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Genome size variation and species relationships in the genus *Hydrangea*

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Abstract Genome size and base composition in 16 species and subspecies of the *Hydrangea*, a woody ornamental genus of *Hydrangeaceae*, were evaluated by flow cytometry in relation to their chromosome number. This is the first such study concerning the genome size of these species together with a karyotype study of the most important species, *Hydrangea macrophylla* subsp. *macrophylla* (Hortensia), from an economical point of view. The 2C DNA content ranged from 1.95 pg in *Hydrangea quercifolia* to 5.00 pg in *Hydrangea involucrata*. The base composition ranged from 39.9% GC in *Hydrangea aspera* subsp. *sargentiana* to 41.1% in *Hydrangea scandens* subsp. *scandens* (significant difference at $p < 0.05$). The smallest genome sizes were those of the three species originating from North or South America. Most of the species studied presented a chromosome number of $2n = 2x = 36$, except for those of the section *Aspereae* which showed 2n = 30, 34 and 36. A primary karyotype has been made for the first time for *H. macrophylla* subsp. *macrophylla*. Phylogenetic relationships between species, the origin of chromosome number and an exploration of the genetic diversity within the genus are discussed.

Keywords *Hydrangea* · *Hydrangeaceae* · DNA content · Chromosome number · Karyotype · Phylogeny

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Introduction

The great ornamental value of *Hydrangea* has made this genus famous. Since the introduction of the most-cultivated species, *Hydrangea macrophylla*, from Japan to England, garderners have improved this species with empirical crosses and selection in England, France, Germany and Switzerland. Compared with wild varieties, cultivars tend towards more-colored flowers, more-rigid and dwarf stems. However, future progress will depend upon a genetic understanding of the genus and on more-elaborate breeding programs to explore genetic resources.

Nevertheless, since the extensive study of McClintock in 1957, few systematic studies have been devoted to *Hydrangea*, a woody genus of the *Saxifragaceae* family. This genus occurs in the temperate regions of eastern Asia and eastern North America and extends southward into the tropics of both hemispheres (McClintock 1957).

From a systematic point of view, the *Saxifragaceae sensu lato* is a very large family composed of 15 to 17 sub-families, one of which is the *Hydrangeoideae*. However, a molecular phylogeny based on 18S rRNA and *rbc*L sequences places the *Hydrangeoideae* sub-family far from the general clade of the *Saxifragaceae*, which can be more-narrowly defined (Soltis and Soltis 1997). Indeed, Cronquist (1981) had already proposed a family, the *Hydrangeaceae*, distinct from the *Saxifragaceae*. Phylogenetic relationships between species within the genus *Hydrangea* have not yet been studied.

According to McClintock's classification (1957) the genus *Hydrangea* is divided into two sections, *Hydrangea* McClint. and *Cornidia* Engl., mainly on the basis of plant habit, leaf texture, and the presence and morphology of floral bracts. These sections comprise several subsections, species and subspecies, essentially and respectively based on the position of the ovary and on seed morphology, the shape of the leaves and the type of inflorescence. Furthermore, there are many varieties created from these species, which have often been named as

distinct species. Consequently, there is a confusion of nomenclature, which complicates the taxonomic treatment. However, McClintock's classification remains the most-integrated one available for the genus *Hydrangea*, with 25 species.

The genome-size evaluation of a species is an important tool in genetic diversity studies as it can contribute towards species identification, and also reveal divergence between genera or within a genus, as illustrated for *Hypochaeris* (Cerbah et al. 1999) or *Musa* (Lysak et al. 1999). In closely related species, it is important to know the origin of DNA variation, the direction of changes and the fraction of DNA involved in these changes (Juan and Petitpierre 1990; Bennett and Leitch 1995). Although some correlations have been found between the genome size of species and life cycle (annuals vs perennials), nucleotypic effects (cell size and cell cycle time), as well as ecological (altitude and latitude) and physiological features of the environment (Cullis 1990; Bennett and Leitch 1995), from a fundamental point of view, the evolutionary significance of variation in genome size is not yet understood. Moreover, only a small part of the nuclear DNA has a coding function and variation of the genome size within major taxonomic groups is not correlated with organismic complexity, a conclusion which is known as the C-value paradox (Thomas 1971). The adaptive significance of this variation in genome size, even at lower levels, remains questionable (review in Bennett 1998). Therefore, genome-size data within related species are still needed to clarify these evolutionary questions. The relation between genome size and base composition (GC%) is also of interest because sometimes it can be related to phenotypic traits (Vinogradov 1998) and contributes to the study of the isochore organization in plants (Montero et al. 1990).

