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Nuclear DNA Amounts of Mulberries (*Morus* spp.) and Related Species

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Abstract

Mulberries (*Morus* spp.) include species that are generally diploid but may also occasionally exist in the triploid state ("diploid" species), and others that naturally have different levels of ploidy ("polyploid" species). In the present study, we measured the nuclear DNA amounts in 271 cultivars or strains of 15 mulberry species (nine diploid and six polyploid species) using flow cytometry. A wide range of interspecific 2C DNA values was observed, with the largest being 10.8 times higher than the smallest. Intraspecific differences in 1Cx values, a measure of the monoploid genome size, were within 11% for all species examined. Interspecific variation in 1Cx values was within 28% for all mulberry species in this study. The variation of 1Cx values in polyploid species was larger than that in diploid species. Among the diploid species, the 1Cx values of species native to the Japanese islands were higher than those that originated from East Asia on the Asian continent. The 2C values of other species of Moraceae and Cannabaceae were also estimated, and a wide variation was found.

Discipline: Genetic resources
Additional key words: C value, Cx value, polyploid, Moraceae, Cannabaceae

Introduction

The genus *Morus* contains species with various levels of ploidy, ranging from diploidy ($2n = 2x = 28$) to docosaploidy ($2n = 22x = 308$). Many diploid species of mulberry are the sole food source of silkworms in sericulture. For instance, *M. alba* is native to China but is also found worldwide on continents where it is not native, such as Europe, Africa, North America and South America, whereas *M. bombycis* is native to main islands of Japan including particularly the areas of Japan subject to regular snowfall.

The genetics of *Morus* species is complicated by the occurrence of polyploidy in the genus. Polyploidization is one of the most important evolutionary processes in higher plants (Otto 2007, Wood et al. 2009). Diploid *Morus* species, such as *M. acidosa*, *M. alba*, *M. atropurpurea*, *M. bombycis*, *M. indica*, *M. kagayamae*, *M. latifolia and M. rotundiloba*, have $2n = 2x = 28$ (Datta 1954, Osawa 1920, Tahara 1909). Although fertile diploid individuals form the large majority in these species, some infertile or poorly fertile triploids occur in their populations (Machii et al. 2001, Osawa 1920). Triploid ($2n = 3x = 42$) and tetraploid ($2n = 4x = 56$) forms have also been identified in *M. laevigata* (Das 1961, Datta 1954). *M. boninensis* is tetraploid (Koyama et al. 1998). Hexaploid species ($2n = 6x = 84$), such as *M. serrata* (Basavaiah et al. 1989) and *M. tiliaefolia* (Seki 1952) are known, and polyploidy can extend to docosaploidy ($2n = 22x = 308$) as in *M. nigra* (Basavaiah et al. 1990, Darlington & La Cour 1947, Seki

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& Oshigane 1960).

Triploid cultivars that occurred naturally are frequently found among traditional and indigenous cultivars of diploid species (Machii et al. 2001, Osawa 1920). Cultivated mulberry varieties and strains are generally propagated by the grafting or cutting of individuals or bud sports selected from wild populations or F_1 populations. Thus, triploid cultivars have been maintained even when infertile. It has been found that the artificial doubling of chromosomes can be induced at relatively high frequencies using gamma irradiation (Katagiri 1976) or colchicine treatment (Tojyo 1985). As polyploidization is an important process in both genome evolution and plant breeding, the study of polyploidy in mulberry species may provide valuable information on genome evolution.

In addition to polyploidization, other evolutionary processes can affect genome size (Bennetzen et al. 2005). Thus, an increase in repetitive sequences may lead to an increase in genome size, whereas after polyploidization genome size can be rapidly reduced within one generation (Eilam et al. 2008, 2009). Both events may lead to changes in the 2C DNA value (the unreplicated genome DNA content). Genome evolution is likely to occur during speciation and dispersion from the original location. The diploid mulberry species *M. alba* has been artificially dispersed from its origin in China to many other countries with the spread of sericulture. Here, we investigate whether this dispersion has affected genome sizes across the genus *Morus*.

