogram of PI fluorescence intensity (count vs. PI-A) of diploid (2x), triploid (3x), tetraploid (4x), hexaploid (6x), and decosaploid (22x) with reference to *Pisum sativum*. (I) Scatterplot (PI-W vs. PI-A) of diploid (2x), triploid (3x), tetraploid (4x), hexaploid (6x), and decosaploid (22x) nuclei, showing they are evenly spaced in respect to fluorescence and represent a well-defined series of areas that correspond to 2C, 3C, 4C, 6C, and 22C nuclei.

Data analysis

1. Metaphase plate images were captured (with an automated measuring scale bar) and a presentation (karyomorphological drawing) was prepared in Keynote, Apple Inc. (Version 10).
2. Genome size (Mbp) was calculated according to the formulae by Lysak and Dolezel (1998) with the conversion of 1 pg equal to 980 Mbp (Dolezel et al., 2003). Finally, the genome size of studied accessions was calculated using the standard reference of *Pisum sativum*. DNA content of the mulberry accessions ranged from 0.85 (diploid) to 8.67 pg (decosaploid) and the coefficient of variation was 3.21 (< 5%; Figure 4H). The floreada.io (https://floreada.io/flow-cytometry-software) online tool was used to generate histograms and scatterplots for FCM analysis.

Notes

1. In step B5, change 3EtOH:1GAA solution once every 30 min for bleaching of chlorophyll.
2. In step B6, incubation for a minimum of two days and resuspending the sample in freshly prepared ice-cold 3EtOH:1GAA solution at 12-h-intervals is essential.
3. In step C9, enzymatic treatment (pectinase, cellulase, and pectolyase) for 4 h at 37 °C in the dark is recommended for karyotype analysis of higher ploidy level (2n = 3x, 4x, 6x, and 22x). For general cytological analysis and chromosome study, treating only with pectinase for 6 h at 37 °C is optimal.

Recipes

1. PDB

Reagent	Final concentration	Amount
PDB	Saturated	10 g
H_2O	n/a	95 mL
Total	n/a	100 mL

2. 70% ethanol

Reagent	Final concentration	Amount
Ethanol (absolute)	70%	70 mL
H_2O	n/a	30 mL
Total	n/a	100 mL

3. 0.002 M HQ

Reagent	Final concentration	Amount
HQ	0.002 M	0.073 g
ddH_2O	n/a	250 mL
Total	n/a	250 mL

4. 45% GAA

Reagent	Final concentration	Amount
GAA	45%	45 mL
ddH_2O	n/a	55 mL
Total	n/a	100mL

5. Potassium chloride (KCl)

Reagent	Final concentration	Amount
KCl	1 M	7.5 g
ddH_2O	n/a	100 mL
Total	n/a	100 mL

6. EtOH (3):GAA (1)

Reagent	Final concentration	Amount
Ethanol	3 parts	75 mL
GAA	1 part	25 mL
Total	n/a	100 mL

7. Enzyme cocktail

Reagent	Final concentration	Amount
KCl	75 mM	7.5 mL
Cellulase	2%	2.0 g
Pectinase	2.5%	2.5 g
Pectolyase	1%	1.0 g
EDTA (0.5 M)	7.5 mM	1.5 mL
ddH_2O	n/a	91 mL
Total	n/a	100 mL

8. 1% Aceto-orcein

Reagent	Final concentration	Amount
GAA	45%	45 mL
Orcein	1%	1.0 g
ddH_2O	n/a	55 mL
Total	n/a	100 mL

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Competing interests

The authors declare that they have no competing interests.

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