Genome size is an important parameter in handling molecular tools for the improvement of agricultural and horticultural features. For example, correlations have been found between genome size and seed size, leaf width and leaf length in soybean (Chung et al. 1998). On an industrial scale, genome size is used to screen production by in vitro techniques in order to check ploidy levels or uniformity in clones (Marie and Brown 1993). It is also a useful guide for cross-breeding possibilities in horticulture, as in *Actinidia* (Blanchet et al. 1992).

The UPOV (international union for the protection of new varieties of plants) collection in Angers (France) is an important genetic resource in Europe with 16 species and more than 450 infraspecific categories. With the aim to describe and analyse the relationship between these species, we have undertaken a cytometric analysis of DNA content and base composition in 16 species and subspecies of *Hydrangea* in relation to their chromosome number. This first approach was completed with a karyotype study of the economically most important species, *H. macrophylla* subsp. *macrophylla*. This establishes a basis for further analysis of the genus to investigate phylogenetic relationships and to elaborate future breeding programs in order to introduce new original characters.

Table 1 Origin of samples

Taxon	Taxonomic section/subsection[a]	Geographical origin	Accession number of individuals
H. arborescens L.	*Hydrangea/Americanae*	North America	147,166, 345, 370, 394
H. quercifolia Bartr.	*Hydrangea/Americanae*	North America	93, 94, 205; 207, 393
H. involucrata Sieb.	*Hydrangea/Asperae*	Japan	164, 355, 372, 418, 419
H. aspera Don subsp. *aspera* McClint.	*Hydrangea/Asperae*	Taiwan	352, 443
H. aspera Don subsp. *strigosa* McClint.	*Hydrangea/Asperae*	China	346, 375
H. aspera Don subsp. *robusta* McClint. (= *H. longipes* Franch.)	*Hydrangea/Asperae*	China	396
H. aspera Don subsp. *sargentiana* (Rehder) McClint.	*Hydrangea/Asperae*	China	154, 188,
H. anomala D. Don subsp. *anomala* McClint.	*Hydrangea/Calyptranthae*	Himalaya	344
H. anomala subsp. *petiolaris* Sieb. & Zucc.	*Hydrangea/ Calyptranthae*	Japan, China	140, 141, 142, 347
H. scandens (L.f.) subsp. *scandens* McClint.	*Hydrangea/Petalantae*	Japan	9718 S
H. scandens (L.f.) subsp. *luikinensis* (Nakai) McClint.	*Hydrangea/Petalantae*	Japan	145, 449, 9713 S
H. paniculata Sieb.	*Hydrangea/ Heteromallae*	Japan, China	262, 263, 264, 399, 422
H. heteromalla D. Don	*Hydrangea/ Heteromallae*	North East China, Himalaya	139, 350, 351, 354, 973S
H. macrophylla (Thunb.) Ser. subsp. *macrophylla* McClint.	*Hydrangea/ Macrophyllae*	Japan	170, 212, 234, 250, 309
H. macrophylla (Thunb.) Ser. subsp. *serrata* (Thunb.) Makino	*Hydrangea/ Macrophyllae*	Japan, South Korea	165, 257, 259, 349, 382
H. seemannii Riley	*Cornidia/ Monosegia*	Chile	143, 155, 9515 S

[a] According to McClintock (1957)

Materials and methods

Origin of samples

All samples used for analysis originated from the UPOV collection, growing in the regional arboretum of Angers (France). Accessions are represented by clones, either botanical varieties introduced from original countries or cultivars. The taxonomic treatment, the geographical origin of species and the accession number of the samples examined are given in Table 1. All taxonomic subsections of the section *Hydrangea* were represented, but only one species from the section *Cornidia* (sub-section *Monosegia*) was added for comparison.

Determination of nuclear DNA content and base composition by flow cytometry

The total DNA amount in nuclei was assessed by flow cytometry using *Pisum sativum* cv Long Express (Truffaut SA) (2C = 8.37 pg, 40.5% GC: Marie and Brown 1993) as an internal reference according to the technique adapted from Brown et al. (1991). Briefly, selected tissues were excised, and chopped with a razor blade in a plastic Petri dish in cold buffer (Galbraith et al. 1983), modified to 0.5% Triton X-100 with 10 mM of sodium metabisulphite added freshly. The cell suspension was filtered through 48 µm-mesh nylon. Nuclei in the filtrate were stained with different fluorochromes: ethidium bromide (Sigma), a DNA intercalating dye used with RNase, the GC-specific dye mithramycin (Serva), and the AT-specific dye bisbenzimide Hoechst 33342 (Aldrich Chimie), used respectively at 30, 50, and 5 µg/ml.