With respect to genome size evolution by polyploidization, the concepts of holoploid genome size (1C; 2C is the DNA content of the whole chromosome complement) and monoploid genome size (1Cx DNA content of basic chromosomes) are important. In this report, we use the terms "1C and 1Cx values" as defined by Greilhuber et al. (2005). Genome doubling changes the 1C (2C) value but does not change the 1Cx value. A change in 1Cx value, if the chromosome number does not change, indicates variation at the sequence level, such as an increase in repetitive sequences. Different types of genomic evolution in two independent populations (or species) from one ancestor may lead to the hybridization difficulty between them. Knowledge about the genome size in each population or species will provide information helpful for the selection of parental strains and species for cross breeding, as a large difference in genome sizes between the parents likely reduces hybridization affinity.

Flow cytometry has been used for rapid and accurate estimation of nuclear DNA amounts in many plant species (Bennett & Leitch 1995, 2005a, 2005b, Bennett et al. 2000, Doležel 1991, Doležel & Bartoš 2005). In a previous flow cytometry study, we reported that some diploid *Morus* species possess relatively low 1C (Yamanouchi et al. 2010); however, there is a dearth of information from other *Morus* species, particularly those that are polyploid. In this report, we describe the nuclear DNA amounts in diploid and polyploid mulberry species, and in other Moraceae and Cannabaceae species closely related to the mulberry genus for comparison.

Materials and methods

1. Plant materials

A total of 271 cultivars and strains of the *Morus* species were investigated for 2C and 1Cx values: *M. acidosa*, *M. alba*, *M. atropurpurea*, *M. bombycis*, *M. boninensis*, *M. celtidifolia*, *M. indica*, *M. kagayamae*, *M. latifolia*, *M. laevigata*, *M. nigra*, *M. notabilis*, *M. rotundiloba*, *M. serrata* or *M. tiliaefolia* (Table 1 and Table 2). Of these, *M. acidosa*, *M. alba*, *M. atropurpurea*, *M. bombycis*, *M. indica*, *M. kagayamae*, *M. latifolia and M. rotundiloba* are called diploid species here because they mainly exist in diploid form, and rare triploids are infertile. *M. notabilis* was reported to be "haploid" in nature ($2n = 2x = 14$) (He et al. 2013); however, we call it a diploid species in this report as the cultivars of *M. notabilis* maintained in the Japanese Gene Bank are mainly diploid ($2n = 2x = 28$). *M. boninensis*, *M. celtidifolia*, *M. laevigata*, *M. nigra*, *M. serrata*, and *M. tiliaefolia* are polyploid species. Among the 271 cultivars and strains, 240 cultivars are registered in the Japanese Gene Bank.

We also measured the 2C values of 18 species of Moraceae and three species of Cannabaceae: *Artocarpus altilis*, *A. heterophyllus*, *Broussonetia kazinoki*, *B. kazinoki* × *B. papyrifera*, *B. papyrifera*, *Cudrania tricuspidata*, *Fatoua villosa*, *Ficus benjamina*, *F. carica*, *F. elastica*, *F. erecta*, *F. microcarpa*, *F. pumila*, *F. religiosa*, *F. sarmentosa* var. *nipponica*, *F. superba var. japonica*, *F. tinctoria*, *Maclura tricuspidata*, *Humulus japonicus*, *H. lupulus* and *H. lupulus* var. *cordifolius* (Table 3). The mulberry varieties and strains and *B. kazinoki* × *B. papyrifera*, *B. papyrifera*, *C. tricuspidata* and *F. carica* and some *F. benjamina*, *F. elastica*, *F. microcarpa* and *F. pumila* were cultivated at the National Institute of Agrobiological Sciences (NIAS) of Japan. Samples of the species *A. altilis*, *A. heterophyllus*, *F. benghalensis*, *F. erecta*, *F. religiosa*, *F. superba* var. *japonica*, *F. superba* var. *japonica*, and *F. tinctoria*, and some plants of *F. benjamina*, *F. elastica*, *F. microcarpa* and *F. pumila* were kindly provided by the Tsukuba Botanical Garden, the National Science Museum of Japan. Five cultivars of *H. lupulus* were kindly provided by the Central Laboratories

