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Ploidy level and their relationship with vegetative traits of mulberry (*Morus* spp.) species in Taiwan

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ABSTRACT

To accelerate the mulberry breeding program and examine the genome size and ploidy levels of 27 mulberry accessions of promising lines in Taiwan, nuclei suspensions were prepared from unfolded young leaves and analyzed by a flow cytometer. The genome size of each accession was estimated by referring to the standard genome size of chicken erythrocyte nuclei (CEN, 2.5 pg). Our results indicated that 25 among 27 investigated accessions in *M. bombycis*, *M. alba*, *M. latifolia*, *M. australis*, *M. formosnensis,* and *M. atropurpurea* are diploids with genome sizes ranging from 0.61 to 0.71 pg. 'Elongated mulberry No. 1' (*M. laevigata*) with a genome size of 1.06 pg is a triploid. Two populations were identified in the accession '67C001' (*M. australis* Poir.) with a genome size of either 0.63 pg or 0.98 pg. Due to their similarity in appearance, we suspected that they are probably from the same origin, thus warranting further investigations. Several important vegetative characters of diploids and triploids have been estimated, including leaf length, leaf width, petiole length, petiole width, leaf thickness, and internode length, to document their correlation using the unpaired *t*-test. Subsequently, the statistical relationship between diploid and triploid species on some of these important characters, including leaf width, petiole width and internode length, was determined through binary regression analysis. We suggested that the internode length could provide a beneficial index to distinguish individuals with different ploidy levels in the field. Results of this study provide useful information to clarify the taxonomy of *Morus* and improve breeding programs aiming to advance horticulture and sericulture.

1. Introduction

Mulberry (*Morus* spp.) is an important economical crop that has versatile utilizations. In traditional folks, it provides forage for rearing silkworm (*Bombyx mori*) and has implications in the pharmaceutical industry (Asano et al., 1994), cosmetic industry (Wang et al., 2016), dye, wood, and for landscaping (Chang, 2006; Aroonpong and Chang, 2015). Besides, the production and economic value of mulberry has increased as it now is recognized for the nutritional and healthy value due to its abundance in phytochemicals which imparts oxidative properties to the fruits (Chen et al., 2004; Lee and Hwang, 2017; Vijayan et al., 2011). Hence, in addition to being cultivated for fresh or process consumption, it also has a high demand for use in the agri-food industry.

Mulberry varies greatly in its appearance and adapts well to various environments due to its dioecious and cross-pollination nature (Chang, 2006; Vijayan et al., 2011). Some wild mulberry species, i.e., *M. serrata* and *M. laevigata*, are quite different from domesticated varieties and have better resistance to biotic and abiotic stresses (Tikader and Dandin, 2007). It is essential to preserve and utilize these wild species for breeding program because these can sustain drastic climate changes. Seven mulberry species, including the introduced (*M. alba*, *M. latifolia, M. laevigata*, *M. atropurpurea,* and *M. bombycis)* and the indigenous (*M. australis,* and *M. formosensis*) can be found in Taiwan (Chang, 2006). Numerous varieties have been selected from these species. Within these varieties, some are distinctive in their vegetative size, inability of fruiting, suspicious polyploidy level (Chang, 2006).

Polyploid plants differ from diploids usually in morphological

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characteristics, cytological characters, and metabolic activities. Even-number polyploids may be fertile, whereas plants with odd ploidy levels are often sterile, because defective gametes due to abnormal chromosome pairing in meiosis (Jones, 2003). *Morus* spp. is one of the interesting genera with diverse ploidy levels. Cytological research on mulberry was firstly conducted by Tahara (1910), who indicated that most mulberry species are diploids with 28 chromosomes. Since then, mulberry species with 14 (He et al., 2013), 42, 56, 84, 140, and even 308 chromosomes (Osawa, 1920; Janaki-Ammal, 1948) have been identified. However, ploidy levels may vary within species (Chu and Sun, 1986). Therefore, it is essential for breeders to clarify the ploidy levels in the germplasm for parental selection.

Traditional ploidy estimation has been conducted by chromosome counting. The chromosome number is examined in the mitotic metaphase cells which are prepared from active mitosis tissues such as apical meristems of young roots or shoot tips. Chromosome data could be supplemented by the size or density of stomata or pollen mother cells. In recent decades, DNA flow cytometry has been developed as a rapid, accurate, and convenient tool to measure plant nuclear DNA content (Galbraith et al., 1983; Michaelson et al., 1991).

DNA content has been known in proportion to ploidy level as previously reported in the genera *Ipomoea* (Ozias-Akins and Jarret, 1994), *Musa* (Oselebe et al., 2006), *Rubus* (Meng and Finn, 2002), and *Vaccinium* (section *Cyanococcus*; Costich et al., 1993). In the method of flow cytometry, DNA content is calculated based on the ratio of sample to standards with known DNA content, while the ploidy level is determined by referring to the DNA content of a known diploid (De Laat et al., 1987; Costich et al., 1993; Galbraith et al., 1983; Ozias-Akins and Jarret, 1994; Lin et al., 2001; Meng and Finn, 2002).

Conventionally, female flower traits, particularly style length, have been the principal characters used in the classification of mulberries with respect to female accessions (Koidzume, 1917; Minamizawa, 1976; Chang, 2006). However, recently, selected quantitative vegetative traits derived from numerical taxonomic analysis have been proven to be useful for identifying different sexual forms to facilitate plant classification in mulberry (Chang et al., 2014). Understanding such taxonomic characters may help determine the association of ploidy with morphology in mulberry.