Total DNA content was determined by the ratio of the modal position of 2C stained nuclei of samples relative to those of *P. sativum*. One picogram of DNA is 980×10^6 bp (Cavalier-Smith 1985). About five individuals per species were assessed for each analysis, which was made on 5,000 nuclei using an EPICS V cytometer (Beckman Coulter, Roissy). Each measure was repeated two times. Base composition was calculated according to the Godelle et al. (1993) equations, taking into account the number of fixation sites for the fluorescence of each fluorochrome (on average a binding site for 5 A-T and 3 G-C respectively for Hoechst and mithramycin). In this manner, base composition was estimated with two distinct calculations Hoechst for A-T and mithramycin for G-C. The coefficients of variation of the 2C *Hydrangea* nuclei in histograms were about 3–4 % for ethidium bromide, Hoechst and mithramycin. Mean values of samples for total DNA amount and GC% were compared by anova followed by a Tukey Highest Significant Difference (HSD) test for Unequal N. Statistical analyses were made using Statistica sofware 4.0 (StatSoft, France).

Determination of chromosome number

Root-tip meristems were pre-treated with 0.05% (w/v) colchicine in water for 3 h at 25°C, then fixed in 3:1 ethanol:acetic acid for 24–48 h. Chromosome counts were made using the usual method of Feulgen staining.

Only for *H. macrophylla* subsp. *macrophylla*, about ten metaphase plates were digitalized using a colour video camera (JVC) and an image analyzer system (Vision Explorer, Graftek, France). Chromosome measurements (data not shown) and karyotype elaboration were made using Image Tool and Photoshop 4.0 softwares. The chromosome types were determinated according to Levan et al. (1964). Photographs were obtained using a video printer (Sony).

Results

Genome size

Estimations of 2C DNA amount and GC percentages, as well as the chromosome number of the species studied are given in Table 2. Genome size (2C) for these species ranged from 1.95 pg in *Hydrangea quercifolia* to 5.00 pg in *Hydrangea involucrata*. The analysis was highly significant ($p < 0.001$) according to the ANOVA. The base composition ranged from 39.9% GC in *Hydrangea aspera* subsp. *sargentiana* to 41.1% in *Hydrangea scandens* subsp. *scandens* and this analysis was also highly significant ($p < 0.05$). There was no correlation between 2C DNA content and GC% ($r = 0.04$).

A comparison of results with the HSD test for Unequal N showed (Table 2) that for 2C DNA content there were significant differences ($p < 0.05$) between species from different taxonomic subsections, though with many exceptions. For instance, *Hydrangea anomala* from subsection 3, *Hydrangea heteromalla* from subsection 5,

Table 2 DNA content and base composition in 16 taxa of *Hydrangea*

Taxon	S[a]	2C DNA (pg)	Tukey's grouping for 2C ($p = 0.05$)	%GC	2n
H. arborescens	1	2.31	b	40.8	2*x* = 36
H. quercifolia	1	1.95	a	40.7	2*x* = 36
H. involucrata	2	5.00	h	40.7	2*x* = 30
H. aspera subsp. *aspera*	2	4.74	h	40.8	2*x* = 36
H. aspera subsp. *strigosa*	2	3.47	e	40.7	2*x* = 34
H. aspera subsp. *sargentiana*	2	3.12	d	39.9	2*x* = 34
H. aspera subsp. *robusta*	2	3.02	d	40.5	2*x* = 34
H. anomala subsp. *anomala*	3	3.13	d	40.7	2*x* = 36
H. anomala subsp. *petiolaris*	3	2.71	c	40.9	2*x* = 36
H. scandens subsp. *scandens*	4	3.68	ef	41.1	2*x* = 36
H.scandens subsp. *luikinensis*	4	3.82	f	40.9	2*x* = 36
H. paniculata	5	3.77	f	40.4	2*x* = 36
H. heteromalla	5	2.95	d	40.6	2*x* = 36
H. macrophylla subsp. *macrophylla*	6	4.30	g	41.0	2*x* = 36
H. macrophylla subsp. *serrata*	6	3.85	f	40.6	2*x* = 36
H. seemannii	7	2.09	a	40.3	2*x* = 36