Table 1. 1Cx values of *Morus* species estimated by flow cytometry

Species	Ploidy of varieties and strains analyzed	Analyzed number by PI	Mean 1Cx value (pg)	Minimum - maximum 1Cx value (pg)	Analyzed number both by DAPI and by PI	AT content (%)
Morus acidosa	Diploid	28	0.363 de*	0.340 - 0.377	24	67.1
M. alba	Diploid and triploid**	33	0.354 bcd	0.339 - 0.373	29	67.3
M. atropurpurea	Diploid	3	0.352 bcd	0.349 - 0.354	3	67.6
M. bombycis	Diploid, triploid and tetraploid***	83	0.362 cde	0.345 - 0.377	71	67.2
M. indica	Diploid	6	0.352 bcd	0.344 - 0.356	6	67.0
M. kagayamae	Diploid	25	0.368 e	0.357 - 0.376	23	67.5
M. latifolia	Diploid, triploid and mixaploid****	47	0.352 bcd	0.341 - 0.368	38	67.4
M. notabilis	Diploid and triploid**	8	0.350 bc	0.341 - 0.361	7	67.1
M. rotundiloba	Diploid and triploid**	17	0.355 bcd	0.351 - 0.360	15	67.2
M. laevigata	Triploid and tetraploid	9	0.395 f	0.384 - 0.416	0	-
M. boninensis	Tetraploid	2	0.356 bcde	0.354 - 0.357	2	66.6
M. celtidifolia	Hexaploid	3	0.338 ab	0.333 - 0.343	2	66.9
M. serrata	Hexaploid	3	0.415 g	0.410 - 0.419	2	66.6
M. tiliaefolia	Hexaploid	2	0.364 cde	0.363 - 0.364	0	-
M. nigra	Docosaploid	2	0.330 a	0.328 - 0.333	2	66.9

*: The same alphabetic characters indicate no significant difference at the 0.05 level by Ryan's method.

**: Diploid is common while triploid is rare in nature.

***: Diploid is common while triploid is rare in nature. Only one tetraploid cultivar is maintained in the Japanese Gene Bank, except for artificially induced tetraploids.

****: Diploid is common while triploid is rare in nature. Mixaploid is an artificial mixaploid, 'Popberry,' and each diploid and tetraploid peak was analyzed.

Table 2. 2C values and ploidies of *Morus* species estimated by flow cytometry

Species	Mean 2C value (pg)				
	Ploidy	Diploid	Triploid	Tetraploid	Hexaploid	Docosaploid
M. acidosa		0.725				
*M. alba**		0.705	1.077			
M. atropurpurea		0.705				
*M. bombycis***		0.720	1.108	1.450		
M. indica		0.705				
M. kagayamae		0.736				
*M. latifolia**		0.704	1.061			
*M. notabilis**		0.701	1.046			
*M. rotundiloba**		0.710	1.074			
M. laevigata			1.174	1.637		
M. boninensis				1.423		
M. celtidifolia					2.028	
M. serrata					2.489	
M. tiliaefolia					2.181	
M. nigra						7.265

*: Diploid is common while triploid is rare in nature.

**: Diploid is common while triploid is rare in nature. Only one tetraploid cultivar is maintained in the Japanese Gene Bank, except for artificially induced tetraploids.

for Key Technologies, Kirin Co., Ltd. The other samples of Moraceae and Cannabaceae species were collected from wild individual plants grown on the premises of NIAS (Tsukuba or Hitachiomiya, Japan).