Obtaining cytogenetic data on germplasm to help accelerate crop improvement will enable development of more effective breeding strategies and advance genetic conservation. To improve the breeding progress in *Morus*, the aim of this study was to determine the ploidy and genome size of 27 mulberry accessions of germplasm collected in Taiwan, and to identify distinguishable vegetative traits that breeders could easily use in the field to determine ploidy level.

2. Materials and methods

2.1. Plant materials

Twenty-seven accessions randomly selected from seven *Morus* spp., including hybrids and unidentified accessions (Chang, 2009; Chang et al., 2014), were used in this study (Table 1). Of these accessions, *M. alba* 'Shinichinose,' a diploid species with a known chromosome number of 28 (Tahara, 1910), was used as a standard for checking ploidy levels. Samples were collected from 1 to 3 plants which were obtained from 4- to 7-years-old trees in the mulberry germplasm collection at the Miaoli District Agricultural Research and Extension Station (MDARES, 24°49′ N, 120°82′ E), Gongguan, Miaoli, Taiwan, ROC. All trees were grown under regular cultivation practices as reported by Chang and Liou (2006).

2.2. Nuclei isolation of mulberry leaves

Nuclei suspensions were prepared as described by Galbraith et al. (1983). Three growth stages of 'Taisang No. 2' mulberry (*Morus australis*) leaf, which were young folded leaves (≈3 cm), unfolded leaves (50% expansion, ≈6–7 cm), and fully expansion leaves (> 10 cm), were used to determine the proper stages for flow cytometric analysis. All leaf samples of each accession for ploidy and DNA content analysis were later collected based on the unfolded and young stage. Leaf samples were collected in spring of 2008 and 2015 (for re-checking), stored at 4 °C, and processed within five days.

Leaf samples were thoroughly rinsed with distilled water. A 0.79 cm^2 disc free of primary and secondary veins was collected from each leaf sample. Leaf discs were then chopped with a razor blade in 1 ml chopping buffer (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, 1% Triton X-100; and extra 0.5% 2-mercaptoethanol was added just before the buffer was used). The cellular debris, consisting of finely minced tissue fragments, were filtered through 2 layers of 30 µm nylon mesh and stored on ice. The suspensions of plant material and standards were stained for one hour with nucleic acid-intercalating flurorchrome and propidium iodide (50 µg/ml). RNase (10 µg/ml) was added to reduce the interferences of RNA. To estimate the nuclear DNA content, chicken (*Gallus domesticus*) erythrocyte nuclei (CEN, 2C genome size = 2.5 pg, Biosure) were used as a standard. The CEN were also stained with the same PI concentration. A mixture of 240 µl stained nuclei suspension from tested accessions and 30 µl CEN standard suspensions were used to evaluate the genome size.

2.3. Flow cytometry and genome size estimation

Specimens were analyzed on a flow cytometer (EPICS Elite ESP, Beckman Coulter Electronics, Hialeah, Fla.) equipped with a 15-mV argon laser operating at 488 nm. A minimum of 10,000 fluorescent events (suspension nuclei) per sample were acquired and the data were stored as a distribution of frequency accumulation in a single-parameter histogram by WinMDI 2.9 (Flow cytometry Core Facility, Scripps Research Institute; http://facs.scripps.edu/software.html). Measurements of each specimen were repeated with 1–3 replicates. Only data collected from samples with G1/G0 peaks with a coefficient of variation < 5% were used in estimating C-value.

Genome size of each specimen was estimated by referring to the standard genome size of CEN (2.500 pg): sample DNA content = 2.500 × sample relative fluorescence intensity/CEN fluorescence intensity (Fig. 1A).

M. alba 'Shinichinose' (2n = 28; Tahara, 1910) was used as the standard mulberry sample in estimating ploidy, which was calculated using the following formula: relative ratio = mean sample DNA content/DNA content of 'Shinichinose'. The ploidy level was determined using the following formula: ploidy level = 2 × relative ratio.

The variation in DNA content of diploid accessions was tested with the least significant difference (LSD) by COSTAT (ver 6.2, CoHort software, USA).

2.4. Vegetative traits measurement

Quantitative vegetative characters, including leaf length, leaf width, ratio of leaf length to width, petiole length, petiole width, leaf thickness, and internode length were measured. The mean data for each accession were grouped, based on ploidy level, and evaluated using the unpaired *t*-test (Singh and Chaudhary, 1985) on Excel (2006, Microsoft, USA). Subsequently, the statistical relationship between diploid and triploid species on leaf width, petiole width and internode length was determined through binary regression analysis on Excel (2006, Microsoft, USA). The mean data of leaf width, petiole width, and internode length from six diploid species (*M. alba* 'Kairyonezumigaeshi', 'Shinichinose', 'Shidareguwa'; *M. atropurpurea* '46C020', 'Taisang No. 19', 'Miaoli No. 1'; *M. australis* '67C001-A', '58C307', 'Taisang No. 1', *M. bombycis* 'Kenmochi', *M. formosensis* '58C398', '67C002', '58C495'; *M. latifolia* 'Unryuu', 'Astubaroku', 'Mizumihaiteku') and two triploid