[a] S: taxonomic subsection;
1: *Americanae*,
2: *Asperae*,
3: *Calyptranthae*,
4: *Petalantae*,
5: *Heteromallae*,
6: *Macrophyllae*,
7: *Cornidia*/*Monosegia*

and *Hydrangea aspera* subspp. *robusta* and *sargentiana* from subsection 2, all have a similar genome size (Table 2). Within the same subsection, at least several significant differences ($p < 0.05$) appeared, except within the *Calyptranthae* and the *Petalantae*. The single representative of the section *Cornidia*, *Hydrangea seemanii*, has a genome size among the smallest class.

The results obtained for GC% were also compared with an HSD test for Unequal N. Although GC contents showed slight differences between species in the ANOVA, they were not statistically significant at $p < 0.05$.

Chromosome number

Most of the species studied displayed a diploid chromosome number of 2n = 2*x* = 36. Nevertheless, in the subsection *Asperae*, only *H. aspera* subsp. *aspera* showed 2n = 2*x* = 36, a different chromosome number of 2n = 2*x* = 34 being found for the three other subspecies of *H. aspera* while the species *H. involucrata* displayed 2n = 2*x* = 30 chromosomes.

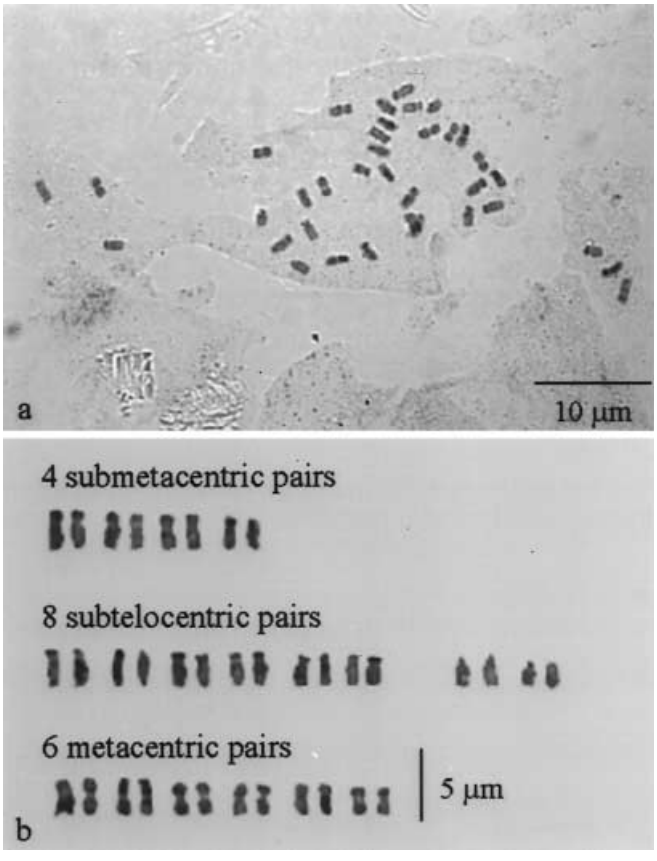

Fig. 1 **a** Metaphase plate (2n = 36) of *H. macrophylla* subsp. *macrophylla* following Feulgen staining of chromosomes. **b** Corresponding karyogram: chromosomes of *H. macrophylla* subsp. *macrophylla* grouped or paired on the basis of total chromosome length and arm ratio

Karyotype of *H. macrophylla* subsp. *macrophylla*

The karyological features of total chromosome length and arm ratio were evaluated (but not described here) to construct a karyotype (Fig. 1a and b) for the species *H. macrophylla* subsp. *macrophylla.* The chromosomes were small (1–2 µm) and with a similar morphology, so that even with the various parameters we could not classify all the chromosomes by pairs. However, we can recognize six metacentric, four submetacentric and eight subtelocentric chromosome pairs (Fig. 1b), with some well-distinguishable. One submetacentric or subtelocentric chromosome pair bears rarely visible satellites and probably nucleolar organizer regions.

Discussion

Genome size variation

For the entire *Saxifragaceae* family, genome size has only been reported for *Saxifraga granulata* with 2C = 1.35 pg (Redondo et al. 1996). This is the first data concerning the *Hydrangeaceae*. The species *H. quercifolia*, from the morphological taxonomic section *Americanae*, had the smallest 2C DNA value (1.95 pg), namely 2C = 1.9×10^9 base pairs, and *H. involucrata* (5.00 pg), from the section *Asperae*, had the largest, giving a 2.5-fold range of variation in this panel of 16 taxa. The genome size also varied between species within each taxonomic subsection. Therefore 2C DNA content cannot be considered as having a taxonomic value for the genus *Hydrangea*.