2. Flow cytometry

Flow cytometry was performed as previously described (Yamanouchi et al. 2008, 2010) using an FA CA-IV flow cytometer (Partec GmbH, Germany) equipped with a 488-nm argon ion laser and HBO lamp for UV excitation. Approximately 10-50 mg of tissue samples, such as young leaves, shoots, stems, roots, inflorescences buds, and young anthers, were prepared with CyStain® PI Absolute P (Partec) or CyStain® UV Precise P (Partec) for propidium iodide (PI) or 4', 6-diamidino-2-phenylindole (DAPI) staining,

Table 3. 2C values of Moraceae and Cannabaceae species

Species	No. of varieties, strains and individuals analyzed	Mean 2C value (pg)	Minimum - maximum 2C value (pg)	Analyzed number by DAPI	AT content (%)
Moraceae					
Artocarpus					
A. altilis	1	1.876	-	1	66.4
A. heterophyllus	1	2.093*	-	1	66.0
Broussonetia					
B. kazinoki	3	0.885	0.881 - 0.891	1	66.6
B. kazinoki × *B. papyrifera*	1	0.955	-	-**	-**
B. papyrifera	3	1.022	1.017 - 1.025	2	66.8
Fatoua					
F. villosa	2	0.754	0.746 - 0.764	1	67.5
Ficus					
F. benghalensis	1	0.854	-	1	68.2
F. benjamina	2	0.940	0.925 - 0.956	2	66.8
F. carica	2	0.676	0.659 - 0.693	2	67.2
*F. elastica****	1	0.800	-	1	67.6
*F. elastica****	1	1.544	-	1	66.4
F. erecta	1	0.682	-	1	66.9
F. microcarpa	1	0.946	-	1	67.0
F. pumila	1	0.676	-	1	67.9
F. religiosa	1	0.828	-	1	65.8
F. sarmentosa var. nipponica	1	0.620	-	1	67.5
F. superba var. japonica	1	0.859	-	1	66.3
F. tinctoria	1	0.694	-	1	67.7
Maclura					
M. tricuspidata	1	4.797	-	-**	-**
Cannabaceae					
Celtis					
C. sinensis	1	1.998	-	-**	-**
Humulus					
H. japonicus	2	4.925	4.908 - 4.941	1	73.3
H. lupulus	5	5.602	5.504 - 5.727	-**	-**
H. lupulus var. cordifolius	3	5.282	5.176 - 5.414	-**	-**

*: Re-estimated 2C value using the same plant of *A. heterophyllus* as in the previous report (Yamanouchi et al. 2010) and *Morus latifolia* 'Popberry' as an internal standard for flow cytometry. The 2C value was close to the previously estimated 2C value of 2.12 pg, for which *Arabidopsis thaliana* Col-0 was used as an internal standard.

**: Not analyzed

***: Two different ranges of 2C values were observed for *F. elastica*.

respectively. Target samples and internal standard samples were prepared together, and nuclei were extracted by chopping the samples on a plastic petri dish with a razor blade in the extraction buffers provided in the kit.

The excitation/emission wavelengths used for DAPI were 365/435 nm using ultraviolet light, and those for PI were 488/610 nm using an argon ion laser beam. The flow cytometry data were analyzed using the software program CA3 supplied with the flow cytometer. Flow cytometry analysis was conducted at least twice.

As an internal standard, various vegetative organs such as the young leaves and stems, young inflorescences, and young anthers of mulberry cultivars were used. When the vegetative organs of diploid, triploid, tetraploid and hexaploid cultivars were used for the internal standard, the 2C peak was used as the internal standard peak. When the vegetative organs of mixaploid cultivars were used, for example, in a mixaploid that consists of diploid (2*x*) and tetraploid (4*x*) cells, the 2*x* or 4*x* peak or both were used as the standard peaks. When young inflorescences and anthers of mulberry cultivars were used for the internal standard, polysomatic peaks were observed, including a 2C peak that was obviously larger than the 4C, 8C, and 16C peaks or higher (Yamanouchi et al. 2008). The peak closest to the target sample peak was used as the internal standard peak. No obvious 1C peaks of gametic cells were observed in this study, although a small 1C peak was apparently observed in rare cases, similar to our previous study (Yamanouchi et al. 2008).