DNA content differed significantly between the subspecies *H. macrophylla* subsp. *macrophylla* and *H. macrophylla* subsp. *serrata*. This result showed that genome size can evolve very rapidly. These two taxa grow at different altitudes: the subspecies *macrophylla* is found at sea level and the subspecies *serrata* in mountains. It is also relatively easy to distinguish them by their morphological characteristics. Significant differences in genome size were likewise found between subspecies and clones in *Musa acuminata* by Lysák et al. (1999). These authors proposed a relation with the geographical origin of accessions. In contrast, Baranyi and Greilhuber (1996) found no genome size variation in different geographical accessions of *Pisum sativum*.

Differences in genome size within a complex of species are often correlated with ecological factors such as altitude (Godelle et al. 1993; Caceres et al. 1998) or can be a response to physical, chemical or genetic stresses as a strategy for adaptation to a changing environment, as was suggested for light effects on *Helianthus annus* by Price and Johnston (1996).

In our study, the smallest genome sizes were those of *H. quercifolia* (2C = 1.95 pg), *H. seemanni* (2C = 2.09 pg) and *H. arborescens* (2C = 2.31 pg), three species originating from North or South America. Most of the species originate from Asia, which may be consid-

ered as the center of origin of the genus, and few species exist in North and South America. The different ecological and geographical selective pressures would appear to have led to a decrease of the DNA content in American species. However, amplification and deletions of DNA sequences for increasing or decreasing the genome size may just reflect the activity of selfish DNA, one explanation of the C-value paradox. The fact that there was a 2.5-fold genome range between the species of *Hydrangea* suggests, nevertheless, the occurrence of important internal chromosome restructurings involving the amounts of tandemly or interspersed DNA repeated sequences. This genomic fraction can evolve rapidly and is responsible for a major change of DNA content (Flavell 1986; Schmidt and Kudla 1996; Uozu et al. 1997).

Polyploid origin of the genus?

Most of the *Hydrangea* species analyzed were diploid with a chromosome number of 2n = 36. Sax (1931) reported chromosome counts for several species in the section *Hydrangea*, all with 2n = 36, and with 2n = 4*x*= 72 found in a horticultural form of *Hydrangea paniculata*. Moreover, Schoennagel (1931) reported the same chromosome number of 2n = 36 for all the species (*Hydrangea arborescens, Hydrangea radiata, Hydrangea aspera, Hydrangea rosea-alba, Hydrangea opuloides*) in his study. However, our analysis is the first to report different chromosome numbers for species of the section *Asperae* (2n = 30 for *H. aspera* subsp. *involucrata*, 2n = 34 for *H. aspera* subsp. *strigosa* and *H. aspera* subsp. *robusta*). These chromosome numbers are considered as high and correspond to the general trend that, in the flora of the temperate zone, trees and shrubs have higher basic numbers and in general lower frequencies of polyploids within a genus than the perennial herbs.

Two explanations may be proposed for the origin of the basic chromosome number of the genus *Hydrangea*. The first hypothesis is that, like many other woody genera, a high primary basic chromosome number, here $x = 18$, is ancestral for the genus. The other and most probable hypothesis is that this genus is an ancient polyploid. That is, the primary basic number of the *Hydrangea* karyotype would be $x = 6$ or $x = 9$ and that $x' = 18$ would be of secondary polyploid origin. Such an example was described for the genus *Epilobium* (Stebbins 1971). In his study of the chromosomal phylogeny of the *Saxifragaceae*, which included the genus *Hydrangea*, Schoennagel (1931) had considered that the basic chromosome number of the *Saxifragaceae* was $x = 7$, from which a basic chromosome number of $x = 9$ would be derived. More recently, Soltis et al. (1993) considered an original basic chromosome number of $x = 7$ or $x = 11$ for the family on the basis of *rbc*L sequences and cpDNA restriction-site phylogenetic trees. Although genera of the *Hydrangeaceae* are distantly related to the *Saxifragaceae sensu stricto*, this is an argument for the hypothesis of an ancient polyploid origin of this genus from $x = 9$ to $x = 18$. Moreover, the similar chromosomal size and morphology observed in this study for groups of chromosomes in *H. macrophylla* subsp. *macrophylla* is in favour of this hypothesis. The number of each chromosome type also supports an autotetraploid origin.