3. Estimation of nuclear DNA amount and AT content

The 2C nuclear DNA amount (double holoploid genome size) was estimated from the relative fluorescence strengths of sample peaks and internal standards as previously described (Yamanouchi et al. 2008, 2010). The relative 2C value of a target sample was estimated by comparison with the internal standard sample. Relative 1Cx value (monoploid genome size) was derived by dividing the 2C value by the ploidy level.

The frequency of AT bases (AT content) in the genome DNA was estimated by the method described in Barow & Meister (2002). We defined the $2C_{AT}$ value as the estimated AT content per holoploid genome. The relative $2C_{AT}$ value was derived from the flow cytometry data after DAPI staining by comparison with the standard in the same manner as the relative 2C value after PI staining. We estimated the AT content using the correlation between the $2C_{AT}$ value of a sample relative to the standard and the 2C value of the sample relative to the standard (Barow & Meister 2002, Yamanouchi et al. 2010) as follows:

(relative $1C_{AT}$ value) / (relative 1C value)

$$= [(1-AT_{sample})\cdot AT^4_{sample}/(1-AT^4_{sample})] / [(1-AT_{standard})\cdot AT^4_{standard}/(1-AT^4_{standard})]$$

For these calculations, R (R Core Team 2015) was used. R was also used for statistical analysis of Ryan's method according to Aoki (2015).

Results

1. Interspecific variation of nuclear DNA amounts in mulberries

The 1Cx values of mulberry species estimated in this report (0.328-0.419 pg, Table 1) were similar to those found in our previous study (0.352-0.376 pg) (Yamanouchi et al. 2010). Estimated 2C values in mulberry species varied about 10.4-fold from 0.701 pg for *M. notabilis* to 7.265 pg for *M. nigra* (Table 2). The 10.4 ratio is close to the quotient of the ploidy levels (22/2 = 11). The minimum 2C value found in this study was 0.678 pg for a cultivar of *M. alba* and the maximum value was 7.322 pg for a cultivar of *M. nigra* (10.8-fold). The variation in 2C values in the mulberry species was mostly due to variation in ploidy levels (Table 2).

The 1Cx values varied by only 28% across the species analyzed in this study: the smallest 1Cx value measured was 0.328 pg (a cultivar of *M. nigra*) and the largest was 0.419 pg (a cultivar of *M. serrata*). The diploid species showed an 11% range of variation of 1Cx values (0.339 pg for a cultivar of *M. alba* to 0.377 pg for a cultivar of *M. bombycis*). The 1Cx values of triploid cultivars and strains that can occur naturally in diploid species were similar to those of the diploid cultivars and strains of the same species. The intraspecific variations in 1Cx values in all species were less than 10%, except for *M. acidosa* and *M. alba* at 10.88% and 10.03%, respectively. The average estimated 1Cx value of all species was approximately 0.360 pg and that of all diploid species was about 0.356 pg. Larger variation in 1Cx values was observed in polyploid species. *M. serrata*, a hexaploid species, and *M. laevigata*, a triploid or tetraploid species, had higher 1Cx values of 0.410-0.419 pg and 0.384-0.416 pg, respectively, while remarkably low 1Cx values were observed in *M. nigra* (0.328 and 0.333pg).

2. 1Cx value and geographic distribution of diploid species

For diploid species, higher 1Cx values were observed for *M. acidosa*, *M. bombycis*, and *M. kagayamae*. The 1Cx values for these species were 0.363, 0.362, and 0.368 pg, respectively, and are higher than the average value of 0.356 pg for diploid species. All three species are native to Japan.

Table 4. Local differences in 1Cx values of diploid species

Cultivar origin		Species	Species origin	No. of varieties and strains analyzed	Mean 1Cx value (pg)	Minimum - maximum 1Cx value (pg)	No. in Fig. 1*
China	(Continent of Asia)	*M. latifolia*	China	9	0.345 a**	0.341 - 0.355	
West Asia and Southwest Asia	(Continent of Asia)	*M. notabilis*	China	6	0.350 ab	0.344 - 0.356	
West Asia and Central Asia	(Continent of Asia)	*M. alba*	China	7	0.350 ab	0.344 - 0.358	
South Asia	(Continent of Asia)	*M. india*	India	6	0.352 abc	0.344 - 0.356	
Main islands of Japan***	(Japan)	*M. latifolia*	China	28	0.355 bc	0.343 - 0.368	1
Main islands of Japan***	(Japan)	*M. alba*	China	26	0.355 bc	0.339 - 0.373	1
Thailand	(Continent of Asia)	*M. rotundiloba*	Thailand	17	0.355 bc	0.351 - 0.360	
Yaeyama Islands	(Japan)	*M. acidosa*	Nansei Islands of Japan	13	0.361 cd	0.340 - 0.370	2
Main islands of Japan***	(Japan)	*M. bombycis*	Main Islands of Japan	83	0.362 d	0.345 - 0.377	1
Amami Islands and Okinawa Islands	(Japan)	*M. acidosa*	Nansei Islands of Japan	8	0.368 de	0.360 - 0.375	3
Izu Islands	(Japan)	*M. kagayamae*	Izu Islands of Japan	25	0.368 e	0.357 - 0.376	4

*: The numbers correspond to those in Fig. 1.

**: The same alphabetic characters indicate no significant difference at the 0.05 level by Ryan's method.

***: This row contains the results for cultivars and strains derived from Hokkaido, Honshu, Shikoku and Kyushu, and distributed to the small neighboring islands.

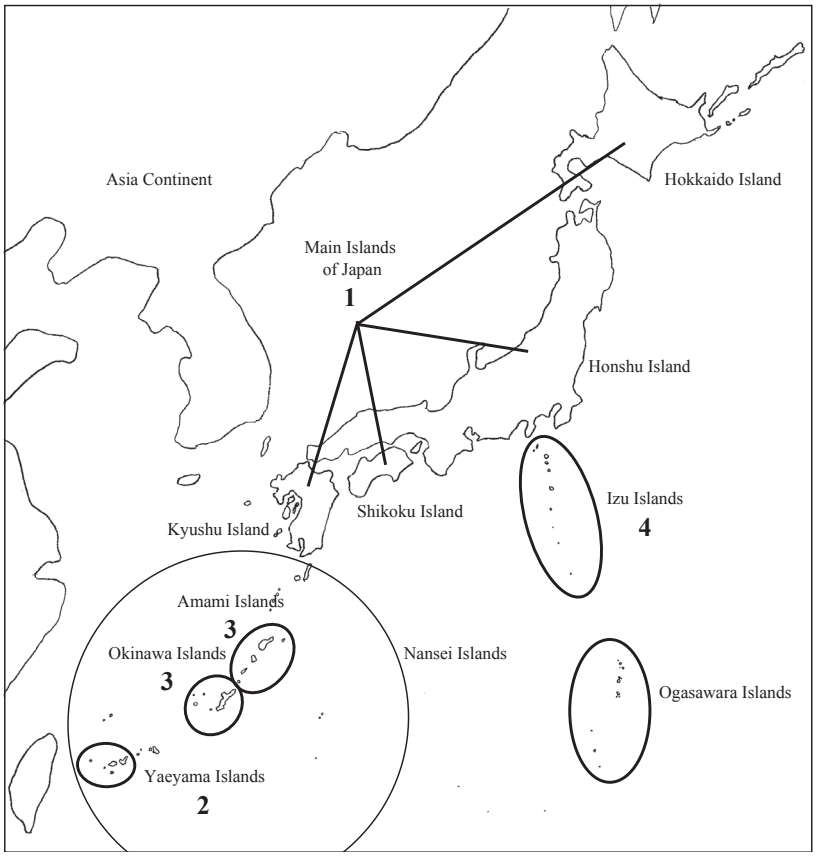

Fig. 1. A rough illustration of the Japanese islands
The numbers 1, 2, 3 correspond to Table 2. The map was modified based on a map by the Geospatial Information Authority of Japan (http://maps.gsi.go.jp/#5/35.362222/138.731389).