From the *rbc*L sequence phylogenetic study of Soltis et al. (1995) for the *Hydrangeaceae*, the basic chromosome number of *Hydrangea* could have been derived either by increasing dysploidy or by an ancient polyploid event. In this latter hypothesis, the various chromosome numbers observed for *H. involucrata* and for the subspecies of *H. aspera* would be the result of a decreasing dysploidy. Polyploidy is considered as a major force in plant evolution by which rapid genome evolution occurs (reviewed in Soltis and Soltis 1999); mechanisms by which gene and genome evolution are processed by polyploidy have been recently reviewed by Wendel (2000). For polyploid-derived species which have become chromosomally diploidized, as expected for species of *Hydrangea*, isozymes or RFLP studies could be valuable tools to argument this hypothesis (Wendel 2000).

H. macrophylla within the genus

The molecular phylogenetic study of Soltis et al. (1995) based on rbcL sequence data, showed that the genus *Hydrangea* is not monophyletic: the five species analysed in their study are positioned in three clades along with other genera of the *Hydrangeaceae*. Namely, one clade is represented by *Hydrangea peruviana*, a South American climber species of the section *Cornidia*, and *H. anomala*, a temperate Asian climber species of the section *Hydrangea*. *H. macrophylla* and *H. arborescens* form another lineage, which is a sister group of the previous clade. *H. quercifolia* is a representative of the third clade.

The variation of nuclear DNA content within the genus follows neither the taxonomic treatment of *Hydrangea* species nor this first phylogenetic grouping of Soltis et al. (1995), but rather underlines, as did the molecular and morphological data, the difficulties of resolving the evolutionary relationships within the genus. Therefore, in this case, the genome-size data cannot be used solely for this kind of study.

According to McClintock, natural hybrids in *Hydrangea* are rare, although a hybrid between *Hydrangea hirta* and *H. scandens* has been reported from the Izu Peninsula of Japan (McClintock 1957). For breeding programs, and particularly for enlarging the genetic diversity in cultivated species, we must consider the phylogenetic relationships between species. The most-closely related species to *H. macrophylla* among the five covered by Soltis et al. (1995) is *H. arborescens*, although, as those authors underlined, surprisingly the closest sister taxon of *H. macrophylla* was *Dichroa febrifuga* (a representive of this other genus). This latter relationship was strongly supported by statistical treatment. Despite the close relationship between *H. macrophylla* and *H. arborescens*, a

sexual incompatibility mechanism prevents reciprocal crosses between them (Kudo and Niimi 1999a). However, experimental crosses were initiated and, with difficulty, useful characters of *H. arborescens* have been introduced into *H. macrophylla* by these last authors (Kudo and Niimi 1999b). Several experimental hybrids, nevertheless entirely sterile, have also been obtained between *H. macrophylla* var. *rosa* and *H. paniculata* and between *H. macro*phylla var. *rosa* and *H. anomala* subsp. *petiolaris* (McClintock 1957), although major genome size differences exist between *H. macrophylla* and these latter two species. Other studies on hybrids between species differing in nuclear DNA content show that, despite large differences in genome size, homoeologous chromosomes pair at pachytene and form chiasmata. Problems with pairing are observed when the DNA differences are very large, of an order of 60% or more (Rees et al. 1984).

In conclusion, this study reports on the genome size and chromosome number of species of the genus *Hydrangea*. For plant-breeding purposes, these data constitute a basic knowledge of genetic resources. For example, a small genome size is an advantageous feature for facilitating molecular approches. Moreover, the choice of species for interspecific experimental hybridization is sometimes critical. When parental species differ sufficiently in nuclear DNA content, it is easier to detect interspecific hybrids according to intermediate values of DNA. Most of the species studied present a chromosome number of $2n = 2x = 36$, except in the section *Aspereae* which revealed unsuspected variability. A primary karyotype has been made for the first time for *H. macrophylla* subsp. *macrophylla*, the most cultivated species of the genus. For a complete karyotype, other chromosome markers are needed to recognize each chromosome pair. For this purpose, we aim to use in situ hybridization of fluorescent DNA probes. In an evolutionary context, we are also undertaking a molecular phylogeny for the genus with an enlarged panel of species, which will be necessary for the understanding of cytological and morphological character evolution.

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