There are many indigenous cultivars of *M. alba* and *M. latifolia* in Japan following their introduction from China. These cultivars are believed to be the offspring of natural crosses in *M. alba* and *M. latifolia*. *M. alba* is widely distributed around the world. We analyzed the 1Cx values of diploid species relative to both geographic distribution and region of origin (Table 4 and Fig. 1). The results showed that among the cultivars found in Japan, those of Chinese origin (*M. alba* and *M. latifolia*) possessed lower 1Cx values. However, some Japanese indigenous cultivars of *M. alba* and *M. latifolia* may be hybrids with *M. bombycis*, which is native to Japan and has a higher 1Cx value. Diploid mulberry species are easily hybridized with each other. Two cultivars from Europe and America had lower 1Cx values, similar to those of the Asian continent (data not shown). The 1Cx values depended on the native area of origin of the species, and not on the area where they were growing.

3. Nuclear DNA amounts of related species

We estimated 2C values in some Moraceae and Cannabaceae species related to mulberry species (Table 3). Except for some Ficus species that showed smaller 2C values than diploid mulberries, the 2C values of the Moraceae species were within the range of 2C values of *Morus*. This indicates that *Morus* is a unique genus in terms of its wide-ranging 2C values.

4. AT content of *Morus* and related species

We estimated the AT content in *Morus* species and some Moraceae species, and Cannabaceae species. The AT content of *Morus* species was similar, in the range of 66.6 - 67.6% (Table 1). The AT content of Moraceae species was also similar, in the range of 66.0 - 68.2% (Table 3). The AT content of *Humulus japonicus*, a Cannabaceae species, was approximately 73.3%, and thus different from Moraceae. The AT content may be stable in the Moraceae species examined in the present study.

Discussion

The 2C values of the diploid and polyploid species studied here were near the expected values estimated by calculation based on their ploidy levels and the previously reported mulberry 1Cx values of 0.352 - 0.376 pg (Yamanouchi et al. 2010). Thus, the interspecific variations in the genome sizes in mulberry were mainly due to polyploidization. *M. notabilis*, first identified in Sichuan province in China, was reported as "a naturally available haploid mulberry species" with a chromosome number of $2n = 14$ (He et al. 2013), which is half that of such well-known diploid mulberry species ($2n = 28$) as *M. alba* (Morus genome database 2013). The genome size in the report of He et al. (2013) is 357 Mb (≈0.365 pg), similar to the 1Cx values found here for diploids at 0.339 - 0.377 pg. The genome of *M. notabilis* suggests the basic chromosome number in *Morus* is 7 based on the genome analysis of *M. notabilis*. In this report, however, we have assumed that 14 is the basic number according to conventional practice. In the Japanese Gene Bank, *M. notabilis* is listed as both diploid and triploid cultivars. Thus, in this report, we used *M. notabilis* as a diploid mulberry species.

The interspecific variation in 1Cx values among diploid species was not large. The range of 1Cx values in the diploid species overlapped. The largest average 1Cx value was 0.368 for *M. kagayamae*, and the smallest was 0.350 pg for *M. notabilis*. However, the smallest 1Cx value of a cultivar of *M. kagayamae* (0.357 pg) was less than the largest value (0.361 pg) of a cultivar of *M. notabilis*. As all polyploid mulberry species probably evolved from an ancestral diploid species, the ancient 1Cx value is likely to be in the range (or similar to the range) of 1Cx values for diploid species (i.e., 0.339-0.377 pg; average of 0.356 pg). We assume that 0.339-0.377 pg is the basal range of 1Cx values for *Morus*.

The variation in interspecific 1Cx values in polyploid species was wider than that in diploid species. The degree and direction of changes in 1Cx values varied among species. Three types of variation were found: 1Cx values larger than those of diploid species; 1Cx values smaller than those of diploid species; and 1Cx values similar to diploid species. Nevertheless, the differences among the species of *Morus* do not seem to be wider than those in related genera, such as *Broussonetia* and *Ficus* of Moraceae, and *Humulus* belonging to Cannabaceae. For example, *H. lupulus* and *H. lupulus* var. *cordifolius* belong to same species but showed different 2C values.

Large increases in 1Cx values were observed in *M. laevigata* (0.395 pg: about 111% of average diploid 1Cx value: 0.356 pg) and *M. serrata* (0.415 pg: about 117% of the average diploid 1Cx value). Meanwhile, *M. tiliaefolia* showed a small increase (0.364: about 102% of the average diploid 1Cx value). These three species were reported to be closely related assessed by fluorescence-based AFLP markers and UPGMA cluster analysis (Sharma et al. 2000). Thus, it is assumed that genome evolution probably occurred in these species, such as through an increase in the copy number of repetitive sequences.

The 1Cx values in *M. nigra* (0.328-0.333 pg) were lower than the basal range of 1Cx values (0.339-0.377 pg), with no overlapping. Genome size reduction may have occurred after polyploidization in *M. nigra*. In

allopolyploids of several Triticeae species, nuclear DNA amounts are significantly smaller than the sum of their parental species (Eilam et al. 2008, 2009). Leitch & Bennett (2004) identified a tendency for 1Cx values of higher ploidy species to be smaller; however, in mulberry, the 1Cx values of three of four hexaploids were larger than those of diploids, although the 1Cx values of the docosaploid and a hexaploid were smaller and slightly smaller, respectively.

The tetraploid species *M. boninensis* (Koyama et al. 1998) showed no change in 1Cx value (0.356 pg) compared to the average diploid 1Cx value (0.356 pg). *M. boninensis* is native to the Ogasawara Islands, which are located southeast of Japan and more than 1,000 km from Tokyo, and represent the most eastern area of *Morus* distribution. *M. boninensis* is a critically endangered species as a result of interspecific crosses with the introduced diploid species *M. acidosa* (Tani et al. 2003). Interspecific cross pollination outcompetes intraspecific pollination in *M. boninensis* due to the production of sterile triploid hybrids. The ease and frequency of interspecific crosses between *M. boninensis* and *M. acidosa* suggest that both species show little evolutionary divergence. However, Sharma et al. (2000) reported that *M. boninensis* had the lowest degree of similarity to other *Morus* species in AFLP marker analysis; their analysis included all species analyzed in the present study. It is possible that no interspecific barrier exists for cross pollination between diploid mulberry species. Pandit et al. (2014) indicated that species with smaller genome sizes were more invasive than those with larger genome sizes. Thus, the predominance of *M. acidosa* over *M. boninensis* in the Ogasawara Islands may be due to the lower 1C value of *M. acidosa*.

Three strains of the hexaploid species *M. celtidifolia* showed only slightly lower 1Cx values (0.333-0.343 pg) compared to the basal range of 1Cx values (0.339-0.377 pg). Unless the species was native to North America, far from the continent of Asia where diploid *Morus* originated, the change in 1Cx values was minimal.

There is some possible evidence for local (regional) changes in 1Cx values in diploid mulberry species. The 1Cx values in *M. acidosa*, *M. bombycis*, and *M. kagayamae*, species native to Japan were the largest among the diploid species. *M. bombycis* in Japan, *M. acidosa* from Amami, Yaeyama and the Okinawa Islands, and *M. kagayamae* from the Izu Islands tended to have higher 1Cx values than the diploid species native to the continent of Asia. In particular, the average 1Cx value of *M. kagayamae* from the Izu Islands was highest among diploid mulberry species. The Izu Islands are the Japanese islands furthest from the continent of Asia (Fig. 1). These Japanese mulberry species are naturally distributed in the most eastern regions of *Morus* native distribution. This geographic isolation may possibly underlie some original aspects of genomic evolution.

There is no interspecific hybridization incompatibility between *M. alba* and *M. kagayamae* (Katsumata 1982). Therefore, the genomic evolution between *M. alba* and *M. kagayamae* is not particularly large. All diploids mulberry species studied in this report are potential parents for crossbreeding.

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